

From the Biochemisches Institut
at Christian-Albrechts-Universität zu Kiel - Kiel University

The role of the palmitoylation site of the A Disintegrin And Metalloprotease 10 (ADAM10)

Dissertation
to acquire the doctoral degree (Dr. med.)
at the Faculty of Medicine
at Christian-Albrechts-Universität zu Kiel - Kiel University

presented by
Maja Grohe
from **Göttingen**

Kiel **2024**

1 st Reviewer:	Prof. Dr. Paul Josef Saftig
2 nd Reviewer:	Prof. Dr. rer. nat. Kirsten Hattermann-Koch
Date of oral examination:	25.11.2025
Approved for printing, Kiel:	13.08.2025

Signed: Prof. Dr. Paul Josef Saftig
(Chairperson of the Examination Committee)

Table of Contents

1 Introduction	1
1.1 Proteases	1
1.1.1 ADAMs	1
1.1.2 ADAM10	2
1.1.3 ADAM10's structure	3
1.1.4 ADAM10's functions	4
1.1.5 Regulation of ADAM10	6
1.1.6 ADAM10 proteolysis	7
1.2 ADAM10 in neurodegenerative diseases	8
1.3 Protein lipidation	9
1.3.1 Protein S-Palmitoylation	10
1.3.2 Effects of Palmitoylation	11
1.3.3 Regulation of Palmitoylation	13
1.3.4 Palmitoylation in disease	15
2 Objectives	17
3 Material and Methods	18
3.1 Material	18
3.1.1 Chemicals	18
3.1.2 Antibodies	18
3.1.3 Cell culture media and additives	19
3.1.4 Kits and ready-made agents	19
3.1.5 Oligonucleotides	20
3.1.6 Expression plasmids	20
3.1.7 Cell lines	21
3.1.8 Buffers	21
3.1.9 Devices	22
3.2 Molecular biological methods	23
3.2.1 Amplification and purification of expression vectors in Escherichia Coli	23
3.2.2 Sequencing of expression vectors	23
3.2.3 Site-directed mutagenesis Polymerase Chain Reaction (PCR)	23
3.3 Cell biological methods	25
3.3.1 Maintenance of cells	25
3.3.2 Cryopreservation of cell lines	25
3.3.3 Transient transfection of cells	25
3.3.4 Inhibitor treatment of cells	25

3.4 Protein biochemical methods	26
3.4.1 Generation of cell lysates for protein extraction	26
3.4.2 Determination of protein concentration	26
3.4.3 Trichloroacetic Acid Precipitation.....	26
3.4.4 SDS polyacrylamide gel electrophoresis.....	27
3.4.5 Western blotting and protein detection.....	27
3.4.6 Co-immunoprecipitation experiments	28
3.4.7 Membrane fractionation of proteins	29
3.4.8 Cell surface biotinylation of proteins	30
3.4.9 Indirect immunofluorescence labelling of cells	30
3.4.10 Acyl-resin Assisted Capture Assay	31
3.4.11 Acyl-PEGyl Exchange Gel Shift (APEGS) Assay	32
3.4.12 Click Chemistry Assay	33
3.5 Statistical Methods.....	34
4 Results	35
4.1 ADAM10 is a palmitoylated protein	35
4.1.1 An acyl-resin assisted capture assay revealed ADAM10 as a palmitoylated protein	36
4.1.2 Confirmation of the palmitoylation of ADAM10 by targeting palmitate insertion using 17-ODYA in a click chemistry reaction	37
4.1.3 Analysis of ADAM10's palmitoylation status	39
4.2 Biochemical characterisation of the palmitoylation-deficient ADAM10 mutant.....	41
4.2.1 Targeting palmitate insertion by fluorophore attachment reveals palmitoylation of human ADAM10 and palmitoylation deficiency of the C699S mutant	43
4.2.2 Analysis of the expression level of the ADAM10 palmitoylation-deficient mutant in comparison with wildtype ADAM10	44
4.3 Intracellular localisation of the palmitoylation-deficient mutant	46
4.3.1 Subcellular localisation of ADAM10	46
4.3.2 Analysis of cell surface expression of wildtype vs palmitoylation-mutant ADAM10	48
4.4 Does the palmitoylation affect ADAM10's substrate shedding capacity?	50
4.4.1 Analysis of the influence of ADAM10's palmitoylation on the proteolytic processing of N-Cadherin.....	51
4.4.2 Analysis of the influence of ADAM10's palmitoylation on the proteolytic processing of APP.....	53
4.5 Characterisation of the ADAM10 carboxyterminal fragment generation	58
4.5.1 Palmitoylation-deficient ADAM10 transfected cells contain less ADAM10 CTF.....	58
4.5.2 Pharmacological inhibition of ADAM10's palmitoylation leads to less ADAM10 CTF generation	61
4.5.3 Selective inhibition of ADAM10 leads to less ADAM10 CTF generation.....	62

4.5.4 Membrane association of ADAM10 CTFs	70
4.6 Investigation of the influence of palmitoylation on ADAM10's interaction partners.....	71
4.6.1 Interactions with SAP-97 and AP-2.....	72
4.6.2 Comparing interaction partners of wildtype and palmitoylation-deficient ADAM10	74
5 Discussion.....	79
5.1 Identification of ADAM10 as a palmitoylated protein	79
5.1.1 Characterisation of the palmitoylation sites in ADAM10	80
5.2 Consequences of the palmitoylation of ADAM10.....	81
5.2.1 The palmitoylation has no apparent effect on the expression and maturation of ADAM10.....	81
5.2.2 The cell surface expression is impaired in the palmitoylation-deficient ADAM10 mutant	82
5.2.3 Palmitoylation of ADAM10 as a substrate-specific regulatory mechanism	84
5.3 ADAM10's palmitoylation regulates its proteolytic processing	85
5.3.1 ADAM10's membrane association is independent of its palmitoylation status.....	88
5.4 ADAM10's palmitoylation affects specific protein-protein interactions	89
6 Conclusions.....	93
7 Summary.....	94
8 References.....	95
9 Acknowledgements	103
10 Declaration	104
11 Publication.....	105

1 Introduction

1.1 Proteases

Proteases are enzymes that break down proteins, enzymes, or peptides by hydrolysing peptide bonds. They are found in all living organisms and have various functions in biological processes. Despite a previous belief that proteases had an unspecific role in protein catabolism, their activity is now known to be carefully regulated, and substrate cleavage is, in most cases, a specific event (Lopez-Otin & Bond, 2008). Protein degradation, but also activation and targeting of proteins as well as processes like wound healing, angiogenesis, and tissue remodelling are regulated by proteases. Therefore, proteases are interesting drug targets when these processes are dysregulated in different pathologies (Turk, 2006).

Proteases can act as endo- or exopeptidases, and they can further be divided according to their catalytic mechanism and the ion or amino acid in the active centres. Examples of subgroups include aspartate, glutamate, cysteine, metallo, serine, threonine, and asparagine proteases. Exopeptidases cleave from either the N-terminal or C-terminal end of a substrate. Endopeptidases cleave within the polypeptide chain (Lopez-Otin & Bond, 2008).

1.1.1 ADAMs

A Disintegrin And Metalloproteases (ADAMs) are type I transmembrane spanning proteases belonging to the group of metalloendoproteases. They have been found in many species ranging from nematodes (*Caenorhabditis elegans*) and yeast (*Schizosaccharomyces pombe*) to vertebrates. In mice, 37 genes encoding for different ADAMs were identified. In humans, 12 of 22 identified genes of the metalloproteinases encode catalytically active enzymes (Edwards et al., 2008). The other ADAMs function as adhesion proteins or receptors but show no catalytic activity (Hsia et al., 2019).

The name reflects the key structural elements which are a disintegrin domain relevant for interactions with integrins and a metalloprotease domain. The catalytic active ADAMs bear a conserved zinc-binding motif HExxHxxGxxH with an ellipsoid shape and three histidine residues in the extracellular metalloprotease domain at the N-terminus (Saftig & Lichtenthaler, 2015; Wolfsberg et al., 1995).

ADAMs generally have a similar domain structure containing an N-terminal signal peptide, a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich

and an EGF-like domain, followed by a transmembrane domain and a short cytoplasmic tail (Edwards et al., 2008). When the expression or function of ADAMs is altered, it can result in various pathological conditions due to their involvement in important physiological processes such as proteolysis, cell adhesion, and cell migration. As a result, certain ADAMs are emerging as potential therapeutic targets for a range of diseases (Camodeca et al., 2019).

1.1.2 ADAM10

One of the best investigated ADAMs is the A Disintegrin And Metalloprotease 10 (ADAM10). It is synthesised in the ER as an inactive proform with a signal peptide directing the protein to the Golgi apparatus and an inhibitory prodomain (Fig. 1, 2). The prodomain is cleaved during the secretory pathway in the Golgi apparatus by furins and the prohormone convertase 7 (Saftig & Lichtenthaler, 2015) (Fig. 1). The inactive proform of ADAM10 has a molecular weight of approximately 100 kDa, but once the prodomain is removed, it becomes active in late secretory compartments and at the plasma membrane as a mature form with a molecular weight of around 70 kDa. ADAM10 is N-glycosylated at four sites in the extracellular domain, which is essential for achieving full enzyme activity (Escrevente et al., 2008).

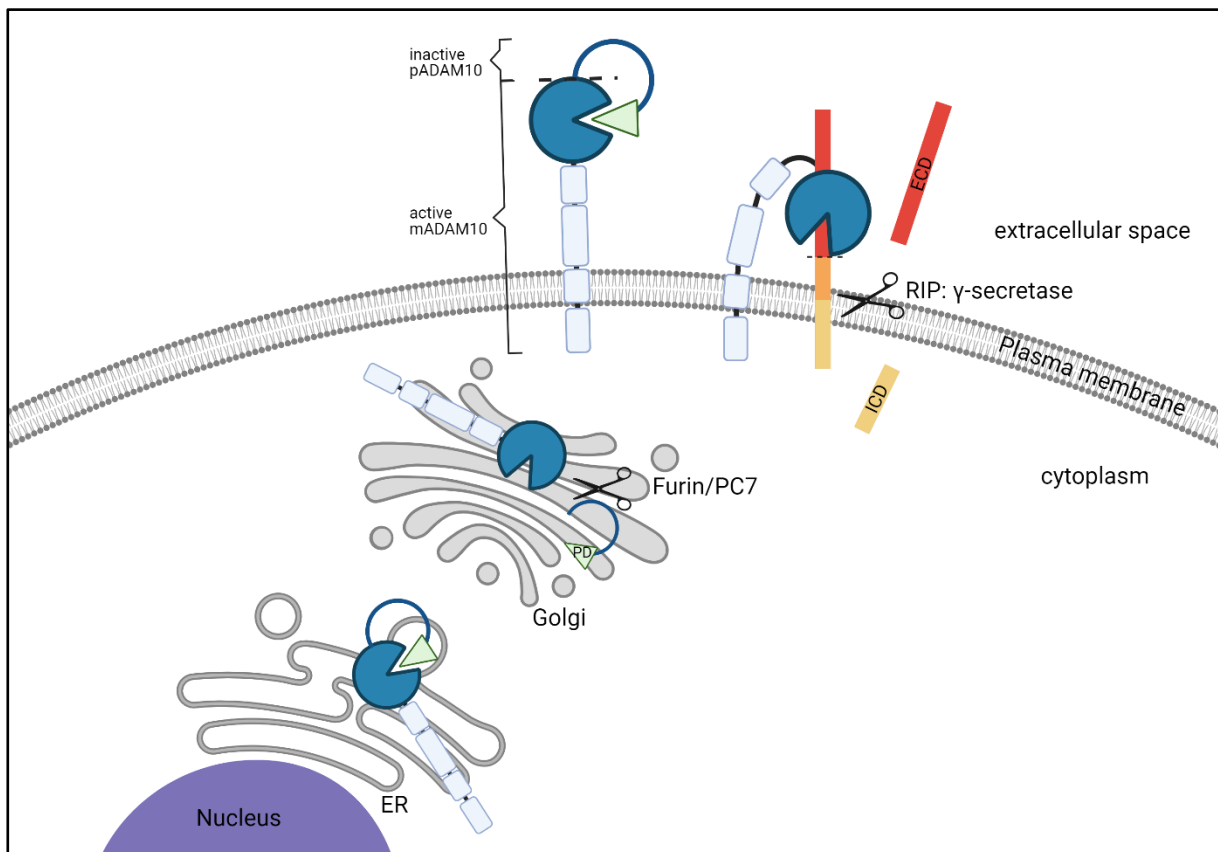


Figure 1: ADAM10 processing and ectodomain shedding. ADAM 10 is synthesised as an

inactive proform (pADAM10) in the endoplasmic reticulum (ER). The prodomain (PD) is cleaved by furin or prohormone caspase 7 (PC7) in the Golgi apparatus (Golgi). ADAM10 is active as its mature form (mADAM10) at the plasma membrane cleaving substrates close to the plasma membrane. This ectodomain shedding releases an extracellular, soluble fragment (ECD) and a membrane-bound part which is subject to further Regulated Intramembranous Proteolysis (RIP) by the γ -secretase complex. The RIP can liberate an intracellular domain (ICD).

1.1.3 ADAM10's structure

The prodomain of ADAM10 plays a crucial role in the correct folding and transport of the protein by acting as an intramolecular chaperone. The prodomain also plays a regulatory role by inhibiting the protease activity of ADAM10. The inactive full-length form including the signal peptide contains 748 amino acids.

The metalloprotease domain of ADAM10 is responsible for the proteolytic activity of the enzyme and is essential for the cleavage of its substrate proteins. The disintegrin and membrane proximal domain are important for substrate recognition and specificity, as well as for the regulation of protease activity (Janes et al., 2005). The membrane proximal domain is further relevant for protein multimerization, which is important for its activity and regulation (Janes et al., 2005). The C-terminal part of ADAM10 contains an ER-retention motif and a basolateral sorting signal that are important for its proper localisation and transport (Maretzky et al., 2015; Wild-Bode et al., 2006). Furthermore, the C-terminal domain also contains interaction sites for other proteins like the clathrin adaptor AP-2, making it crucial for the regulation of ADAM10's activity, localisation, and signaling through interacting regulators (Marcello et al., 2013).

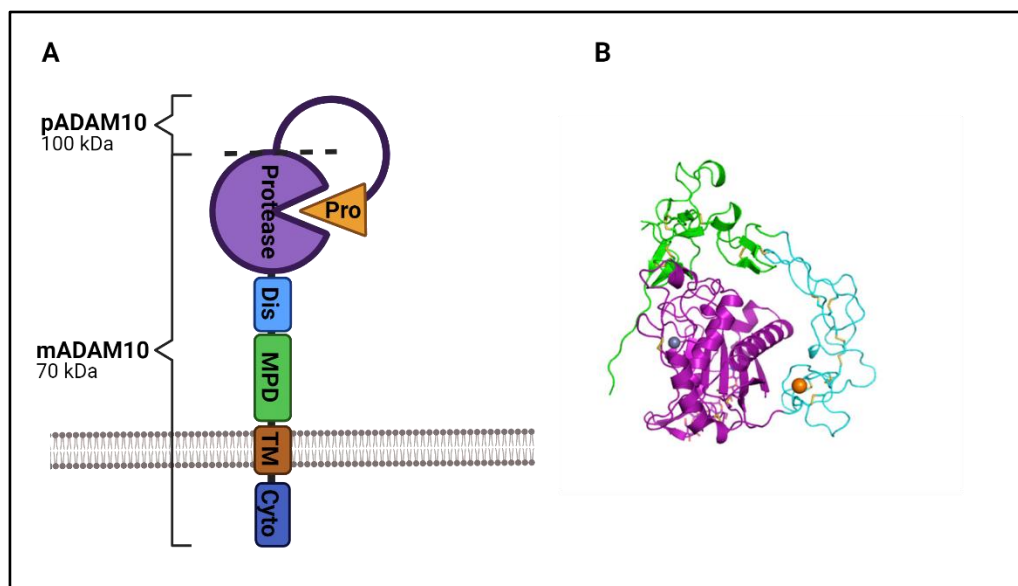


Figure 2: Topology and domain organisation of A Disintegrin And Metalloprotease 10

(ADAM10). **A:** Domain structure of ADAM10, Abbreviations: Pro: Prodomain, Protease: Metalloprotease domain, Dis: Disintegrin domain, MPD: Membrane proximal domain, TM: Transmembrane domain, Cyto: Cytoplasmic tail. The domain colours of the ectodomain of ADAM10 match the coloured X-ray structure in **B:** Architecture of the ectodomain of human mature ADAM10 from (Seegar et al., 2017). The catalytic zinc ion is represented in grey, and a bound calcium ion is in orange.

1.1.4 ADAM10's functions

Substrates of ADAM10 are mostly type I membrane-spanning proteins that are cleaved within their ectodomain near the plasma membrane. This process is called ectodomain shedding and can be followed by subsequent regulated intramembrane proteolysis (RIP) (Fig. 1). Removal of the ectodomain enables the γ -secretase complex to cleave within the transmembrane domain releasing an intracellular fragment. This fragment can be involved in signalling pathways and regulate downstream genes (Edwards et al., 2008).

There is a great variety and still growing number of cell surface proteins that are cleaved by ADAM10. Among these are for example cytokines and adhesion molecules like N-Cadherin or E-Cadherin (Peron et al., 2018). The Notch receptor is another well investigated ADAM10 substrate with multiple functions. It is known as a regulator for cell fate decisions in the embryonic development, but also in homeostasis of adult tissue, and RIP.

The importance of ADAM10 for cellular homeostasis was shown by the lack of ADAM10-mediated Notch processing in an ADAM10 knockout mouse model where mice die at an early stage of embryogenesis at day 9.5. The phenotype has similarities to Notch-deficient mice which have severe defects in the central nervous system, somites and in the cardiovascular system (Hartmann et al., 2002). Further, ADAM10 conditional knockout mice exert epileptic seizures, learning deficits and an altered morphology of postsynaptic structures in the brain (Prox et al., 2013).

In addition to ADAM10's role in developmental processes, the proteolytic processing of other substrates plays important roles for synaptic and neuronal network function. A reduced shedding of adhesion molecules such as the neuronal cadherin (N-Cadherin), neuronal cell adhesion molecule (NCAM) and Neuroligin-1 was shown in ADAM10-deficient neurons (Kuhn et al., 2016). N-Cadherin is an adhesion molecule that is important for synaptic contacts in neurons, more precisely in the hippocampus. It interacts with the actin cytoskeleton via β -catenin, regulating synaptic contact, synaptogenesis, and dendritic spine morphology (Mendez et al., 2010; Uemura et al., 2006).

The amyloid precursor protein (APP) is a transmembrane protein expressed in the brain and has multiple isoforms. APP undergoes proteolytic processing, with different pathways leading to either non-amyloidogenic (Fig. 3) or amyloidogenic products that participate in the regulation of metabolism in neuronal cells and other tissues (Guo et al., 2021). The amyloidogenic pathway involves cleavage by the beta-site amyloid precursor protein cleaving enzyme (β -secretase/BACE), leading to the release of soluble APP β (sAPP β) (Chow et al., 2010). The membrane-bound C-terminus is further processed by the γ -secretase complex which cleaves at one site between amino acids 40 to 44 generating A β , which contributes to the formation of plaques associated with Alzheimer's disease, and an APP intracellular domain (AICD) (O'Brien & Wong, 2011). The α -secretase enzyme, mainly ADAM10, mediates non-amyloidogenic processing, leading to the release of soluble APP α (sAPP α) and an APP C-terminal fragment α (CTF α), which is neuroprotective because the α -site is located within the peptide sequence of A β and therefore prevents the A β plaque formation (Fig. 3) (Postina et al., 2004; Prox et al., 2013).

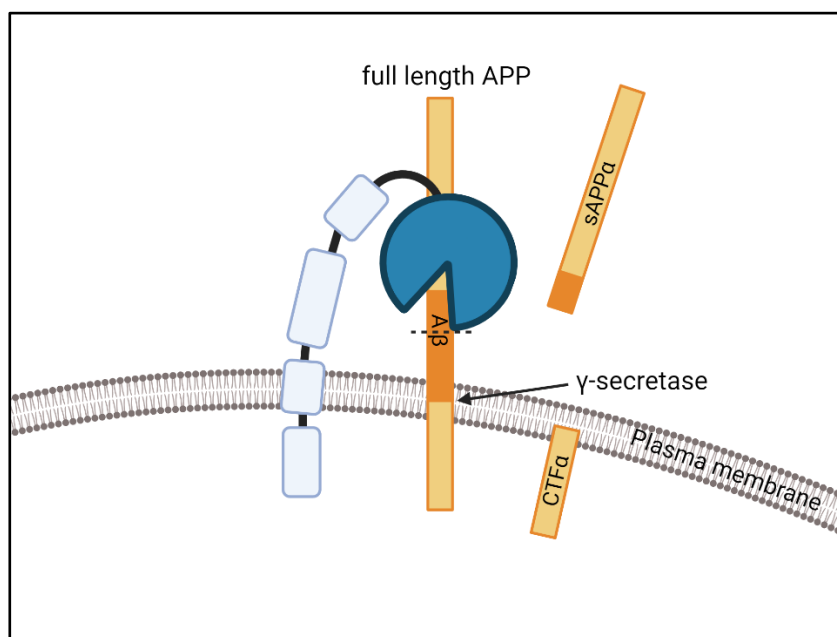


Figure 3: Non-amyloidogenic cleavage of APP. Shown is the non-amyloidogenic cleavage of APP which is initiated by the main α -secretase ADAM10. The ectodomain of APP is cleaved off within the peptide sequence of the A β domain resulting in a soluble APP α (sAPP α) and a membrane-bound fragment. This fragment is further processed by the γ -secretase releasing a C-terminal fragment (CTF α).

In addition to ADAM10's function as a protease, it is a receptor for the α -toxin of *Staphylococcus aureus* which forms pores in the membranes of cells. *S. aureus* is a type of bacteria that can cause a variety of infections in humans, ranging from minor

skin infections to life-threatening conditions. Binding of ADAM10 to the α -toxin initiates shedding of epithelial and endothelial cadherins (E-Cadherins) contributing to bacterial spreading subsequent tissue damage (von Hoven et al., 2019; Wetzel et al., 2017; Wilke & Bubeck-Wardenburg, 2010).

1.1.5 Regulation of ADAM10

Transcriptional regulation

The human ADAM10 gene is spanning 154 kb with 16 exons. Its core promoter region responds to retinoic acid (RA) and is influenced by factors like PPAR α , SIRT1, and XBP-1 (Prinzen et al., 2005).

SIRT1, an NAD⁺ dependent deacetylase in mammals, is known for its potential in age-related and neurodegenerative diseases. By activating ADAM10 transcription through co-activating RXR, SIRT1 increases its expression and reduces A β production (Donmez et al., 2010). Animal models of Alzheimer's disease have shown improvement in brain pathology and behavior deficits with SIRT1 expression (Peron et al., 2018).

XBP-1 is a transcription factor that positively regulates ADAM10 in neuronal cells, and its transcriptional regulation in AD transgenic animal models is linked to ADAM10. Furthermore, XBP-1 indirectly modulates A β production through HRD1 activation and APP degradation, and therefore, it is a critical therapeutic target for neurodegenerative diseases such as AD (Reinhardt et al., 2014).

Other transcription factors are Melatonin, SOX-2, and PAX2 that promote ADAM10 transcription and therefore induce APP non-amyloidogenic processing (Lee et al., 2011; Sarlak et al., 2016; Shukla et al., 2015).

Translational regulators

The ADAM10 mRNA transcript is regulated by several effectors acting on ADAM10 protein expression. The presence of a stable secondary structure of G-quadruplex RNA in the 5'UTR region inhibits ADAM10 translation, and its deletion leads to a large increase in expression (Lammich et al., 2010). MicroRNAs (miRNAs) are also involved in ADAM10 regulation, with miR-122 shown to bind to ADAM10 mRNA and promote its downregulation (Bai et al., 2009).

Posttranslational regulators

ADAM10 activity can be regulated by transcriptional and translational factors but also by interaction with other proteins. Different tetraspanins interact with ADAM10. The subgroup of C8 tetraspanins (TspanC8), defined by eight cysteine residues in their large extracellular loop, is required for the proper trafficking of ADAM10 and enzymatic maturation. Tetraspanin 15 (Tspan15), as an example from the TspanC8 group, pushes ADAM10 from the ER to the plasma membrane and promotes cleavage of N-Cadherin (Matthews et al., 2017).

Interactions of proteins play important roles since a dysfunction of complex interactions and mechanisms often leads to pathologies. ADAM10 is located in postsynaptic densities of excitatory synapses where it is able to cleave APP at the α -site preventing the deposition of A β . There are mechanisms that regulate the localisation of ADAM10 in synaptic membranes and subsequent the ability to process distinct substrates. The synapse-associated protein 97 (SAP-97) interacts with and promotes forward trafficking of ADAM10 (Marcello et al., 2007).

Besides the forward trafficking, a balance of the regulatory mechanisms promoting or inhibiting ADAM10 activity is important. ADAM10 removal from synaptic membranes is clathrin-mediated. The adaptor protein complex 2 (AP-2), a part of the clathrin complex, interacts with the C-terminus of ADAM10 promoting its endocytosis and modulates its surface presence. AP-2 consists of the four different subunits α , β 2, μ 2, and δ . Three of them (α , β 2, μ 2) were shown to interact directly with ADAM10 in human hippocampi (Marcello et al., 2013).

The relevance of these interactions was shown in AD patients showing altered interactions between ADAM10 and SAP-97 and AP-2. In the hippocampi of AD patients, a reduction of the ADAM10/SAP-97 association and an increase of the association of AP-2 with ADAM10 was found (Marcello et al., 2007; Musardo et al., 2014).

1.1.6 ADAM10 proteolysis

In addition to the fact that the clathrin adaptor AP-2 interacts with ADAM10 and is therefore part of the endocytic machinery to reduce the amount of ADAM10 at the plasma membrane, proteolytic cleavage of ADAM10 is another mechanism to regulate ADAM10 activity and expression.

ADAM10 can be cleaved or degraded to form a soluble N-terminal part and a membrane-bound C-terminal part (Peron et al., 2018).

ADAM10 itself is susceptible for proteolytic degradation by members of the ADAM family. The metalloprotease ADAM9 initiates regulated intramembranous proteolysis by cleaving the ectodomain of ADAM10. The remaining membrane-bound part including the cytosolic tail is then further processed by the γ -secretase complex releasing an intracellular domain (ICD) (Toussey et al., 2009). Soluble ADAM10 retains its proteolytic activity which was shown by analysis of the supernatant of mouse embryonic fibroblast (MEF) cells in which soluble ADAM10 is cleaving a synthetic peptide that contains the α -cleavage site of APP (Toussey et al., 2009). The ICD of ADAM10 can translocate to the nucleus and may be involved in gene transcription and signalling pathways (Toussey et al., 2009; Vincent, 2016).

ADAM9 deficiency lowered soluble ADAM10 and CTF levels which was further reduced by ADAM15 deficiency, implicating a role of ADAM15 in ADAM10 shedding. However, other proteases likely contribute to the processing of ADAM10 despite ADAM9 and ADAM15 (Toussey et al., 2009).

1.2 ADAM10 in neurodegenerative diseases

Alzheimer's disease

Alzheimer's disease is the most common neurodegenerative disease accounting for 50 to 75% of dementia cases. It is a condition of the later life as the risk increases in people older than 65 years. It is a global health concern with a huge impact on individuals and on the society considering the expenses for the healthcare system (Lane et al., 2018).

AD pathology begins with cellular alterations in neurons, microglia, and astroglia before cognitive impairment is detectable. Factors such as neuroinflammation, changes in vessels, aging, and a dysfunctional glymphatic clearance pathway contribute to disease development upstream or in parallel (Scheltens et al., 2021). Abnormal accumulation of amyloid β , particularly A β 42, in the extracellular space leads to the formation of A β plaques, which trigger the production and spreading of tau protein and ultimately cause the formation of neurofibrillary tangles (NFT) (Atri, 2019).

Research on Alzheimer's disease (AD) has primarily focused on neurons, with ADAM10 being of significant interest due to its potential to prevent A β formation and to liberate sAPP α which is considered neuroprotective (Postina et al., 2004; Zhang et

al., 2011). However, attempts to characterise ADAM10 as a potential peripheral biomarker of AD have revealed conflicting results, with lower platelet ADAM10 expression (Colciaghi et al., 2004; Manzine et al., 2013) but elevated ADAM10 in plasma and cerebrospinal fluid in AD patients (Pereira Vatanabe et al., 2021). This discrepancy may indicate a decreased amount of functionally active ADAM10 (Pereira Vatanabe et al., 2021). While increasing ADAM10 activity is considered a potential therapeutic approach for AD, concerns arise due to potential negative impacts on other tissue or cell types (Pruessmeyer & Ludwig, 2009). Understanding how ADAM10 activity is related to its localisation and functionality is critical to developing controlled modulation for therapeutic intervention (Musardo et al., 2022).

Huntington's disease

Huntington's disease, caused by a mutation in the huntingtin protein (htt), leads to synaptic dysfunction and cognitive impairment. The huntingtin gene (Hdh) has a variable CAG repeat, translated into a polyglutamine sequence (polyQ) within the protein. If this polyQ sequence exceeds 39 amino acids in length, it results in Huntington's disease, a fatal and inherited neurodegenerative disorder (Vezzoli et al., 2019).

Increased ADAM10 activity and proteolysis of its synaptic substrate N-Cadherin are associated with neurotoxic events in Huntington's disease. The huntingtin protein regulates ADAM10 activity, affecting neurulation and rosette formation in neuroepithelial cells. In zebrafish embryos, defects in neural tube morphogenesis due to htt knockdown were rescued with an ADAM10 inhibitor (Lo Sardo et al., 2012; Vezzoli et al., 2019).

1.3 Protein lipidation

Protein lipidation refers to the posttranslational modification (PTM) of proteins with lipid moieties. This process regulates aspects of physiology, including alterations in hydrophobicity, structural stability, localisation, trafficking between membrane compartments, and interaction with effectors (Fhu & Ali, 2021). Lipids or their metabolites serve as substrates for protein lipidation, and their availability is strictly regulated by cellular metabolism. Lipid modifications are one of the most common types of PTMs, with an estimated 25-40% of proteins being modified by lipids (Main & Fuller, 2022). Palmitoylation and myristoylation are the most frequent types of lipid

modifications of proteins. Evidence of covalent lipid transfer to proteins was first discovered in the 1950s, but the theory of lipid modifications as an essential component of protein trafficking only gained attention decades later (Folch & Lees, 1951). Dysregulation of protein lipidation is associated with a variety of diseases, including metabolic syndromes and cancers (Fhu & Ali, 2021).

1.3.1 Protein S-Palmitoylation

Protein S-Palmitoylation as a fully reversible posttranslational modification is raising interest because of its emerging mechanisms to control the properties and functions of many different proteins. S-Palmitoylation is the most common form of S-acylation where lipids are enzymatically added to a protein (Fig. 4). In the case of palmitoylation, it is the addition of a palmitate residue - a C16 fatty acid - onto an intracellular cysteine residue of a protein through a labile thioester linkage (Main & Fuller, 2022).

Some proteins undergo autopalmitoylation in an intrinsic enzyme-like manner. For example, the TEA domain (TEAD) transcription factor possesses autopalmitoylation functions under physiological concentrations of palmitoyl-CoA (Chan et al., 2016). Though, the palmitoylation process is mostly mediated by palmitoyl acyltransferases (PATs). They are integral membrane proteins with four transmembrane domains and both N- and C-termini exposed to the cytoplasm and a conserved cytoplasmic Asp-His-His-Cys (DHHC) motif (Fig. 4) (Gottlieb & Linder, 2017). 23 different isoforms of PATs have been identified in humans to date.

Integral membrane proteins can only be palmitoylated in the cytoplasmic or intramembranous part (Charollais & Van Der Goot, 2009; Rodenburg et al., 2017). Several palmitoylation events occur close to the transmembrane domain (Reddy et al., 2017). Programs like the CSS-Palm 2.0 have been developed to predict specific palmitoylation sites. However, according to the authors, this program cannot predict juxtamembranous palmitoylation sites (Ren et al., 2008).

Palmitoylation can be detected with different experimental approaches like radiolabelling or chemical labelling of the palmitate to be inserted into a protein. Developed in the 1980s, radiolabelling involves labelling fatty acids in cells with ³H-palmitate (Schlesinger et al., 1980). Click chemistry, developed as an alternative to radiolabelling, uses chemical probes like azido-containing fatty acids that incorporate into acylation sites. The probes are then 'clicked' to a detection system for example an ALEXA fluorophore for analysis (Main & Fuller, 2022).

Techniques like acyl-biotin exchange (ABE) and acyl-resin-assisted capture (Acyl-RAC) involve alkylation of free cysteines, cleavage of the thioester bond of the attached palmitate residue, and immunoprecipitation, but they can't provide information on palmitoylation dynamics or detect changes in palmitoylation states. The cysteine PEGylation assay is a variation of the ABE/Acyl-RAC assay and uses cysteine-reactive molecules to cause a molecular weight shift in the protein through its sensitivity towards hydroxylamine cleavage. Therefore, it can identify the number of sites where palmitoylation occurs. (Main & Fuller, 2022).

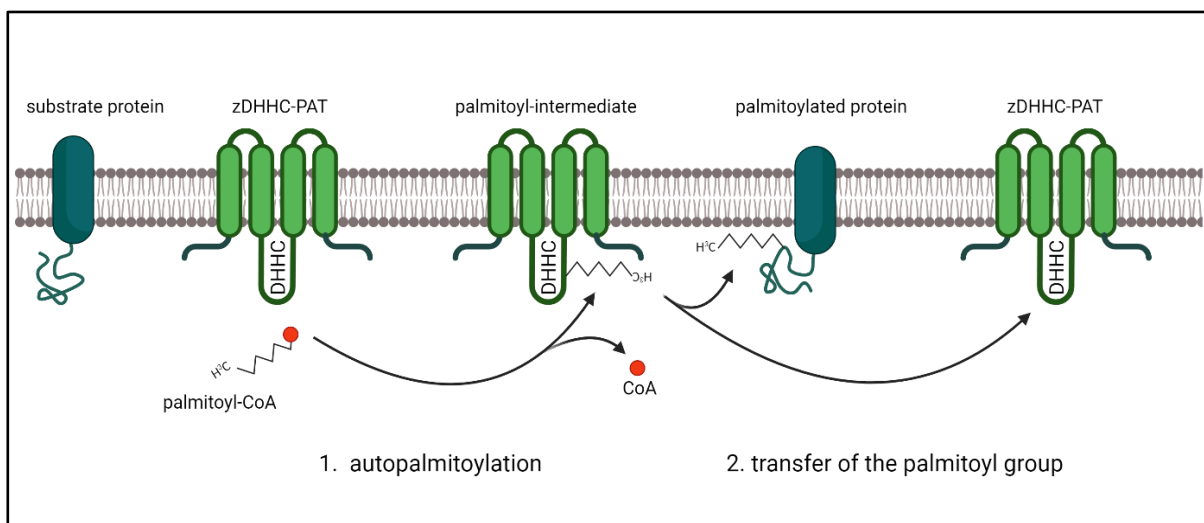


Figure 4: Process of enzymatic protein palmitoylation. Shown is the process of protein palmitoylation by a zDHHC palmitoyltransferase (zDHHC-PAT). First, palmitoyl-CoA binds to the catalytic active centre of the zDHHC-PAT which is the DHHC motif. The autopalmitoylation of the zDHHC-PAT leading to the palmitoyl-intermediate is followed by the second step: The palmitoyl-group is transferred to the substrate.

1.3.2 Effects of Palmitoylation

Protein S-Palmitoylation can affect its substrate in different manners which cannot be predicted yet. The added palmitate increases the hydrophobicity of the protein and the affinity to non-polar structures like lipid bilayers. Subsequently, the structure or localisation of a protein can change. Effects like raft association, tilting of the transmembrane domain or altered trafficking of the palmitoylated protein were already reported (Main & Fuller, 2022).

Lipid raft microdomains are proposed to be functional membrane heterogeneities enriched with liquid ordered molecules such as cholesterol and sphingolipids (Charollais & Van Der Goot, 2009). They have the ability to associate in large, stable structures. Because palmitate is a saturated fatty acid it is intended to favour ordered

lipids which increases the affinity of palmitoylated proteins to these raft-like domains (Fig. 5A). However, not all palmitoylated proteins localise to rafts as it is the case for the transferrin receptor. Some proteins like the anthrax toxin receptor TEM8 are even held back from raft association via palmitoylation (Charollais & Van Der Goot, 2009). The presence of a hydrophobic, saturated fatty acid in close proximity to the transmembrane domain (TMD) can have a significant impact on the physical properties of the TMD. It can increase the overall hydrophobicity of the TMD, increase its effective hydrophobic length, and alter its orientation by inducing a tilt (Fig. 5B). TMDs of proteins may not always be optimally matched to the thickness of the bilayer membrane in which they reside, leading to targeting into other domains or adoption of alternative conformations. Palmitoylation is an important factor in enabling proper insertion of TMDs into lipid bilayers (Charollais & Van Der Goot, 2009). This mechanism can have various downstream effects on a protein's targeting, localisation, interactions, and function.

Different possible mechanisms underly the reported effects. As mentioned, one mechanism could be the localisation of palmitoylated proteins into different microdomains, while another could involve alterations in protein-protein interactions (Charollais & Van Der Goot, 2009). Palmitoylation is likely to affect the conformation and localisation of parts of the cytoplasmic domain due to the increased hydrophobicity that follows the addition of palmitate. This can lead to changes in cytoplasmic interactions between the palmitoylated protein and its interaction partner (Fig. 5C). Palmitoylation-induced alterations in protein interactions are mostly negative, such as in the case of the endothelial signalling molecule phospholipase C β 1, whose palmitoylation can decrease interactions between leukocytes and the endothelium, downregulating microvascular leak (Jin et al., 2021). However, some interactions can be positively regulated, as seen in the interaction of the mannose-6-phosphat-receptor (MPR) with retromers that are involved in trafficking from endosomes to the trans golgi network (McCormick et al., 2008; Schweizer et al., 1996).

The importance of palmitoylation for the regulation of protein platforms can be seen in the example of tetraspanin-enriched microdomains (TEMs). TEMs are dynamic multiprotein complexes containing tetraspanins, integrins, signal molecules, and DHHC PATs. Several proteins involved in TEMs are palmitoylated, particularly tetraspanins. Palmitoylation of these molecules influences tetraspanin interactions and stabilises the association of different tetraspanins. A loss of cohesion of TEMs can lead to altered adhesion and cell spreading (Charrin et al., 2002).

Palmitoylation can also influence oligomerisation of specific proteins. For instance, a palmitoylation-dependence for oligomerisation was observed for the cell death receptor CD95 (McCormick et al., 2008). In contrast, the palmitoylation of CD44, a transmembrane glycoprotein involved in angiogenesis, bone metastasis, and cell migration, can disturb its oligomerisation (Ma et al., 2022).

The effects of palmitoylation described above, frequently result in a change in protein function. Nevertheless, determining whether palmitoylation is directly required for protein function is challenging.

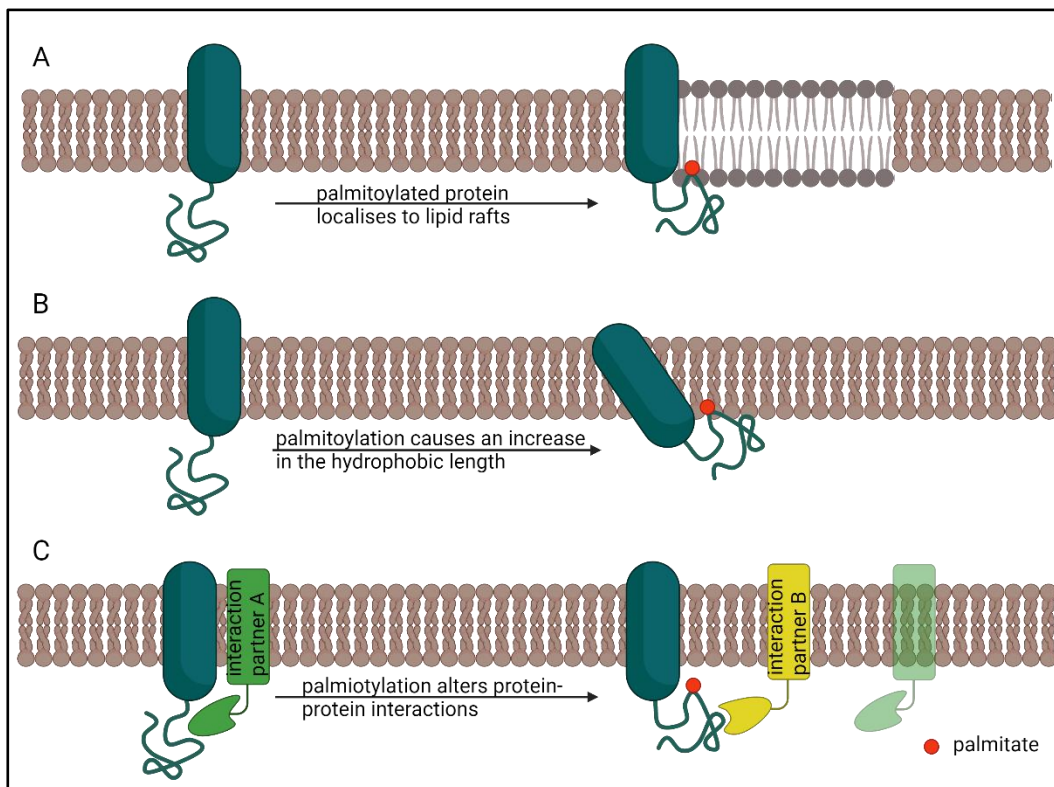


Figure 5: Schematic presentation of the potential effects of protein palmitoylation. Palmitoylation of integral membrane proteins close to the transmembrane domain (TMD) can have various effects. It can cause association with ordered lipids such as lipid rafts (**A**), can increase the hydrophobic lengths and therefore cause a tilt of the TMD (**B**). But it can also alter interactions between the palmitoylated protein and its interaction partners (**C**)

1.3.3 Regulation of Palmitoylation

S-Palmitoylation is a reversible feature that can influence the cell metabolism repeatedly by undergoing a cycle of palmitoylation and depalmitoylation. The common palmitoylating enzymes are the group of PATs whereas the depalmitoylating enzymes belong to different protein families.

zDHHC-PATs are enzymes that catalyse protein palmitoylation and share a common DHHC motif. Though they are expressed ubiquitously, they are specific for different

organelles and some PATs are present in more than one organelle (Jin et al., 2021). For example, the DHHC-PATs 6 and 13 localise to the ER, whereas DHHC 7 and DHHC 8 are present in the Golgi apparatus (Main & Fuller, 2022). Further, some PATs seem to be highly specific and palmitoylate single proteins, but a few PATs also show an overlapping substrate spectrum (Martin et al., 2011). They exhibit different preferences for acyl-CoA chain length and substrate proteins, but the mechanism of substrate specificity is not yet fully understood as no consensus palmitoylation sequence motifs have been identified (Jennings & Linder, 2012).

Depalmitoylation is the hydrolytically catalysed or non-enzymatic removal of the thioester linkage from the cysteine residue. Only few depalmitoylating enzymes have been identified so far. Acyl protein thioesterases 1 and 2 (APT1, 2) have the ability to self-depalmitoylate and contain a hydrophobic pocket for cleaving off fatty acids from acylated proteins. They have similar structures but different substrate specificities and cellular localisations (Kathayat et al., 2018).

Palmitoyl protein thioesterase 1 (PPT1), another enzyme that depalmitoylates proteins, primarily functions as a hydrolase in lysosomes and late endosomes. However, recent data suggest additional functions in other compartments like the nucleus, the ER and Golgi apparatus, synaptic vesicles, cilia, and axons (Sapir et al., 2019). The gene encoding for PPT1 is CLN1, one of 14 genes associated with a group of lysosomal storage diseases called neuronal ceroid lipofuscinoses (NCL), which are characterised by the accumulation of lysosomal storage material, neurodegeneration, and childhood mortality. PPT1 may be involved in vesicular depalmitoylation, followed by lysosomal degradation of S-palmitoylated proteins (Kollmann et al., 2013; Won et al., 2018).

The α/β hydrolase domain 17 (ABHD17) family of enzymes are presumed to be important for the depalmitoylation of Ras family members, GTPases and synaptic proteins, especially the postsynaptic density-95 protein (PSD-95) (Lin & Conibear, 2015). They are themselves S-palmitoylated which is required for their plasma membrane association and to get in proximity to their potential S-palmitoylated substrates (Won et al., 2018).

Depalmitoylation contributes to the balance of the palmitoylation cycle which is important for signalling, growth, and cell organisation. Regulation of the (de-)palmitoylation cycle could be a mechanism to control membrane sampling or trafficking of proteins to the plasma membrane (Rocks et al., 2010; Won et al., 2018).

1.3.4 Palmitoylation in disease

Some mutations or aberrant activities of palmitoyl transferases are closely related to several diseases ranging from cancer and immune deficiency conditions to neurological disorders.

Cancer

Palmitoylation plays key roles in the genesis of four hallmarks of cancer: activation of cell invasion and metastasis, sustained cell proliferation signalling, induction of angiogenesis and resistance to cell death. More than 70 cancer-driving proteins have been identified as being palmitoylated, including the tumour suppressor protein Scribble (Scrib), which is typically palmitoylated by zDHHC7 (Hernandez et al., 2017). Upregulation of the epithelial-to-mesenchymal transcription factor SNAIL was reported to reduce palmitoylation of Scrib and therefore its tumour-suppressing properties through upregulation of APT2 and downregulation of some PATs (Hernandez et al., 2017).

Metabolism

The translocation of insulin-regulated Glut4-receptor is dependent on its palmitoylation at cysteine 223. Therefore, palmitoylation is presumed to play a role in lipid metabolism and glucose homeostasis. zDHHC7 is the major PAT that palmitoylates Glut4. zDHHC7 deficient knockout mice developed type II diabetes symptoms like glucose intolerance, insulin resistance and rising blood sugar (Du et al., 2017; Ren et al., 2015).

Inflammatory signalling

Nucleotide oligomerization domain–like receptors 1 and 2 (NOD 1, 2) are intracellular pattern recognition proteins that are involved in pathogen sensing and immune response. Recruitment to membranes that contain bacterial patterns is required for their antibacterial responses (Barnich et al., 2005; Kufer et al., 2008). NOD 1 and 2 are palmitoylated on multiple cysteine residues by zDHHC5 which is important for proper initialising of signalling pathways through membrane localisation that leads to an effective immune response (Lu et al., 2019). A defective palmitoylation can be linked disease-associated mutations as some of the NOD 2 mutants that are associated with Crohn's disease (3020insC, R702W, L248R, A612T, A755V, and R1019stop) show a reduction of S-palmitoylation of 70 to 90% (Lu et al., 2019).

Alzheimer's disease

The aggregation of A β protein generated during the amyloidogenic pathway of APP cleavage plays a crucial role in the pathophysiology of AD. Approximately 10 % of APP in cells was found to be palmitoylated at two sites in the luminal domain at C186 and C187. Palmitoylated APP preferentially localises to lipid rafts and is then more likely to undergo amyloidogenic processing of APP because it is easily accessible to the β -secretase BACE1 (Cho & Park, 2016). The zDHHC-PATs 7 and 21 were identified to be responsible for the palmitoylation of APP (Bhattacharyya et al., 2013). Interestingly, in a case study of AD in a Chinese family, a mutation of zDHHC 21 was found in the AD-diseased family members but not in the healthy ones. This mutation led to an increase in the palmitoylation of APP (Li et al., 2023).

Huntington's disease

The expansion of the CAG nucleotide repeat in the huntingtin protein (htt) leads to an unstable form of htt (Cho & Park, 2016) causing the symptoms of HD. The zDHHC-PATs 13 and -17 interact with htt which is palmitoylated at cysteine 214 and this interaction correlates inversely to the CAG expansion. Furthermore, the overexpression of zDHHC 17 was shown to increase the palmitoylation of mutated htt in neurons and led to fewer aggregate formation of htt (Sanders & Hayden, 2015). Mice lacking zDHHC 17 exhibit deterioration in medium spiny neurons (MSN) where htt is highly expressed and show symptoms similar to HD, including motor and sensorimotor gating dysfunction and loss of response inhibition (Hornemann, 2015). However, htt palmitoylation was independent of zDHHC 17 suggesting htt to regulate the activity of zDHHC 17 which plays an important role in the pathogenesis of HD (Sanders & Hayden, 2015).

In conclusion, S-palmitoylation plays a critical role in various physiological processes, and its dysregulation is associated with numerous pathological conditions. Examples such as Scrib as a palmitoylated tumour suppressor, the metabolism-mediating Glut4-receptor and NOD 2 whose disease-associated mutations show reduced levels of palmitoylation highlight the significance of S-palmitoylation regulation. Interestingly, palmitoylation was even found to be involved in neurological disorders such as Huntington's and Alzheimer's disease.

2 Objectives

ADAM10 has been identified as a key player in many physiological and pathophysiological processes, including neurodevelopment, neuroinflammation, cancer, and Alzheimer's disease (AD). One of its substrates is the amyloid precursor protein (APP) for which ADAM10 is the main α -secretase. This α -cleavage of APP protects against the generation of pathological A β -plaques that are associated with AD (Tang, 2020). While ADAM10's structure and functions are already extensively investigated, a distinct insight into the regulation of its expression and activity is still in progress.

Protein S-palmitoylation is a posttranslational modification that occurs on cysteine residues of several proteins. The range of effects on the palmitoylated protein cannot yet be predicted. Previous work of Yang et al. showed ADAM10 as a potential candidate for S-palmitoylation in a palmitome study (Yang et al., 2010). ADAM10 occurs to have one cysteine residue in its intracellular C-terminal part which could be subject to palmitoylation.

The central aim of the following work was to investigate the significance of palmitoylation for the function and biochemistry of the metalloproteinase ADAM10. The first objective was to validate the S-palmitoylation of ADAM10 in mammalian cells with approaches like an acyl-resin capture assay. Another critical part of this thesis was to analyse an ADAM10 palmitoylation-deficient mutant to understand the effects of palmitoylation on ADAM10's expression and function. A possible influence of the palmitoylation on the substrate shedding of ADAM10 was investigated by analysing the known substrates N-cadherin and APP.

Since effects on the cleavage of ADAM10 itself were found during the course, this phenomenon was investigated in more detail. Various experiments for the characterisation of the C-terminal fragments of ADAM10 were performed. To give further insight into the function of palmitoylation as regulator for interactions between ADAM10 and other proteins, immunoprecipitation assays were combined with mass spectrometry analysis to identify proteins that interact differently with ADAM10 dependent on its palmitoylation status.

3 Material and Methods

3.1 Material

3.1.1 Chemicals

Table 1 List of frequently used chemicals.

<i>Chemicals</i>	<i>Provider</i>
30 % H ₂ O ₂	Roth
Acrylamide	Roth
Agar-agar	Roth
Agarose	Lonza
Ampicillin	Melford
APS	Sigma
Bromophenol blue	Roth
DMSO	Roth
Dry milk powder	Roth
DTT	Roth
EDTA	Roth
GelCode Blue Stain	Thermo Fisher
Glycin	Roth
Methanol	Roth
β -Mercaptoethanol	Roth
NaCl	Roth
PEI	Polyscience
SDS	Roth
TEMED	Roth
Tris	Roth
Tween20®	Roth
TX-100	Sigma

3.1.2 Antibodies

Table 2 List of frequently used antibodies.

<i>Antigen</i>	<i>Host</i>	<i>Dilution</i>	<i>No.</i>	<i>Provider</i>
<i>Actin</i>	Rabbit	IB (1:2500)	A2066	Sigma-Aldrich
<i>ADAM10 (C-term.)</i>	Rabbit	IB (1:1000)	EPR5622	Abcam
<i>ADAM10 (C-term.)</i>	Rabbit	IP (1:500)	Pin-1	Pineda
<i>Adaptin α</i>	Mouse	IB (1:1000)	sc-17771	Santa Cruz
<i>APP (C-term.)</i>	Rabbit	IB (1:1000)	A8717	Sigma-Aldrich
<i>sAPPα</i>	Rabbit	IB (1:500)	SIG-39151	Covance
<i>KDEL</i>	Mouse	IF (1:500)	10C3	Enzo
<i>LAMP-2</i>	Mouse	IB (1:100)	H4B4	DSHB

<i>LIMP-2</i>	Rabbit	IB (1:100)	L2T2	(Trad et al., 2011)
<i>Myc</i>	Mouse	IB (1:2500)	9B11	Santa Cruz
<i>Flag</i>	Rabbit	IB (1:2500)	D6W5B	Cell Signaling
<i>HA</i>	Rat	IB (1:2500)	3F10	Roche
<i>N-Cadherin</i>	Mouse	IB (1:1000)	610921	BD Transduction Laboratories
<i>Prion Protein (PrPc)</i>	Mouse	IB (1:2500)	6D11	BioLegend
<i>Shed PrPc (sPrPc)</i>	Rabbit	IB (1:500)	shPrP	(Linsenmeier et al., 2018)
<i>SAP-97</i>	Mouse	IB (1:1000)	RPI 197.4	Enzo
<i>Transferrin</i>	Rabbit	IB (1:2500)	ab84036	Abcam
<i>Tspan15</i>	Rabbit	IB (1:2500)	1-92540	Novusbio

Table 3 List of frequently used secondary antibodies.

<i>Secondary Antibodies</i>	<i>Conjugated to</i>	<i>Host</i>	<i>No.</i>	<i>Provider</i>
<i>Anti-goat</i>	Horseradish peroxidase	Rabbit	305-035-003	Dianova
<i>Anti-mouse</i>		Goat	115-035-166	
<i>Anti-rabbit</i>		Goat	111-035-144	
<i>Anti-rabbit</i>	ALEXA Fluor®-488	Goat	A11034	Thermo Fisher Scientific
<i>Anti-mouse</i>		Donkey	A21206	
<i>Anti-rabbit</i>		Goat	A11029	
<i>Anti-mouse</i>	ALEXA Fluor®-594	Goat	A11037	
<i>Anti-rabbit</i>		Donkey	A21207	
<i>Anti-mouse</i>		Goat	A11032	
		Donkey	A21203	

3.1.3 Cell culture media and additives

Table 4 Material used in cell culture.

<i>Cell culture</i>	<i>Provider</i>
<i>DMEM Dulbecco's Modified Eagle Medium</i>	Gibco
<i>Opti-MEM Reduced Serum Medium</i>	Gibco
<i>FCS</i>	Biochrom
<i>PenStrep</i>	PAA
<i>Trypsin/EDTA</i>	Roth

3.1.4 Kits and ready-made agents

Table 5 Frequently used commercial ready-made reagents and kits.

<i>Kit</i>	<i>Provider</i>
<i>cOmplete Protease Inhibitor Cocktail</i>	Roche
<i>GelPurification PCR Kit</i>	Roche
<i>GeneJet Plasmide Miniprep Kit</i>	Thermo Fisher

<i>MES SDS buffer</i>	Thermo Fisher
<i>PureYield Plasmide Midiprep system</i>	Promega
<i>Pierce BCA Protein Assay Kit</i>	Thermo Fisher
<i>SEA blocking buffer</i>	Thermo Fisher
<i>Western Blotting HRP Substrate (TMA-6)</i>	Lumigen

3.1.5 Oligonucleotides

Table 6 List of oligonucleotides used for sequencing and mutagenesis PCR.

<i>Name</i>	<i>Sequence 5'→3'</i>
<i>CMV for</i>	CGCAAATGGGCGGTAGGCGTG
<i>BGH rev</i>	TAGAAGGCACAGTCGAGG
<i>SP6</i>	ATTTAGGTGACACTATAG
<i>SV40PA-R</i>	CTCTGACTTGAGCGTCGATTTT
<i>Murine ADAM10</i>	GAGTGTGAACAC-
<i>G2099C forward</i>	TGGAAATCTTGATAAATCCAGCCATTAAC
<i>Murine ADAM10</i>	GTTAATGGCTGGAT-
<i>G2099C reverse</i>	TTATCAAGATTTCCAGTGTTCCACTC
<i>Human ADAM10</i>	GGAGTATGAACAC-
<i>G2096C forward</i>	TGGATATCTTAATAAATCCAGCCATTAGCAT
<i>Human ADAM10</i>	ATGCTAATGGCTGGATTTATTAAGATATCCAG-
<i>G2096C reverse</i>	TGTTCACTACTCC

3.1.6 Expression plasmids

Table 7 List of frequently used expression plasmids.

<i>Plasmid</i>	<i>Resistance</i>	<i>Reference</i>
<i>pcDNA3.1</i>	Ampicillin	Thermo Fisher Scientific
<i>hADAM10-cmyc tag</i>	Ampicillin	https://www.addgene.org/31717/
<i>hADAM10C699S-cmyc tag</i>	Ampicillin	site directed mutagenesis of hADAM10-cmyc by L. Seipold
<i>hADAM10E/A-cmyc tag</i>	Ampicillin	site directed mutagenesis of hADAM10-cmyc by F. Scharfenberg
<i>hADAM10E/AC/S-cmyc tag</i>	Ampicillin	site directed mutagenesis of hADAM10E/A-cmyc generated during this work according to section 1.2.3.
<i>mADAM10</i>	Ampicillin	(Reiss et al., 2005)
<i>mADAM10C700S</i>	Ampicillin	site directed mutagenesis of mADAM10 by L. Seipold
<i>mADAM10-cHA tag</i>	Ampicillin	(Maretzky et al., 2015)
<i>mADAM10C700S-cHA tag</i>	Ampicillin	site directed mutagenesis of mADAM10-cHA generated during this work according to section 1.2.3.

<i>mADAM10E/A-cHA tag</i>	Ampicillin	site directed mutagenesis of mADAM10-cHA by F. Scharfenberg
<i>mADAM10E/AC700S-cHA tag</i>	Ampicillin	site directed mutagenesis of mADAM10E/A-cHA generated during this work according to section 1.2.3.
<i>mTspan15-nflag tag</i>	Ampicillin	(Koo et al., 2020)
<i>mTspan15-myc tag</i>	Ampicillin	(Prox, Willenbrock, et al., 2012)

3.1.7 Cell lines

Table 8 List of the used cell lines and bacteria strains.

<i>Strain</i>	<i>Provider</i>
<i>E. coli XL1 blue</i>	Stratagene
<i>Human cervix carcinoma HeLa cells (Henrietta Lacks)</i>	DMSZ, established cell line
<i>HeLa ADAM10 knockout</i>	(Wohner et al., 2023)
<i>Murine neuroblastoma 2a</i>	(Olmsted et al., 1970)

3.1.8 Buffers

Table 9 Composition of frequently used buffers.

<i>Buffer</i>	<i>Composition</i>
<i>10x SDS running buffer</i>	0.25 M Tris/HCl 0.2 M Glycin 1% (w/v) SDS
<i>5x Laemmli buffer</i>	625 mM Tris/HCl 50% (v/v) Glycerol 5% (w/v) SDS 5% (v/v) β -Mercaptoethanol traces of bromophenol blue at pH 6.8
<i>ADAM10-Lysis buffer</i>	1 mM EGTA 5 mM Tris 250 mM Saccharose 1% Triton X-100 0,04% cOmplete at pH 7,4
<i>EBC buffer</i>	120 mM NaCl 50 mM Tris-HCl 1% NP-40 at pH 7.4
<i>Blocking solution</i>	5% dry milk powder in TBS-T
<i>Detection solution A</i>	0.1 M Tris/HCl 0.025% (w/v) luminol at pH 8.6
<i>Detection solution B</i>	0.11% p-Coumaricacid in DMSO

<i>Mild Stripping buffer</i>	100 mM Glycin 20 mM Mg-Acetate · 4 H ₂ O 50 mM KCl at pH 2.2
<i>Harsh Stripping Buffer</i>	62,5mM Tris 2% SDS 0,83% 2-Mercaptoethanol at pH 6,8
<i>LB agar</i>	1.5% (w/v) Agar-agar in LB medium
<i>LB medium</i>	1% (w/v) Trypton/Pepton 0.5% (w/v) yeast extract 1% (w/v) NaCl at pH 7.0
<i>DABCO</i>	20% (w/v) DABCO in PBS
<i>Mowiol</i>	25% (w/v) Mowiol 4-88 5% (v/v) Glycerol in PBS
<i>PB</i>	77.4 mM Na ₂ HPO ₄ 22.6 mM NaH ₂ PO ₄ 100 mM Na ₂ HPO ₄
<i>PBS</i>	18 mM KH ₂ PO ₄ 1.37 M NaCl 27 mM KCl at pH 6.8
<i>Separating gel buffer</i>	1.5 M Tris/HCl 0.4% (w/v) SDS at pH 8.8
<i>Stacking gel buffer</i>	0.5 M Tris/HCl 0.4 % (w/v) SDS at pH 6.8
<i>10x Tank Blot</i>	250 mM Tris 2 M Glycin
<i>1x Tank Blot</i>	10% 10x Tank Blot 20% Methanol 70% ddH ₂ O
<i>TBS with Tween® (TBST)</i>	20 mM Tris/HCl 150 mM NaCl 0.1% (v/v) Tween® 20 at pH 7.0

3.1.9 Devices

Table 10 List of frequently used equipment.

<i>Equipment</i>	<i>Provider</i>
<i>Aekte Purifier system</i>	GE Healthcare
<i>Amersham Imager680</i>	GE Healthcare
<i>Branson Sonifier 450</i>	Marshall Scientific
<i>Centrifuge 5425 R</i>	Eppendorf
<i>FACS Canto II</i>	BD Bioscience
<i>Flex Cycler2</i>	Analytics Jena

<i>FV1000 confocal laser microscope</i>	Olympus
<i>Leica DMI8 Microscope</i>	Leica
<i>LSM980 Airyscan 2</i>	Zeiss
<i>Multiplate Tecan Reader</i>	BioTek
<i>Power Pac 200</i>	Biorad
<i>SDS-PAGE gel chamber</i>	Biorad
<i>Thermomixer 5436</i>	Eppendorf
<i>Vortex</i>	Heidolph

3.2 Molecular biological methods

3.2.1 Amplification and purification of expression vectors in Escherichia Coli

To amplify expression plasmids, 1 µl of plasmid DNA was added to chemically competent E. Coli cells and incubated for 20 min at 4°C. The bacteria were then subjected to heat-shock for 40 seconds at 42°C and incubated for 5 min on ice. 300 µl of LB medium at room temperature were added and the cell suspension further incubated for 1 hour at 37°C on a shaker. 100 µl of the cell suspension was then plated on LB agar plates containing selection antibiotics (50 µg/ml ampicillin) and incubated at 37°C overnight. The following day, single colonies were picked and grown in 3 ml or 200 ml LB medium with selection antibiotics (50 µg/ml ampicillin) at 37°C overnight in a shaking incubator. The DNA was isolated and purified using the GeneJet Plasmid miniprep kit or the PureYield Plasmid Midiprep System for small or large volumes, respectively. The isolation was carried out according to the manufacturers' protocols.

3.2.2 Sequencing of expression vectors

The sequencing was performed by Eurofins using the Sanger chain-termination method. Therefore, 5 µl of the plasmid with a concentration of 100 ng/µl was mixed with 5 µl of appropriate forward and reverse sequencing primer at a concentration 5 µM (Tab. 6).

3.2.3 Site-directed mutagenesis Polymerase Chain Reaction (PCR)

Table 11 Pipetting scheme for mutagenesis PCR.

<i>Premix</i>	<i>µl</i>
<i>Oligo 1 [10µM]</i>	2,5
<i>Oligo 2 [10µM]</i>	2,5
<i>5x Phusion HF buffer</i>	10,0

<i>dNTPs [10mM]</i>	1,0
<i>DMSO</i>	2,5
<i>H₂O</i>	29,4
<i>DNA [500ng]</i>	1,6
<i>Phusion Polymerase</i>	0,5
<i>Total Volume</i>	50,0

Table 12 Program for mutagenesis PCR

<i>PCR Program</i>	<i>Time</i>	<i>T (°C)</i>	<i>Cycle</i>
<i>Initial denaturation</i>	30 s	98	1
<i>Denaturation</i>	10 s	98	
<i>Annealing</i>	30 s	72	25
<i>Elongation</i>	8 min	72	
<i>Final Extension</i>	0 – 5 min	72	1
<i>End</i>	∞	10	1

Different ADAM10 expression plasmids underwent site-directed mutagenesis replacing guanine with cytosine at position 2099 or 2096 in murine or human ADAM10, respectively. This led to an amino acid exchange of cysteine to serine resulting in the absence of a sulfhydryl group which prevents a potential palmitoylation (Fig. 6).

The PCR reaction mix was prepared following the pipetting scheme (Tab. 11). This mix included two specific oligonucleotides that frame the DNA sequence coding for the region intended for mutation (Tab. 6). The mutagenesis PCR involved distinct phases, guided by temperature variations according to the PCR program (Tab. 12).

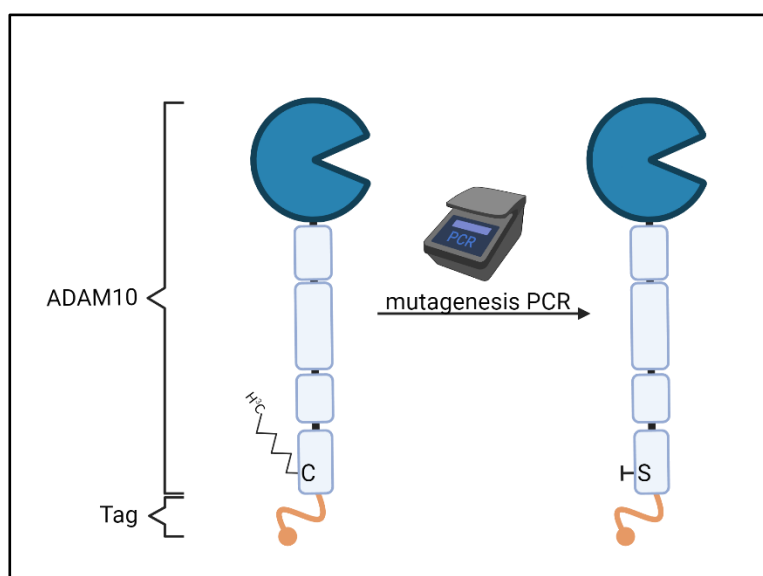


Figure 6: structure and alteration of ADAM10 and its palmitoylation-deficient mutant. Shown is the mature form of ADAM10 with the protein-tag. The mutation is located in the C-terminal domain at site 700 (mADAM10) or 699 (hADAM10) of the amino acid sequence. This

mutated ADAM10 expression plasmid lacks the sulfhydryl group which is necessary for palmitoylation of the protein.

3.3 Cell biological methods

3.3.1 Maintenance of cells

Cells were kept under sterile conditions in an incubator at 37°C and 5% CO₂ atmosphere in 10ml plates with DMEM containing 4.5 g/L of D-glucose and L-glutamine (Thermo Fischer Scientific) supplemented with 10% (v/v) FBS and 1% (v/v) Penicillin/Streptomycin. For cultivation, cells were split two times a week reaching 80% confluency. Therefore, handling was done under a laminar flow bench to ensure sterile conditions. The medium was removed, the attached cells were washed with PBS and then detached with Trypsin/EDTA. The cells were resuspended in cultivation medium and seeded in a 1:5 or 1:10 dilution.

3.3.2 Cryopreservation of cell lines

For long-term storage, cells were preserved as cryo-cultures in liquid nitrogen. Cells were detached from the dishes using 1ml Trypsin/EDTA, resuspended in 9 ml medium and spun down at 300 x g for 5 min at room temperature. The cells were resuspended in 1 ml DMEM containing 20% (v/v) FBS and 10% (v/v) DMSO. After freezing at -80°C, they were transferred to liquid nitrogen.

3.3.3 Transient transfection of cells

For overexpression experiments, cells were transfected with different DNA expression plasmids 24 h after seeding. They were incubated for 5-6 h in one third of the cell culture medium. Polyethylenimine (PEI) was used as transfection reagent because lipofection was the chosen transfection method. For the transfection mixture, 1-2 µg DNA and PEI were mixed in a 1:3 ratio and added to 100-200 µl of DMEM. The mixture was incubated for 15 minutes at room temperature and added to the cells. After 5-6 hours of incubation time, the medium was exchanged and filled up to 10 ml. 48 hours after transfection, the cells were harvested and analysed.

3.3.4 Inhibitor treatment of cells

For inhibitor treatment of cells, 24 hours after seeding the cell culture medium was removed and the cells were incubated for 16 hours with the inhibitors GI254023X,

GM6001 and Inhibitor X in different dishes. Each inhibitor was diluted to a concentration of 3 μ M in 1.5 or 3 ml of Opti-MEM medium in 6 or 10 cm culture dishes, respectively. The ADAM10 stimulating reagent Ionomycin was used at a concentration of 5 μ M. After the incubation period, 1 to 2 ml of the protein-containing supernatant were collected for analysis. The cells were then harvested and analysed.

3.4 Protein biochemical methods

3.4.1 Generation of cell lysates for protein extraction

Cell culture medium was removed from the culture dishes and cells were washed three times with ice-cold PBS. 500 μ l of PBS containing cOmplete protease inhibitor cocktail were added, and the cells were harvested with a cell scraper. The suspension was transferred into an Eppendorf tube and centrifuged at 4000 rpm at 4°C for 10 minutes. The cells were resuspended in ADAM10 lysis buffer (Tab. 9) or lysis buffer according to the protocol, sonicated twice at 4°C (15-20 pulses, 90%, level 4), further lysed on ice for 60 minutes and then again sonicated twice. The cell suspension was centrifuged at 13300 rpm for 10 min at 4°C and the protein-containing supernatant was transferred into a new tube for determination of protein concentration.

3.4.2 Determination of protein concentration

The protein concentration of lysates was determined using the Pierce BCA Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The samples were diluted 1:10 with distilled water. Duplicates of the samples and a calibration series with defined concentrations of bovine serum albumin (BSA) ranging from 0 to 2.0 mg/ml were incubated with 200 μ l of the assay mixture for 30 min at 37°C. The absorbance was measured at 562 nm using a Synergy HT (BioTek) microplate reader.

3.4.3 Trichloroacetic Acid Precipitation

Trichloroacetic acid precipitation was performed to collect proteins that are secreted to the supernatant during cell metabolism. To condition the supernatant, the cell culture medium DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin was exchanged by 1.5 or 3 ml of Opti-MEM in 6 or 10 cm culture dishes and incubated for 24 hours. 1 ml of the conditioned media was collected and supplemented with protease inhibitor. To remove cells and debris, the samples were centrifuged and transferred to new tubes twice. First, they were centrifuged at 500g for 5 min at 4°C transferring 900

µl of the media to new tubes and then at 5000g for 5 min, retaining 800 µl in the final tube. To sample 2% DOC were added in a 1:100 dilution. Samples were mixed and incubated for 30-minutes on ice. Next, TCA was added in a 1:10 ratio and samples were vortexed followed by another 30-minute incubation on ice. Centrifugation at 15000g for 15 min at 4 degrees was performed separating the protein-containing pellet from the media. The media was carefully removed to eliminate trichloroacetic acid. 95 µl 1x Laemmli buffer diluted with ADAM10 lysis buffer and supplemented with 1-2 µl 2 M NaOH to neutralize remainings of TCA were added to the protein pellets. The samples were incubated at 50°C until the pellets were dissolved. Subsequently, they were prepared for SDS-PAGE analysis by boiling for 5 min at 95°C.

3.4.4 SDS polyacrylamide gel electrophoresis

Table 13 Components of SDS-Polyacrylamide gels.

	<i>Separating gel 10%</i>	<i>Stacking gel 5,25%</i>
<i>Separating buffer</i>	2,6 ml	
<i>Stacking buffer</i>		337,5 µl
<i>30% Acrylamide/bisa crylamide rotophorese gel</i>	3,3 ml	437,5 µl
<i>H2O</i>	4 ml	1,7125 ml
<i>10% ammonium persulfate (APS)</i>	60 µl	15 µl
<i>Tetramethylethylenediamine (TEMED)</i>	30µl	7,5 µl

The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins according to their molecular weight. Therefore, samples were adjusted to equal amounts of protein concentration and prepared with denaturing loading buffer (5x Laemmli) at 60°C for 20 min.

The prepared samples were loaded to either self-made 10% gels (Tab. 13) or precasted Bis-Tris 4-12% gels (Thermo Fisher Scientific) next to a protein standard ladder (PageRuler Plus). The gel was exposed to a constant voltage of 80-120V in 1x SDS running buffer (Tab. 9).

3.4.5 Western blotting and protein detection

The Western blot method detects specific proteins in samples by first separating them using SDS-PAGE and then transferring them onto a nitrocellulose membrane. This membrane is probed with primary and secondary antibodies, the last of which is linked

to Horseradish peroxidase (HRP). HRP catalyses a reaction with luminol and H_2O_2 to emit chemiluminescence, visualizing the proteins.

The proteins were transferred from the polyacrylamide gel onto a nitrocellulose membrane (GE Healthcare) using a wet blot system. The gel, the nitrocellulose membrane and 2 pieces of Whatman-filter paper were equilibrated in transfer buffer and placed between two electrodes inside of a wet blot chamber. The proteins were transferred under specific electrical conditions depending on the protein size: 800 mA for 2 hours at 4°C for most proteins (>20 kDa), and 250 mA or 400 mA for 2 hours at 4°C for smaller proteins (<20 kDa). Afterwards, the membrane was incubated in a blocking solution (TBST + 5% (w/v) milk powder) for 60 min to reduce unspecific antibody signals. The membrane was then incubated with the primary antibody diluted in blocking solution in gentle agitation overnight at 4°C.

At the next day, the membrane was washed three times with TBST for 10 min at RT before incubating with a HRP-coupled secondary antibody diluted in blocking solution at RT for 60 min and then washed again three times with TBST for 10 min. The peroxidase activity could then be detected using an AmershamTM Imager 680 with 400 µl of detection solution (containing 1 ml detection solution A, 100 µl detection solution B, 2 µl H_2O_2 , 100 µl Lumigen ECL A, 100µl Lumigen ECL B) which was carefully pipetted on top and evenly spread over the membrane. The quantification of chemiluminescence signals was then accomplished using the ImageJ Software.

3.4.6 Co-immunoprecipitation experiments

Immunoprecipitation with Protein G coupled Dynabeads

To analyse protein-protein interactions, co-immunoprecipitation experiments were performed. One of the interaction partners is pulled out of total cell lysates using a specific antibody against the protein and magnetic beads that bind to the antibody. Therefore, cell lysates were prepared as described above using 500 µl EBC lysis buffer (120 mM NaCl, 50 mM Tris-HCl, 1% Nonidet P-40, pH 7.4 + cOmplete protease inhibitor). 50 µl of the total lysates were kept to control for protein expression. Equal amounts of the residual lysates were incubated with primary antibodies overnight at 4°C under continuous rotation. To validate the results, a control for unspecific antibody and bead binding were prepared which contained only lysis buffer and antibody for the antibody control and cell lysate without antibody for the bead control.

On the same day, magnetic Protein G coupled Dynabeads (Thermo Fisher Scientific) were three times washed with EBC lysis buffer and incubated with SEA-blocking buffer

(Thermo Fisher Scientific) overnight at 4°C to saturate unspecific binding sites. The next day, Dynabeads were resuspended in EBC buffer and 50 µl per sample was added to the protein-antibody mix and all controls. They were incubated for 30 minutes in a rotation wheel at room temperature (RT). After incubation, the supernatant was discarded using a magnet. The beads were washed 6 times for 5 minutes with EBC buffer on a rotation wheel at RT. After the last washing step, reducing Laemmli buffer was added to the beads to elute proteins by heating the samples to 60°C for 20 min. Precipitates and all controls were further analysed by SDS-PAGE and immunoblotting.

Immunoprecipitation with myc trap beads

The magnetic myc trap beads bind to a myc tag containing protein which can therefore be precipitated. The day before cell lysing, 50 µl of myc trap beads per sample (including antibody control) were washed in EBC buffer twice and incubated in SEA-blocking buffer overnight at 4°C in a rotation wheel.

At the next day, cells were lysed in EBC lysis buffer. 50 µl of the total lysates were kept to control for protein expression and equal amounts of protein in 500 µl of buffer were used for precipitation. The myc trap beads were resuspended in EBC buffer and added to the protein-antibody mix and all controls. After incubation for 30 minutes, the beads were washed and proteins were prepared for SDS-PAGE analyses and immunoblotting as described above.

3.4.7 Membrane fractionation of proteins

To separate a soluble and a membrane-bound protein fraction of total cell lysates, they were centrifuged several times at different speed.

First, cells were sonicated in a fractionation buffer (10 mM Tris at pH 7,5 + 140 mM NaCl + 250 mM Sucrose + cOmplete protease inhibitor). For a total input fraction, 50 µl of the cell suspension was taken off and lysed in fractionation buffer containing 1% Triton X-100. To extract the soluble fraction, the cell suspension was ultracentrifuged at 100.000 x g for 2 hours at 4°C. The supernatant which contains the soluble protein fraction was prepared for SDS-PAGE with 5x Laemmli buffer for 20 min at 60°C.

The remaining pellet was washed twice and then resuspended in fractionation buffer, sonicated, and ultracentrifuged at 100.000 x g for 1 hour at 4°C. The resulting pellet comprising the membrane-bound protein fraction was resuspended in 100 µl of fractionation buffer with 1% Triton X-100 and then prepared for SDS-PAGE with 5x Laemmli buffer for 20 min at 60°C.

3.4.8 Cell surface biotinylation of proteins

To analyse the cell surface expression of proteins, biotin solution was used to label and precipitate cell surface proteins with streptavidin/agarose beads. All steps were carried out at 4°C to maximise protein stability. The cells attached to the culture dish were washed three times with PB-CM before being incubated 30 minutes with a biotin solution (1mg/ml Biotin EZ-Link Sulfo-NHS-SS-Biotin Thermo Fisher). Afterwards, the cells were incubated with quenching buffer for 10 min and washed three times to remove unbound biotin. Cells were lysed in EBC(B)-buffer (150 mM NaCl, 50 mM Tris/HCl, pH 7.4, 1 % Triton-X100, cOmplete protease inhibitor) and equal amounts of protein were used for precipitation. Therefore, lysates were incubated with 75 µl of washed Streptavidin Agarose beads (Thermo Fisher Scientific) per sample for 1 hour in a rotation wheel. After three washing steps, proteins were eluted from the beads and prepared for SDS-PAGE analyses with reducing Laemmli buffer at 60°C for 20 minutes.

3.4.9 Indirect immunofluorescence labelling of cells

Table 14 Components of immunofluorescence labelling solutions.

Fixation solution	4% Paraformaldehyde (PFA) in PBS
Permeabilization/washing solution	0.2% Saponin in PBS
Quenching solution	0.12% Glycin 0.2% Saponin in PBS
Blocking solution	10% FCS 0.2% Saponin in PBS
Mounting medium	Dabco/Mowiol + Dapi

To visualize the subcellular localisation of proteins, indirect immunofluorescence labelling was performed. Specific primary antibodies that can be labelled by fluorophore-coupled secondary antibodies target the protein of interest and marker proteins for subcellular compartments. Cells were cultured and transfected on coverslips and left to grow for 48 hours. Afterwards, they were washed two times with sterile PBS and then fixated with 4% (w/v) paraformaldehyde (PFA) in PBS for 20 min. After three additional washing steps with PBS, a solution of 0.2% (w/v) saponin in PBS was added for 5 min to permeabilise cells. After that, cells were incubated in quenching

solution for 10 min and rinsed with PBS three times. To prevent unspecific binding of antibodies, the samples were incubated with blocking solution for 1 hour at room temperature. The samples were incubated with a primary antibody mix (1:100 dilution in blocking solution) overnight at 4°C. The coverslips were then washed 5 times and incubated for 1 hour with AlexaFluor fluorophore-coupled secondary antibodies (1:500 dilution in blocking solution). After incubation with secondary antibodies, the coverslips were washed 5 times with 0.2% (w/v) saponin in PBS and then twice with distilled water. They were then mounted onto microscope slides with mounting medium containing 1 µl/ml DAPI (4-,6-diamidino-2-phenylindole) to stain nuclei. To analyse the subcellular localisation of the protein, two different microscopes were used:

Table 15 Used microscopes.

<i>Microscope</i>	<i>Manufacturer</i>	<i>Objective magnification</i>	<i>Objective name</i>
<i>LSM980 Airyscan2</i>	Zeiss	20x	Plan-Apo 10x/0.8 M27
		40x	C-Apo 40x/1.20 W Corr
		63x	Plan-Apo 63x/1.40 O DIC M27
<i>DMi8</i>	Leica	5x	N PLAN
		20x	HCX PL Fluotar L `Corr0-2`
		40x	HCX PL Fluotar `0.17`
		63x	HCX PL APO `0.17`

3.4.10 Acyl-resin Assisted Capture Assay

Table 16 Component of buffers used for acyl-resin assisted capture assays.

<i>Blocking Buffer</i>	100mM HEPES
	1mM EDTA
	2,5% SDS
	0,1% freshly added S-Methyl Methanethiosulfonate (MMTS)
	pH 7,5
<i>Binding Buffer</i>	100mM HEPES
	1mM EDTA
	1% SDS
	pH 7,5
<i>Cleavage reagent</i>	2M NH ₂ OH
	pH 7,5
<i>Preservation reagent</i>	2M NaCl

To detect S-acylation of ADAM10, the acyl-resin assisted capture (Acyl-RAC) assay was performed, followed by SDS-PAGE and Western blotting for sample analysis. During this process, the palmitate residue from the target protein is cleaved and the protein captured by thiopropyl sepharose beads.

Cells were washed and collected in PBS containing cOmplete protease inhibitor. They were then lysed in blocking buffer without MMTS, sonicated twice for 20 seconds, and subsequently treated with MMTS, followed by a 4-hour incubation at 40°C with constant shaking. Proteins were precipitated using acetone, adding three times the sample volume and chilling at -20°C for 20 minutes. After centrifuging at 16,000 x g for 5 minutes, the pellet was washed five times with 70% acetone, vortexed, and centrifuged to isolate the protein pellet. The pellet was then air-dried and stored at -20°C or immediately processed. For resuspension, the pellet was shaken in binding buffer at 40°C until dissolved. Concurrently, thiopropyl sepharose beads were prepared in binding buffer for one hour rotating, then centrifuged and resuspended in 60 µl of binding buffer per sample.

After resuspension and brief centrifugation at 16,000 x g, 30 µl of the lysate was removed, mixed with 5x Laemmli buffer, and heated to 60°C for 20 minutes. The remaining lysate was split into experimental and negative control samples. Prewashed beads were added to both, followed by cleavage reagent for experimental samples and preservation reagent for control samples, and incubated for 3 hours at room temperature on a rotation wheel. After centrifugation, supernatants were collected - cleaved unbound fraction (cUB) from the experimental sample and preserved unbound fraction (pUB) from control samples - and treated with 2x Laemmli buffer. The beads were washed five times with binding buffer and eluted with 30 µl of 2x Laemmli buffer at 60°C for 10 minutes. The resulting supernatants represented the cleaved bound fraction (C) from the experimental sample and the preserved bound fraction (P) from the controls.

3.4.11 Acyl-PEGyl Exchange Gel Shift (APEGS) Assay

Table 17 Component of buffers used for APEGS assays.

<i>PEG</i>	mPEG-5k (SUNBRIGHT ME-050MA, NOF corporation, Japan)
<i>Buffer A</i>	4% SDS 5 mM EDTA in PBS
<i>Buffer B</i>	5 mM EDTA in PBS
<i>Buffer C</i>	2% SDS in PBS
<i>Buffer D (for dilution)</i>	1% SDS in PBS
<i>Buffer H</i>	1.33 M Hydroxylamine (pH 7.0) 5 mM EDTA

<i>Buffer T</i>	13 mM Tris(2-carboxyethyl)phosphine (TCEP) 1.33 M Tris-HCl (pH 7.0) 5 mM EDTA 13 mM TCEP
-----------------	---

To assess the ratio of palmitoylated to non-palmitoylated ADAM10, the acyl-PEGylation exchange assay was used. Palmitate groups are replaced with PEG molecules, inducing a molecular weight shift that reveals different palmitoylation states of the protein.

Initially, cells were washed and collected in PBS containing cOmplete protease inhibitor, then lysed in buffer A at 37°C for 10 minutes. Buffer B was added to achieve a final concentration of 1% SDS in 5 mM EDTA. 50 µl of the lysate was reserved as an input control and the remaining lysate was further processed. Samples were diluted to 0.5 mg/ml protein concentration. To protect all free thiol groups, 0.5 M Tris(2-carboxyethyl)phosphine (TCEP) was added (final concentration of 10 mM) and incubated on a rotation wheel for 30 minutes at room temperature. Subsequently, N-Ethylmaleinimid (NEM) was added, and the incubation continued for three hours at room temperature. Proteins were precipitated using 100% ice-cold acetone for 20 minutes at -20°C, followed by centrifugation at 13300 rpm and three washes with 70% acetone. After air-drying, buffer A was used to dissolve the pellet at 37°C.

For the cleaved fraction, buffer H was added, and for the control, buffer T, followed by a 1-hour incubation at 37°C. A second protein precipitation was performed as described before, and the pellet was resuspended in buffer C at 37°C, followed by a 5-minute centrifugation at 13300 rpm at room temperature. After adjusting the protein concentration to 0.5 mg/ml, TCEP was added to 100 µl of the lysate. Then, 10 µl of 200 mM mPEG (or 200 mM NEM for controls) were added, and samples were mixed for 1 hour at room temperature. A third protein precipitation followed the same procedure as described above. Finally, the samples were prepared for SDS-PAGE with 1x Laemmli sample buffer and heating to 95°C for 5 minutes.

3.4.12 Click Chemistry Assay

To analyse protein S-acylation dynamics, a click chemistry method was performed, incorporating 'clickable' palmitate and methionine analogues into cells. These molecules can be tagged with fluorophores for detection. Specifically, the methionine

analogue tracks the protein's degradation, while the palmitate analogue indicates its palmitoylation status.

Cells were seeded and upon reaching 80% confluency, subjected to a starvation protocol. They were washed twice with sterile PBS and incubated for one hour in starvation medium (DMEM high glucose, 200 μ M L-cysteine, 4 mM glutamine, 1 mM sodium pyruvate, 10% dialysed FCS). The pulse phase began with the addition of 50 μ M L-Azidohomoalanine (AHA) and 50 μ M 17-Octadecynoic acid (17-ODYA) to the medium. Following a 3-hour pulse, cells were harvested at various times to determine optimal pulse times for detecting palmitoylated ADAM10 or chased for different durations in DMEM supplemented with 10% FCS, 2 mM L-methionine, and 500 μ M palmitic acid.

For protein analyses, an immunoprecipitation was conducted using Protein G-coupled Dynabeads and phosphate lysis buffer (pH 7.4) with 1% Triton X-100 and cOmplete protease inhibitor. The lysates were incubated with primary antibody targeting the protein of interest and Protein G-coupled Dynabeads were blocked in 5% BSA-containing phosphate lysis buffer overnight. The following day, beads were washed, added to the lysate-antibody mix for 30 minutes, and subsequently washed three times. To rebuffer the beads, they were additionally washed twice with click reaction buffer (phosphate buffer at pH 7.0). The "CuAAC Biomolecule Reaction Buffer Kit" from Jena Bioscience was used for labelling 17-ODYA with AlexaFluorophore647-azide and AHA with Alexa Fluorophore488-alkyne, following the manufacturer's instructions. After labelling, beads were washed three more times in click reaction buffer, shielded from light, and finally resuspended in 1x Laemmli buffer with 5% β -mercaptoethanol for 10 minutes at room temperature before analyses.

3.5 Statistical Methods

Quantitative results are presented as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 8.0.1. For comparisons between two groups, statistical significance was determined using an unpaired t-test. For comparisons involving three or more groups, a one-way analysis of variance (ANOVA) was applied. A p-value of less than 0.05 was considered statistically significant (* p < 0.05; ** p < 0.005; **** p < 0.0001).

4 Results

4.1 ADAM10 is a palmitoylated protein

The metalloprotease ADAM10 is ubiquitously expressed in mice and humans. Proteases undergo different modifications that contribute to a diversity of biochemical regulatory mechanisms. Lipidation is such a posttranslational modification and raised general interest in the last decades. Increasing the hydrophobicity of a modified protein, lipidation can be involved in different mechanisms through association in distinct membrane microdomains or altered interactions with other proteins. A typical lipidation event is S-palmitoylation, which is a common modification that raised attention because of its reversibility. Consequently, S-palmitoylation can regulate protein activity, localisation, and trafficking in a dynamic, changeable way (Main & Fuller, 2022).

A high-throughput characterization of S-acylated proteins revealed ADAM10 among many other proteins as a potentially palmitoylated protein. Wei Yang et al. developed a new large-scale proteomic approach for palmitoyl protein identification and site characterization (PalmPISC). ADAM10 was proposed to be palmitoylated at a medium confidence level in this study (Yang et al., 2010).

No.	Acc. Number	Gene Symbol	Description	Fraction	P-value	17-ODYA
25	IPI00013897	ADAM10	ADAM10 precursor	Non-Raft	0.089	M
	IPI00013897	ADAM10	ADAM10 precursor	Raft		

Figure 7: Extract from the results of the PalmPISC study. In the protein-based procedure performed as described in the paper from Wei Yang et al. the proform of ADAM10 (ADAM10 precursor) was identified to be a potentially palmitoylated protein with a medium confidence (M) level and a p-value of 0.089. Investigated were lipid raft enriched membrane domains (Fraction Raft) and non-raft membrane domains (Fraction Non-Raft). 17-ODYA: 17-Octadecynoid Acid. Adapted from Yang et al.

The actual palmitoylation of ADAM10 remains to be experimentally validated and further investigated. S-Palmitoylation might contribute to substrate-specific regulations of ADAM10 in case of shedding activity, cellular localisation, or protein-protein interactions. Because ADAM10 utilises substrates that play important roles in physiological and pathophysiological processes it is of pivotal importance to gain knowledge of possible regulatory mechanisms.

4.1.1 An acyl-resin assisted capture assay revealed ADAM10 as a palmitoylated protein

To verify that ADAM10 is indeed S-acylated an acyl-resin assisted capture (acyl-RAC) assay was performed. Using this method, the endogenous existing fatty acid residue is hydrolysed, and the resulting free thiol group is bound to beads which are eluted and analysed (Tewari et al., 2020).

The acyl-RAC assay was performed on lysates of Human cervix carcinoma Henrietta Lacks (HeLa), murine neuroblastoma Neuro-2a (N2a), mouse embryonal fibroblast cells (MEF) and mouse brain tissue to verify the S-acylation of ADAM10 in different tissue and cells. Proteins present in fraction “C” (cleaved) are acylated because the fatty acid residue is cleaved and then bound to thiopropyl sepharose beads for precipitation, which works only for proteins that have a lipid modification. In the preserved fraction “P” that serves as a negative control, samples were not treated with the hydrolysing reagent. The palmitoylated protein, i.e. ADAM10, should not be precipitated by the beads in this fraction (Fig. 8A-D). The Immunoblot analyses of the precipitates revealed prominent signals at a molecular weight of 100 kDa and 70 kDa, corresponding to pro- (pADAM10) and mature ADAM10 (mADAM10), respectively. ADAM10 is precipitated in fraction “C” in all tested cell lines and mouse brain tissue. There is an unspecific binding above the detected pADAM10 in the acyl-RAC assays of N2a and HeLa cells mainly in the cleaved unbound fractions that present the supernatants of the cell-bead suspension after binding reactions and serve as controls. Lysosomal integral membrane protein 2 (Limp2) is also a palmitoylated protein (unpublished data from S. Heybrock, CAU Kiel) which was used as a positive control. Clear signals at a molecular weight above 70 kDa corresponding to Limp2 were detected in the cleaved fraction and the input control (Fig. 8C, D). Lysosome-associated membrane protein 2 (Lamp2) is predicted not to be palmitoylated as it has no cysteine in the cytosolic C-terminus and could indeed not be recovered in the cleaved fraction and was therefore used as a negative control (Rudnik et al., 2022) (Fig. 8D). The total cell lysate (Input) controls the expression of ADAM10, Limp2, and Lamp2, respectively.

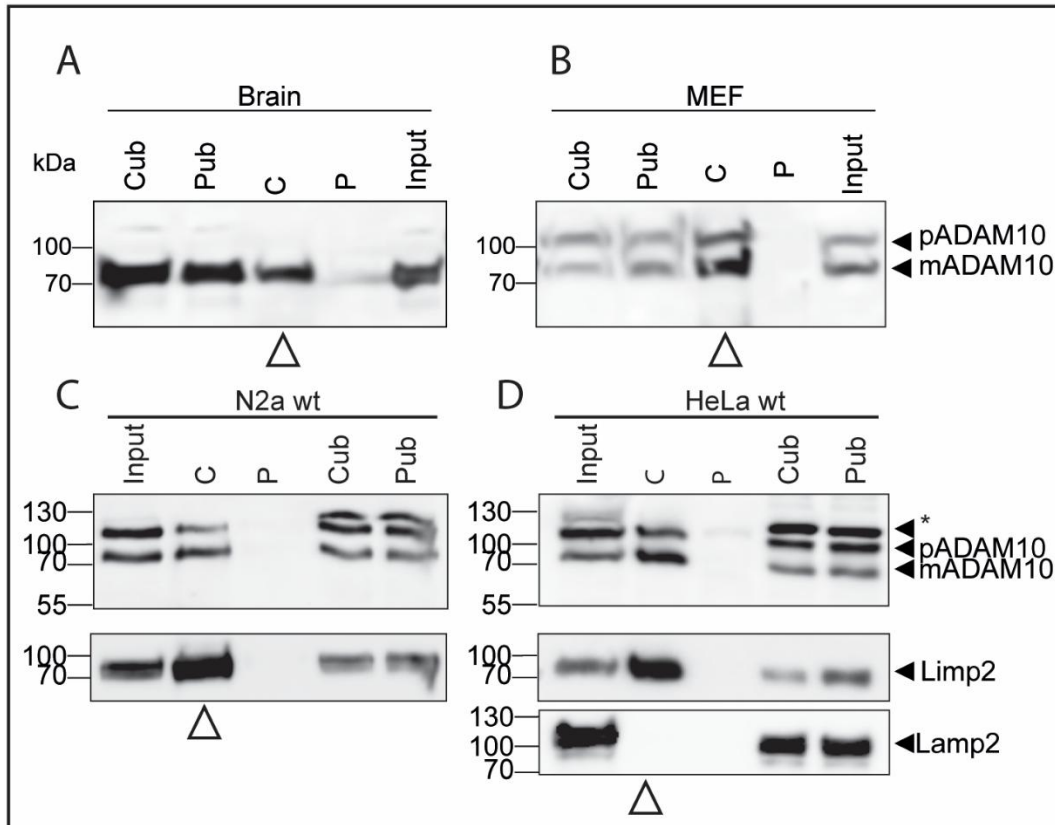


Figure 8: Detection of S-acylated ADAM10. An Acyl-resin assisted capture (Acyl-RAC) assay was used to detect S-acylation of ADAM10 in different tissue and cell lines. Immunoblot analyses of **A**: mouse brain tissue, **B**: mouse embryonal fibroblast cells (MEF), **C**: murine N2A neuroblastoma cells (N2a), and **D**: Human cervix carcinoma Henrietta Lacks (HeLa) cells. The membranes were incubated with antibodies directed against the C-terminus of ADAM10. The proform of ADAM10 (pADAM10) and the active, mature form of ADAM10 (mADAM10) are indicated by arrowheads. The open arrowhead indicates the cleaved fraction (C) where palmitoylated proteins are detected. The preserved fraction (P) where acylation sites were not cleaved but preserved with sodium chloride served as a negative control. The Input fraction (Input) shows protein expression of ADAM10, Limp2, and Lamp2 in untreated total cell lysates. Residual protein that was not bound to the used beads is shown in the cleaved unbound (Cub) and preserved unbound (Pub) fractions. Limp2 serves as positive control (C, D) whereas Lamp2 serves as a negative control (D). (Acyl-RAC assay of brain and MEF cells was provided by Dr. Lisa Seipold and Dr. Sönke Rudnik.) * shows unspecific binding.

4.1.2 Confirmation of the palmitoylation of ADAM10 by targeting palmitate insertion using 17-ODYA in a click chemistry reaction

The acyl-RAC assay has limits in discriminating between different fatty acids (Tewari et al., 2020). To verify the assumption that specifically S-palmitoylation occurs on ADAM10, an independent approach was chosen where fluorescence-labelled palmitoyl-residues are directly detected using a click chemistry method.

First, HeLa cells were starved for one hour in a cell culture medium without palmitate, so that a labelled palmitate analogon 17-ODYA which is added after starvation can be incorporated (pulse). This pulse period is followed by a chase of different periods without 17-ODYA in the cell culture medium. After removing the clickable palmitate

analogon, the cell metabolism keeps working, and either labelled proteins stay palmitoylated and stable, which can be detected, or undergo depalmitoylation or degradation, which would lead to a decreased fluorescence signal after SDS-PAGE analysis. Cells were harvested and 50µl of the cell lysate was saved as input control before proceeding with the immunoprecipitation (IP) using an ADAM10-specific antibody. To visualize the 17-ODYA, that was inserted into ADAM10, IPs were treated with a fluorescent Alexa fluorophore 647 (AF 647), which is linked to 17-ODYA in a consequent click chemistry reaction. Lysates and IPs were separated on an SDS-PAGE gel which was further analysed by fluorescence imaging detecting fluorescence at 647nm (Fig. 9A). The immunoblot of the SDS-PAGE gel, stained with an antibody directed against ADAM10 (Fig. 9B), reveals a prominent signal at around 70 and 100 kDa demonstrating the successful IP of ADAM10. The input fraction, that confirms the proper expression of ADAM10 in cell lysates, shows clear signals at the same molecular weights of 100 and 70 kDa corresponding to the pro- and mature form of ADAM10, respectively. Pro-, as well as the mature form of ADAM10, were also detected after fluorescence imaging, indicating a successful incorporation of the 17-ODYA into the ADAM10 protein.

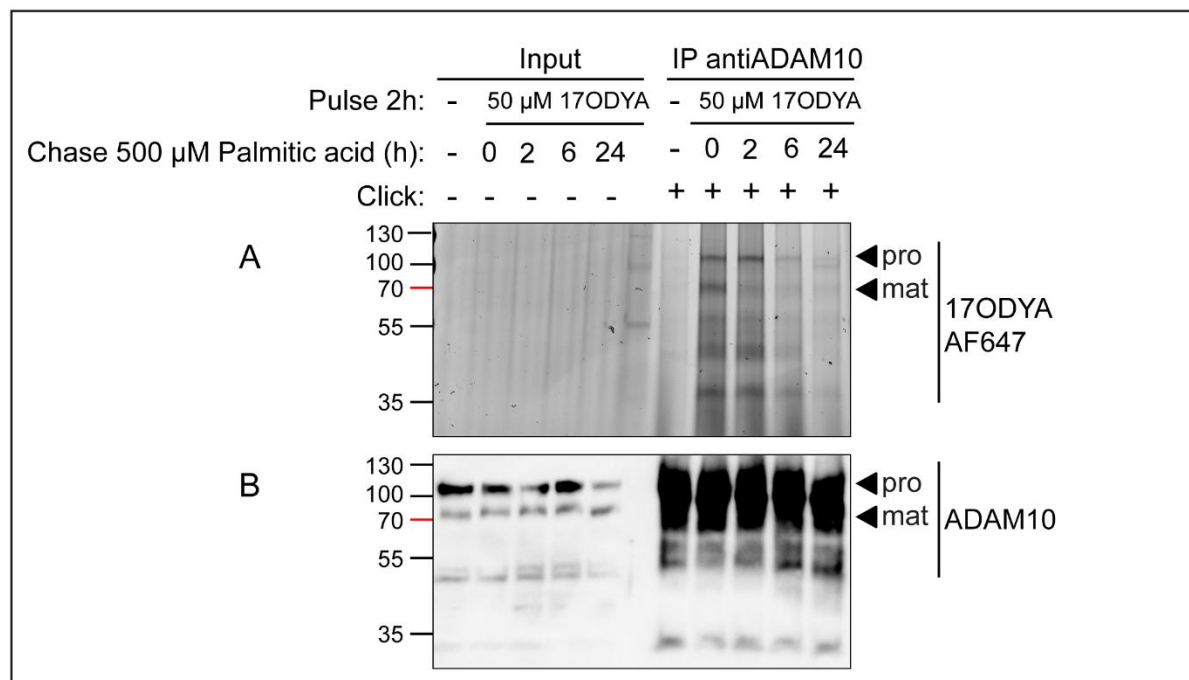


Figure 9: Click Chemistry Assay in HeLa cells shows ADAM10 is a palmitoylated protein. ADAM10 in HeLa cells is palmitoylated as the Click Chemistry method reveals. A pulse of 2 hours with 50µM 17-Octadecynoic Acid (17-ODYA) and indicated periods of chase were conducted. **A:** The integrated 17-ODYA which is used for labeling palmitoylation substrates was linked to an ALEXA fluorophore 647 (AF647) for detection by fluorescence imaging after SDS-PAGE. **B:** Immunoblot analysis of the total protein lysate (Input) and the immunoprecipitation (IP) for ADAM10. The blots were incubated with antibodies directed

against the C-terminus of ADAM10. The proform of ADAM10 (pADAM10) and the active, mature form of ADAM10 (mADAM10) are indicated by arrowheads. Experiment in cooperation with S. Rudnik

4.1.3 Analysis of ADAM10's palmitoylation status

Another method to analyse S-palmitoylation is an acyl-PEGylation exchange assay which is an adjustment of the acyl capturing assay. The acylation is also hydrolysed using hydroxylamine. The resulting free thiol group is not captured by beads but instead linked to a maleimide polyethylene glycol (PEG) molecule with a molecular weight of around 5 kDa (Specia & Diaz, 2020). Consequently, palmitoylated proteins are detected by an SDS-PAGE detectable molecular weight shift. Since the molecular weight of the investigated protein increases for every palmitoylated site, this method is applied to analyse the number of palmitoylated sites. (Main & Fuller, 2022).

Since the thioester bond between a cysteine residue and a palmitate is a reversible linkage, the protein may undergo multiple palmitoylations in its life cycle (Ko & Dixon, 2018). Therefore, the relation between palmitoylated and non-palmitoylated states of the protein can also be revealed using the acyl-PEGylation exchange assay which was performed to investigate the palmitoylation status of ADAM10 (Fig. 10).

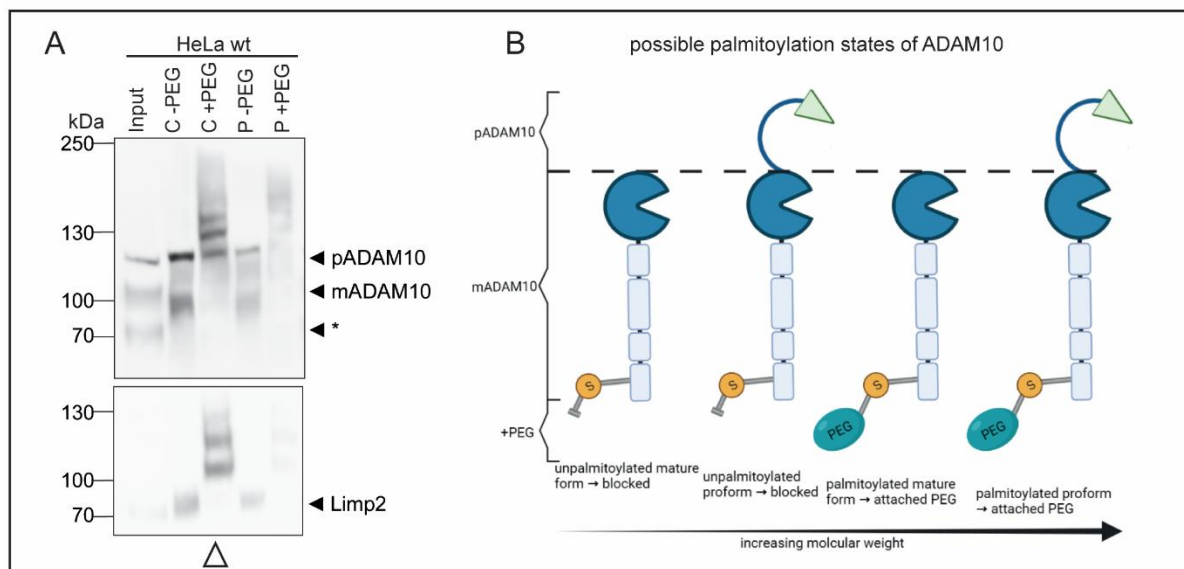


Figure 10: Palmitoylation states of human ADAM10 in HeLa cells. An Acyl-PEG exchange assay was used to detect possible different palmitoylation states of ADAM10 **A:** Immunoblot analysis of HeLa cells. The blot was incubated with an antibody directed against the C-terminus of ADAM10. The proform of ADAM10 (pADAM10) and the active, mature form of ADAM10 (mADAM10) in total cell lysates (Input) are indicated by arrowheads. The open arrowhead indicates the cleaved fraction (C) that is treated with hydroxylamine for cleaving thioester bonds and with polyethyleneglycol (PEG) molecules that attach to the resulting free thiol groups. In this fraction, each palmitoylated site causes a shift to a higher molecular weight. Limp2 served as a positive control which is palmitoylated at one site causing two bands in the immunoblot analysis in fraction "C +PEG". As a control, samples were also treated with

hydroxylamine but not with PEG molecules (C -PEG). Further negative controls were included which were preserved with sodium chloride and either treated with PEG molecules (P +PEG) or not (P -PEG). Because S-acylation is a reversible modification and ADAM10 has a proform and an active, mature form there are four possible states of palmitoylation as indicated in **B**: Unpalmitoylated pro and mature ADAM10 are blocked by Tris(2-carboxyethyl)phosphine (TCEP) and not attached to PEGs whereas palmitoylated ADAM10 is attached to a PEG molecule. * indicates a possible unspecific binding.

Endogenous human ADAM10 in HeLa cells was analysed by immunoblotting and detection with an antibody directed against the C-terminus of ADAM10. The resulting immunoblot in Figure 10 shows three shifted bands at a molecular weight of around 130 kDa in the “cleaved fraction” that was incubated with the PEG molecules (C +PEG). The different bands indicate different palmitoylation states of ADAM10. ADAM10’s cytoplasmic region contains only one cysteine but ADAM10 can be observed as a proform and a mature form containing one possible palmitoylation site each. Since the two upper bands, migrating slightly above and below 130 kDa, in the PEGylated fraction show a clear shift to a higher molecular weight than in the non-PEGylated fraction, they possibly correspond to the palmitoylated proform and palmitoylated mature form, respectively. The lower band at a molecular weight between 100 and 130 kDa likely corresponds to a non-palmitoylated form of ADAM10. Limp2, a lysosomal membrane protein, which was detected at a molecular weight of 70 kDa in the input and non-PEGylated fractions, served as a positive control for a palmitoylated protein. In the cleaved and PEGylated fraction, this signal disappears and separates into two bands at a higher molecular weight between 100 and 130 kDa. The input fraction reveals the cellular expression of the analysed proteins ADAM10 and Limp2.

The two clearly shifted bands of ADAM10 and the band running below indicate the use of at least three different palmitoylation states. The lower band is at a similar molecular weight as compared to the band in the non-PEGylated fraction which indicates the existence of ADAM10 in a non-palmitoylated state. The other two shifted bands correspond to two forms of palmitoylated ADAM10 (Fig. 10A). This, in turn, might imply that the palmitoylation is not a steady state but a dynamic modification on ADAM10 for at least one of the pro- or mature forms.

4.2 Biochemical characterisation of the palmitoylation-deficient ADAM10 mutant

ADAM10 was found to be palmitoylated in different cell lines and mouse brain tissue (Fig. 8). This palmitoylation is a reversible modification and seems to be dynamic (Fig. 10). The amino acid sequence of ADAM10 contains only one possible palmitoylation site in the cytoplasmic C-terminus which is the cysteine residue at position 699 in human ADAM10 (Fig. 11A). Cysteine has the thiol group which is necessary for protein S-palmitoylation.

To analyse the palmitoylation of ADAM10 and its potential functions, an ADAM10 mutant, deficient for the palmitoylation site, was available. To modify wildtype ADAM10 so that it is deficient for palmitoylation, the cysteine was replaced by serine. The guanine in the triplet that encodes cysteine was replaced by a cytosine resulting in serine which lacks the thiol group that is responsible for the thioester linkage of the palmitate. However, serine is similar to cysteine in size, charge, and polarity. Human and murine ADAM10 mutants (hADAM10C699S-myc, mADAM10C700S) were sequenced and aligned with the wildtype human and murine ADAM10 expression plasmids (Fig. 11B).

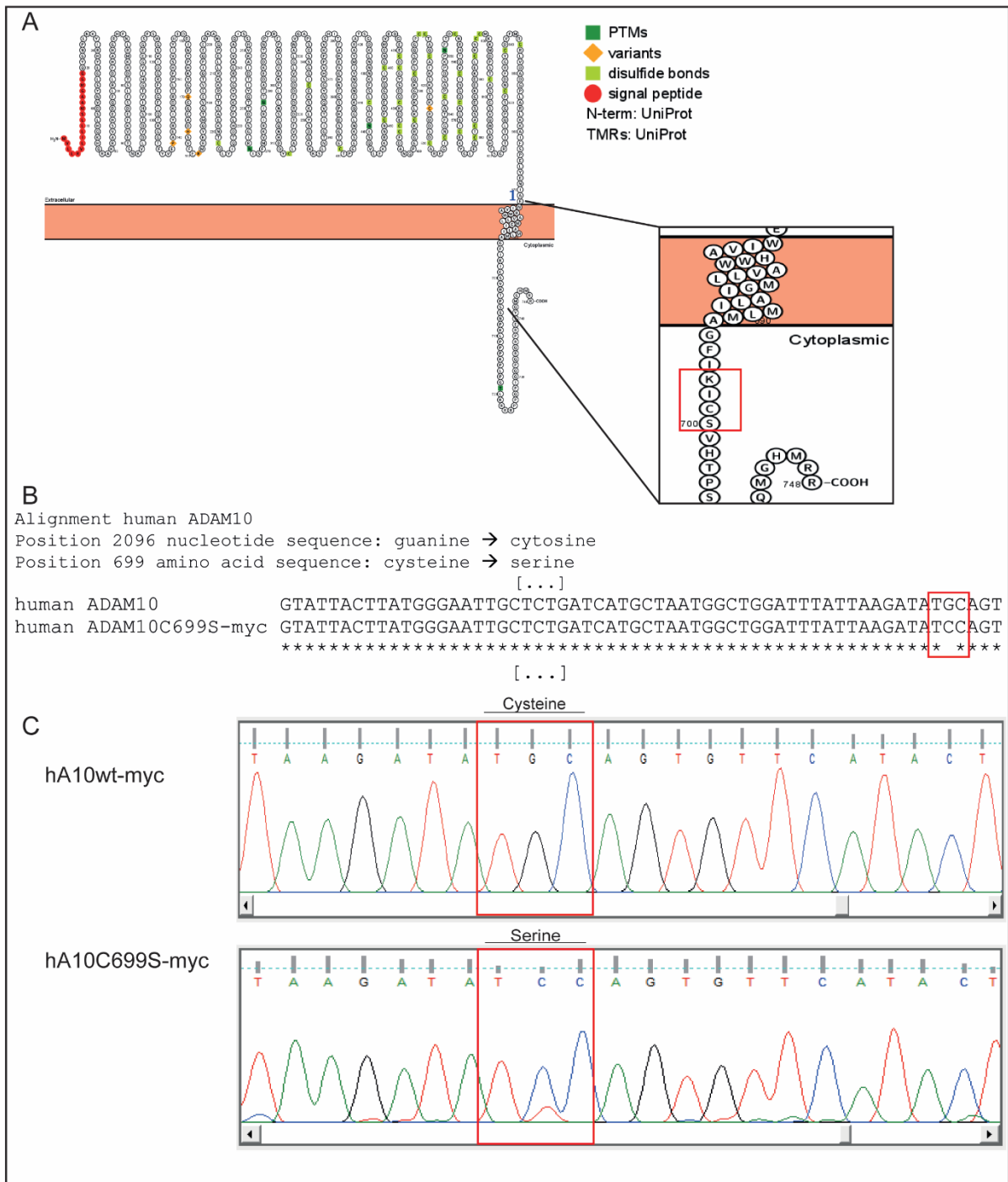


Figure 11: Predicted topology of human ADAM10 with the mutation site of the palmitoylation-deficient mutant. A: The predicted topology of ADAM10 and the amino acid sequence of human ADAM10 (created by Uniprot and Protter). **B:** Alignment of the human palmitoylation-deficient (hA10C699S-myc) and wildtype ADAM10 (hA10wt-myc) expression plasmid. **C:** The chromatogram of the indicated plasmids. Indicated with red boxes is the mutation site of ADAM10 where the cysteine (TGC) is replaced by a serine (TCC).

The ADAM10 mutant is mutated at position 699 for human (C699S) and position 700 for murine (C700S) ADAM10. After sequencing and alignment of the wildtype and mutated ADAM10 expression plasmids, they were analysed using the Click chemistry

method as described below to investigate if palmitoylation occurs on the transfected wildtype ADAM10 and to investigate the palmitoylation deficiency of the mutant.

4.2.1 Targeting palmitate insertion by fluorophore attachment reveals palmitoylation of human ADAM10 and palmitoylation deficiency of the C699S mutant

To determine the palmitoylation status of ADAM10, HeLa cells that are deficient for ADAM10 (HeLa A10ko cells) were transfected with either human wildtype ADAM10 or the mutated ADAM10 expression construct and the same click chemistry method as described above was used (Fig. 12). Cells were starved for one hour in a cell culture medium without palmitate after transfection of the expression plasmids to increase the probability of inserting the labelled palmitic analogon 17-ODYA afterward. The culture medium was then enriched with 50µM 17-ODYA and added to cells for 2 to 24 hours (Pulse). Cells were harvested and an amount of 50µl of the cell lysate was saved as input control before immunoprecipitating (IP) ADAM10. To visualize the 17-ODYA that was potentially attached to ADAM10, IPs were treated with a fluorescent Alexa fluorophore 647 (AF 647), which is linked to 17-ODYA in a click chemistry reaction.

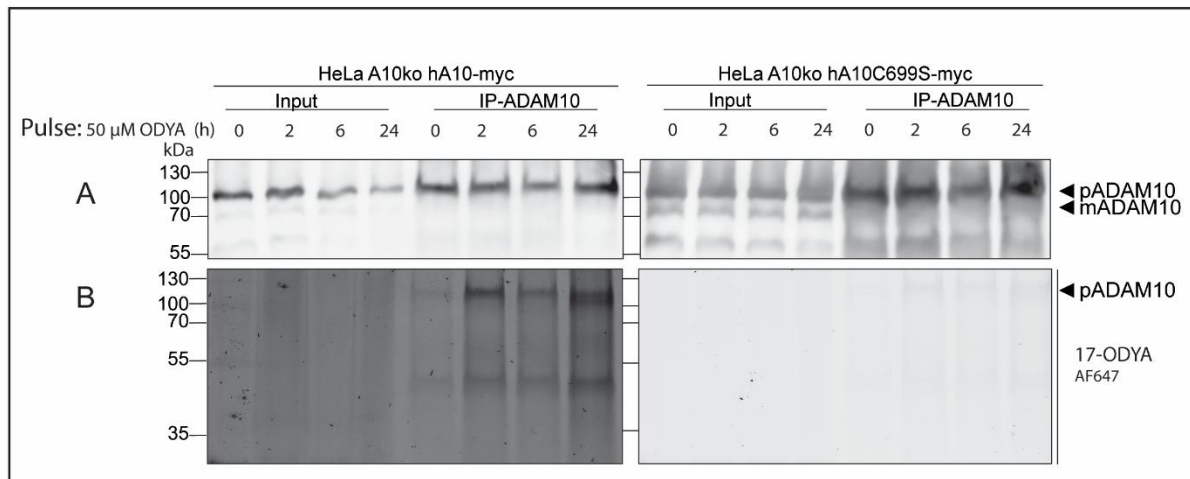


Figure 12: Click Chemistry Assay in HeLa ADAM10 knockout cells reveals palmitoylation of transfected wildtype ADAM10 and confirms palmitoylation deficiency of the human ADAM10 C699S mutant. Immunoblot analyses of cell lysates (Input) of HeLa ADAM10 knockout cells (HeLaA10ko) and the immunoprecipitation (IP) for ADAM10 are shown in **A**. Cells were either transfected with a wildtype (hA10-myc) or mutated (hA19C699S-myc) version of human ADAM10. The blots were incubated with antibodies directed against the C-terminus of ADAM10. The proform of ADAM10 (pADAM10) and the active, mature form of ADAM10 (mADAM10) are indicated by arrowheads. The integrated 17-ODYA which was used to label palmitoylation substrates was linked to an ALEXA fluorophore 647 (AF647) for detection by fluorescence imaging after SDS-PAGE is shown in **B**. Cells were first starved for one hour which was followed by a pulse of 50µM 17-ODYA for different periods as indicated to determine the best fitting pulse duration. The arrowhead at around 100 kDa indicates the

palmitoylated wildtype human ADAM10 whereas the proposed palmitoylation-deficient mutant is not palmitoylated.

The immunoblots were stained with an antibody directed against ADAM10 (Fig. 12A). The input fraction, that shows the proper expression of ADAM10 in cell lysates, reveals clear signals at a molecular weight of 70 and 100 kDa corresponding to the mature and proform of ADAM10, respectively. The prominent signals in the IP fraction at a molecular weight of 100 kDa corresponding to the proform of ADAM10 demonstrate the successful immunoprecipitation of ADAM10 (Fig.12A).

The proform of ADAM10 was detected at a molecular weight around 100 kDa in the wildtype transfected samples using a fluorescence imager at 647 nm, indicating a successful insertion of the 17-ODYA. In contrast, the human ADAM10 C699S construct was equally expressed and immunoprecipitated. However, no 17-ODYA could be detected, indicating that no palmitoylation occurred (Fig. 12B). This mutant was therefore used in subsequent experiments to evaluate the consequences of ADAM10's S-palmitoylation.

In summary, there is evidence that endogenous ADAM10 (Fig. 8, 9, 10), as well as the protein product of the wildtype ADAM10 expression plasmid (Fig. 12), are palmitoylated in contrast to the ADAM10 C699S mutant (Fig. 12).

4.2.2 Analysis of the expression level of the ADAM10 palmitoylation-deficient mutant in comparison with wildtype ADAM10

The consequences of protein palmitoylation are currently not predictable and the various possibilities include improper folding in the endoplasmic reticulum or retention in the Golgi during the cellular pathway, which can lead to an altered expression or maturation of the protein. To address the emerging question, if the palmitoylation of ADAM10 has any influence on the expression of pro- or mature ADAM10, ADAM10 overexpression experiments were performed in different cell types. HeLa cells were used as a human cell line. In addition, murine neuroblastoma, Neuro 2A, cells were investigated to obtain information about the role of ADAM10's palmitoylation in neuronal cells. Cells were either transfected with the murine or human version of ADAM10 and its mutant. Samples transfected with an empty vector, were included as controls.

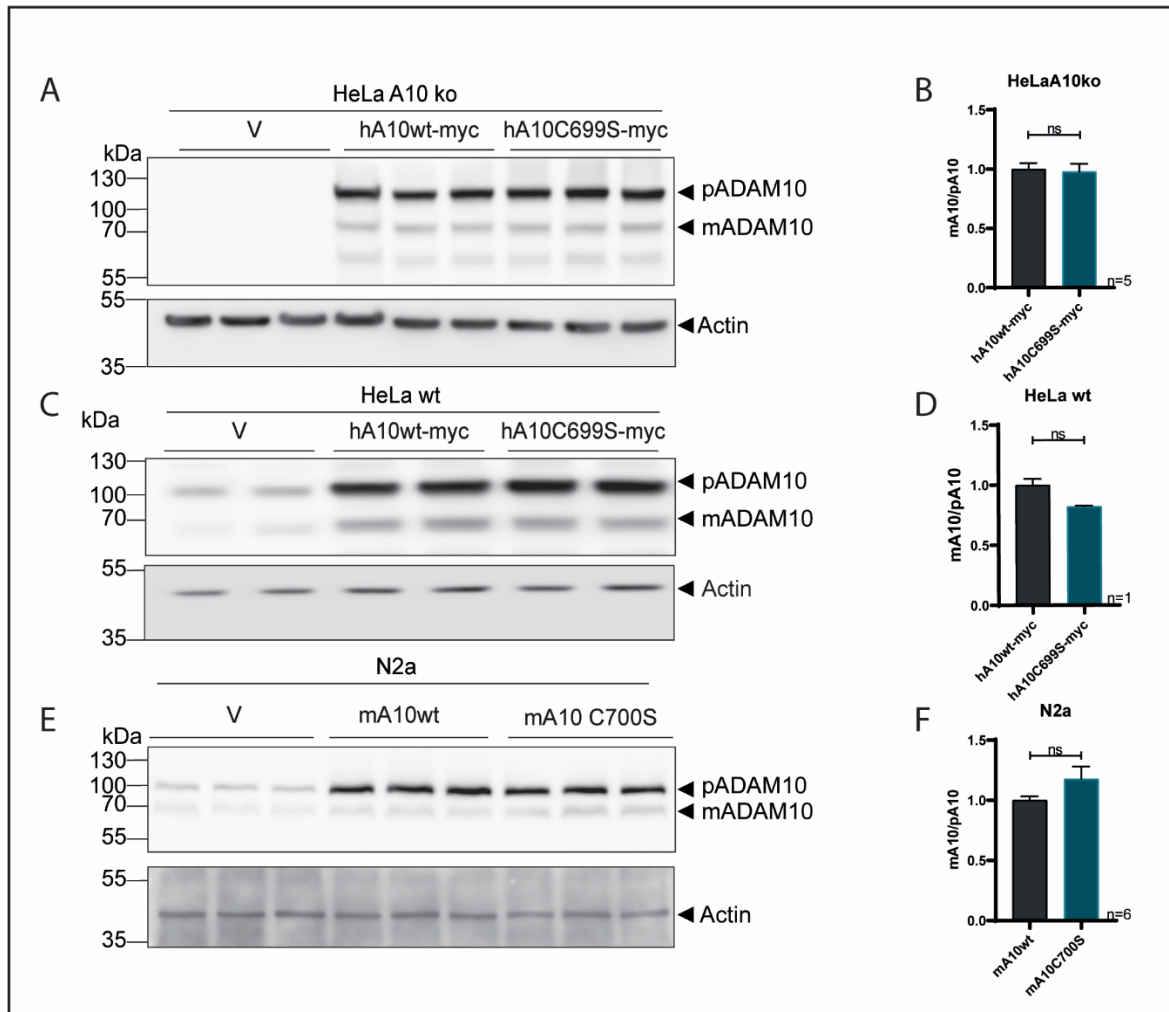


Figure 13: Lack of ADAM10's palmitoylation site does not affect ADAM10 maturation or ADAM10 expression levels. Immunoblot analyses of cell lysates of HeLa cells, HeLa ADAM10 knockout cells (HeLaA10ko), and murine Neuro 2a neuroblastoma cells (N2a) are shown. **A:** HeLaADAM10ko cells, **C:** HeLa wt cells **E:** N2a cells. The blots were incubated with antibodies directed against the C-terminus of ADAM10 and actin, respectively. Cells were either transfected with an empty vector (V), wildtype hA10-myc/mA10 (wt), or mutated hA10C699S-myc/mA10C700S. The proform of ADAM10 (pADAM10) and the active, mature form of ADAM10 (mADAM10) are indicated. Equal protein loading was controlled by the detection of actin. **B, D,** and **F** depict quantifications that show the ratio of mature and proform of ADAM10 (**B:** n=5, **D:** n=1, and **F:** n=6 independent experiments). Values are normalized to wt ADAM10 transfection level and shown as mean $\pm$ SEM. Significance was tested using an unpaired t-test (* $p < 0.05$, ** $p < 0.005$).

Immunoblot analyses of HeLa ADAM10 knockout cells (HeLaA10ko), HeLa cells, and murine neuroblastoma cells (N2a) were transfected with pcDNA (V), wildtype ADAM10 and the ADAM10 C699S or C700S mutant expression constructs, respectively (Fig. 13). The clear bands at a molecular weight of 70 and 100 kDa correspond to the mature and proform of ADAM10, respectively. Signal intensities of the proform and the active, mature form of ADAM10 in cells were similar comparing the transfections with wildtype or the palmitoylation-deficient mutant expression constructs. HeLa ADAM10 knockout cells were used to rule out any effects of artificial cell processes caused by endogenous

wildtype ADAM10. Equal protein loading was verified by similar signal intensities in all samples after actin staining. Quantification of the ratio of mature and pro ADAM10 visualizes equal maturation levels in all three investigated cell lines (Fig. 13B, D, F). These Immunoblot analyses show the expression level of total cell lysates. To capture possible alterations in the intracellular localisation of pro- or mature ADAM10, further experiments were conducted.

4.3 Intracellular localisation of the palmitoylation-deficient mutant

ADAM10 is synthesised in the ER as a proform and then transported to the Golgi apparatus. The prodomain is cleaved on its way to the plasma membrane where ADAM10 becomes activated (Saftig & Lichtenthaler, 2015). To address the question, whether the palmitoylation influences the intracellular localisation of ADAM10, an immunohistochemistry assay and a biotinylation approach were performed comparing wildtype and palmitoylation-deficient ADAM10.

4.3.1 Subcellular localisation of ADAM10

For the immunohistochemistry assay, cells were transfected with the human wildtype or C699S mutant ADAM10, permeabilized, and stained for the myc tag which is attached to the expressed ADAM10 plasmid. KDEL was co-stained for analysing ER colocalisation (Fig. 14).

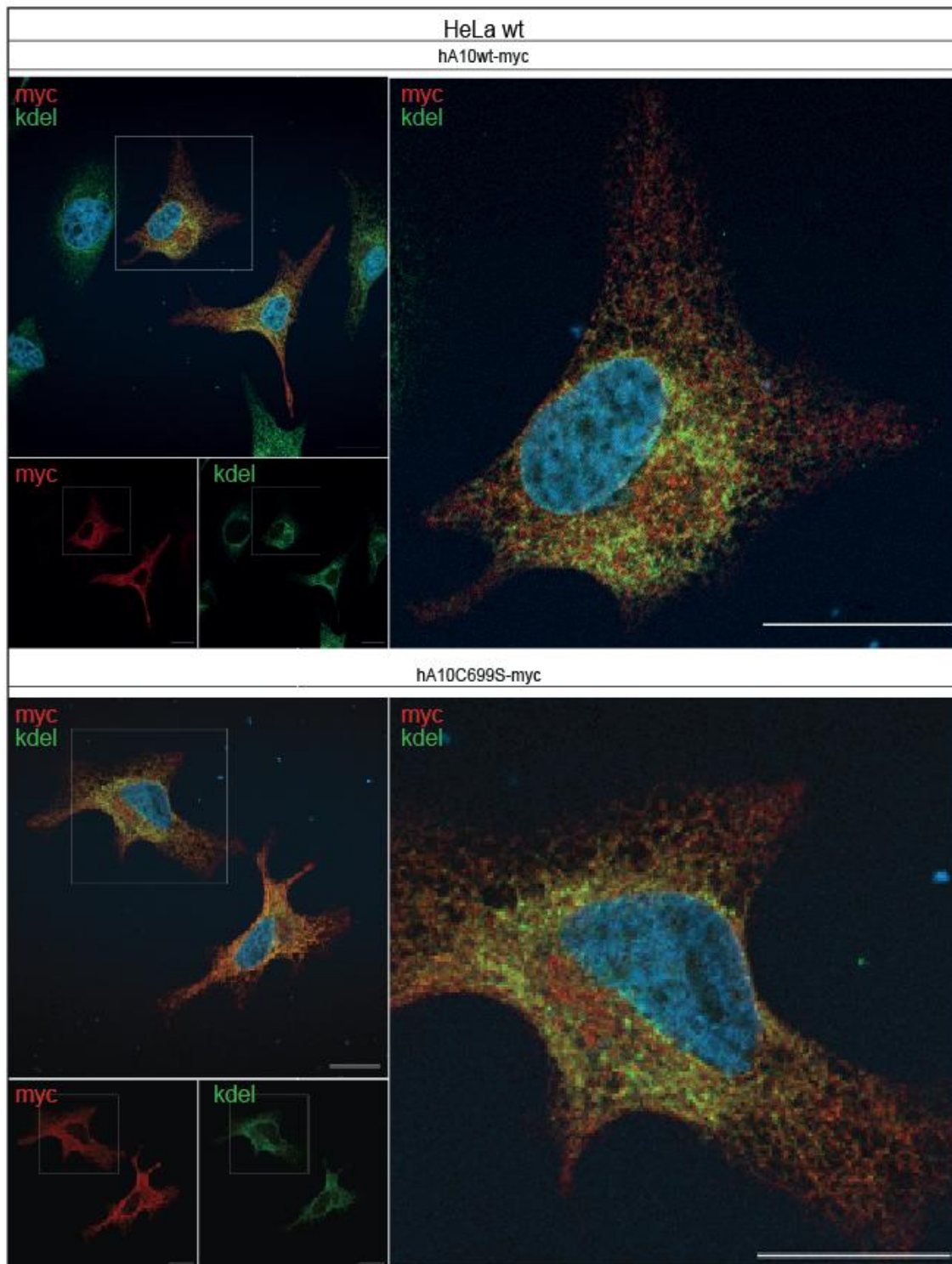


Figure 14: Analysis of the intracellular localisation of transfected wildtype and palmitoylation-deficient ADAM10 in HeLa cells. Wildtype (wt) HeLa cells were either transfected with a wildtype or mutated C699S version of human (h) ADAM10. The intracellular localisation of ADAM10 was revealed in immunohistochemical stainings using an antibody directed against the myc-tag (ALEXA 594 in red). Costaining was conducted using an antibody directed against kdel (ALEXA 488 in green) to visualize ER-staining. The nucleus is shown in blue (DAPI). The right picture shows an enlarged extract from the overview on the left. Both types of ADAM10 are localised within the ER, in intracellular membranes, and in part at the plasma membrane. The scale bars represent 20µm.

The intracellular localisation of ADAM10 wildtype and C699S is shown in figure 14. The signal after myc-staining corresponding to ADAM10 is present in the whole cell

and also in part visible at the cell surface. A punctual colocalisation of ADAM10 (red) with the ER (green) is evident. This experiment showed no obvious differences in the subcellular localisation when wildtype and palmitoylation-deficient ADAM10 were compared (Fig. 14).

4.3.2 Analysis of cell surface expression of wildtype vs palmitoylation-mutant ADAM10

Surface localisation of ADAM10 could not be accurately determined using immune stainings of the cells. To address this question by a more quantitative approach, a biotinylation experiment was chosen to compare the surface appearance of ADAM10 in cells expressing wildtype and palmitoylation-deficient ADAM10 (Fig. 15).

Palmitoylation can alter protein-protein interactions (Jin et al., 2021). Tspan15 is an important interaction partner for ADAM10 promoting the maturation and trafficking of ADAM10 to the plasma membrane (Prox, Willenbrock, et al., 2012). To analyse if Tspan15 is still binding to ADAM10 and is also able to accelerate ER to plasma membrane transport of ADAM10, a co-transfection with either the wildtype or the C699S version of ADAM10 in combination with a Tspan15 expression plasmid was included in the experimental set up.

To reduce background effects of endogenous ADAM10, transfected HeLa ADAM10 knockout cells were used. Cells were transfected with either an empty vector, the wildtype, or mutated version of human ADAM10. Additionally, co-transfections with Tspan15 and wildtype ADAM10 or the ADAM10 C699S mutant were performed. Cells were not permeabilized and surface proteins were stained in the cold with a biotin solution (+Biotin) or left untreated (-Biotin). After cell lysis, a small amount of the total lysate was kept for validation of proper protein expression (Fig. 15A, right panel). The remaining biotinylated surface proteins were then precipitated with streptavidin-agarose beads (IP) (Fig. 15A, left panel).

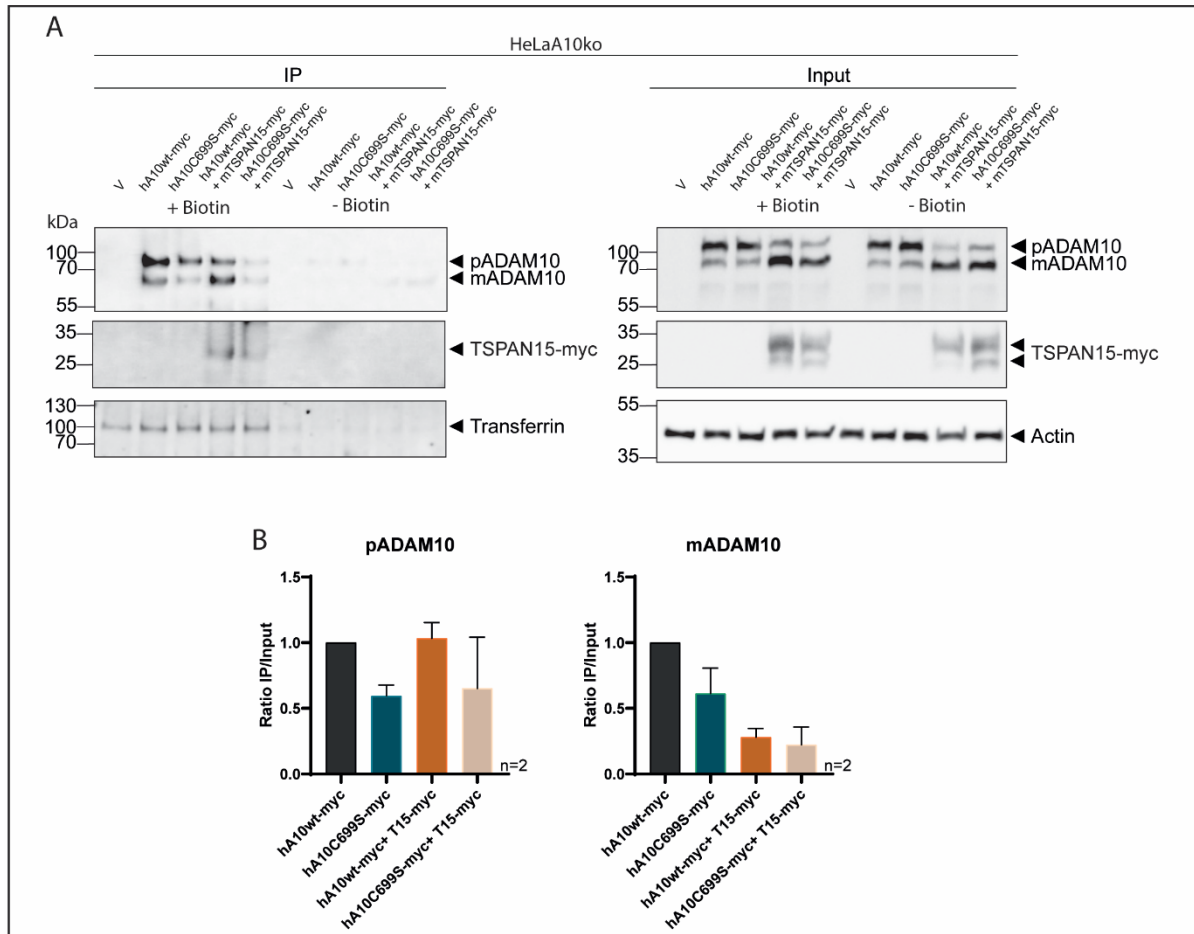


Figure 15: Analysis of the cell surface expression of transfected wildtype and palmitoylation-deficient ADAM10 in HeLa ADAM10 knockout (HeLaA10ko) cells. HeLa ADAM10ko cells were either transfected with a wildtype (wt) or mutated version (C699S) of human (h) ADAM10. For increased maturation and transport of ADAM10 to the plasma membrane, an additional co-transfection of either wildtype or mutated hADAM10 with Tspan15 expression plasmids was performed. As negative control cells were transfected with an empty vector (V). Surface biotinylation was performed and pro (p) and mature (m) ADAM10 cell surface expression was analysed by precipitation (IP) using streptavidin agarose beads. **A:** Immunoblot analysis shows the cell surface expression of pro and mature ADAM10 and Tspan15 in IP fractions of biotin-labelled (+Biotin), but not in untreated (-Biotin) samples. Expression of ADAM10 and Tspan15 was also detected in total cell lysates (Input) with antibodies directed against ADAM10 and myc tag for Tspan15 detection, respectively. Equal protein loading was controlled by the detection of transferrin in the IP fraction and actin in the Input fraction. **B** depicts quantifications of pro and mature ADAM10 surface expression levels in relation to total pro and mature ADAM10 expression levels (Ratio IP/Input) compared between the different transfections of two (n=2) independent experiments. Values are normalized to wt ADAM10 transfection level and shown as mean $\pm$ SEM. Significance was tested using a one-way ANOVA test (* $p < 0.05$). No statistical significance was achieved.

The specificity of the biotin-streptavidin pulldown was confirmed after immunoblotting revealing the pro- and mature form of wildtype ADAM10 and the ADAM10 C699S mutant at a molecular weight of 100 and 70 kDa in the biotin treated but not in the untreated IP fractions. In the Tspan15 co-transfected IP samples treated with biotin, a band at a molecular weight between 25 and 35 kDa could be detected using a myc-

specific antibody likely corresponding to myc tagged Tspan15. ADAM10 and Tspan15 were also detected in total cell lysates confirming sufficient and equal protein expression (Input). Actin was used to determine equal protein loading in the input fraction. Transferrin as a cell surface protein was used to demonstrate that surface labelling with biotin worked and to determine equal protein loading in the IP fraction. In this experiment (Fig. 15), the ADAM10 pro- and mature forms after the expression of the palmitoylation-deficient ADAM10 mutant were reduced at the surface of transfected HeLaA10ko cells (Fig. 15A, left panel). Furthermore, analysing Tspan15 and ADAM10 co-transfected samples, the surface levels of palmitoylation-deficient ADAM10 remain reduced in contrast to the wildtype ADAM10. Tspan15, known to promote ADAM10 maturation, cannot rescue the reduced surface expression of ADAM10, despite being able to lead to more mature ADAM10 as visualized in the input samples (Fig. 15A, right panel). To rule out any effects caused by unequal expression levels of ADAM10 in the total cell lysates, results were set in relation to the amount of ADAM10 in the input fraction (Fig. 15B).

In conclusion, ADAM10 is palmitoylated in different cell types. ADAM10's palmitoylation has no obvious effects on ADAM10 expression in cells but possibly causes an accelerated transport to the cell surface. In other words, the palmitoylation-deficient mutant was less efficiently transported to and labelled at the cell surface. Whether this finding has any influence on the function of ADAM10 was investigated in the following parts.

4.4 Does the palmitoylation affect ADAM10's substrate shedding capacity?

Since ADAM10 is known to cleave many proteins like the amyloid precursor protein (APP) or N-Cadherin and since it was described to be involved in neuronal diseases like Alzheimer Disease (Saftig & Lichtenthaler, 2015), it was interesting to investigate possible regulations towards ADAM10's shedding activity. A palmitoylation may exert possible indirect effects on ADAM10's shedding activity through alteration of subcellular localisation, protein-protein interactions, or protein trafficking (Jin et al., 2021). It might also affect the shedding activity directly. To address the question if the palmitoylation of ADAM10 influences protease activity, two known ADAM10 substrates (N-Cadherin and APP) were chosen.

4.4.1 Analysis of the influence of ADAM10's palmitoylation on the proteolytic processing of N-Cadherin

N-Cadherin is an important cell adhesion protein involved in neuronal development, differentiation and long-term potentiation. It is regulated by the shedding of ADAM10 which is essential for further processing by intramembrane cleaving secretases (Reiss et al., 2005; Uemura et al., 2006). N-Cadherin and its C-terminal cleavage product (CTF1) is expressed and reliably detectable in HeLa cells, making this cell line suitable for the following experiment. To address the question if palmitoylation has any effects on ADAM10-mediated cleavage of N-Cadherin, either wildtype or C699S ADAM10 were expressed in HeLa ADAM10 knockout cells. Cells were further lysed, and the samples were analysed by immunoblotting.

Tspan15 expression is important for the proteolytic processing of N-Cadherin by ADAM10 since the appearance of the ADAM10-generated C-terminal fragment of N-Cadherin was decreased in Tspan15 knockout mice brains (Seipold et al., 2018). To analyse whether a Tspan15 interaction with ADAM10 can compensate for possible palmitoylation-dependent alterations in N-cadherin shedding, HeLa cells were also transfected with both ADAM10 and Tspan15.

It was first analysed if the palmitoylation deficiency causes altered interactions between ADAM10 and Tspan15. Therefore, HeLa ADAM10 knockout cells were either transfected with human wildtype ADAM10 or the human ADAM10 C699S mutant, together with human Tspan15. A sample transfected with an empty vector was prepared as a negative control. Cells were lysed and an immunoprecipitation (IP) was performed by precipitating ADAM10 with an antibody directed against its C-terminus (Fig. 16A). The Fc domain of the antibody was then precipitated by protein G coupled dynabeads. To ensure proper protein expression, 50µl of the total cell lysates (Input) were kept before proceeding with the IP.

A prominent band at a molecular weight of 100 kDa and a weaker band at a molecular weight of 70 kDa corresponding to the pro- and mature form of ADAM10, respectively, revealed the successful immunoprecipitation of ADAM10 (Fig. 16A). The specificity of the immunoprecipitation was confirmed using the antibody (AB-ctrl) and bead control (Bead-ctrl), where no signals were detectable. Tspan15 was detected in the immunoprecipitates of ADAM10 at a molecular weight of 25 and 30 kDa confirming co-precipitation of the glycosylated tetraspanin. No difference in the signal intensities of the bands corresponding to Tspan15 was observed comparing wildtype and the C699S

mutant ADAM10. Sufficient and equal expression of ADAM10 and Tspan15 in cells was confirmed by analysing the total cell lysates (Input).

In the following experiment (Fig. 16B), N-Cadherin full-length expression levels and its processed C-terminal fragment (CTF) were analysed when wildtype ADAM10 or its palmitoylation-deficient mutant were expressed either with or without additional Tspan15 expression.

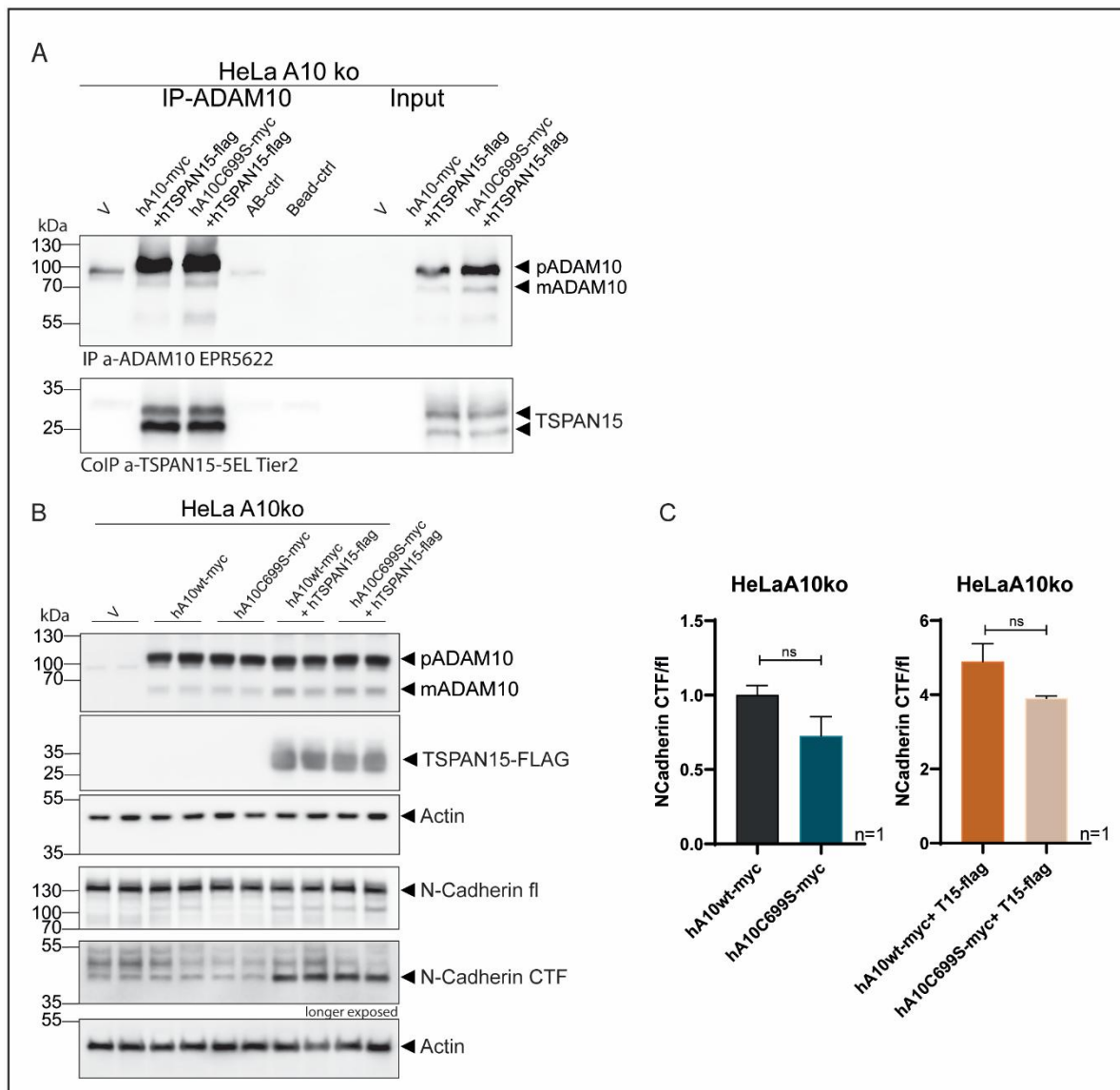


Figure 16: Expression of palmitoylation-deficient human ADAM10 does not alter N-Cadherin shedding. Immunoblot analyses of immunoprecipitations of ADAM10 in cell lysates of HeLa ADAM10 knockout cells (HeLaA10ko) are shown in **A**. The membranes were incubated with antibodies directed against the C-terminus of ADAM10 and against Tspan15. HeLaA10ko cells were either transfected with an empty vector (V), wildtype (wt) or the mutated version (C699S) of human (h) ADAM10 with additional co-transfection of Tspan15. Immunoprecipitation (IP-ADAM10) was performed with an antibody directed against the C-terminus of ADAM10. Lysis buffer incubated with antibody (AB-ctrl) and cell lysate without antibody (bead-ctrl) were used as a negative control. The proform of ADAM10 (pADAM10) and the active, mature form of ADAM10 (mADAM10) are indicated by arrowheads. Tspan15 co-immunoprecipitation (CoIP) is shown below. Immunoblot analyses of cell lysates of HeLa

ADAM10 knockout cells are shown in **B**: HeLaA10ko. Cells were either transfected with an empty vector (V), wildtype (wt), or mutated version (C699S) of human (h) ADAM10. For increased maturation and transport of ADAM10 to the plasma membrane, where it is predominantly active, an additional co-transfection of either wildtype or mutated hADAM10 with Tspan15 expression plasmids was performed. The blots were incubated with antibodies directed against the C-terminus of ADAM10 and N-Cadherin, respectively. Equal protein loading was controlled by detection of actin and Tspan15 expression verified by detection of flag tag. The proform of ADAM10 (pADAM10) and the active, mature form of ADAM10 (mADAM10) as well as the full-length form of N-Cadherin (fl) and the generated C-terminal fragment (CTF) are indicated by the arrowheads. Quantifications of signal intensities of N-Cadherin CTF in relation to the full-length form (fl) are shown in **C**. Values are normalized to wt ADAM10 transfection level and shown as mean $\pm$ SEM. Significance was tested using an unpaired t-test (* $p < 0.05$). No significant difference in N-cadherin shedding was observed comparing wildtype to mutated ADAM10 transfection either with or without co-expression of Tspan15.

The expression of pro- and mature ADAM10 corresponding to the clear signals at a molecular weight of 100 and 70 kDa, respectively, was validated by immunoblotting (Fig. 16B). Tspan15 expression corresponding to the prominent signal at a molecular weight between 25 and 35 kDa was confirmed using an anti-FLAG antibody. Equal protein loading was verified by actin staining.

Because Tspan15 increases ADAM10-mediated shedding of N-Cadherin (Noy et al., 2016; Seipold et al., 2018), Tspan15 overexpression was used to identify the C-terminal fragment of N-Cadherin (N-Cadherin CTF) that is specifically produced by ADAM10. It could be identified between 35 and 55 kDa when co-expressing ADAM10 and Tspan15 (Fig. 16B). Furthermore, as reported (Seipold et al., 2018) an increase in the mature form of ADAM10 was detected in all Tspan15 transfected samples.

A slight decrease of the ADAM10-mediated N-Cadherin CTF related to the full-length form of N-Cadherin was observed comparing wildtype ADAM10 with C699S mutant transfected samples. However, no significance was achieved (Fig. 16C). An additional band above the band corresponding to the ADAM10-mediated N-Cadherin CTF was observed. This band with an origin of unknown nature was also slightly affected by the palmitoylation deficiency of ADAM10.

4.4.2 Analysis of the influence of ADAM10's palmitoylation on the proteolytic processing of APP

As a further approach, an additional ADAM10 substrate was analysed to address the question if the palmitoylation has any effects on ADAM10-mediated cleavage. The role in neuronal diseases like Alzheimer's makes APP an interesting substrate of ADAM10 which produces a membrane-bound and a soluble extracellular fragment that can specifically be attributed to ADAM10-mediated cleavage (Prox, Rittger, et al., 2012).

Transfected HeLa ADAM10 knockout cells were used to have a clear comparison between wildtype and palmitoylation-deficient ADAM10 with no residual activity of endogenous ADAM10 (Fig. 17). Murine neuroblastoma N2a cells were used since APP cleavage is important in neuronal cells (Fig. 18). Both cell lines were transfected to express either wildtype or the mutated version of ADAM10. Cells were further lysed, and the samples were analysed by immunoblotting. Furthermore, similar to the experiment described before, additional co-transfections with ADAM10 and Tspan15 expression plasmids were performed.

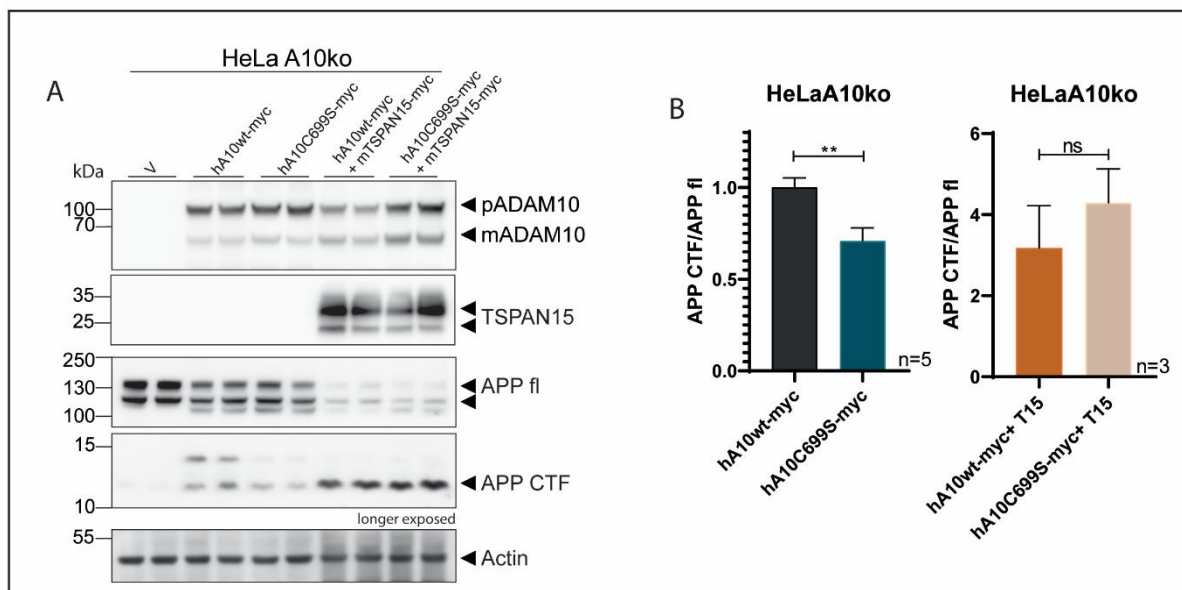


Figure 17: Expression of palmitoylation-deficient human ADAM10 alters APP processing but Tspan 15 rescues the effects. Immunoblot analyses of cell lysates of HeLa ADAM10 knockout cells are shown in **A**: HeLaA10ko. Cells were transfected either with an empty vector (V), wildtype (wt), or mutated version (C699S) of human (h) ADAM10. For increased maturation and transport of ADAM10 to the plasma membrane, where it is predominantly active, an additional co-transfection of either wildtype or mutated hADAM10 with Tspan15 expression plasmids was performed. The blots were incubated with antibodies directed against the C-terminus of ADAM10 and APP, respectively. Equal protein loading was controlled by detection of actin and Tspan15 expression was verified by detection of Tspan15. The proform of ADAM10 (pADAM10) and the active, mature form of ADAM10 (mADAM10) as well as the full-length form of APP (fl) and the generated C-terminal fragment (CTF) are indicated by arrowheads. Quantifications of signal intensities of APP CTF in relation to the full-length form (fl) are shown in **B** as evaluated from n=5 and n=3 independent experiments, respectively. **A** shows the results of one representative experiment. Values are normalized to wt ADAM10 transfection level and shown as mean $\pm$ SEM. Significance was tested using an unpaired t-test (* $p < 0.05$, ** $p < 0.005$). A significant decrease in APP shedding was observed comparing wildtype to mutated ADAM10 transfection without co-expression of Tspan15. Additional co-transfection with Tspan15 rescued the observed effects.

The heterologous expression of pro- and mature ADAM10 in HeLa ADAM10 ko cells revealed clear signals at a molecular weight of 100 and 70 kDa, respectively (Fig. 17). Prominent bands after analysing with an anti-Tspan15 antibody between 25 and 35

kDa were observed in the Tspan15-transfected samples. Actin was used to control for equal protein loading. The endogenous and glycosylated APP full-length forms were detected at around 130 kDa whereas an APP carboxy-terminal fragment (APP CTF) was detected above 10 kDa.

ADAM10 as the major α -secretase for APP generates the membrane-bound CTF C83 of APP (Asai et al., 2003; Prox, Rittger, et al., 2012). An increased turnover of APP in ADAM10 and Tspan15 co-expressing cells was observed leading to less full-length form of APP and increased levels of the C83 APP CTF (Fig. 17A).

Comparing cell lysates of cells transfected with either wildtype ADAM10 or ADAM10 C699S, there was no obvious difference observed in the full-length levels of APP. However, analysing the detected APP CTF C83 in relation to its full-length forms, a statistically significant difference was observed. Transfection with the human ADAM10 C699S expression plasmid led to a decrease of the APP turnover and the level of APP CTF C83 compared to the samples transfected with the human wildtype ADAM10 expression plasmid. This indicates that shedding activity towards APP may be altered in cells expressing the palmitoylation-deficient ADAM10 mutant. Interestingly, after co-transfection of Tspan15, this decrease in the APP shedding could be rescued (Fig. 17A, B). To further address this finding in another cell line, the same experiment was performed in murine neuronal neuroblastoma (N2a) cells (Fig. 18).

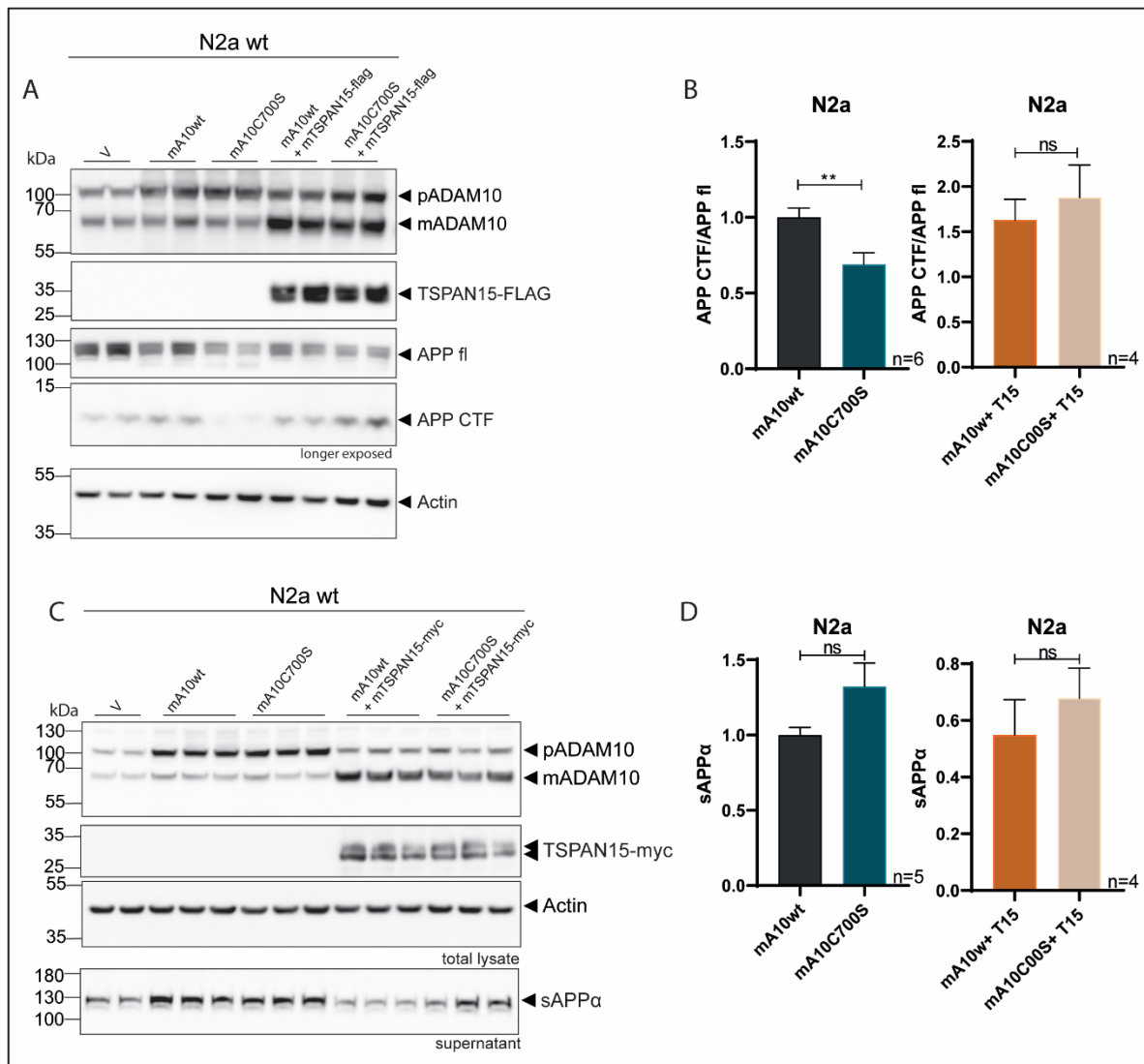


Figure 18: Expression of palmitoylation-deficient murine ADAM10 alters proteolytic APP processing. Immunoblot analyses of cell lysates and supernatants of murine N2A neuroblastoma cells are shown in **A** and **C**: N2a cells. Cells were either transfected with an empty vector (V), wildtype (wt), or mutated version (C700S) of murine (m) ADAM10. For increased maturation and transport of ADAM10 to the plasma membrane, where it is predominantly active, an additional co-transfection of either wildtype or mutated murine ADAM10 with Tspan15 expression plasmids was performed. The blots were incubated with antibodies directed against the C-terminus of ADAM10 and APP, respectively. Equal protein loading was controlled by detection of actin and Tspan15 expression verified by detection of myc tag. The proform of ADAM10 (pADAM10) and the active, mature form of ADAM10 (mADAM10) as well as the full-length form of APP (fl), the generated C-terminal fragment (CTF), and the soluble APP α fragment are indicated by arrowheads. Quantifications of signal intensities of APP CTF in relation to the full-length form (fl) are shown in **B** as evaluated from n=6 and n=4 independent experiments, respectively. Quantifications of sAPP α levels are shown in **D** as evaluated from n=5 and n=4 independent experiments, respectively. **A** and **C** show the results of one representative experiment each. Values are normalized to wt ADAM10 transfection level and shown as mean $\pm$ SEM. Significance was tested using an unpaired t-test (* $p < 0.05$, ** $p < 0.005$).

Again, pro and mature ADAM10 were identified using an ADAM10-specific antibody, as well as Tspan15 transfections were verified using an anti-myc antibody (Fig. 18A,

C). APP full length and CTF levels were detected at a molecular weight between 130 kDa and 100 kDa and below 15 kDa, respectively.

An additional transfection of Tspan15 led to an increase of the mature form of ADAM10 in all Tspan15 transfected samples (Fig. 18A, C). In the murine ADAM10 C700S mutant transfected samples a significant decrease in APP CTF levels in relation to its full-length form could be observed (Fig. 18A). When comparing wildtype or palmitoylation-deficient ADAM10 co-transfection with Tspan15, no change in APP CTF levels in the murine ADAM10 C700S transfected samples compared to wildtype ADAM10 could be observed. This was also shown with the human constructs in HeLaA10ko cells (Fig. 17) before.

Because soluble APP alpha (sAPP α) is a direct cleavage fragment of APP after ADAM10-mediated shedding, sAPP α levels were investigated in the supernatants of N2a cells. ADAM10 and Tspan15 expression in cell lysates was confirmed (Fig. 18C). Proteins in the supernatants were analysed after trichloroacetic acid (TCA) precipitation and further immunoblotting. Using an sAPP α -neo epitope specific antibody (Cuchillo-Ibanez et al., 2015), immunoreactive bands at a molecular weight of 130 kDa were detected corresponding to soluble and α -secretase (ADAM10) released sAPP α (Fig. 18C). No significant difference in sAPP α levels in the supernatants of ADAM10 C700S or ADAM10 wildtype transfected N2a cells even with or without co-transfection of Tspan15 were observed (Fig. 18D).

In conclusion, palmitoylation deficiency of ADAM10 has no apparent effect on the interaction with Tspan15 in co-immunoprecipitation experiments (Fig. 16A). There was no clear difference observed between wildtype and palmitoylation-deficient ADAM10 regarding N-Cadherin cleavage (Fig. 16B, C). Furthermore, palmitoylation of ADAM10 seems not to affect the production of soluble APP α (Fig. 18C). However, a significant decrease in the expression of the ADAM10-mediated APP CTF in the samples transfected with the palmitoylation-deficient ADAM10 could be observed compared to wildtype ADAM10 transfected samples indicating an influence on the stability of this fragment. An additional co-transfection with Tspan15 rescued these effects (Fig. 17, 18).

4.5 Characterisation of the ADAM10 carboxyterminal fragment generation

ADAM10 is synthesized as an inactive zymogen and processed to the active, mature form (Peron et al., 2018). Further proteolytic processing of ADAM10 can result in N-terminal, extracellular fragments, or C-terminal, intracellular fragments. There are different cleaving processes described in the literature. ADAM10 seems to be subject to proteolysis by other ADAMs like ADAM9 or -15. Also γ -secretase is involved in the subsequent intramembranous cleavage of ADAM10 (Prox, Rittger, et al., 2012). Both processes as well as a possible autoproteolysis by ADAM10 (Brummer et al., 2018) itself may result in different C-terminal fragments at different molecular weights. To better understand these ADAM10-specific fragments and the involved proteases (Fig.12A) and how far palmitoylation at the cytosolic side of ADAM10 affects this processing events, an analysis of wildtype ADAM10 and palmitoylation deficient ADAM10 was performed.

4.5.1 Palmitoylation-deficient ADAM10 transfected cells contain less ADAM10 CTF

An influence of the palmitoylation on the general expression of ADAM10 could not be observed in cell lysates (Fig. 13). To further address a possible influence of the palmitoylation on ADAM10, the C-terminal fragments (CTF) of the two different versions of ADAM10, i.e. wildtype and palmitoylation-deficient ADAM10 were analysed. Because, as described, Tspan15 is known to play a role in the maturation and trafficking of ADAM10 to the plasma membrane (Prox, Willenbrock, et al., 2012), the question if Tspan15 co-transfection leads to a stabilisation of wildtype or palmitoylation-deficient ADAM10 was also addressed.

Murine ADAM10-myc and ADAM10 C700S-myc were expressed in N2a cells. Human ADAM10-myc, ADAM10 C699S-myc and Tspan15-flag were expressed in HeLa ADAM10 knockout cells. Cells transfected with an empty vector served as negative control. Following cell lysis, the generation of ADAM10-specific C-terminal cleavage products (ADAM10 CTFs) was analysed by immunoblotting using an antibody directed against the cytoplasmic carboxyterminal domain of ADAM10 (Fig.19 B, D).

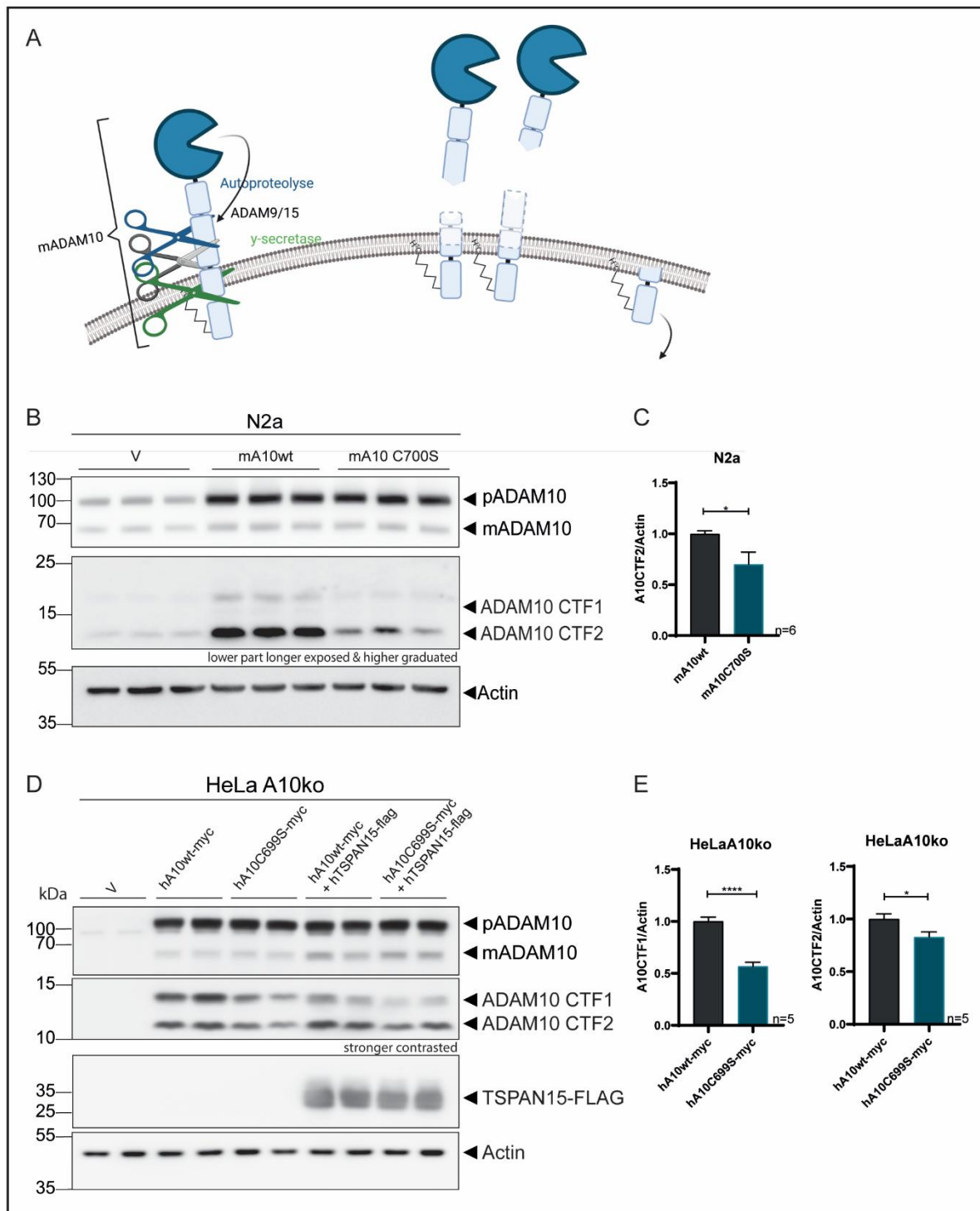


Figure 19: Expression of palmitoylation-deficient murine or human ADAM10 alters ADAM10 CTF generation. Immunoblot analyses of cell lysates of murine neuroblastoma Neuro 2a cells (N2a) and HeLa ADAM10 knockout cells (HeLaA10ko) are shown. **A:** N2a cells, **C:** HeLa A10ko cells. The blots were incubated with antibodies directed against the C-terminus of ADAM10, actin, and flag tag, respectively. Cells were either transfected with an empty vector (V), wildtype human A10-myc/murine A10 (wt,) or mutated hA10C699S-myc/mA10C700S. For increased maturation and transport of ADAM10 to the plasma membrane, an additional co-transfection of either wildtype or mutated hADAM10 with Tspan15 expression plasmids was performed in HeLaA10ko cells (**C**). The proform of ADAM10 (pADAM10) and the active, mature form of ADAM10 (mADAM10) are indicated by arrowheads. Equal protein loading was controlled by detection of actin. **B** and **D** depict quantifications that show the ADAM10 CTF expression level in relation to actin as evaluated from (**B**: n=6 and **D**: n=5) independent

experiments. Values are normalized to wt ADAM10 transfection level and shown as mean $\pm$ SEM. Significance was tested using an unpaired t-test (* $p < 0.05$, **** $p < 0.0001$).

Expression of wildtype or palmitoylation-deficient ADAM10 in transfected N2a and HeLa ADAM10 knockout cells (HeLaA10ko) was confirmed with an anti-ADAM10 antibody showing comparable signals of pro and mature ADAM10 at a molecular weight of 100 and 70 kDa, respectively (Fig. 19B, D). Proper expression levels of Tspan15 were verified by staining with an antibody directed against the FLAG-tag, resulting in equal signal intensities in Tspan15 transfected cells (Fig. 19D). The additional transfection of Tspan15 in HeLaA10ko cells led to an increase of mature ADAM10 as described previously (Fig. 16, 17).

Interestingly, two C-terminal fragments (CTFs) of ADAM10 could be detected at about 10 to 20 kDa in N2a cells as well as in HeLa cells. The signal intensities of both CTFs were clearly decreased in the palmitoylation-deficient mutant transfected cells compared to cells transfected with the wildtype version of ADAM10.

In N2a cells, the signal of the smaller C-terminal fragment of ADAM10 (ADAM10 CTF 2) was more prominent than of the larger one (ADAM10 CTF 1), which was not reliably detectable in all experiments. When ADAM10 was deficient for the palmitoylation, the signal intensity of ADAM10 CTF 2 decreased more than that of ADAM10 CTF 1. A significant reduction of this CTF was observed in ADAM10 C700S transfected N2a cells while pro and mature forms of ADAM10 remained on equal levels (Fig. 19C).

In HeLa ADAM10 knockout cells, the above-described effects on the signal intensities of the ADAM10 CTFs could also be observed. In contrast to the ADAM10 CTF 1 in N2a cells, both CTFs could be detected in all experiments in HeLaA10ko cells. Transfection of ADAM10 C699S-myc led to a significant reduction by half of the signal intensity of ADAM10 CTF 1 compared to the wildtype transfected ADAM10-myc. The signal intensity of ADAM10 CTF 2 decreased less than that of ADAM10 CTF 1. Furthermore, it was observed that the ADAM10 CTF 1 is reduced in all Tspan15 co-transfected samples from HeLaA10ko cells but with a similar fold change comparing wildtype and palmitoylation-deficient ADAM10 without Tspan15 co-expression. The ADAM10 CTF 2 was not prominently affected by the additional co-expression of Tspan15.

4.5.2 Pharmacological inhibition of ADAM10's palmitoylation leads to less ADAM10 CTF generation

To support that the impaired ADAM10 CTF generation is a result of the palmitoylation-deficiency of ADAM10, an experiment was performed where palmitoylation is ubiquitously and pharmacologically inhibited in analysed cells. 2-bromopalmitic acid (2-BP) was used for inhibition of palmitoylation (Jennings et al., 2009). After transfection of the wildtype ADAM10 expression construct and treatment with 2-BP, cells were lysed and further analysed by immunoblotting. The previously established click assay that labels palmitoylation by detecting inserted 17-ODYA with a fluorescent Alexa fluorophore 647 (AF647) was used to verify the inhibited palmitoylation on wildtype transfected ADAM10.

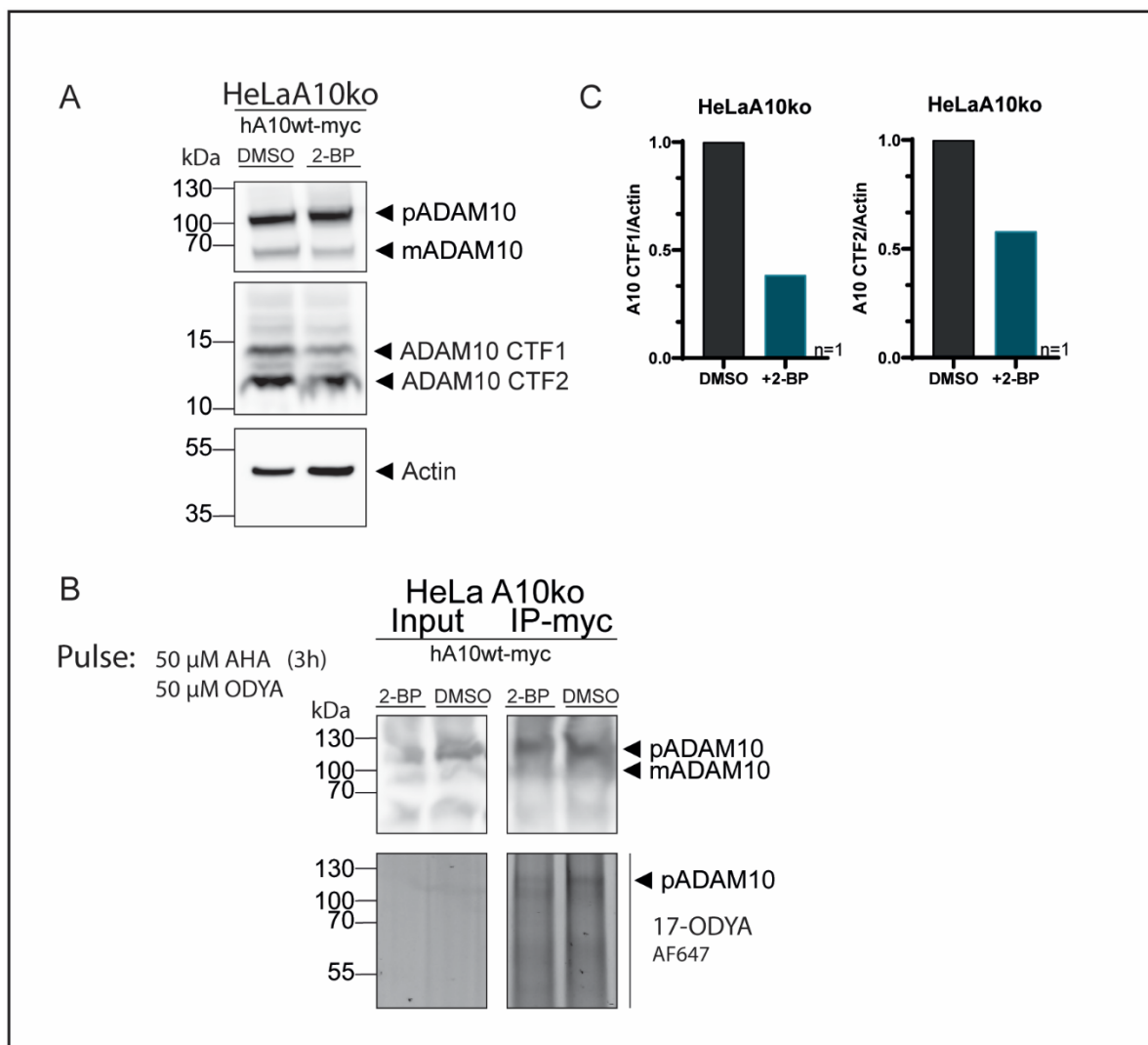


Figure 20: Impaired ADAM10 CTF 1 generation in transfected HeLa ADAM10 knockout cells can be traced to leaking palmitoylation of ADAM10. Immunoblot analysis of HeLa ADAM10 knockout cells (HeLaA10ko) is shown in **A**. Cells were transfected with human wildtype ADAM10 (hA10wt-myc) and either treated with 2-Bromopalmitic acid (2-BP) as palmitoylation inhibitor or DMSO as a control. The blots were incubated with antibodies

directed against the C-terminus of ADAM10 or actin, respectively. The control of the palmitoylation inhibitor is shown in **B** using the click chemistry method. HeLaA10ko cells were transfected with the wildtype ADAM10 which was either treated with 2-BP or DMSO. In each case, the total protein lysate (Input) and the immunoprecipitation (IP) for ADAM10 with the implemented click assay are shown. The blots were incubated with antibodies directed against the C-terminus of ADAM10. The proform (pADAM10) and the active, mature form of ADAM10 (mADAM10) are indicated by arrowheads. After starvation of the cells for one hour, a pulse of 50µM 17-ODYA was performed for 3 hours. The integrated 17-Octadecynoic Acid (17-ODYA) which is used for labeling palmitoylation substrates was linked to an ALEXA fluorophore 647 (AF647) for detection by fluorescence imaging. The reduced palmitoylation of the transfected wildtype ADAM10 treated with 2-BP compared to the untreated sample is shown. **C** depicts quantifications of ADAM10 CTF 1 and 2 in relation to actin as a loading control. Values are normalized to the wt ADAM10 transfection level.

Analysis of the immunoblot shows the pro- and mature form of ADAM10 in cell lysates (Input) using an anti-ADAM10 antibody which confirms proper expression of the protease. The CTFs of ADAM10 were detected corresponding to the two bands at a molecular weight between 10 and 15 kDa (Fig. 20A). The signal intensities of both CTFs seem reduced in the samples treated with 2-BP which is set in relation to actin as loading control (Fig. 20C). Further, the detected bands in the immunoprecipitated fractions (IP-myc) correspond to the pro- and mature form of ADAM10 confirming sufficient precipitation (Fig. 20B). The fluorescence analysed SDS gel of the click assay shows a band at around 100 kDa in the IP fraction corresponding to the proform of ADAM10. This fluorescent signal in the 2-BP treated sample is reduced in comparison to the DMSO treated sample (Fig. 20B) indicating that the palmitoylation of ADAM10 is reduced but does not completely disappear.

In conclusion, the expression of a palmitoylation-deficient ADAM10 mutant led to decreased levels of ADAM CTFs in N2a and HeLaA10ko cell lines. Moreover, the effects of the reduced CTFs could also be shown by pharmacological inhibition of palmitoylation in HeLaA10ko cells.

4.5.3 Selective inhibition of ADAM10 leads to less ADAM10 CTF generation

Palmitoylation-deficient ADAM10 leads to a reduced generation of the C-terminal fragments of ADAM10. To further address if non-palmitoylated ADAM10 is less active and therefore might inhibit its self-cleavage, the following experiment was performed.

Cells endogenously expressing ADAM10 were treated with different inhibitors to see if any of them have effects on the CTF generation of ADAM10. The used inhibitors were GI254023X (GI) for selective inhibition of ADAM10 (Hoettecke et al., 2010), GM 6001

(GM) for inhibition of a broad spectrum of metalloproteases especially ADAM10 and -17, and Inhibitor X (carbamic acid tert-butyl ester L-685,458, Calbiochem) for inhibition of the γ -secretase. Ionomycin, as a calcium ionophore, was used to induce ADAM10 activity (Le Gall et al., 2009). HeLa and N2a cells were treated with a concentration of 3 μ M of the different inhibitors and 5 μ M of ionomycin for about 16 hours. After cell lysis, samples were separated on SDS-PAGE gels, and immunoblots were prepared. ADAM10 was detected with an antibody directed against its C-terminus to visualize the CTFs. APP and sAPP α were detected to control the function of the inhibitors since APP is cleaved by ADAM10 (Asai et al., 2003; Hoettecke et al., 2010). The γ -secretase is responsible for further regulated intramembrane proteolysis of shedding products of APP resulting in APP CTFs (Hitzenberger et al., 2020). Therefore, detection of the APP CTF can be used to control for sufficient inhibition of the γ -secretase by Inhibitor X (Barthet et al., 2018). sAPP α is a direct cleavage product of ADAM10 that can be used to evaluate the efficiency of the applied ADAM10 inhibitors. Actin was used to determine equal protein loading.

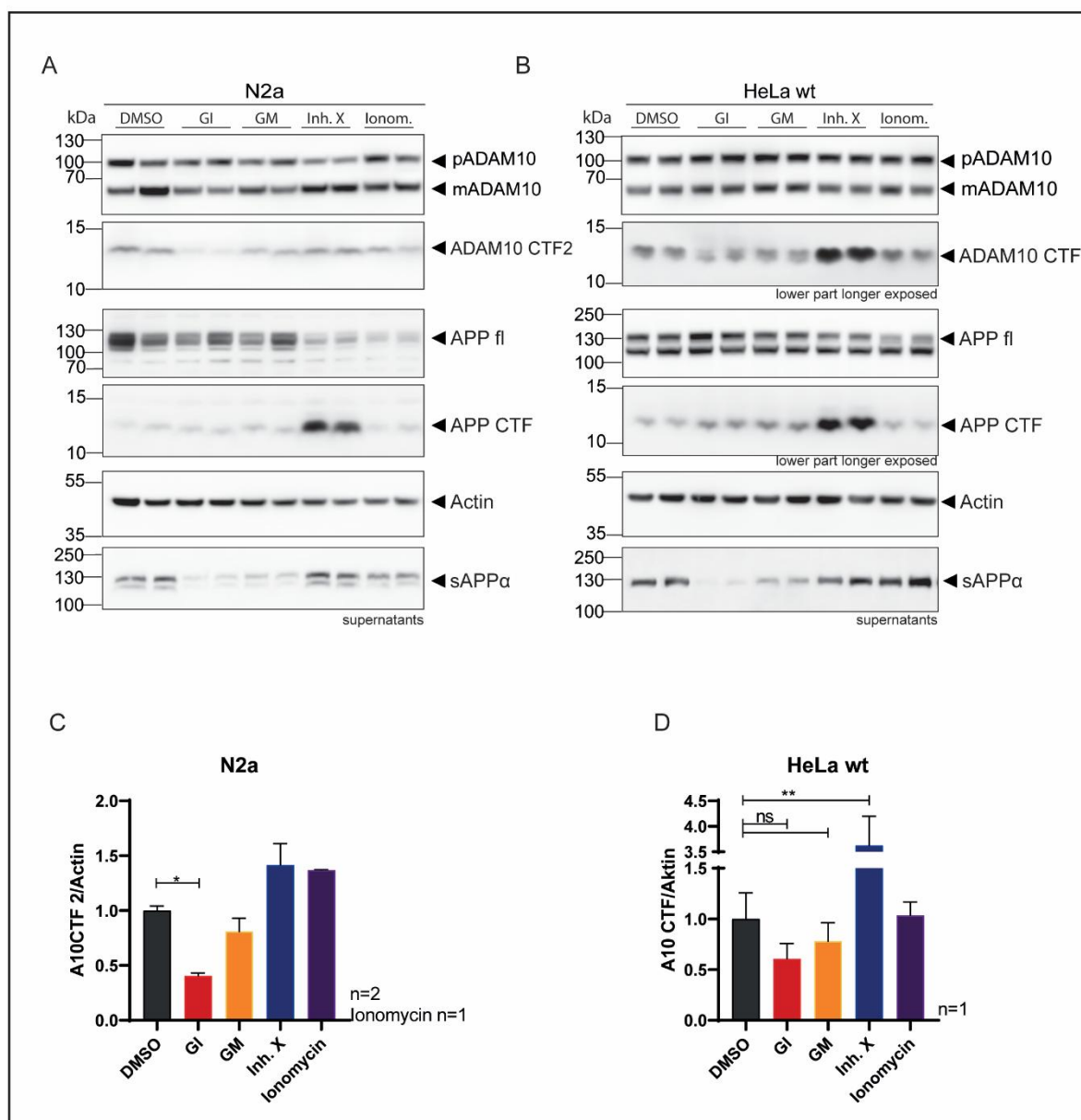


Figure 21: Inhibition of metalloproteinases in N2a and HeLa cells indicates ADAM10 CTF generation as a function of self shedding. Immunoblot analyses of cell lysates of murine N2A neuroblastoma cells (N2a) and HeLa ADAM10 knockout cells (HeLaA10ko) are shown. **A:** Cell lysates and related supernatants of N2a cells. **B:** Cell lysates and related supernatants of HeLa cells. Both were treated by indicated Inhibitors with a concentration of 3 μ M or a stimulator with a concentration of 5 μ M are shown. DMSO serves as untreated control. GI is an inhibitor for selective inhibition of ADAM10, GM inhibits metalloproteinases like ADAM10 and -17, Inhibitor X (Inh. X) inhibits the gamma(γ)-secretase, and Ionomycin (Ionon.) was used to induce ADAM10. As a control of the inhibitory effects of GI and GM and the inducing effects of Ionomycin, the supernatants were analysed for soluble APP alpha (sAPP α) whereas Inhibitor X was verified by detecting APP CTF which is further processed by the γ -secretase. **C** and **D** depict quantifications of the expression of ADAM10 CTF 2 in N2a cells and ADAM10 CTF 1 in HeLa cells to actin as a loading control. Quantification in B is evaluated from two independent experiments (n=2) except the ionomycin-treated sample (n=1). Quantification in D is evaluated from the shown experiment (n=1). Values are normalized to DMSO treatment level and shown as mean $\pm$ SEM. Significance was tested using a one-way ANOVA test (* p <0.05, ** p <0.005). ADAM10 CTF 2 generation was significantly impaired by inhibition of GI.

Murine pro and mature ADAM10 expression was visualized in N2a cells corresponding to the bands at a molecular weight of 100 and 70kDa, respectively (Fig. 21A). In this experiment, only one ADAM10 CTF was resolved below 15 kDa most likely corresponding to the ADAM10 CTF 2 which was also more affected by the palmitoylation deficiency in previous experiments (Fig.19B). A significant reduction of the signal intensities of the ADAM10 CTF 2 is shown in the GI treated samples after selective inhibition of ADAM10 (Fig. 21C). The use of the broad band metalloproteinase inhibitor GM resulted only in a slight reduction of ADAM10 CTF 2 whereas application of Inhibitor X led to an accumulation of the CTF. The sAPP α levels in the GI and GM treated samples were strongly reduced indicating that both inhibitors led to a successful reduction of ADAM10 activity. GM as a broad-spectrum metalloprotease inhibitor led to less sufficient sAPP α reduction. The APP CTF expression is increased in the Inhibitor X-treated samples indicating that the γ -secretase was successfully inhibited and was not able to further process this CTF. Treatment with ionomycin did not alter signal intensities of the APP CTF and sAPP α . From the reduced ADAM10 CTF 2 expression level in the GI and GM treated samples it can be concluded that ADAM10 itself contributes to the generation of one CTF and this function is impaired in the palmitoylation-deficient mutant of ADAM10.

In HeLa cells, a similar trend was observed (Fig. 21B). Pro and mature ADAM10 and an ADAM10 CTF were detected using an antibody directed against the C-Terminus of ADAM10. At an endogenous level, the previously observed two CTFs could not be clearly separated. One band at a molecular weight between 10 and 15 kDa is labelled as ADAM10 CTF in this experiment. Application of GI and GM led to a slight decrease of the signal intensities of this ADAM10 CTF. This effect was not significant although the sAPP α control shows sufficiently reduced levels in the GI and GM treated samples. However, a significant accumulation of the ADAM10 CTF when treated with Inhibitor X is shown (Fig. 21D). The function of this inhibitor was verified by detection of the APP CTF which was also found to be accumulated in the Inhibitor X-treated samples.

The experiments that revealed the impaired ADAM10 CTF generation in the palmitoylation-deficient mutant, the selective inhibition of ADAM10 that provided evidence for a similar reduction of the ADAM10 CTF levels and the experiment using the palmitoylation inhibitor 2-BP that also led to an impaired ADAM10 CTF generation contribute to the understanding of the proteolytic fragmentation processes occurring within the ADAM10 protein. The γ -secretase seems to be involved in processing of the

carboxyterminal fragments of ADAM10. It was also noted that ADAM10 itself is an important factor of its auto-proteolytic cleavage.

The following experiment aimed to investigate in more detail the role of ADAM10 autoproteolysis and palmitoylation in the generation of the ADAM10 CTF(s). Two questions were addressed: whether the inactive ADAM10 mutant produces as little ADAM10 CTF as the palmitoylation-deficient mutant which would support the hypothesis that ADAM10 CTF generation is directly related to ADAM10 autoproteolysis and whether autoproteolysis is inhibited in the palmitoylation-deficient mutant. The experiment compared the ADAM10 CTF levels of cells transfected with wildtype ADAM10 to cells transfected with different mutant forms of ADAM10.

The inactivating mutation in the ADAM10 protease region involves replacing the amino acid glutamate with an alanine at position 384 or 385 in the human or murine version with the catalytic active site of ADAM10, respectively. To generate palmitoylation-deficient mutants, a mutagenesis PCR protocol was used (Fig. 22), which involved mutating the palmitoylation site at position 699 or 700 in the human or murine mutant, respectively, by replacing the cysteine with a serine, as previously described in section 3.2.

The resulting expression plasmids carry mutations in both the protease region and the palmitoylation site, rendering them inactive and palmitoylation-deficient. The mutant forms included an inactive mutant (hA10E/A-myc, mA10E/A-HA; kindly provided by Dr. Franka Scharfenberg, Biochemical Institute, CAU Kiel), a protease inactive mutant with further palmitoylation deficiency (hA10E/A C/S-myc, mA10E/A C/S-HA), and proteolysis active palmitoylation-deficient mutant (hA10C/S-myc, mA10C/S-HA) (Fig. 22).

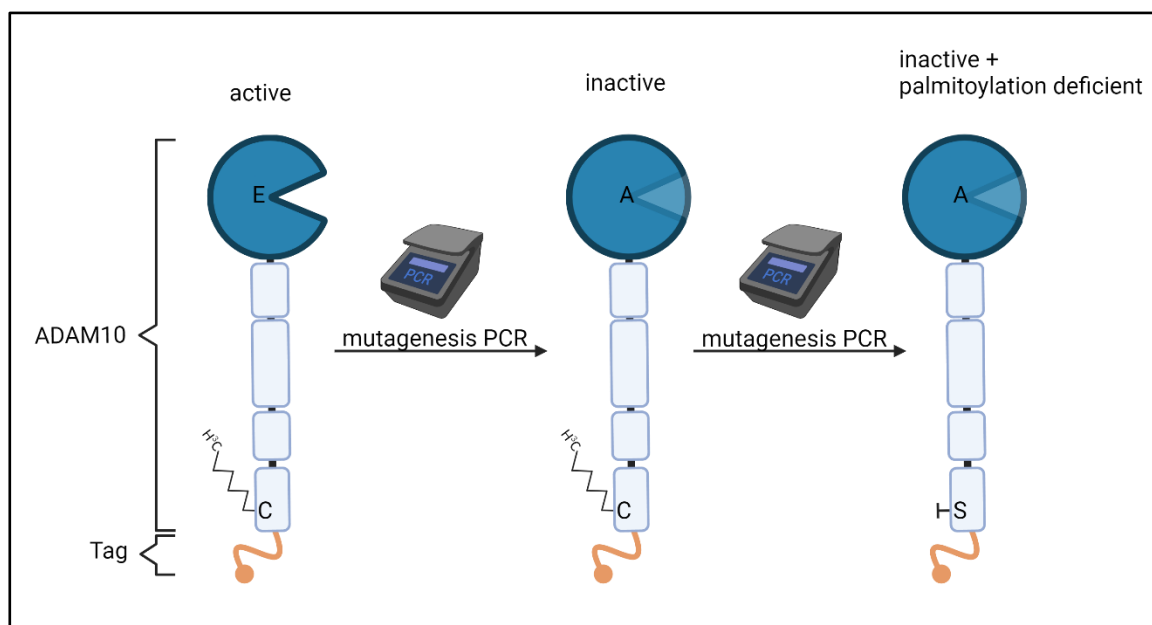


Figure 22: ADAM10 and the generation of inactive mutants. The inactive ADAM10 mutant (mA10E/A-HA, hA10E/A-myc) which is mutated within the protease region replacing the amino acid glutamate with an alanine at position 384 or 385 in the human or murine version, respectively, was then further mutated to abolish the putative palmitoylation site within the C-terminus of ADAM10. The mature form of ADAM10 (mADAM10) is shown with a tag that is either a myc-tag in the human version or an HA-tag in the murine version.

Lysates of N2a and HeLa ADAM10 knockout cells were transfected and further analysed by immunoblotting (Fig. 23). Murine, HA-tagged plasmids of the different ADAM10 versions as indicated above were transfected in biological triplicates. As control, lysates of cells transfected with an empty vector (V) were prepared in biological duplicates. Pro and mature ADAM10 were detected with an antibody directed against the C-terminus of ADAM10 confirming comparable expression levels. Actin was detected to determine equal protein loading. Two ADAM10 CTFs could be detected in this experiment corresponding to the bands at a molecular weight of 20 (ADAM10 CTF1) and 15 kDa (ADAM10 CTF2), respectively.

The ADAM10 CTF 2 is more prominent than ADAM10 CTF 1 in all samples of lysed N2a cells (Fig. 23A) as also noticed in previous experiments (Fig. 19B). The transfection of the active ADAM10 C700S palmitoylation mutant reduced the levels of ADAM10 CTF 2 compared to the wildtype version confirming our observations in previous experiments (Fig. 19C). Importantly, the protease inactive mutants, regardless of the palmitoylation status (mA10E/A-HA, mA10E/A C/S-HA), also reduced the levels of ADAM10 CTF 2. ADAM10 CTF 1 was hardly detectable, with no differences between samples with different protease versions. These findings suggest that the ADAM10 CTF 2 levels are affected by both protease inactive ADAM10 and

palmitoylation-deficient ADAM10 mutants in a similar manner leading to the conclusion that the palmitoylation deficiency might impair the ability for ADAM10-mediated autoproteolysis.

The same experiment was conducted in HeLa ADAM10 knockout cells transfected with the human, myc-tagged expression plasmids in biological triplicates (Fig. 23B). Cells transfected with an empty vector (V) in duplicates served as negative control. Pro and mature, as well as the ADAM10 CTFs, were detected with an anti-myc antibody. Actin served as loading control.

The two observed ADAM10 CTFs correspond to signals at a molecular weight of 15 (ADAM10 CTF1) and around 12 kDa (ADAM10 CTF2). In all samples with expressed protease inactive ADAM10 mutants (hA10E/A-myc, hA10E/A C/S-myc), the 15kDa ADAM10 CTF 1 disappeared completely. The expression of the 12 kDa ADAM10 CTF 2 was slightly reduced in samples transfected with the protease active but palmitoylation-deficient mutant (hA10C/S-myc). This reduction was more pronounced in samples transfected with the protease inactive ADAM10 mutant (hA10E/A-myc). In samples transfected with the protease inactive, palmitoylation-deficient mutant (hA10E/A C/S-myc), the expression of the ADAM10 CTF 2 was increased. However, the expression level of the full-length forms of ADAM10 was also higher in those samples and the increased expression level of the CTFs can result from the increased level of full-length forms. These results indicate that ADAM10 CTF 1 levels are influenced by both protease-inactive ADAM10 and palmitoylation-deficient ADAM10 mutants. The effect of protease inactivation is more significant, leading to the hypothesis that the lack of palmitoylation may reduce the capacity for ADAM10-mediated autoproteolysis but does not completely eliminate it.

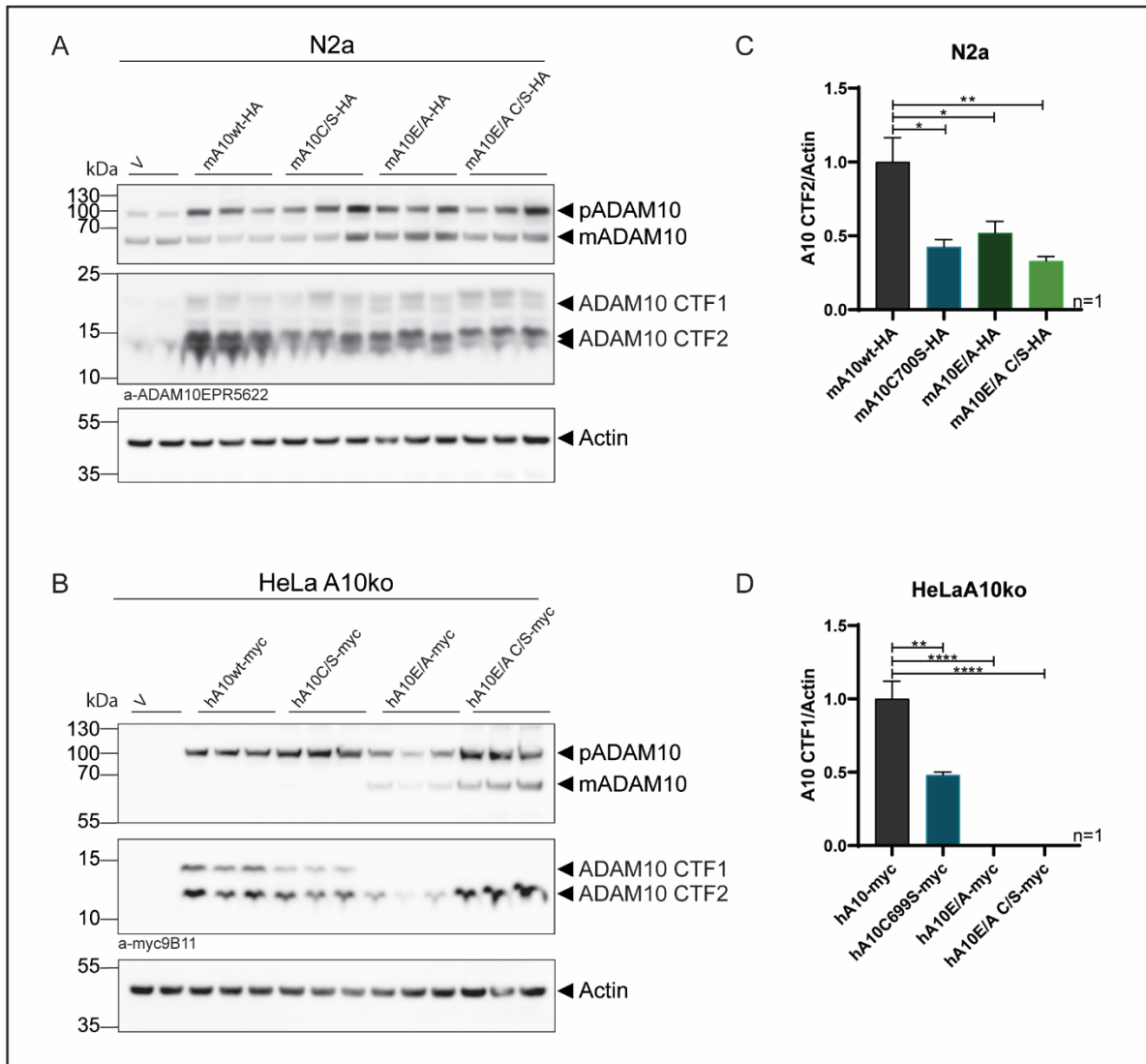


Figure 23: Analyses of transfected N2a and HeLaADAM10 knockout cells indicate ADAM10 CTF generation as a function of self-shedding which is impaired after transfection of a palmitoylation-deficient mutant and an inactive ADAM10 mutant. Immunoblot analyses of cell lysates of murine N2A neuroblastoma cells (N2a) are shown in **A** and HeLa ADAM10 knockout cells (HeLaA10ko) in **B**. Cells were either transfected with an empty vector (V), wildtype hA10-myc/mA10-HA (wt), mutated hA10C699S-myc/mA10C700S-HA or inactive ADAM10 mutants either with (hA10E/AC/S-myc or mA10E/AC/S-HA) or without mutation (hA10E/A-myc or mA10E/A-HA) of the palmitoylation site. The blots were incubated with antibodies directed against the C-terminus of ADAM10 and actin, respectively. **C** and **D** depict quantifications of ADAM10 CTF expression in relation to actin as loading control. Values are normalized to wildtype ADAM10 transfection level and shown as mean $\pm$ SEM. Significance was tested using a one way ANOVA test (* $p < 0.05$, ** $p < 0.005$). ADAM10 CTF 2 generation in N2a cells was significantly impaired in cell lysates of the inactive mutant transfected cells and as already shown in the palmitoylation mutant transfected cells. ADAM10 CTF 1 in HeLaA10ko cells is not detectable when expressing the inactive mutant.

In summary, the experiments suggest that ADAM10 autoproteolysis and palmitoylation could be linked, as impaired autoproteolysis is responsible for the altered ADAM10 CTF generation after expression of the palmitoylation-deficient ADAM10 mutant. This is supported by similar findings observed in cells overexpressing an inactive ADAM10

mutant (Fig. 23) and in cells with endogenous ADAM10 after selective inhibition of ADAM10 that led to a reduction of the ADAM10 CTF levels (Fig. 21).

4.5.4 Membrane association of ADAM10 CTFs

ADAM10 as a transmembrane spanning protein can be proteolysed to form a soluble N-terminal and a membrane-bound C-terminal part (Peron et al., 2018) which is further processed into soluble and intracellular fragments. Characterising the location and potential functions of ADAM10's C-terminal fragments can provide valuable insights into the complex biology, fate and half-life of this protein and may be helpful to understand the role of C-terminal palmitoylation on the generation of these fragments. Because the palmitoylation could cause different alterations that could also lead to altered subcellular locations of the ADAM10 CTFs, a fractionation of membrane and soluble compartments was performed to isolate proteins in their respective compartments. HeLa ADAM10 knockout cells were transfected with either wildtype or palmitoylation-deficient ADAM10 or an empty vector as negative control. After cell lysis using sonication in a fractionation buffer, the lysates containing either cells expressing the wildtype or palmitoylation-deficient ADAM10 mutant were ultracentrifuged to separate soluble proteins in the supernatant from membrane-bound proteins in the pellet. The supernatant was collected and prepared for SDS-PAGE analysis of soluble fractions. Two centrifugation steps lead to the membrane-bound fraction in the remaining pellet. An SDS-PAGE and immunoblotting was performed to determine the distribution of proteins between the fractions.

Clear signals of ADAM10, GAPDH, and EGFR could be detected in total cell lysates confirming sufficient protein expression. GAPDH as a soluble protein and EGF-Receptor as a membrane-bound protein verified proper compartment separation. The vector control, wildtype, and palmitoylation-deficient ADAM10 transfected samples are indicated in all fractions, respectively (Fig. 24).

Based on the immunoblot analysis, it appears that both the full-length form of ADAM10 and the ADAM10 C-terminal fragments are membrane-bound, as indicated by the presence of prominent signals in the membrane-bound fraction at molecular weights of 100, 70, 20, and 15 kDa, respectively. Furthermore, no signal of any ADAM10 fragment was detected in the soluble fractions of both wildtype and palmitoylation-deficient ADAM10 (Fig. 24). This suggests that the CTFs as well as the full-length form of ADAM10 are associated with the cellular membrane. However, it is unclear from this

experimental setting whether the CTFs and the full-length form of ADAM10 are localised to different membrane-bound compartments.

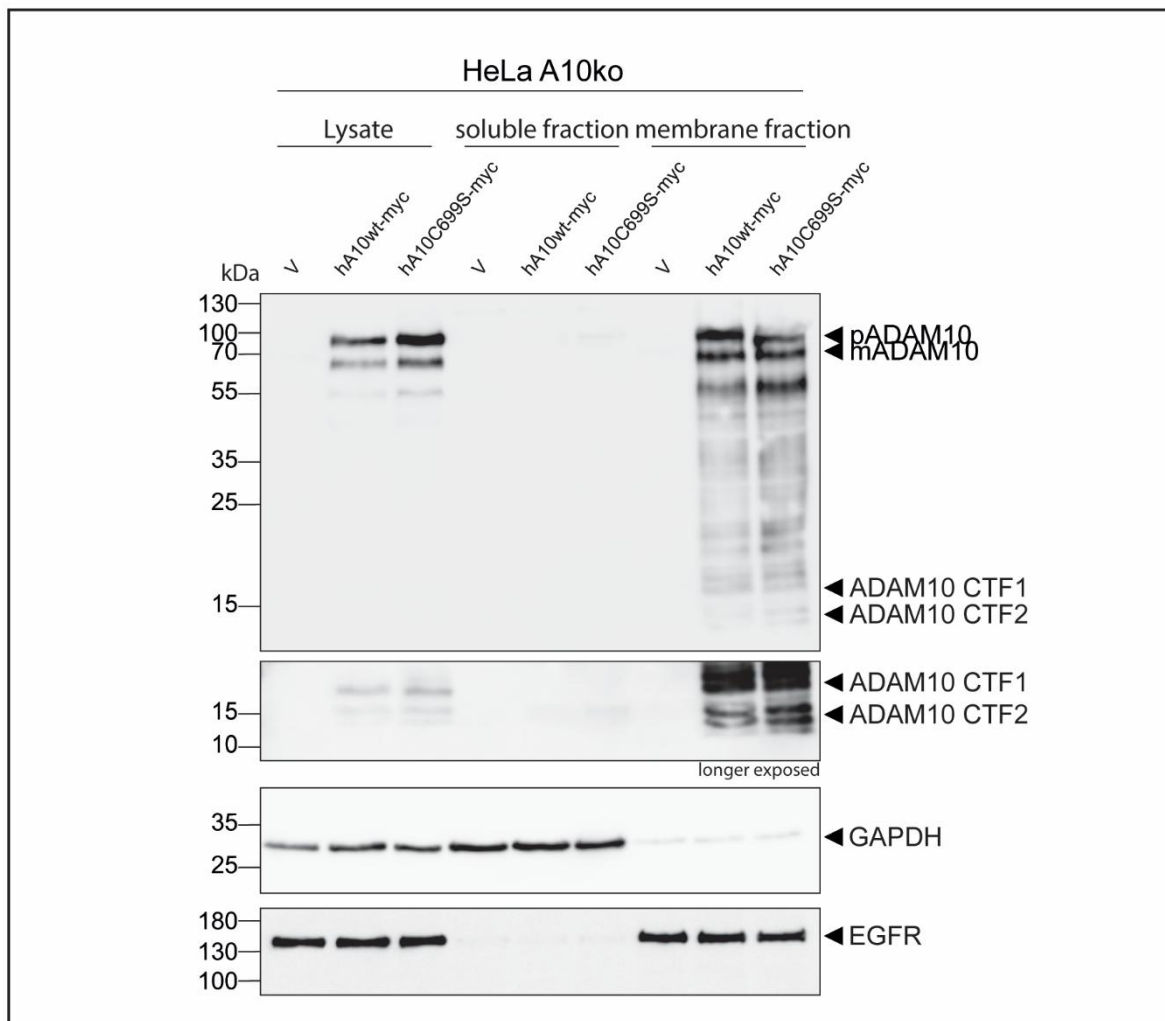


Figure 24: Localisation of ADAM10 CTFs in membranes. Immunoblot analyses of cell lysates of HeLa ADAM10 knockout (HeLaA10ko) cells are shown. Cells were transfected either with an empty vector (V), wildtype (wt), or mutated version (C699S) of human (h) ADAM10. The proform (pADAM10), the active, mature form (mADAM10), and both CTFs of ADAM10 are indicated by arrowheads. Compartment association and equal protein loading were controlled by GAPDH for the soluble fractions and EGF-Receptor was used to demonstrate its presence in membranous fractions.

4.6 Investigation of the influence of palmitoylation on ADAM10's interaction partners

Proteins often do not function as a single protein but in a complex of proteins. Protein-protein interactions are important for many biological and biochemical processes that ensure cell metabolism and survival (Rabbani et al., 2018).

4.6.1 Interactions with SAP-97 and AP-2

Since palmitoylation can affect protein-protein interactions it is interesting to find out whether this is also the case for ADAM10 and its interaction partners. First, already identified interaction partners of ADAM10 were studied.

The proteins SAP-97 and AP-2 regulate the surface levels and activity of ADAM10. SAP-97 is a scaffolding protein expressed in neuronal synapses, and its interaction with ADAM10 is important for ADAM10's localisation and activity (Marcello et al., 2013). The AP-2 adaptor complex promotes ADAM10 endocytosis from the plasma membrane (Marcello et al., 2013). SAP-97 and AP-2 have a converse role in regulating ADAM10 maintenance at excitatory synapses, where SAP-97 mediates trafficking of ADAM10 from the Golgi apparatus to the surface during long-term potentiation, while AP-2 mediates ADAM10 endocytosis during long-term depression. Since both SAP-97 and AP-2 bind to the C-terminal part of ADAM10, it is interesting to investigate if the C-terminal located palmitoylation of ADAM10 has any direct influence on the binding of these proteins. The potential cellular localisation where interactions between ADAM10 and SAP-97 and AP-2 may occur are indicated (Fig. 25A).

To investigate the effect of palmitoylation on ADAM10's interaction with SAP-97 and AP-2, immunoprecipitation experiments were conducted (Fig. 25). Since SAP-97 and AP-2 are highly expressed in synapses of neuronal cells, the murine neuroblastoma cell line N2a was utilised. An HA-tagged, murine ADAM10 expression plasmid was transfected into N2a cells to compare the interaction with palmitoylated and palmitoylation-deficient ADAM10. Immunoprecipitation was performed using an antibody against the HA-tag.

After immunoblotting using an antibody against the C-terminus of ADAM10, detection of ADAM10 in the wildtype and palmitoylation-deficient immunoprecipitate resulted in a clear signal at a molecular weight of 100 kDa corresponding to the full-length proform of ADAM10. No ADAM10 was detected in the vector-transfected control (V), antibody (AB-ctrl), and bead control (bead-ctrl) confirming specific precipitation of ADAM10. Detection of ADAM10 in the input fraction confirmed sufficient protease expression. The ADAM10 CTF is reduced in the palmitoylation-deficient ADAM10 transfected total cell lysate and IP fraction as expected from previous experiments (Fig. 25).

Co-immunoprecipitations (CoIP) of SAP-97 and AP-2 were observed in the wildtype and palmitoylation-deficient ADAM10 immunoprecipitates, using antibodies directed against SAP-97 and against adaptin α as a subunit of the AP-2 complex. Considerable expressions of SAP-97 and adaptin α were detected in the input fraction. SAP-97

appeared to be present in higher amounts in the ADAM10 C700S-HA precipitated sample. However, the palmitoylation deficiency seems not to directly influence co-precipitation of AP-2 since signal intensities of adaptin α are similar comparing wildtype and palmitoylation-deficient precipitates.

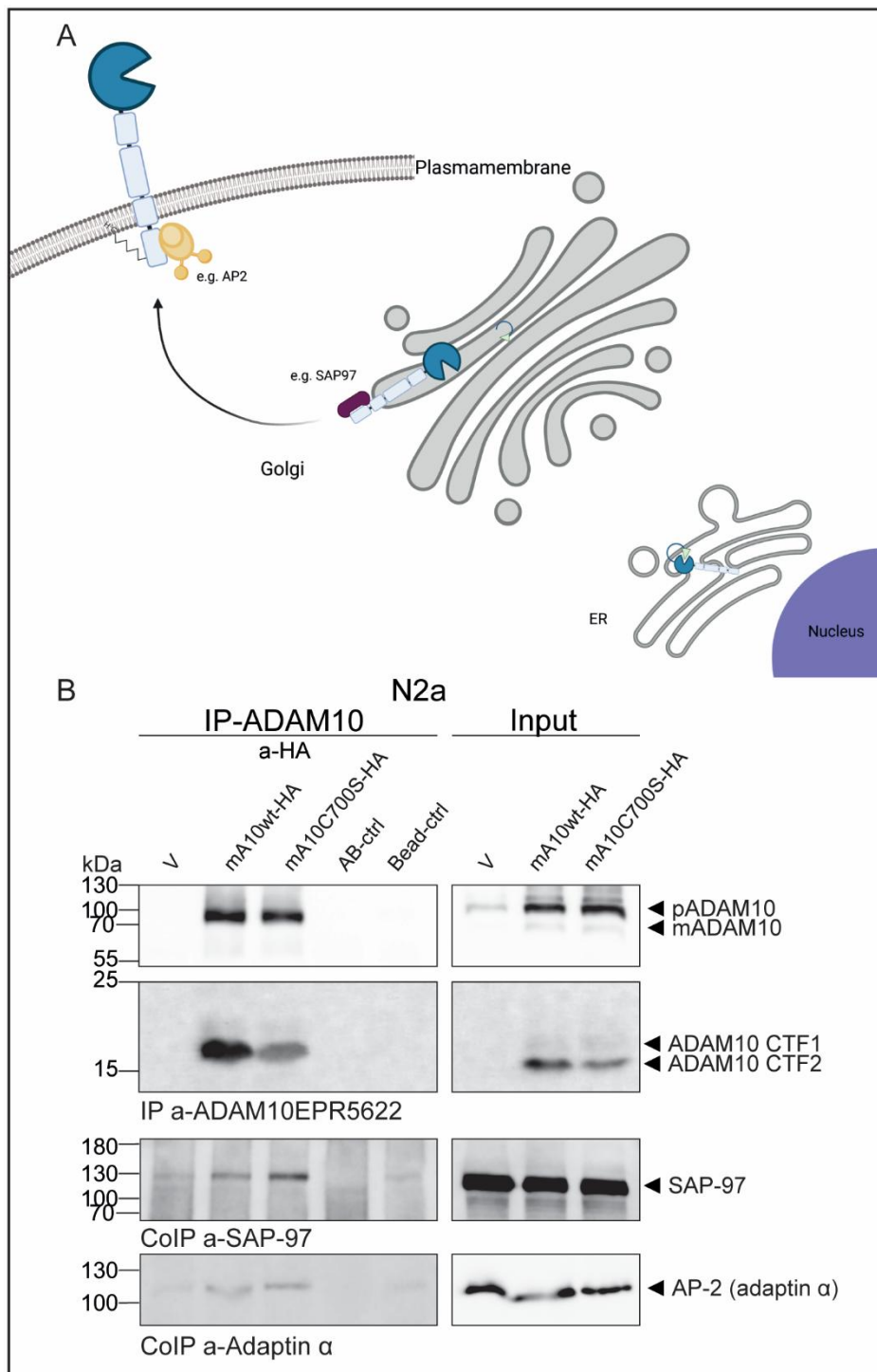


Figure 25: Expression of wildtype vs palmitoylation-deficient murine and co-immunoprecipitation experiments to analyse ADAM10 interactions with known interactors. A schematic overview of two known interaction partners of ADAM10 is shown in **A**. Immunoblot analyses of immunoprecipitations of ADAM10 in cell lysates of murine N2A neuroblastoma cells (N2a) are shown in **B**. The blots were incubated with antibodies directed against the C-terminus of ADAM10 and potential interaction partners as indicated,

respectively. N2a cells were either transfected with an empty vector (V), wildtype (wt), or mutated version (C700S) of murine (m) ADAM10. Immunoprecipitation (IP-ADAM10) was performed with an antibody directed against the HA-tag. Lysis buffer incubated with antibody (AB-ctrl) and cell lysate without antibody (bead-ctrl) were used as negative control. The proform of ADAM10 (pADAM10) and the active, mature form of ADAM10 (mADAM10) are indicated by arrowheads. SAP-97 and adaptin α co-immunoprecipitations (CoIP) are shown in **B** with differences in coprecipitation of SAP-97 and no differences when adaptin α as a subunit of AP-2 was co-precipitated comparing wildtype and palmitoylation-deficient ADAM10 expression.

4.6.2 Comparing interaction partners of wildtype and palmitoylation-deficient ADAM10

To further address proteins that interact differently with ADAM10 when it is palmitoylated or not, a mass spectrometric analysis was performed after precipitation of ADAM10 from cell lysates.

To this end, HeLa ADAM10 knockout cells were transfected with either wildtype or palmitoylation-deficient ADAM10 in triplicates. Cells transfected with an empty vector were used to control for unspecific binding. An immunoprecipitation (IP) was carried out as described previously and then analysed via mass spectrometry (MS) (Fig. 26A). The MS-part including the evaluation of the data was performed in collaboration with Dr. Tomas Kouldelka and Prof. Dr. Andreas Tholey (Institute of Experimental Medicine, CAU Kiel)) (Fig. 26B).

Shown are comparisons between precipitated proteins of wildtype ADAM10 immunoprecipitations and controls and of ADAM10 C699S precipitates and controls (Fig. 26B). The log₂ of differences is depicted on the x-axis and the -log₁₀ of the p-value on the y-axis. Hits with a red dot had significant t-tests with a p-value below 0.05. The proteins that bound specifically to ADAM10 or its palmitoylation-deficient mutant, respectively, are depicted on the right of each volcano plot. The left side shows proteins that bound unspecifically to beads.

Proteins that specifically co-precipitated with either wildtype or palmitoylation-deficient ADAM10, as opposed to the control, and exhibited different abundance levels between the ADAM10 variants were identified. Therefore, t-tests were conducted to compare proteins co-precipitated from immunoprecipitations of wildtype or palmitoylation-deficient ADAM10 to those in the control precipitates, enabling the extraction of unspecifically bound proteins. For the remaining proteins that specifically bound to wildtype or palmitoylation-deficient ADAM10, another t-test was performed comparing hits in the wildtype and palmitoylation-deficient ADAM10 precipitates (Fig. 26C). A

significant t-test result was achieved with a p-value below 0.05 and a cut-off of a two standard deviation (SD) fold change.

Only a few proteins had significant differences in the binding affinity to the palmitoylation-deficient ADAM10. These proteins were matched to three different groups:

1. Proteins highlighted with a red dot were significantly more detected in both versions of ADAM10 than in the controls and they had significant differences in the amount of co-precipitated protein. This means that the proteins bind to both ADAM10 and ADAM10 C699S but with a different abundance.
2. The green highlighted proteins, on the one hand, only bound specifically to the wildtype ADAM10 and were significantly different in the comparison between both ADAM10 versions which indicates binding to wildtype but not palmitoylation-deficient ADAM10.
3. Proteins highlighted with an orange dot, on the other hand, were significantly more detected in the IPs of ADAM10 C699S than in the control and not in the IPs of wildtype ADAM10 meaning that they specifically bind to the palmitoylation-deficient mutant but not to the wildtype version.

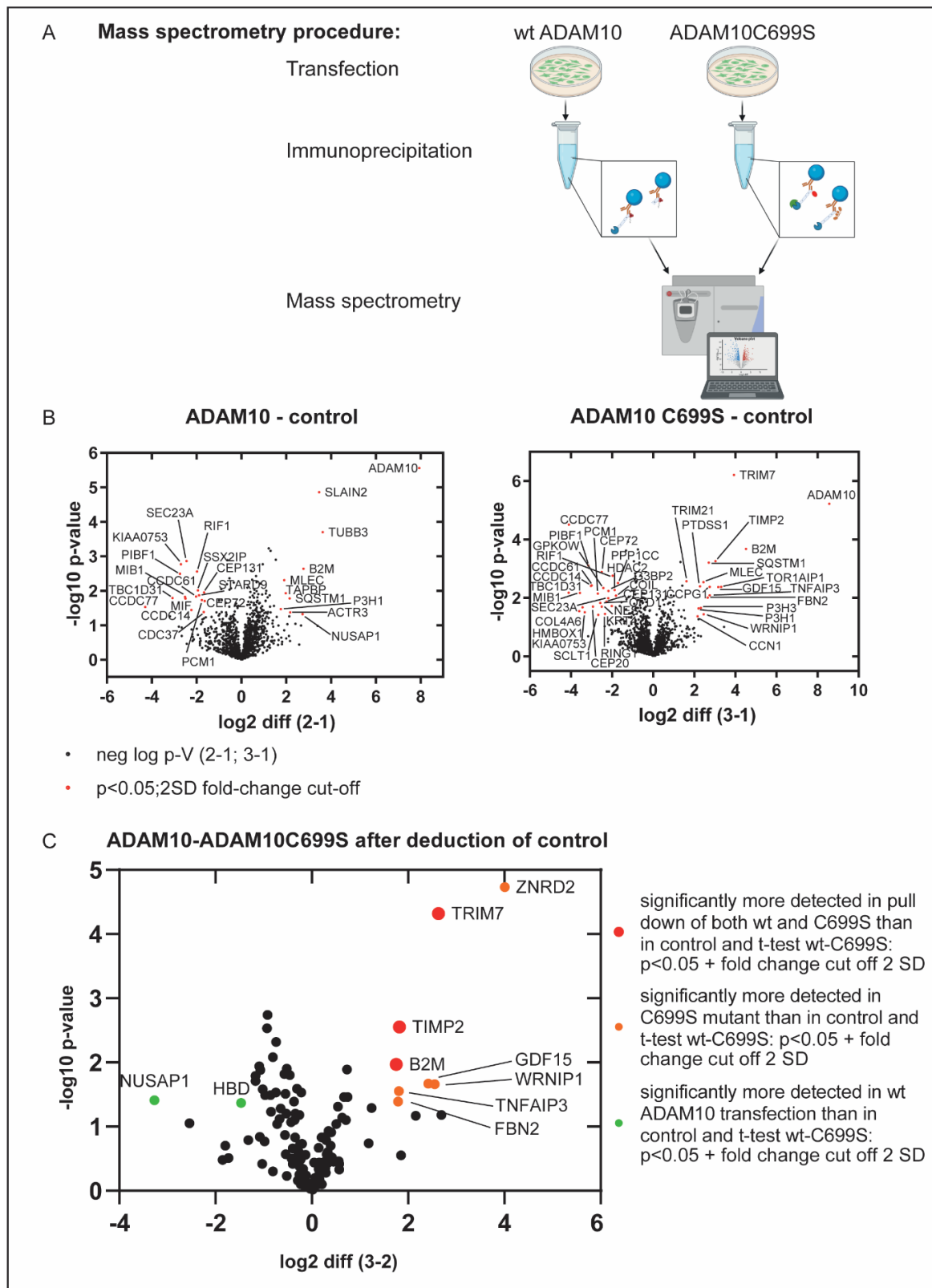


Figure 26: Identification of differences in interactions with ADAM10 or ADAM10 palmitoylation-deficient mutant. Mass spectrometry analyses of immunoprecipitations of ADAM10 are shown. **A** depicts a schematic overview of the implemented procedures leading to the volcano plots. HeLaADAM10 knockout cells were either transfected with ADAM10, ADAM10C699S, or an empty vector for control of unspecific binding. Immunoprecipitations were kindly analysed by T. Koudelka via mass spectrometry. **B** shows the result of the mass spectrometry as volcano plots of protein lysates. ADAM10 or ADAM10C699S were compared to the control vector. The right part of each plot shows the proteins that bound more to ADAM10

than to the control vector. The red dots mark the proteins that were filtered for a p-value of <0,05 and a fold change cut-off of 2 SD. **C** presents the comparison between proteins that bound to ADAM10 and ADAM10C699S after deduction of the unspecifically bound proteins that were also present in the control. Red dots show the proteins that were present in both wt and C699S immunoprecipitations but had a significant difference in the amount of co-precipitation. Green dots mark the proteins that were significantly detected in the wt ADAM10 IP and had a significant difference comparing wt and palmitoylation-deficient ADAM10 IP. The orange dots on the other side show proteins that were significantly detected in the ADAM10C699S IP and had a significant difference comparing wt and palmitoylation-deficient ADAM10 IP. 1: vector control, 2: wildtype ADAM10, 3: ADAM10 C699S.

Table 18 displays those proteins identified through this MS analysis of the IPs that are of interest due to their specific function or subcellular location. Among the new candidates for interaction partners of both wildtype and palmitoylation-deficient ADAM10 in the red group, tissue inhibitor of metalloproteinases 2 (TIMP2) was found to bind more to the palmitoylation-deficient version. It preferentially inhibits MMPs 2 and 14 but TIMPs are in general known to also interact with and inhibit ADAMs (Amour et al., 2000). Additionally, tripartite motif containing protein 7 (TRIM7), an E3 ubiquitin ligase that may promote tumour proliferation (Chakraborty et al., 2015), was found in this group. Tumour necrosis factor alpha-induced protein 3 (TNFAIP3), a ubiquitin-editing protein involved in anti-inflammatory regulation and cancer biology (Mooney & Sahingur, 2021), was found to only interact with the palmitoylation-deficient ADAM10. In the green labelled group (Fig. 26C), nucleolar and spindle-associated protein 1 (NUSAP1) which binds and stabilises microtubules (Qian et al., 2018), only binds to wildtype ADAM10 and not the palmitoylation-deficient version.

Protein	Function	Localisation	References
TRIM7	Tripartite motif containing protein 7 - E3 ubiquitin ligase: polyubiquitination of RACO-1 - involved in tumour cell proliferation, glycogen metabolism - antiviral roles in norovirus infection -IFN induction; pro-viral in ZIKA virus infection	Golgi apparatus, cytoplasm	(Giraldo, Hage, et al., 2020)
TIMP2	tissue inhibitor of metalloproteinases 2 - inhibits MMP-2 and-14 preferentially	extracellular	(Hayakawa et al., 1994; Kaczorowska et al., 2020)

TNFAIP3	tumour necrosis factor alpha-induced protein 3; A20	- Ubiquitin-editing protein, ends nuclear factor (NF)-κB activation → anti-inflammatory - possibly anti-apoptotic because of NF-κB inhibition → involved in cancer biology	lysosome, nucleus, cytoplasm	(Mooney & Sahingur, 2021)
NUSAP1	Nucleolar and spindle-associated protein 1	- binds and stabilizes microtubules - linked to poor prognosis of several forms of cancer	Intracellular, cytoskeleton, cytosol, nucleus	(Qian et al., 2018)

Table 18: Function and localisation of proteins detected in the mass spectrometry analysis of differences in potential ADAM10 and ADAM10 C699S interaction partners

5 Discussion

The function of ADAM10 is well investigated in physiological and pathophysiological processes. However, how to control and regulate its activity remains largely unclear. Although several proteins, such as Tspan15, interact with ADAM10 and regulate the protease—Tspan15, for example, promotes the maturation and trafficking of ADAM10 to the cell surface (Prox, Willenbrock, et al., 2012)—further investigations are needed to understand how (posttranslational) modifications of ADAM10 itself contribute to its regulation. The present study aimed to elucidate the role of protein S-palmitoylation, a specific regulatory posttranslational modification, for ADAM10 function.

Protein S-palmitoylation has raised interest since it is a reversible modification with widespread effects. This lipid modification increases the hydrophobicity potentially localising the modified protein to distinct membrane compartments such as lipid rafts or causing a tilt in the transmembrane domain leading to additional functional changes. The attached palmitate residue can also change the association with interacting proteins (Jin et al., 2021).

5.1 Identification of ADAM10 as a palmitoylated protein

Technical advances in the detection of palmitoylated proteins allowed assessment in proteome-wide scale characterisations. A large-scale proteomic approach for palmitoyl protein identification and site characterisation was used, revealing among many other proteins ADAM10 as a potentially palmitoylated protein with a medium confidence level (Yang et al., 2010). Preliminary work confirmed such findings and described ADAM10 in mouse brain tissue and MEF cells, as well as in HeLa cells, palmitoylated (Fig. 8A, B, 9).

The results presented in this study substantiate that ADAM10 is indeed palmitoylated. In fact, using three different methods, an acyl-resin assisted capture (acyl-RAC) assay, an acyl-PEGylation exchange assay, and a Click chemistry method, the palmitoylation of ADAM10 could be confirmed (Fig. 8C, D, 10, 12). An acyl-RAC assay was performed to verify the S-acylation of ADAM10 in murine neuroblastoma N2a cells and HeLa cells. The results showed that ADAM10 is indeed acylated, as it was precipitated by the beads in the fraction containing acylated proteins but not in the negative control fraction (Fig. 8). The Click assay confirmed that the acylation is specifically an S-palmitoylation showing the inserted fluorescent palmitate analogon 17-ODYA in HeLa and HeLa ADAM10 knockout cells transfected with an ADAM10 expression plasmid (Fig. 9, 12).

5.1.1 Characterisation of the palmitoylation sites in ADAM10

Palmitoylation typically occurs on cysteine residues in the cytoplasmic domains of proteins or towards the inner membrane within transmembrane domains (Heiler et al., 2015; Main & Fuller, 2022), as these regions are accessible to palmitoyltransferases. In the case of ADAM10, which exists in both pro and mature forms within cells, its cytosolic C-terminus contains only one cysteine residue (Fig. 11), leading to four possible forms of ADAM10, a palmitoylated or non-palmitoylated proform and a palmitoylated or non-palmitoylated mature form (Fig. 10B). While the acyl-RAC and the Click assay allow qualitative assessment of protein S-palmitoylation (Gao & Hannoush, 2018; Main & Fuller, 2022), the acyl-PEGylation exchange assay (Fig. 10) can reveal different palmitoylation states in a semi-quantitative manner (Specia & Diaz, 2020). The use of an acyl-PEGylation assay revealed three distinct forms of ADAM10, as indicated by three clear bands in the PEG-linked fraction (Fig. 10A). Depending on the level of palmitoylation, ADAM10 may appear as either one or two distinct bands per form, with the lower band representing the non-palmitoylated protein and the band with increased molecular weight indicating palmitoylation.

The explicit correlation of the shifted bands with the potential palmitoylation states of the pro- and mature form of ADAM10 was difficult because of the fuzziness of the bands. The three bands indicate that one form of ADAM10, the pro- or mature form, is present in only one palmitoylation state whereas the other one is present in a palmitoylated and a non-palmitoylated version. No band at the height of the mature form of ADAM10 could be observed in the cleaved fraction with attached PEG molecules indicating that the mature form of ADAM10 is palmitoylated.

There are potential reasons why one variant is fully palmitoylated while the other is not. One idea is that ADAM10, after being synthesised as the proform, may undergo palmitoylation in the Golgi apparatus, and subsequently remain palmitoylated as a mature form. Thus, the proform could exist in two distinct states: one that is newly synthesised within the ER and not yet palmitoylated, and another one most likely having exported out of the ER that is already palmitoylated. The mature form would only exhibit the palmitoylated state.

5.2 Consequences of the palmitoylation of ADAM10

Protein palmitoylation plays an important role in different cellular processes such as regulating protein stability, subcellular localisation, membrane trafficking, and enzymatic activity. Palmitoylation or depalmitoylation can cycle on timescales ranging from seconds to hours, allowing proteins to shuttle between specific membrane compartments and microdomains (Won & Martin, 2018). Dysregulated palmitoylation has been linked to many human diseases, such as viral infections, cancer, diabetes, and neurodegenerative disorders (Jin et al., 2021).

5.2.1 The palmitoylation has no apparent effect on the expression and maturation of ADAM10

The prodomain of ADAM10 not only keeps the protease in its inactive state but is also important for proper protein folding (Anders et al., 2001). It is cleaved by proprotein convertases in the Golgi apparatus (Roghani et al., 1999). This maturation into its active form is an essential step for the activation of the protease and its shedding ability. Downregulated maturation by altering the prodomain can lead to impaired ADAM10-mediated α -secretase activity and increased A β levels which are associated with the development of senile plaques in Alzheimer's disease (Suh et al., 2013).

The levels of pro and mature ADAM10 were equal in the wildtype and palmitoylation-deficient ADAM10-transfected samples in N2a, HeLa, and HeLa ADAM10 knockout cells (Fig. 13) indicating that the palmitoylation deficiency of ADAM10 had no apparent effect on the expression of ADAM10 or its maturation. It is possible that the palmitoylation which occurs at the cytosolic C-terminus of the protein does not affect the maturation because the prodomain is cleaved from the lumenally located N-terminus. These separated positions can explain that both processes can proceed without affecting each other.

Because a palmitoylation is known to impair compartment localisation, it was further investigated, if the subcellular localisation of ADAM10 is impaired in the palmitoylation-deficient mutant. The immunofluorescence staining of HeLa cells comparing transfected wildtype and palmitoylation-deficient ADAM10 showed no obvious differences in the subcellular location of ADAM10 (Fig. 14). Probably due to the overexpression of the ADAM10 plasmids, the ER staining is pronounced and little post ER structures were detected.

5.2.2 The cell surface expression is impaired in the palmitoylation-deficient ADAM10 mutant

The localisation of a protease at the plasma membrane plays an important role in its activity and regulatory processes. There are different mechanisms how the palmitoylation of ADAM10 can alter its membrane localisation. In general, a palmitoylation can contribute to membrane stability because of the increased hydrophobicity but can also be involved in protein-protein interactions that lead to distinct membrane localisations (Charollais & Van Der Goot, 2009). Furthermore, a palmitoylation deficiency could cause a retention in the ER or Golgi apparatus.

As mentioned above, no differences in the localisation of palmitoylation-deficient ADAM10 compared to wildtype ADAM10 in immunohistochemistry analyses were noted and the maturation and cellular expression of ADAM10 seemed unaffected by the palmitoylation (Fig. 13, 14). Importantly, the level of surface expression of the palmitoylation-deficient ADAM10 mutant was found decreased when analysed by surface biotinylation experiments (Fig. 15).

The TetraspaninC8 group is known to enhance the maturation and surface presence of ADAM10. Tspan15 as a member of the TspanC8 family interacts with ADAM10 already in the ER likely masking the ER retention motif of ADAM10 in the C-terminal domain and contributing to localisation and stability at the cell surface (Prox, Willenbrock, et al., 2012). Tspan15 also promotes the maturation of ADAM10 (Prox, Willenbrock, et al., 2012).

Direct disruption of Tspan15/ADAM10 interaction by the palmitoylation of ADAM10 would likely lead to an alteration of both surface trafficking and maturation and is therefore unlikely to be involved in an isolated decrease in surface expression of the palmitoylation-deficient ADAM10. Conclusive with the unaltered ER colocalisation and maturation of ADAM10, immunoprecipitations of wildtype and palmitoylation-deficient ADAM10 showed no differences in the interaction with Tspan15 (Fig. 16A).

Tspan15 interacts with ADAM10 through the extracellular stalk region (Noy et al., 2016). The functional association between ADAM10 and Tspan15 is primarily mediated by the large extracellular loop domain, with potential additional contacts between the transmembrane regions. Particularly, the interaction of ADAM10 and Tspan15 is reduced but not eliminated by mutating the conserved N166A/Y168A/H169A site of Tspan15. This was evidenced by the partial maturation of ADAM10 and the increased expression of Tspan15 when co-expressed with ADAM10 (Lipper et al., 2022). The extracellular located binding site of ADAM10 and

Tspan15 could explain why the palmitoylation at the cytoplasmic C-terminus has no apparent influence on the binding affinity of Tspan15 and ADAM10.

Several other mechanisms to enhance the trafficking of ADAM10 to the plasma membrane are linked to proteins interacting at the C-terminus such as the interaction with SAP-97 and the clathrin adaptor AP-2. In the studies of Marcello et al., the role of SAP-97 in neurons as an interaction partner that directly binds to the C-terminus of ADAM10 was shown. The Golgi exit and trafficking of ADAM10 to the plasma membrane are promoted by this interaction leading to increased proteolytic processing of APP generating the α -secretase product sAPP α (Marcello et al., 2007).

Marcello et al. further identified that the residues 735R and 737R which are located within the last 15 amino acids of ADAM10's C-terminal domain are required for the AP-2 interaction with ADAM10, which is required for ADAM10 endocytosis and modulation of its plasma membrane levels. The ADAM10/AP-2 association was increased in the hippocampi of AD patients, and activity-dependent synaptic plasticity induced ADAM10 endocytosis through AP-2 association (Marcello et al., 2013).

Interestingly, the interaction of ADAM10 with SAP-97 was increased in the palmitoylation-deficient ADAM10 precipitate from N2a cells and the interaction with AP-2 showed no apparent alterations comparing wildtype and palmitoylation-deficient ADAM10 (Fig. 25B).

The increased co-precipitation of SAP-97 in the ADAM10 palmitoylation-deficient precipitate stands somewhat in contrast to the decreased surface expression of the palmitoylation-deficient mutant making more complicated regulatory mechanisms likely. An increased interaction of the palmitoylation-deficient ADAM10 with SAP-97 could show insufficient compensatory mechanisms to enhance the decreased surface expression of the ADAM10 mutant. What should not be left unmentioned is that the surface biotinylation was conducted twice and the immunoprecipitation only once and further approaches such as a flow cytometry method are necessary to confirm the decreased surface expression of the palmitoylation-deficient ADAM10.

However, Marcello et al.'s data suggests that the binding site of ADAM10 for SAP-97 and AP-2 are separate, with AP-2 binding at a distance from SAP-97. While the last 15 amino acids of ADAM10's cytoplasmic tail are crucial for proper AP-2 binding, the membrane proximal proline-rich domain is essential for SAP-97 binding (Marcello et al., 2013). The palmitoylation site of ADAM10 is closer to the SAP-97 binding site which could explain that the interaction between ADAM10 and SAP-97 is affected by ADAM10's palmitoylation but not the interaction with AP-2.

In conclusion, ADAM10 is expressed independently from its palmitoylation status in cells but surface expression of the palmitoylation-deficient ADAM10 mutant is reduced. An altered cellular localisation between the expression of the palmitoylation-deficient ADAM10 and the wildtype version was not observed in immunohistochemistry experiments. That doesn't mean an alteration due to the palmitoylation of ADAM10 in subcellular compartments or within membrane segments like lipid rafts is not existing. Further investigation is necessary to investigate the localisation within the cellular pathway and membrane sections.

5.2.3 Palmitoylation of ADAM10 as a substrate-specific regulatory mechanism

The activity of a protease is not only dependent on the function of the protease active domain but often essentially dependent on other domains. Therefore, it is difficult to obtain information if palmitoylation is required for the direct function of a protease. The regulation of the surface expression of a given protein is for example an important mechanism to control the function of a membrane expressed protein, e.g. a receptor. Further, there are other possible mechanisms such as the trafficking into intramembranous segments like lipid rafts, or the proximity of substrate and protease that contribute to more substrate-specific regulations (Charollais & Van Der Goot, 2009).

This study aimed to investigate whether ADAM10's palmitoylation contributes to regulatory mechanisms of its shedding activity. Wildtype or palmitoylation-deficient ADAM10 were expressed in HeLa cells and the effects of ADAM10's palmitoylation on the shedding of N-Cadherin and APP, two substrates of ADAM10 were analysed. The results revealed that there was no significant difference in the degree of shedding of N-Cadherin between the wildtype and palmitoylation-deficient ADAM10 (Fig. 16). The ADAM10-mediated cleavage product of N-Cadherin could be identified through co-expression of Tspan15 increasing its generation which aligns with findings from previous studies (Jouannet et al., 2016).

Interestingly, there was a notable decrease in the ADAM10-mediated APP CTF (Chow et al., 2010) generation relative to the full-length APP form in cell lysates that were transfected with the ADAM10 mutant deficient in palmitoylation (Fig. 17). Additionally, the co-expression of Tspan15 resulted in an increase of the APP cleavage product (CTF) independently from the palmitoylation, and it even restored its generation in cells

that were transfected with the palmitoylation-deficient mutant (Fig.17B). The ADAM10-mediated cleavage of APP describes a non-amyloidogenic pathway producing an N-terminal soluble, neuroprotective APP α fragment (sAPP α) and a membrane-bound C-terminal fragment of APP (APP C83) (Chow et al., 2010). Further RIP by the γ -secretase releases an intracellular APP C-terminal fragment (O'Brien & Wong, 2011). ADAM10 is mainly active at the plasma membrane, where it cleaves its substrates. Interestingly, ADAM10's palmitoylation deficiency affects APP cleavage but not the shedding of N-Cadherin which suggests that ADAM10's palmitoylation may regulate the cleavage of specific substrates. Substrate recognition plays a crucial role in substrate-specific activity of a protease. The palmitoylation could alter ADAM10's conformation and therefore cause less efficient interaction with APP but not its interaction with N-Cadherin.

Protein-protein interactions can also contribute to the substrate-dependent activity of a protease. ADAM10 interacts with several proteins that modulate its activity, localisation, and substrate specificity. ADAM10-mediated cleavage of both N-Cadherin and APP can be enhanced by interaction with Tspan15 (Jouannet et al., 2016; Seipold et al., 2018). However, the interaction of ADAM10 and Tspan15 was independent from ADAM10's palmitoylation. Considering the wide range of substrates that ADAM10 targets (Reiss & Saftig, 2009), it is possible that ADAM10's palmitoylation could still impact its activity towards specific substrates and further studies are necessary to elaborate possible effects.

5.3 ADAM10's palmitoylation regulates its proteolytic processing

ADAM10 is processed to the active, mature form, which can then be further proteolytically processed to generate N- and C-terminal fragments. One of the topics of this study was the impact of palmitoylation on the generation of ADAM10's C-terminal fragments (CTFs). ADAM10 itself is subject to sheddases and is further processed by regulated intramembrane proteolysis (RIP) (Tousseyn et al., 2009). ADAM9 and -15 contribute to the ectodomain shedding of ADAM10 resulting in a 10 kDa C-terminal fragment. While ADAM9 and -15 deficiency in MEF cells and brain homogenates reduced the generation of this fragment (Tousseyn et al., 2009), it did not completely disappear, suggesting the involvement of other proteases in the shedding process.

Wildtype and palmitoylation-deficient versions of ADAM10 were expressed in N2a cells and HeLa cells lacking endogenous ADAM10. Both cell lines generated two CTFs of ADAM10, of about 10 to 20 kDa, which were decreased in cells expressing palmitoylation-deficient ADAM10 compared to cells expressing wildtype ADAM10. The smaller CTF (ADAM10 CTF 2) was more prominent in N2a cells, whereas both CTFs were detectable in HeLa cells. In N2a cells, the reduction in signal intensity of ADAM10 CTFs was more pronounced for ADAM10 CTF 2 than for ADAM10 CTF 1 (Fig. 19B). In HeLa ADAM10ko cells, the reduction of the ADAM10 CTF 1 was more pronounced (Fig. 19D).

Using 2-bromopalmitic acid (2-BP) to pharmacologically inhibit palmitoylation in cells transfected with a wildtype ADAM10 expression construct, the signal intensities of both CTFs were reduced (Fig. 20). This finding aligns with decreased ADAM10 CTF levels in N2a and HeLaA10ko cells transfected with palmitoylation-deficient mutants. Taken together, these results suggest that palmitoylation of ADAM10 is necessary for the generation of ADAM10 CTFs with its absence impairing CTF generation. Co-expression of Tspan15 with ADAM10 led to reduced ADAM10 CTF1 (Fig. 19D). The assumption of ADAM10 CTF as a shedding product of ADAM10 suggests that Tspan15 may stabilise ADAM10 unaffected by palmitoylation status, as evidenced by the lower expression level of ADAM10 C699S CTFs with Tspan15 overexpression compared to wildtype.

The effect of ADAM10 inhibition and palmitoylation on the generation of ADAM10 CTFs was investigated in cells endogenously expressing ADAM10 (Fig. 21). The results show that selective inhibition of ADAM10 using GI254023X led to a significant reduction in the levels of one of the ADAM10 CTFs, which was also affected by palmitoylation deficiency. This suggests that ADAM10 itself contributes to the generation of this CTF and that its activity is impaired in the palmitoylation-deficient mutant of ADAM10.

In contrast, inhibition of the γ -secretase resulted in an accumulation of the ADAM10 CTF, indicating that the γ -secretase is responsible for further processing of this CTF. These findings are in line with the studies from Tousseyn et al. who already revealed the involvement of the metalloproteases ADAM9 and -15 and the γ -secretase in the proteolytic processing of ADAM10 (Tousseyn et al., 2009).

For further investigations, an experiment overexpressing a protease inactive ADAM10 mutant was performed. The levels of ADAM10 CTF 2 were affected by both protease inactive ADAM10 and palmitoylation-deficient ADAM10 mutants in N2a cells. In HeLa

ADAM10 knockout cells, the ADAM10 CTF 1 disappeared completely in all samples with expressed inactive ADAM10 mutants (Fig. 23). The results confirm that impaired autoproteolysis in the palmitoylation-deficient mutant may be responsible for the altered ADAM10 CTF generation.

In conclusion, this study provides evidence for a link between ADAM10 autoproteolysis and palmitoylation in ADAM10 CTF generation (Fig. 27). Impairment of either process can lead to altered ADAM10 CTF generation, with impaired autoproteolysis having a more pronounced effect.

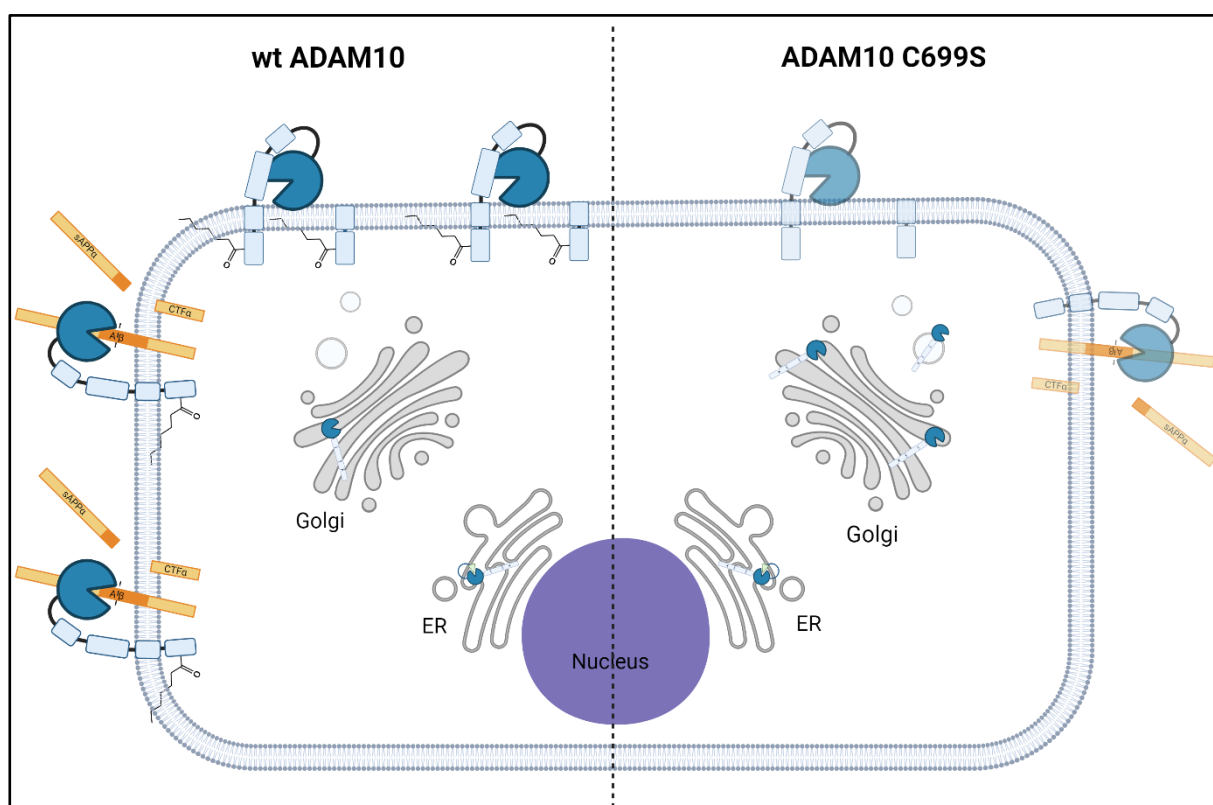


Figure 27: Schematic representation of the effects of palmitoylation on ADAM10. The diagram presents a side-by-side comparison of wildtype ADAM10 and the palmitoylation-deficient mutant in a cellular system, including the plasma membrane, nucleus, ER, and Golgi apparatus. On the left, wildtype ADAM10 is depicted with a palmitate residue in its skeletal formula, while on the right, the palmitoylation-deficient ADAM10 lacks this residue. Based on the results from this study, the surface expression of ADAM10 is reduced in the palmitoylation-deficient mutant, indicated by a smaller quantity of ADAM10 protein on the right. Additionally, the expression of ADAM10 C699S resulted in lower levels of ADAM10 CTF and APP CTF compared to wildtype ADAM10, illustrated by decreased shedding of ADAM10 itself and APP on the right side.

Inhibition of dynamin-mediated endocytosis causes an accumulation of immature and mature ADAM10 but also increased the appearance of a 16 kDa ADAM10 CTF in HEK cells (Carey et al., 2011). This suggests that the cleavage of ADAM10 occurs at the cell surface which is consistent with previous reports that ADAM10 can be processed by the sheddases ADAM9 and -15 (Toussey et al., 2009). ADAM10 also cleaves its

substrates at the plasma membrane and can therefore contribute to its autoproteolysis. Since protein-protein interactions are commonly regulated by palmitoylation, an alteration in the interaction of ADAM10 with proteins like dynamin due to the palmitoylation deficiency could also lead to the observed reduction of the ADAM10 CTF. A palmitoylation deficiency might drive interaction-mediated endocytosis or trafficking into other subcellular compartments where it cannot be shed. The reduced surface expression of the palmitoylation-deficient full-length form of ADAM10 in biotinylation experiments (Fig. 15) supports this assumption.

The correlation of the CTF levels of the inactive ADAM10 mutant and the GI254023X-mediated inhibition of ADAM10 with the CTF levels of the palmitoylation-deficient ADAM10 mutant are indications for a regulation of the autoproteolytic processing of ADAM10 by the palmitoylation. However, an interaction-mediated reduced surface expression as the actual cause of the CTF reduction in ADAM10 C699S transfected cells cannot be excluded. Further studies are needed to elucidate the mechanisms underlying the observed effects of palmitoylation on ADAM10's proteolytic processing.

5.3.1 ADAM10's membrane association is independent of its palmitoylation status

Since palmitoylation increases the hydrophobicity of a protein which can lead to distinct membrane association, it was necessary to investigate whether palmitoylation of ADAM10 influences its membrane association and particularly the appearance of ADAM10's CTFs. HeLa ADAM10 knockout cells were transfected with either wildtype or palmitoylation-deficient ADAM10, or an empty vector as negative control. Dividing membrane and soluble cell compartments, the results suggest that both the full-length form of ADAM10 and its CTFs are membrane-bound, as indicated by their presence in the membrane-bound fraction (Fig. 24). This finding is consistent with previous studies that have reported the membrane-bound nature of ADAM10 CTFs (Peron et al., 2018; Wetzel et al., 2017). Furthermore, both wildtype and palmitoylation-deficient ADAM10 were present in the membrane-bound fraction, indicating that palmitoylation does not obviously influence the membrane association of ADAM10 and its CTFs.

Previous studies have suggested that ADAM10 is localised to different membrane compartments through interaction with tetraspanins such as Tspan5 and -15 (Jouannet et al., 2016) as well as its surface expression may be dependent on ADAM10's activity (Seifert et al., 2021). Therefore, it is also possible that the CTFs and the full-length

form of ADAM10 are localised to different membrane-bound compartments and may have distinct functions depending on their palmitoylation status.

5.4 ADAM10's palmitoylation affects specific protein-protein interactions

Protein-Protein interactions play an important role in the function and regulation of proteases. ADAM10's maturation and trafficking are, for example, promoted by distinct proteins from the TetraspaninC8 group (Jouannet et al., 2016). Further, the interaction with SAP-97 and AP-2 are important regulatory mechanisms for transport to the plasma membrane and endocytic processes in neuronal synapses (Marcello et al., 2013). Since there exist no techniques to predict the effects of palmitoylation of specific proteins, immunoprecipitation experiments and mass spectrometry analyses were conducted to explore how interactions with known and new partners are affected that differentially interact with ADAM10 due to its palmitoylation. The data presented in this study provide insights into the role of palmitoylation in modulating ADAM10's interactions with various proteins. This analysis shows that palmitoylation can influence the interaction of ADAM10 with its partners, such as SAP-97, TRIM7, TIMP2, TNFAIP3, and NUSAP1 (Fig. 25, 26). These proteins have diverse roles in cellular processes such as trafficking, endocytosis, tumour progression, inhibition of proteases, inflammation, and microtubule stabilisation.

The interaction between ADAM10 and SAP-97 or AP-2 is particularly interesting as both proteins have a converse role in regulating ADAM10 maintenance at excitatory synapses. The results suggest that palmitoylation may not have a direct influence on the interaction between ADAM10 and AP-2, but it appears to affect the interaction with SAP-97. This observation highlights the importance of palmitoylation in modulating protein-protein interactions and consequently the subcellular localisation and activity of ADAM10. Although the palmitoylation-deficient ADAM10 mutant interacted more abundantly with SAP-97 in the chosen experimental conditions, ADAM10 was less present at the plasma membrane. This contrasts with the established function of the SAP-97/ADAM10 interaction, which drives ADAM10 towards postsynaptic membranes in hippocampal neurons (Marcello et al., 2007). The results indicate that SAP-97-mediated trafficking is not reduced in the palmitoylation-deficient mutant and AP-2-mediated endocytosis remains unaffected. The cause of the reduced surface expression of the ADAM10 mutant remains unclear and requires further investigation.

Further, the interactions with proteins from the TetraspaninC8 group were addressed to regulate substrate specificity for ADAM10 via subcellular localisation. ADAM10 is stabilised at the plasma membrane and directed to lateral junctions through interaction with Tspan15 (Harrison et al., 2021). Tspan15 expression seems to be dependent on the sufficient expression of ADAM10 as it was shown that ADAM10 knockdown led to a reduction of Tspan15 expression by 80% (Harrison et al., 2021). The interaction between ADAM10 and Tspan15 is an important factor especially for the cleavage of N-Cadherin (Seipold et al., 2018). Tspan15 was equally expressed and co-precipitated with wildtype ADAM10 and the palmitoylation-deficient mutant from transfected HeLa ADAM10 knockout cells (Fig. 16A) indicating that the palmitoylation has no influence on the binding ability of Tspan15. It was published that the binding site of both proteins is within their extracellular domains (Lipper et al., 2022). The intracellularly located palmitoylation does not seem to impact ADAM10's structure or folding to the extent that it cannot bind to Tspan15, consistent with the finding that N-Cadherin cleavage is not altered in cells co-transfected with Tspan15 and ADAM10 C699S compared to Tspan15 and wildtype ADAM10 co-transfected cells.

Furthermore, the study identifies new interaction partners for ADAM10 that are differentially associated with the palmitoylated or palmitoylation-deficient ADAM10. These partners include TRIM7, TIMP2, TNFAIP3, and NUSAP1. The differential binding affinities of these proteins to the wildtype and palmitoylation-deficient ADAM10 suggest a potential regulatory role for palmitoylation in the context of tumour proliferation, protease inhibition, inflammation, and microtubule stabilisation (Chakraborty et al., 2015; Mooney & Sahingur, 2021; Qian et al., 2018). TNFAIP3, TIMP2, and TRIM7 are proteins that were identified to interact more with the palmitoylation-deficient ADAM10 after mass spectrometric analysis although TIMP2 and TRIM7 were identified to interact also with wildtype ADAM10.

Tripartite motif containing protein 7 (TRIM7) is an E3 ubiquitin ligase that is involved in the polyubiquitination of RACO-1 which is a coactivator of AP-1. This activation is a possible mechanism to promote tumour proliferation (Chakraborty et al., 2015). Because it is also described that ADAM10 is involved in cancer progression by cleaving other substrates that in turn promote tumour growth and spreading (Smith et al., 2020), it was interesting if the palmitoylation deficiency might lead to more association with the tumour promoting TRIM7. A potential subcellular compartment where both proteins would have the ability to interact is the Golgi apparatus. TRIM7 is mainly located at the

Golgi apparatus (Giraldo, Xia, et al., 2020), where ADAM10 is processed from its proform to the active, mature form (Fig. 28).

Tissue inhibitor of metalloproteinases 2 (TIMP2) preferentially inhibits MMPs 2 and 14 but TIMPs are in general known to also inhibit ADAMs. A recombinant soluble form of the catalytic domain of ADAM10 is for example inhibited by TIMP1 and -3 (Amour et al., 2000). Because the ADAM10 CTF generation was decreased in cells expressing the palmitoylation-deficient ADAM10 mutant it needs to be further addressed whereas an interaction with TIMP2 might lead to a selective decrease in the activity towards autoproteolysis. TIMP2 is extracellularly located and inhibits the catalytic active regions of MMPs (Hayakawa et al., 1994) which indicates a potential interaction site with ADAM10 to be at the catalytic active region (Fig. 28).

Tumour necrosis factor alpha-induced protein 3 (TNFAIP3) is a ubiquitin-editing protein that terminates the activation of the nuclear factor (NF)- κ B. This mechanism leads to an involvement in anti-inflammatory regulations but on the other side also to involvement in cancer biology because of its antiapoptotic impact (Mooney & Sahingur, 2021). TNFAIP3 is only an interacting partner for ADAM10 in cells expressing the palmitoylation-deficient ADAM10. TNFAIP3 can be located at several sites in the cytoplasm where an interaction with ADAM10 is possible (Fig. 28) but it can also be directed to lysosomes and the nucleus (Mooney & Sahingur, 2021).

Nucleolar and spindle-associated protein 1 (NUSAP1) is the only protein that was detected to interact more with wildtype ADAM10 than with its palmitoylation-deficient mutant. It binds and stabilises microtubules and can be located in different compartments. Its function is mainly localised in the nucleus but also in parts in the cytosol stabilising microtubules. It is linked to a poor prognosis of several cancer forms (Qian et al., 2018). Fragments of degraded ADAM10 can be directed to the nucleus (Toussey et al., 2009) and possibly interact with NUSAP1 (Fig. 28).

Further investigations using different approaches like immunoprecipitations or crosslinking of protein complexes are necessary to confirm the distinct interactions with palmitoylated or non-palmitoylated ADAM10 and their potential consequences.

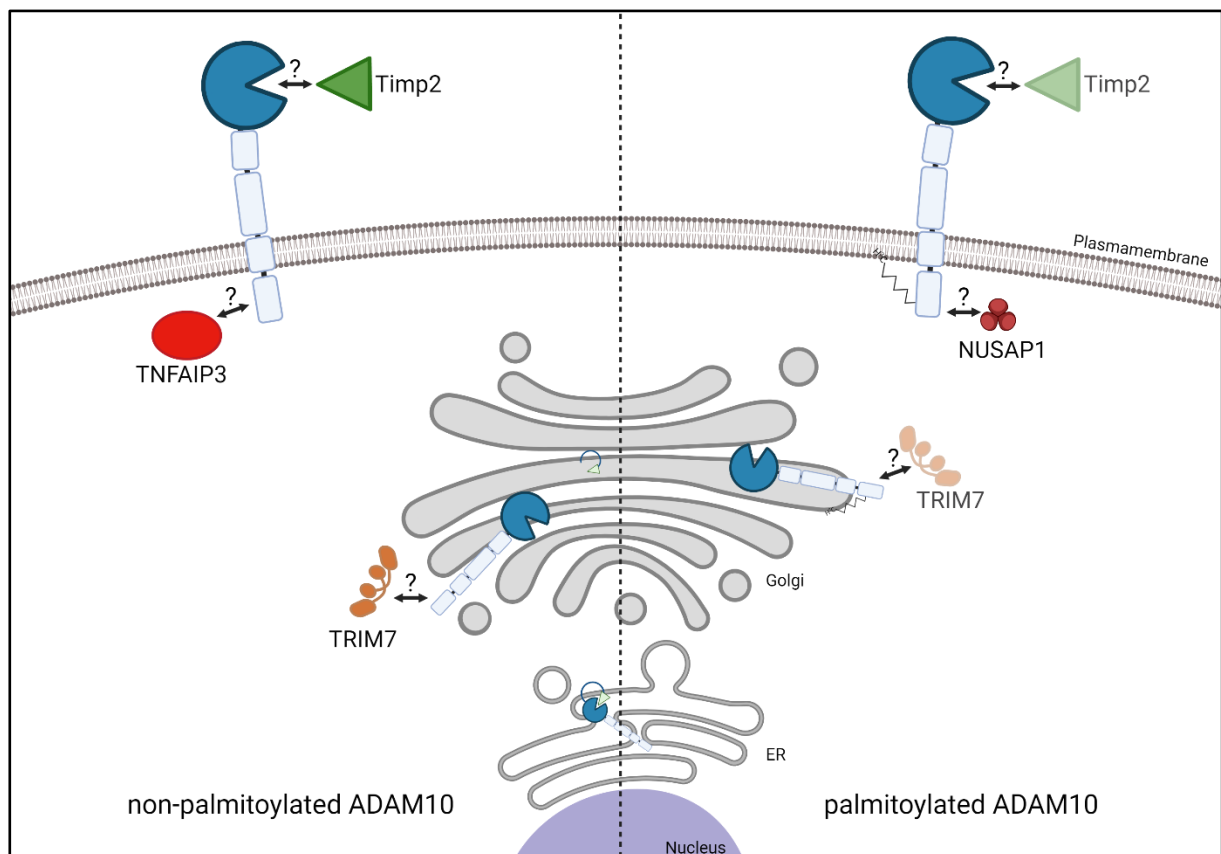


Figure 28: Possible locations of the interactions between ADAM10 and its potential interaction partners. Shown is a schematic overview of ADAM10's way to the plasma membrane. Possible interactions of proteins with ADAM10 detected by mass spectrometry analyses of ADAM10 immunoprecipitations (IPs) are depicted with arrows and question marks. On the left, interactions with non-palmitoylated ADAM10 are shown and on the right, interactions with palmitoylated ADAM10 are shown. The interaction partners are shown in the compartment that they're known to be present in from literature analyses. Transparency of proteins represents that interaction in the IP of palmitoylated ADAM10 was detected but less abundant than in the IP of non-palmitoylated ADAM10. TIMP2: Tissue inhibitor of metalloproteinases 2, TNFAIP3: Tumour necrosis factor alpha-induced protein 3, TRIM7: Tripartite motif containing protein 7, NUSAP1: Nucleolar and spindle-associated protein 1.

6 Conclusions

In conclusion, the palmitoylation of ADAM10 is a significant post-translational modification that can influence its function and interactions in a complex manner. While ADAM10 is palmitoylated, its expression levels remain independent of this modification. However, palmitoylation seems to play an important role in the surface expression of ADAM10, as evidenced by the reduced surface presence of palmitoylation-deficient mutants. The ADAM10-mediated shedding of N-Cadherin appears unaffected by palmitoylation status. Conversely, the processing of APP is notably reduced in cells expressing palmitoylation-deficient mutants, highlighting the importance of palmitoylation in ADAM10's ability to interact with and process certain substrates. The question of whether palmitoylation significantly affects ADAM10's interaction with specific partners remains open and requires further investigation.

Moreover, the generation of ADAM10 C-terminal fragments, potentially through ADAM10's autoproteolytic activity, is reduced in the absence of palmitoylation. This reduction suggests that ADAM10's autoproteolytic activity might be dependent on its palmitoylation, further underlining the multifaceted role of this modification in regulating ADAM10's functions.

7 Summary

The A Disintegrin and Metalloprotease 10 (ADAM10) has been recognized as a pivotal player in various physiological and pathophysiological processes, encompassing neurodevelopment, neurodegenerative diseases like Alzheimer's disease (AD), and cancer. Despite considerable knowledge about ADAM10's structure and functions, investigations into the regulation of its expression and activity remain limited. One aspect of interest is ADAM10's potential palmitoylation, a reversible posttranslational modification occurring on cysteine residues of various proteins. Effects of Palmitoylation on proteins are diverse and can impact activity, localisation, and trafficking.

In the present work, the significance of ADAM10's palmitoylation site on its function and biochemistry was elucidated. Through techniques like acyl-resin capture assays, the palmitoylation of ADAM10 was confirmed in various cell types. Interestingly, palmitoylation did not affect ADAM10 expression levels but possibly influenced its transport to the cell surface, as a palmitoylation-deficient mutant was less efficiently transported to and labelled at the cell surface. Furthermore, the study provides evidence for a link between ADAM10 autoproteolysis and palmitoylation since impairment of either process led to altered generation of the carboxy-terminal fragment of ADAM10 (CTF). This finding supports the conclusion that ADAM10 itself contributes to the generation of its CTF and that its palmitoylation is important for the autoproteolytic activity of ADAM10.

Investigations into ADAM10's shedding activity revealed potential effects of palmitoylation on the cleavage of distinct substrates. While the shedding of APP was reduced in cells expressing the palmitoylation deficient ADAM10 mutant, no significant impact was observed for N-cadherin shedding. Additionally, the study explored ADAM10's interactions with proteins like SAP-97 and AP-2, known regulators of ADAM10 activity, with preliminary results suggesting potential alterations in binding affinity due to palmitoylation.

In conclusion, ADAM10 is S-palmitoylated which plays a role in regulating its function and biochemistry. The palmitoylation of ADAM10 promotes its expression at the cell surface and the generation of ADAM10 C-terminal fragments. Additionally, the palmitoylation may modulate the shedding of distinct substrates such as APP.

8 References

- Amour, A., Knight, C. G., Webster, A., Slocombe, P. M., Stephens, P. E., Knauper, V., Docherty, A. J., & Murphy, G. (2000). The in vitro activity of ADAM-10 is inhibited by TIMP-1 and TIMP-3. *FEBS Lett*, 473(3), 275-279. [https://doi.org/10.1016/s0014-5793\(00\)01528-3](https://doi.org/10.1016/s0014-5793(00)01528-3)
- Anders, A., Gilbert, S., Garten, W., Postina, R., & Fahrenholz, F. (2001). Regulation of the alpha-secretase ADAM10 by its prodomain and proprotein convertases. *FASEB J*, 15(10), 1837-1839. <https://doi.org/10.1096/fj.01-0007fje>
- Asai, M., Hattori, C., Szabo, B., Sasagawa, N., Maruyama, K., Tanuma, S., & Ishiura, S. (2003). Putative function of ADAM9, ADAM10, and ADAM17 as APP alpha-secretase. *Biochem Biophys Res Commun*, 301(1), 231-235. [https://doi.org/10.1016/s0006-291x\(02\)02999-6](https://doi.org/10.1016/s0006-291x(02)02999-6)
- Atri, A. (2019). The Alzheimer's Disease Clinical Spectrum: Diagnosis and Management. *Med Clin North Am*, 103(2), 263-293. <https://doi.org/10.1016/j.mcna.2018.10.009>
- Bai, S., Nasser, M. W., Wang, B., Hsu, S. H., Datta, J., Kutay, H., Yadav, A., Nuovo, G., Kumar, P., & Ghoshal, K. (2009). MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. *J Biol Chem*, 284(46), 32015-32027. <https://doi.org/10.1074/jbc.M109.016774>
- Barnich, N., Aguirre, J. E., Reinecker, H. C., Xavier, R., & Podolsky, D. K. (2005). Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor-kappaB activation in muramyl dipeptide recognition. *J Cell Biol*, 170(1), 21-26. <https://doi.org/10.1083/jcb.200502153>
- Barthet, G., Jorda-Siquier, T., Rumi-Masante, J., Bernadou, F., Muller, U., & Mulle, C. (2018). Presenilin-mediated cleavage of APP regulates synaptotagmin-7 and presynaptic plasticity. *Nat Commun*, 9(1), 4780. <https://doi.org/10.1038/s41467-018-06813-x>
- Bhattacharyya, R., Barren, C., & Kovacs, D. M. (2013). Palmitoylation of amyloid precursor protein regulates amyloidogenic processing in lipid rafts. *J Neurosci*, 33(27), 11169-11183. <https://doi.org/10.1523/JNEUROSCI.4704-12.2013>
- Brummer, T., Pignoni, M., Rossello, A., Wang, H., Noy, P. J., Tomlinson, M. G., Blobel, C. P., & Lichtenthaler, S. F. (2018). The metalloprotease ADAM10 (a disintegrin and metalloprotease 10) undergoes rapid, postlysis autocatalytic degradation. *FASEB J*, 32(7), 3560-3573. <https://doi.org/10.1096/fj.201700823RR>
- Camodeca, C., Cuffaro, D., Nuti, E., & Rossello, A. (2019). ADAM Metalloproteinases as Potential Drug Targets. *Curr Med Chem*, 26(15), 2661-2689. <https://doi.org/10.2174/0929867325666180326164104>
- Carey, R. M., Blusztajn, J. K., & Slack, B. E. (2011). Surface expression and limited proteolysis of ADAM10 are increased by a dominant negative inhibitor of dynamin. *BMC Cell Biol*, 12, 20. <https://doi.org/10.1186/1471-2121-12-20>
- Chakraborty, A., Diefenbacher, M. E., Mylona, A., Kassel, O., & Behrens, A. (2015). The E3 ubiquitin ligase Trim7 mediates c-Jun/AP-1 activation by Ras signalling. *Nat Commun*, 6, 6782. <https://doi.org/10.1038/ncomms7782>
- Chan, P., Han, X., Zheng, B., DeRan, M., Yu, J., Jarugumilli, G. K., Deng, H., Pan, D., Luo, X., & Wu, X. (2016). Autopalmitoylation of TEAD proteins regulates transcriptional output of the Hippo pathway. *Nat Chem Biol*, 12(4), 282-289. <https://doi.org/10.1038/nchembio.2036>
- Charollais, J., & Van Der Goot, F. G. (2009). Palmitoylation of membrane proteins (Review). *Mol Membr Biol*, 26(1), 55-66. <https://doi.org/10.1080/09687680802620369>
- Charrin, S., Manie, S., Oualid, M., Billard, M., Boucheix, C., & Rubinstein, E. (2002). Differential stability of tetraspanin/tetraspanin interactions: role of palmitoylation. *FEBS Lett*, 516(1-3), 139-144. [https://doi.org/10.1016/s0014-5793\(02\)02522-x](https://doi.org/10.1016/s0014-5793(02)02522-x)
- Cho, E., & Park, M. (2016). Palmitoylation in Alzheimer's disease and other neurodegenerative diseases. *Pharmacol Res*, 111, 133-151. <https://doi.org/10.1016/j.phrs.2016.06.008>

- Chow, V. W., Mattson, M. P., Wong, P. C., & Gleichmann, M. (2010). An overview of APP processing enzymes and products. *Neuromolecular Med*, 12(1), 1-12. <https://doi.org/10.1007/s12017-009-8104-z>
- Colciaghi, F., Marcello, E., Borroni, B., Zimmermann, M., Caltagirone, C., Cattabeni, F., Padovani, A., & Di Luca, M. (2004). Platelet APP, ADAM 10 and BACE alterations in the early stages of Alzheimer disease. *Neurology*, 62(3), 498-501. <https://doi.org/10.1212/01.wnl.0000106953.49802.9c>
- Cuchillo-Ibanez, I., Lopez-Font, I., Boix-Amoros, A., Brinkmalm, G., Blennow, K., Molinuevo, J. L., & Saez-Valero, J. (2015). Heteromers of amyloid precursor protein in cerebrospinal fluid. *Mol Neurodegener*, 10, 2. <https://doi.org/10.1186/1750-1326-10-2>
- Donmez, G., Wang, D., Cohen, D. E., & Guarente, L. (2010). SIRT1 suppresses beta-amyloid production by activating the alpha-secretase gene ADAM10. *Cell*, 142(2), 320-332. <https://doi.org/10.1016/j.cell.2010.06.020>
- Du, K., Murakami, S., Sun, Y., Kilpatrick, C. L., & Luscher, B. (2017). DHHC7 Palmitoylates Glucose Transporter 4 (Glut4) and Regulates Glut4 Membrane Translocation. *J Biol Chem*, 292(7), 2979-2991. <https://doi.org/10.1074/jbc.M116.747139>
- Edwards, D. R., Handsley, M. M., & Pennington, C. J. (2008). The ADAM metalloproteinases. *Mol Aspects Med*, 29(5), 258-289. <https://doi.org/10.1016/j.mam.2008.08.001>
- Escrevente, C., Morais, V. A., Keller, S., Soares, C. M., Altevogt, P., & Costa, J. (2008). Functional role of N-glycosylation from ADAM10 in processing, localization and activity of the enzyme. *Biochim Biophys Acta*, 1780(6), 905-913. <https://doi.org/10.1016/j.bbagen.2008.03.004>
- Fhu, C. W., & Ali, A. (2021). Protein Lipidation by Palmitoylation and Myristoylation in Cancer. *Front Cell Dev Biol*, 9, 673647. <https://doi.org/10.3389/fcell.2021.673647>
- Folch, J., & Lees, M. (1951). Proteolipides, a new type of tissue lipoproteins; their isolation from brain. *J Biol Chem*, 191(2), 807-817. <https://www.ncbi.nlm.nih.gov/pubmed/14861226>
- Gao, X., & Hannoush, R. N. (2018). A Decade of Click Chemistry in Protein Palmitoylation: Impact on Discovery and New Biology. *Cell Chem Biol*, 25(3), 236-246. <https://doi.org/10.1016/j.chembiol.2017.12.002>
- Giraldo, M. I., Hage, A., van Tol, S., & Rajsbaum, R. (2020). TRIM Proteins in Host Defense and Viral Pathogenesis. *Curr Clin Microbiol Rep*, 7(4), 101-114. <https://doi.org/10.1007/s40588-020-00150-8>
- Giraldo, M. I., Xia, H., Aguilera-Aguirre, L., Hage, A., van Tol, S., Shan, C., Xie, X., Sturdevant, G. L., Robertson, S. J., McNally, K. L., Meade-White, K., Azar, S. R., Rossi, S. L., Maury, W., Woodson, M., Ramage, H., Johnson, J. R., Krogan, N. J., Morais, M. C., . . . Rajsbaum, R. (2020). Envelope protein ubiquitination drives entry and pathogenesis of Zika virus. *Nature*, 585(7825), 414-419. <https://doi.org/10.1038/s41586-020-2457-8>
- Gottlieb, C. D., & Linder, M. E. (2017). Structure and function of DHHC protein S-acyltransferases. *Biochem Soc Trans*, 45(4), 923-928. <https://doi.org/10.1042/BST20160304>
- Guo, Y., Wang, Q., Chen, S., & Xu, C. (2021). Functions of amyloid precursor protein in metabolic diseases. *Metabolism*, 115, 154454. <https://doi.org/10.1016/j.metabol.2020.154454>
- Harrison, N., Koo, C. Z., & Tomlinson, M. G. (2021). Regulation of ADAM10 by the TspanC8 Family of Tetraspanins and Their Therapeutic Potential. *Int J Mol Sci*, 22(13). <https://doi.org/10.3390/ijms22136707>
- Hartmann, D., de Strooper, B., Serneels, L., Craessaerts, K., Herreman, A., Annaert, W., Umans, L., Lubke, T., Lena Illert, A., von Figura, K., & Saftig, P. (2002). The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. *Hum Mol Genet*, 11(21), 2615-2624. <https://doi.org/10.1093/hmg/11.21.2615>
- Hayakawa, T., Yamashita, K., Ohuchi, E., & Shinagawa, A. (1994). Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). *J Cell Sci*, 107 (Pt 9), 2373-2379. <https://doi.org/10.1242/jcs.107.9.2373>
- Heiler, S., Mu, W., Zoller, M., & Thuma, F. (2015). The importance of claudin-7 palmitoylation on membrane subdomain localization and metastasis-promoting activities. *Cell Commun Signal*, 13, 29. <https://doi.org/10.1186/s12964-015-0105-y>

- Hernandez, J. L., Davda, D., Cheung See Kit, M., Majmudar, J. D., Won, S. J., Gang, M., Pasupuleti, S. C., Choi, A. I., Bartkowiak, C. M., & Martin, B. R. (2017). APT2 Inhibition Restores Scribble Localization and S-Palmitoylation in Snail-Transformed Cells. *Cell Chem Biol*, 24(1), 87-97. <https://doi.org/10.1016/j.chembiol.2016.12.007>
- Hitzenberger, M., Gotz, A., Menig, S., Brunschweiler, B., Zacharias, M., & Scharnagl, C. (2020). The dynamics of gamma-secretase and its substrates. *Semin Cell Dev Biol*, 105, 86-101. <https://doi.org/10.1016/j.semcdb.2020.04.008>
- Hoettecke, N., Ludwig, A., Foro, S., & Schmidt, B. (2010). Improved synthesis of ADAM10 inhibitor GI254023X. *Neurodegener Dis*, 7(4), 232-238. <https://doi.org/10.1159/000267865>
- Hornemann, T. (2015). Palmitoylation and depalmitoylation defects. *J Inherit Metab Dis*, 38(1), 179-186. <https://doi.org/10.1007/s10545-014-9753-0>
- Hsia, H. E., Tushaus, J., Brummer, T., Zheng, Y., Scilabra, S. D., & Lichtenthaler, S. F. (2019). Functions of 'A disintegrin and metalloproteases (ADAMs)' in the mammalian nervous system. *Cell Mol Life Sci*, 76(16), 3055-3081. <https://doi.org/10.1007/s00018-019-03173-7>
- Janes, P. W., Saha, N., Barton, W. A., Kolev, M. V., Wimmer-Kleikamp, S. H., Nievergall, E., Blobel, C. P., Himanen, J. P., Lackmann, M., & Nikolov, D. B. (2005). Adam meets Eph: an ADAM substrate recognition module acts as a molecular switch for ephrin cleavage in trans. *Cell*, 123(2), 291-304. <https://doi.org/10.1016/j.cell.2005.08.014>
- Jennings, B. C., & Linder, M. E. (2012). DHHC protein S-acyltransferases use similar ping-pong kinetic mechanisms but display different acyl-CoA specificities. *J Biol Chem*, 287(10), 7236-7245. <https://doi.org/10.1074/jbc.M111.337246>
- Jennings, B. C., Nadolski, M. J., Ling, Y., Baker, M. B., Harrison, M. L., Deschenes, R. J., & Linder, M. E. (2009). 2-Bromopalmitate and 2-(2-hydroxy-5-nitro-benzylidene)-benzo[b]thiophen-3-one inhibit DHHC-mediated palmitoylation in vitro. *J Lipid Res*, 50(2), 233-242. <https://doi.org/10.1194/jlr.M800270-JLR200>
- Jin, J., Zhi, X., Wang, X., & Meng, D. (2021). Protein palmitoylation and its pathophysiological relevance. *J Cell Physiol*, 236(5), 3220-3233. <https://doi.org/10.1002/jcp.30122>
- Jouannet, S., Saint-Pol, J., Fernandez, L., Nguyen, V., Charrin, S., Boucheix, C., Brou, C., Milhiet, P. E., & Rubinstein, E. (2016). TspanC8 tetraspanins differentially regulate the cleavage of ADAM10 substrates, Notch activation and ADAM10 membrane compartmentalization. *Cell Mol Life Sci*, 73(9), 1895-1915. <https://doi.org/10.1007/s00018-015-2111-z>
- Kaczorowska, A., Miekus, N., Stefanowicz, J., & Adamkiewicz-Drozynska, E. (2020). Selected Matrix Metalloproteinases (MMP-2, MMP-7) and Their Inhibitor (TIMP-2) in Adult and Pediatric Cancer. *Diagnostics (Basel)*, 10(8). <https://doi.org/10.3390/diagnostics10080547>
- Kathayat, R. S., Cao, Y., Elvira, P. D., Sandoz, P. A., Zaballa, M. E., Springer, M. Z., Drake, L. E., Macleod, K. F., van der Goot, F. G., & Dickinson, B. C. (2018). Active and dynamic mitochondrial S-depalmitoylation revealed by targeted fluorescent probes. *Nat Commun*, 9(1), 334. <https://doi.org/10.1038/s41467-017-02655-1>
- Ko, P. J., & Dixon, S. J. (2018). Protein palmitoylation and cancer. *EMBO Rep*, 19(10). <https://doi.org/10.15252/embr.201846666>
- Kollmann, K., Uusi-Rauva, K., Scifo, E., Tynnela, J., Jalanko, A., & Bräulke, T. (2013). Cell biology and function of neuronal ceroid lipofuscinosis-related proteins. *Biochim Biophys Acta*, 1832(11), 1866-1881. <https://doi.org/10.1016/j.bbadis.2013.01.019>
- Koo, C. Z., Harrison, N., Noy, P. J., Szyrocka, J., Matthews, A. L., Hsia, H. E., Muller, S. A., Tushaus, J., Goulding, J., Willis, K., Apicella, C., Cragoe, B., Davis, E., Keles, M., Malinova, A., McFarlane, T. A., Morrison, P. R., Nguyen, H. T. H., Sykes, M. C., . . . Tomlinson, M. G. (2020). The tetraspanin Tspan15 is an essential subunit of an ADAM10 scissor complex. *J Biol Chem*, 295(36), 12822-12839. <https://doi.org/10.1074/jbc.RA120.012601>
- Kufer, T. A., Kremmer, E., Adam, A. C., Philpott, D. J., & Sansonetti, P. J. (2008). The pattern-recognition molecule Nod1 is localized at the plasma membrane at sites of bacterial interaction. *Cell Microbiol*, 10(2), 477-486. <https://doi.org/10.1111/j.1462-5822.2007.01062.x>
- Kuhn, P. H., Colombo, A. V., Schusser, B., Dreytmüller, D., Wetzel, S., Schepers, U., Herber, J., Ludwig, A., Kremmer, E., Montag, D., Müller, U., Schweizer, M., Saftig, P., Bräse, S., & Lichtenthaler, S.

- F. (2016). Systematic substrate identification indicates a central role for the metalloprotease ADAM10 in axon targeting and synapse function. *Elife*, 5. <https://doi.org/10.7554/eLife.12748>
- Lammich, S., Buell, D., Zilow, S., Ludwig, A. K., Nuscher, B., Lichtenthaler, S. F., Prinzen, C., Fahrenholz, F., & Haass, C. (2010). Expression of the anti-amyloidogenic secretase ADAM10 is suppressed by its 5'-untranslated region. *J Biol Chem*, 285(21), 15753-15760. <https://doi.org/10.1074/jbc.M110.110742>
- Lane, C. A., Hardy, J., & Schott, J. M. (2018). Alzheimer's disease. *Eur J Neurol*, 25(1), 59-70. <https://doi.org/10.1111/ene.13439>
- Le Gall, S. M., Bobe, P., Reiss, K., Horiuchi, K., Niu, X. D., Lundell, D., Gibb, D. R., Conrad, D., Saftig, P., & Blobel, C. P. (2009). ADAMs 10 and 17 represent differentially regulated components of a general shedding machinery for membrane proteins such as transforming growth factor alpha, L-selectin, and tumor necrosis factor alpha. *Mol Biol Cell*, 20(6), 1785-1794. <https://doi.org/10.1091/mbc.e08-11-1135>
- Lee, S. B., Doberstein, K., Baumgarten, P., Wieland, A., Ungerer, C., Burger, C., Hardt, K., Boehncke, W. H., Pfeilschifter, J., Mihic-Probst, D., Mittelbronn, M., & Gutwein, P. (2011). PAX2 regulates ADAM10 expression and mediates anchorage-independent cell growth of melanoma cells. *PLoS One*, 6(8), e22312. <https://doi.org/10.1371/journal.pone.0022312>
- Li, W., Pang, Y., Wang, Y., Mei, F., Guo, M., Wei, Y., Li, X., Qin, W., Wang, W., Jia, L., & Jia, J. (2023). Aberrant palmitoylation caused by a ZDHHC21 mutation contributes to pathophysiology of Alzheimer's disease. *BMC Med*, 21(1), 223. <https://doi.org/10.1186/s12916-023-02930-7>
- Lin, D. T., & Conibear, E. (2015). ABHD17 proteins are novel protein depalmitoylases that regulate N-Ras palmitate turnover and subcellular localization. *Elife*, 4, e11306. <https://doi.org/10.7554/eLife.11306>
- Linsenmeier, L., Mohammadi, B., Wetzels, S., Puig, B., Jackson, W. S., Hartmann, A., Uchiyama, K., Sakaguchi, S., Endres, K., Tatzelt, J., Saftig, P., Glatzel, M., & Altmeyer, H. C. (2018). Structural and mechanistic aspects influencing the ADAM10-mediated shedding of the prion protein. *Mol Neurodegener*, 13(1), 18. <https://doi.org/10.1186/s13024-018-0248-6>
- Lipper, C. H., Gabriel, K. H., Seegar, T. C. M., Durr, K. L., Tomlinson, M. G., & Blacklow, S. C. (2022). Crystal structure of the Tspan15 LEL domain reveals a conserved ADAM10 binding site. *Structure*, 30(2), 206-214 e204. <https://doi.org/10.1016/j.str.2021.10.007>
- Lo Sardo, V., Zuccato, C., Gaudenzi, G., Vitali, B., Ramos, C., Tartari, M., Myre, M. A., Walker, J. A., Pistocchi, A., Conti, L., Valenza, M., Drung, B., Schmidt, B., Gusella, J., Zeitlin, S., Cotelli, F., & Cattaneo, E. (2012). An evolutionary recent neuroepithelial cell adhesion function of huntingtin implicates ADAM10-Ncadherin. *Nat Neurosci*, 15(5), 713-721. <https://doi.org/10.1038/nn.3080>
- Lopez-Otin, C., & Bond, J. S. (2008). Proteases: multifunctional enzymes in life and disease. *J Biol Chem*, 283(45), 30433-30437. <https://doi.org/10.1074/jbc.R800035200>
- Lu, Y., Zheng, Y., Coyaudo, E., Zhang, C., Selvakumaran, A., Yu, Y., Xu, Z., Weng, X., Chen, J. S., Meng, Y., Warner, N., Cheng, X., Liu, Y., Yao, B., Hu, H., Xia, Z., Muise, A. M., Klip, A., Brumell, J. H., . . . Neculai, D. (2019). Palmitoylation of NOD1 and NOD2 is required for bacterial sensing. *Science*, 366(6464), 460-467. <https://doi.org/10.1126/science.aau6391>
- Ma, Z., Shi, S., Ren, M., Pang, C., Zhan, Y., An, H., & Sun, F. (2022). Molecular mechanism of CD44 homodimerization modulated by palmitoylation and membrane environments. *Biophys J*, 121(14), 2671-2683. <https://doi.org/10.1016/j.bpj.2022.06.021>
- Main, A., & Fuller, W. (2022). Protein S-Palmitoylation: advances and challenges in studying a therapeutically important lipid modification. *FEBS J*, 289(4), 861-882. <https://doi.org/10.1111/febs.15781>
- Manzine, P. R., Barham, E. J., Vale Fde, A., Selistre-de-Araujo, H. S., Iost Pavarini, S. C., & Cominetti, M. R. (2013). Correlation between mini-mental state examination and platelet ADAM10 expression in Alzheimer's disease. *J Alzheimers Dis*, 36(2), 253-260. <https://doi.org/10.3233/JAD-130125>
- Marcello, E., Gardoni, F., Mauceri, D., Romorini, S., Jeromin, A., Epis, R., Borroni, B., Cattabeni, F., Sala, C., Padovani, A., & Di Luca, M. (2007). Synapse-associated protein-97 mediates alpha-

- secretase ADAM10 trafficking and promotes its activity. *J Neurosci*, 27(7), 1682-1691. <https://doi.org/10.1523/JNEUROSCI.3439-06.2007>
- Marcello, E., Saraceno, C., Musardo, S., Vara, H., de la Fuente, A. G., Pelucchi, S., Di Marino, D., Borroni, B., Tramontano, A., Perez-Otano, I., Padovani, A., Giustetto, M., Gardoni, F., & Di Luca, M. (2013). Endocytosis of synaptic ADAM10 in neuronal plasticity and Alzheimer's disease. *J Clin Invest*, 123(6), 2523-2538. <https://doi.org/10.1172/JCI65401>
- Maretzky, T., Evers, A., Le Gall, S., Alabi, R. O., Speck, N., Reiss, K., & Blobel, C. P. (2015). The cytoplasmic domain of a disintegrin and metalloproteinase 10 (ADAM10) regulates its constitutive activity but is dispensable for stimulated ADAM10-dependent shedding. *J Biol Chem*, 290(12), 7416-7425. <https://doi.org/10.1074/jbc.M114.603753>
- Martin, B. R., Wang, C., Adibekian, A., Tully, S. E., & Cravatt, B. F. (2011). Global profiling of dynamic protein palmitoylation. *Nat Methods*, 9(1), 84-89. <https://doi.org/10.1038/nmeth.1769>
- Matthews, A. L., Szyroka, J., Collier, R., Noy, P. J., & Tomlinson, M. G. (2017). Scissor sisters: regulation of ADAM10 by the TspanC8 tetraspanins. *Biochem Soc Trans*, 45(3), 719-730. <https://doi.org/10.1042/BST20160290>
- McCormick, P. J., Dumaesq-Doiron, K., Pluviose, A. S., Pichette, V., Tosato, G., & Lefrancois, S. (2008). Palmitoylation controls recycling in lysosomal sorting and trafficking. *Traffic*, 9(11), 1984-1997. <https://doi.org/10.1111/j.1600-0854.2008.00814.x>
- Mendez, P., De Roo, M., Poglia, L., Klauser, P., & Muller, D. (2010). N-cadherin mediates plasticity-induced long-term spine stabilization. *J Cell Biol*, 189(3), 589-600. <https://doi.org/10.1083/jcb.201003007>
- Mooney, E. C., & Sahingur, S. E. (2021). The Ubiquitin System and A20: Implications in Health and Disease. *J Dent Res*, 100(1), 10-20. <https://doi.org/10.1177/0022034520949486>
- Musardo, S., Marcello, E., Gardoni, F., & Di Luca, M. (2014). ADAM10 in synaptic physiology and pathology. *Neurodegener Dis*, 13(2-3), 72-74. <https://doi.org/10.1159/000354233>
- Musardo, S., Therin, S., Pelucchi, S., D'Andrea, L., Stringhi, R., Ribeiro, A., Manca, A., Balducci, C., Pagano, J., Sala, C., Verpelli, C., Grieco, V., Edefonti, V., Forloni, G., Gardoni, F., Meli, G., Di Marino, D., Di Luca, M., & Marcello, E. (2022). The development of ADAM10 endocytosis inhibitors for the treatment of Alzheimer's disease. *Mol Ther*, 30(7), 2474-2490. <https://doi.org/10.1016/j.ymthe.2022.03.024>
- Noy, P. J., Yang, J., Reyat, J. S., Matthews, A. L., Charlton, A. E., Furmston, J., Rogers, D. A., Rainger, G. E., & Tomlinson, M. G. (2016). TspanC8 Tetraspanins and A Disintegrin and Metalloprotease 10 (ADAM10) Interact via Their Extracellular Regions: EVIDENCE FOR DISTINCT BINDING MECHANISMS FOR DIFFERENT TspanC8 PROTEINS. *J Biol Chem*, 291(7), 3145-3157. <https://doi.org/10.1074/jbc.M115.703058>
- O'Brien, R. J., & Wong, P. C. (2011). Amyloid precursor protein processing and Alzheimer's disease. *Annu Rev Neurosci*, 34, 185-204. <https://doi.org/10.1146/annurev-neuro-061010-113613>
- Olmsted, J. B., Carlson, K., Klebe, R., Ruddle, F., & Rosenbaum, J. (1970). Isolation of microtubule protein from cultured mouse neuroblastoma cells. *Proc Natl Acad Sci U S A*, 65(1), 129-136. <https://doi.org/10.1073/pnas.65.1.129>
- Pereira Vatanabe, I., Peron, R., Mantellatto Grigoli, M., Pelucchi, S., De Cesare, G., Magalhaes, T., Manzone, P. R., Figueredo Balthazar, M. L., Di Luca, M., Marcello, E., & Cominetti, M. R. (2021). ADAM10 Plasma and CSF Levels Are Increased in Mild Alzheimer's Disease. *Int J Mol Sci*, 22(5). <https://doi.org/10.3390/ijms22052416>
- Peron, R., Vatanabe, I. P., Manzone, P. R., Camins, A., & Cominetti, M. R. (2018). Alpha-Secretase ADAM10 Regulation: Insights into Alzheimer's Disease Treatment. *Pharmaceuticals (Basel)*, 11(1). <https://doi.org/10.3390/ph11010012>
- Postina, R., Schroeder, A., Dewachter, I., Bohl, J., Schmitt, U., Kojro, E., Prinzen, C., Endres, K., Hiemke, C., Blessing, M., Flamez, P., Dequenue, A., Godaux, E., van Leuven, F., & Fahrenholz, F. (2004). A disintegrin-metalloproteinase prevents amyloid plaque formation and hippocampal defects in an Alzheimer disease mouse model. *J Clin Invest*, 113(10), 1456-1464. <https://doi.org/10.1172/JCI20864>

- Prinzen, C., Muller, U., Endres, K., Fahrenholz, F., & Postina, R. (2005). Genomic structure and functional characterization of the human ADAM10 promoter. *FASEB J*, 19(11), 1522-1524. <https://doi.org/10.1096/fj.04-3619fje>
- Prox, J., Bernreuther, C., Altmeyen, H., Grendel, J., Glatzel, M., D'Hooge, R., Stroobants, S., Ahmed, T., Balschun, D., Willem, M., Lammich, S., Isbrandt, D., Schweizer, M., Horre, K., De Strooper, B., & Saftig, P. (2013). Postnatal disruption of the disintegrin/metalloproteinase ADAM10 in brain causes epileptic seizures, learning deficits, altered spine morphology, and defective synaptic functions. *J Neurosci*, 33(32), 12915-12928, 12928a. <https://doi.org/10.1523/JNEUROSCI.5910-12.2013>
- Prox, J., Rittger, A., & Saftig, P. (2012). Physiological functions of the amyloid precursor protein secretases ADAM10, BACE1, and presenilin. *Exp Brain Res*, 217(3-4), 331-341. <https://doi.org/10.1007/s00221-011-2952-0>
- Prox, J., Willenbrock, M., Weber, S., Lehmann, T., Schmidt-Arras, D., Schwanbeck, R., Saftig, P., & Schwake, M. (2012). Tetraspanin15 regulates cellular trafficking and activity of the ectodomain sheddase ADAM10. *Cell Mol Life Sci*, 69(17), 2919-2932. <https://doi.org/10.1007/s00018-012-0960-2>
- Pruessmeyer, J., & Ludwig, A. (2009). The good, the bad and the ugly substrates for ADAM10 and ADAM17 in brain pathology, inflammation and cancer. *Semin Cell Dev Biol*, 20(2), 164-174. <https://doi.org/10.1016/j.semcdb.2008.09.005>
- Qian, Z., Li, Y., Ma, J., Xue, Y., Xi, Y., Hong, L., Dai, X., Zhang, Y., Ji, X., Chen, Y., Sheng, M., Sheng, Y., Yang, L., Liu, J., Dai, X., Shi, J., Xie, T., & Dong, J. (2018). Prognostic value of NUSAP1 in progression and expansion of glioblastoma multiforme. *J Neurooncol*, 140(2), 199-208. <https://doi.org/10.1007/s11060-018-2942-1>
- Rabbani, G., Baig, M. H., Ahmad, K., & Choi, I. (2018). Protein-protein Interactions and their Role in Various Diseases and their Prediction Techniques. *Curr Protein Pept Sci*, 19(10), 948-957. <https://doi.org/10.2174/1389203718666170828122927>
- Reddy, K. D., Malipeddi, J., DeForte, S., Pejaver, V., Radivojac, P., Uversky, V. N., & Deschenes, R. J. (2017). Physicochemical sequence characteristics that influence S-palmitoylation propensity. *J Biomol Struct Dyn*, 35(11), 2337-2350. <https://doi.org/10.1080/07391102.2016.1217275>
- Reinhardt, S., Schuck, F., Grosgen, S., Riemenschneider, M., Hartmann, T., Postina, R., Grimm, M., & Endres, K. (2014). Unfolded protein response signaling by transcription factor XBP-1 regulates ADAM10 and is affected in Alzheimer's disease. *FASEB J*, 28(2), 978-997. <https://doi.org/10.1096/fj.13-234864>
- Reiss, K., Maretzky, T., Ludwig, A., Tousseyn, T., de Strooper, B., Hartmann, D., & Saftig, P. (2005). ADAM10 cleavage of N-cadherin and regulation of cell-cell adhesion and beta-catenin nuclear signalling. *EMBO J*, 24(4), 742-752. <https://doi.org/10.1038/sj.emboj.7600548>
- Reiss, K., & Saftig, P. (2009). The "a disintegrin and metalloprotease" (ADAM) family of sheddases: physiological and cellular functions. *Semin Cell Dev Biol*, 20(2), 126-137. <https://doi.org/10.1016/j.semcdb.2008.11.002>
- Ren, J., Wen, L., Gao, X., Jin, C., Xue, Y., & Yao, X. (2008). CSS-Palm 2.0: an updated software for palmitoylation sites prediction. *Protein Eng Des Sel*, 21(11), 639-644. <https://doi.org/10.1093/protein/gzn039>
- Ren, W., Sun, Y., & Du, K. (2015). Glut4 palmitoylation at Cys223 plays a critical role in Glut4 membrane trafficking. *Biochem Biophys Res Commun*, 460(3), 709-714. <https://doi.org/10.1016/j.bbrc.2015.03.094>
- Rocks, O., Gerauer, M., Vartak, N., Koch, S., Huang, Z. P., Pechlivanis, M., Kuhlmann, J., Brunsfeld, L., Chandra, A., Ellinger, B., Waldmann, H., & Bastiaens, P. I. (2010). The palmitoylation machinery is a spatially organizing system for peripheral membrane proteins. *Cell*, 141(3), 458-471. <https://doi.org/10.1016/j.cell.2010.04.007>
- Rodenburg, R. N. P., Snijder, J., van de Waterbeemd, M., Schouten, A., Granneman, J., Heck, A. J. R., & Gros, P. (2017). Stochastic palmitoylation of accessible cysteines in membrane proteins revealed by native mass spectrometry. *Nat Commun*, 8(1), 1280. <https://doi.org/10.1038/s41467-017-01461-z>

- Roghani, M., Becherer, J. D., Moss, M. L., Atherton, R. E., Erdjument-Bromage, H., Arribas, J., Blackburn, R. K., Weskamp, G., Tempst, P., & Blobel, C. P. (1999). Metalloprotease-disintegrin MDC9: intracellular maturation and catalytic activity. *J Biol Chem*, 274(6), 3531-3540. <https://doi.org/10.1074/jbc.274.6.3531>
- Saftig, P., & Lichtenthaler, S. F. (2015). The alpha secretase ADAM10: A metalloprotease with multiple functions in the brain. *Prog Neurobiol*, 135, 1-20. <https://doi.org/10.1016/j.pneurobio.2015.10.003>
- Sanders, S. S., & Hayden, M. R. (2015). Aberrant palmitoylation in Huntington disease. *Biochem Soc Trans*, 43(2), 205-210. <https://doi.org/10.1042/BST20140242>
- Sapir, T., Segal, M., Grigoryan, G., Hansson, K. M., James, P., Segal, M., & Reiner, O. (2019). The Interactome of Palmitoyl-Protein Thioesterase 1 (PPT1) Affects Neuronal Morphology and Function. *Front Cell Neurosci*, 13, 92. <https://doi.org/10.3389/fncel.2019.00092>
- Sarlak, G., Htoo, H. H., Hernandez, J. F., Iizasa, H., Checler, F., Konietzko, U., Song, W., & Vincent, B. (2016). Sox2 functionally interacts with betaAPP, the betaAPP intracellular domain and ADAM10 at a transcriptional level in human cells. *Neuroscience*, 312, 153-164. <https://doi.org/10.1016/j.neuroscience.2015.11.022>
- Scheltens, P., De Strooper, B., Kivipelto, M., Holstege, H., Chetelat, G., Teunissen, C. E., Cummings, J., & van der Flier, W. M. (2021). Alzheimer's disease. *Lancet*, 397(10284), 1577-1590. [https://doi.org/10.1016/S0140-6736\(20\)32205-4](https://doi.org/10.1016/S0140-6736(20)32205-4)
- Schlesinger, M. J., Magee, A. I., & Schmidt, M. F. (1980). Fatty acid acylation of proteins in cultured cells. *J Biol Chem*, 255(21), 10021-10024. <https://www.ncbi.nlm.nih.gov/pubmed/7430112>
- Schweizer, A., Kornfeld, S., & Rohrer, J. (1996). Cysteine34 of the cytoplasmic tail of the cation-dependent mannose 6-phosphate receptor is reversibly palmitoylated and required for normal trafficking and lysosomal enzyme sorting. *J Cell Biol*, 132(4), 577-584. <https://doi.org/10.1083/jcb.132.4.577>
- Seegar, T. C. M., Killingsworth, L. B., Saha, N., Meyer, P. A., Patra, D., Zimmerman, B., Janes, P. W., Rubinstein, E., Nikolov, D. B., Skiniotis, G., Kruse, A. C., & Blacklow, S. C. (2017). Structural Basis for Regulated Proteolysis by the alpha-Secretase ADAM10. *Cell*, 171(7), 1638-1648 e1637. <https://doi.org/10.1016/j.cell.2017.11.014>
- Seifert, A., Dusterhoft, S., Wozniak, J., Koo, C. Z., Tomlinson, M. G., Nuti, E., Rossello, A., Cuffaro, D., Yildiz, D., & Ludwig, A. (2021). The metalloproteinase ADAM10 requires its activity to sustain surface expression. *Cell Mol Life Sci*, 78(2), 715-732. <https://doi.org/10.1007/s00018-020-03507-w>
- Seipold, L., Altmeppen, H., Koudelka, T., Tholey, A., Kasperek, P., Sedlacek, R., Schweizer, M., Bar, J., Mikhaylova, M., Glatzel, M., & Saftig, P. (2018). In vivo regulation of the A disintegrin and metalloproteinase 10 (ADAM10) by the tetraspanin 15. *Cell Mol Life Sci*, 75(17), 3251-3267. <https://doi.org/10.1007/s00018-018-2791-2>
- Shukla, M., Htoo, H. H., Wintachai, P., Hernandez, J. F., Dubois, C., Postina, R., Xu, H., Checler, F., Smith, D. R., Govitrapong, P., & Vincent, B. (2015). Melatonin stimulates the nonamyloidogenic processing of betaAPP through the positive transcriptional regulation of ADAM10 and ADAM17. *J Pineal Res*, 58(2), 151-165. <https://doi.org/10.1111/jpi.12200>
- Smith, T. M., Jr., Tharakan, A., & Martin, R. K. (2020). Targeting ADAM10 in Cancer and Autoimmunity. *Front Immunol*, 11, 499. <https://doi.org/10.3389/fimmu.2020.00499>
- Specia, D. J., & Diaz, E. (2020). Acyl-PEGyl Exchange Gel Shift Assay for Quantitative Determination of Palmitoylation of Brain Membrane Proteins. *J Vis Exp*(157). <https://doi.org/10.3791/61018>
- Suh, J., Choi, S. H., Romano, D. M., Gannon, M. A., Lesinski, A. N., Kim, D. Y., & Tanzi, R. E. (2013). ADAM10 missense mutations potentiate beta-amyloid accumulation by impairing prodomain chaperone function. *Neuron*, 80(2), 385-401. <https://doi.org/10.1016/j.neuron.2013.08.035>
- Tang, B. L. (2020). Enhancing alpha-secretase Processing for Alzheimer's Disease-A View on SFRP1. *Brain Sci*, 10(2). <https://doi.org/10.3390/brainsci10020122>
- Tewari, R., West, S. J., Shayahati, B., & Akimzhanov, A. M. (2020). Detection of Protein S-Acylation using Acyl-Resin Assisted Capture. *J Vis Exp*(158). <https://doi.org/10.3791/61016>

- Toussey, T., Thathiah, A., Jorissen, E., Raemaekers, T., Konietzko, U., Reiss, K., Maes, E., Snellinx, A., Serneels, L., Nyabi, O., Annaert, W., Saftig, P., Hartmann, D., & De Strooper, B. (2009). ADAM10, the rate-limiting protease of regulated intramembrane proteolysis of Notch and other proteins, is processed by ADAMS-9, ADAMS-15, and the gamma-secretase. *J Biol Chem*, 284(17), 11738-11747. <https://doi.org/10.1074/jbc.M805894200>
- Turk, B. (2006). Targeting proteases: successes, failures and future prospects. *Nat Rev Drug Discov*, 5(9), 785-799. <https://doi.org/10.1038/nrd2092>
- Uemura, K., Kihara, T., Kuzuya, A., Okawa, K., Nishimoto, T., Ninomiya, H., Sugimoto, H., Kinoshita, A., & Shimohama, S. (2006). Characterization of sequential N-cadherin cleavage by ADAM10 and PS1. *Neurosci Lett*, 402(3), 278-283. <https://doi.org/10.1016/j.neulet.2006.04.018>
- Vezzoli, E., Caron, I., Talpo, F., Besusso, D., Conforti, P., Battaglia, E., Sogne, E., Falqui, A., Petricca, L., Verani, M., Martufi, P., Caricasole, A., Bresciani, A., Cecchetti, O., Rivetti di Val Cervo, P., Sancini, G., Riess, O., Nguyen, H., Seipold, L., . . . Zuccato, C. (2019). Inhibiting pathologically active ADAM10 rescues synaptic and cognitive decline in Huntington's disease. *J Clin Invest*, 129(6), 2390-2403. <https://doi.org/10.1172/JCI120616>
- Vincent, B. (2016). Regulation of the alpha-secretase ADAM10 at transcriptional, translational and post-translational levels. *Brain Res Bull*, 126(Pt 2), 154-169. <https://doi.org/10.1016/j.brainresbull.2016.03.020>
- von Hoven, G., Qin, Q., Neukirch, C., Husmann, M., & Hellmann, N. (2019). Staphylococcus aureus alpha-toxin: small pore, large consequences. *Biol Chem*, 400(10), 1261-1276. <https://doi.org/10.1515/hsz-2018-0472>
- Wetzel, S., Seipold, L., & Saftig, P. (2017). The metalloproteinase ADAM10: A useful therapeutic target? *Biochim Biophys Acta Mol Cell Res*, 1864(11 Pt B), 2071-2081. <https://doi.org/10.1016/j.bbamcr.2017.06.005>
- Wild-Bode, C., Fellerer, K., Kugler, J., Haass, C., & Capell, A. (2006). A basolateral sorting signal directs ADAM10 to adherens junctions and is required for its function in cell migration. *J Biol Chem*, 281(33), 23824-23829. <https://doi.org/10.1074/jbc.M601542200>
- Wilke, G. A., & Bubeck Wardenburg, J. (2010). Role of a disintegrin and metalloprotease 10 in Staphylococcus aureus alpha-hemolysin-mediated cellular injury. *Proc Natl Acad Sci U S A*, 107(30), 13473-13478. <https://doi.org/10.1073/pnas.1001815107>
- Wohner, B., Li, W., Hey, S., Drobny, A., Werny, L., Becker-Pauly, C., Lucius, R., Zunke, F., Linder, S., & Arnold, P. (2023). Proteolysis of CD44 at the cell surface controls a downstream protease network. *Front Mol Biosci*, 10, 1026810. <https://doi.org/10.3389/fmolb.2023.1026810>
- Wolfsberg, T. G., Primakoff, P., Myles, D. G., & White, J. M. (1995). ADAM, a novel family of membrane proteins containing A Disintegrin And Metalloprotease domain: multipotential functions in cell-cell and cell-matrix interactions. *J Cell Biol*, 131(2), 275-278. <https://doi.org/10.1083/jcb.131.2.275>
- Won, S. J., Cheung See Kit, M., & Martin, B. R. (2018). Protein depalmitoylases. *Crit Rev Biochem Mol Biol*, 53(1), 83-98. <https://doi.org/10.1080/10409238.2017.1409191>
- Won, S. J., & Martin, B. R. (2018). Temporal Profiling Establishes a Dynamic S-Palmitoylation Cycle. *ACS Chem Biol*, 13(6), 1560-1568. <https://doi.org/10.1021/acscchembio.8b00157>
- Yang, W., Di Vizio, D., Kirchner, M., Steen, H., & Freeman, M. R. (2010). Proteome scale characterization of human S-acylated proteins in lipid raft-enriched and non-raft membranes. *Mol Cell Proteomics*, 9(1), 54-70. <https://doi.org/10.1074/mcp.M800448-MCP200>
- Zhang, Y. W., Thompson, R., Zhang, H., & Xu, H. (2011). APP processing in Alzheimer's disease. *Mol Brain*, 4, 3. <https://doi.org/10.1186/1756-6606-4-3>

9 Acknowledgements

At this point I would like to thank everyone who has helped and supported me through this journey. It was an incredible time learning and developing this thesis.

First of all, I would like to thank my supervisors Prof. Paul Saftig and Dr Lisa Seipold-Zimmermann who helped and guided me every step of the way with a huge amount of knowledge and advice.

Furthermore, I would like to express a special thanks to the second reviewer for reviewing this thesis.

I am grateful to Dr Sönke Rudnik for his collaboration and help during my project especially helping me with the several palmitoylation assays. Also, I thank my collaborators from the Institute of Experimental Medicine of the CAU Kiel Prof. Andreas Tholey and Dr Tomas Koudelka for the mass spectrometry data and their time helping me interpret them.

I am lucky I was able to be part of the AG Saftig with all the members and past members who created a perfect environment to work on this thesis. I will miss the strength of the community in this working group which made me remember this time of my studies as great as it was. I would like to thank (in no order) Lisa, Lina, another (equally great) Lisa, Marie, Mara, Maria, Sönke, Katharina, Ceddi, Seb, Pranji, Valentin, Anne, Florian, Saskia, Jascha, Patrick, Stine, Alessandro and Marlies. I enjoyed the several after-work events, always cheerful lunches and especially coffee breaks with all of you.

Lisa, you have taught me, a clueless medical student, how to research properly. You helped and encouraged me whenever necessary. The other Lisa and Lina, you were always there laughing, grumbling, and drinking coffee with me. I am beyond grateful for working with you and knowing you.

Last but not least, I would like to thank my family and friends for always being there for me, for putting up with me sometimes and for all the good and not-so-good times. I would not be where I am without you. You know how much I appreciate you.

10 Declaration

Herewith I declare that, apart from the supervisor's guidance, I have prepared the present thesis on my own and according to the Rules of Good Scientific Practice of the German Research Foundation. Concepts and ideas that were taken from other sources are indicated as such.

This thesis has not been submitted partially or wholly as part of a doctoral degree to another examining body.

Kiel, 01.09.2024

Maja Grohe

11 Publication

Bar, J., Fanutza, T., Reimann, C. C., Seipold, L., **Grohe, M.**, Bolter, J. R., Delfs, F., Bucher, M., Gee, C. E., Schweizer, M., Saftig, P., & Mikhaylova, M. (2024). Non-canonical function of ADAM10 in presynaptic plasticity. *Cell Mol Life Sci*, 81(1), 342.