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# The Protein Lipidation and its Analysis

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**Abstract**

Protein Lipidation is essential not only for membrane binding but also for the interaction with effectors and the regulation of signaling processes, thereby playing a key role in controlling protein localization and function. Cholesterylation, the attachment of the glycosylphosphatidylinositol anchor, as well as N-myristoylation, S-prenylation and S-acylation are among the most relevant protein lipidation processes. Little is still known about the significance of the high diversity in lipid modifications as well as the mechanism by which lipidation controls function and activity of the proteins. Although the development of new strategies to uncover these and other unexplored topics is in great demand, important advances have already been achieved during the last years in the analysis of protein lipidation. This review will highlight the most prominent lipid modifications encountered in proteins and will provide an overview of the existing methods for the analysis and identification of lipid modified proteins.

## Introduction

Biological cell membranes are typically formed by mixtures of lipids and proteins. Whereas the major lipid components are glycerophospholipids, cholesterol and sphingolipids, proteins located in the membrane can be divided in two main classes, integral proteins and associated proteins. In contrast to the permanently-bound integral proteins, associated proteins, which account for 25-40% of all eukaryotic proteins, are temporarily attached either to the lipid bilayer or to other integral proteins by a combination of hydrophobic, electrostatic and other non-covalent interactions [1]. This transient membrane association can be accomplished in different ways. In some cases it occurs by interaction with membrane lipids such as phospholipids or sphingolipids [2] or mediated by conserved lipid binding domains [3]. Alternatively, it can be also achieved by increasing protein hydrophobicity via cotranslational or posttranslational attachment of lipid groups. The most common lipidation processes are the equipment of proteins with a glycosylphosphatidylinositol anchor (GPI) as well as N-myristoylation, S-prenylation and S-acylation, also known as S-palmitoylation. Except the labile thioesters resulting from S-acylation, all these modifications are permanently installed into the proteins. Other known lipidations are the cholesterylation of members of the Hedgehog family or the uncommon conjugation of the ubiquitin-like yeast Autophagy-related protein 8 (Atg 8) with phosphatidylethanolamine [4,5].

Protein lipidation is not only essential for binding and partitioning in different membrane microdomains, [6,7] but also for the interaction with effectors and the regulation of signaling processes, thereby playing a key role in controlling protein localization and function [8]. However, the significance of the high diversity in lipid modifications as well as the mechanism by which lipidation controls function and activity remains poorly understood. Furthermore, misregulation has been often encountered in diseases [9,10]. Hence, lipidated proteins are crucial for bacterial, viral and parasite infection processes [10] and alterations in the lipidation pattern of membrane associated proteins have been detected in cancer [11,12] as well as in neurological disorders [13]. For all these reasons, the development of strategies enabling the study of known and new posttranslational modifications and especially of the dynamic changes of the reversible palmitoylation would be of great interest. Although much progress has already been achieved in this direction, new approaches are still required to circumvent the limitations of the current protocols. The increasing number of research groups from a wide variety of disciplines that have recently joined this field, apart from clearly demonstrating the growing interest on protein lipidation, may have an important contribution to the development of new tools and strategies. As such, this review will highlight the most prominent lipid modifications encountered in proteins and will provide an overview of the existing methods for the analysis and identification of lipid modified proteins detailing their advantages and limitations.

## Types of Lipidation

**Glycosylphosphatidylinositol anchor (GPI)**: The pottranslational attachment of a glycosylphosphatidylinositol (GPI) at the C-terminus of proteins occurs in the endoplasmic reticulum (ER) and the modified proteins are then translocated to the membrane where they remain anchored to the outer leaflet of the cell membrane. It is predicted that 0.5% of all the eukaryotic proteins may include this modification [14] and this could increase up to 10% in the case of parasites [15]. Glycosylphosphatidylinositol is a structurally complex molecule formed by a phosphoethanolamine, a glycan core, phosphoinositol and lipid residues. The complexity of the GPI unit relies on its enormous structural diversity. Hence, the glycan core can be decorated with several side-chain modifications and at the phosphoinositol unit different lipid moieties can be similarly attached (diacylglycerol, alkylacylglycerol, arachidonic acid, etc.), that may contain in turn additional lipid residues. All in all, the different combinations result in high structural diversity which complicates its study (recently reviewed in [16,17]). Several roles have been suggested for the GPI posttranslational modification apart from the membrane anchoring of proteins. Hence, GPI anchors may be involved in cell contact and adhesion and have a role in cellular communication, signal transduction, oncogenesis [18] and immune response [19]. Furthermore, this lipid modification has been shown to be relevant for prion disease pathogenesis and for parasites, which use the GPI anchor to increase their infectivity [15]. Despite this complexity in structure and function, some methods have been established for the detection of GPI-anchored proteins as well as for the structural analysis of the GPI units (the reader is referred to a

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| Lipid modifications and relevant examples | Function | Methods |
|---|---|---|
| **C-GPI**<br><br>reviewed in [16,17] | -cellular communication<br>-oncogenesis [18]<br>-immune response [19]<br>-parasite infection [15] | -reviewed in [20]<br>-MS (GPI-anchored proteins [21]<br>GPI units [22]) |
| **C-Cholesterylation**<br>sonic Hedgehog (Shh)[28] | -development | -radioactivity [28]<br>-biorthogonal reactions [29] |
| **N-Myristoylation**<br><br>-Bid, [32] actin,[34] PAK, Gelsolin [33,34]<br>-HIV-1 Gag,[38] Nef [40]<br>-Arf,[66] MARCKS, [67] Src family kinases [134] | -reviewed in [10]<br>-regulation of apoptosis<br>-virus, bacterial infectivity [37, 38]<br>-signaling<br>-misregulated in cancer [41] and neurological disorders [42] | -radioactivity<br>tritiated fatty acids, [33,34,81.85]<br>iodinated fatty acids [82,83]<br>-MS [88,89]<br>-bioorthogonal reactions (reviewed in [106,135])<br>Staudinger ligation [121]<br>CuAAC [128,132] |
| **C-Isoprenylation**<br><br>Ras superfamily of small GTPases [12]<br>**Farnesylation**: Ras proteins<br>**Geranylgeranylation**: Rab proteins, Rho proteins | -membrane association<br>-interaction with partners [136]<br>-signaling [12]<br>-cell migration, adhesion [138]<br>-membrane trafficking [45] | -radioactivity [138]<br>-MS [90]<br>-bioorthogonal reactions<br>(reviewed in [106,135])<br>Staudinger ligation [113,114,115,120] CuAAC [116,117,131] |
| **Acylation**<br><br>**S-acylation**:<br>Integral membrane proteins such as G-protein coupled receptor, ion channels<br>Soluble proteins such as: SNAP-25,[62] N-Ras, H-Ras, [59] src family kinases,[64] Gα proteins [65]<br>**N-acylation**: $G\alpha_s$ subunit, [49] Shh protein [27]<br>**O-acylation**: Wnt proteins [50] | -affinity for raft-like domains[17]<br>-ion channels regulation, maturation and trafficking[52]<br>-distribution of palmitoylated peripheral membrane proteins[57]<br>-regulation of synaptic plasticity[13]<br>-trafficking and activity of signaling proteins[59] | -radioactivity [139]<br>Iodinated fatty acids [82,83]<br>-MS [51,87]<br>MudPIT, [93] GC/MS [97]<br>-Acyl Biotin Exchange (ABE), [98-101,102,140] PalmPISC [58]<br>-bioorthogonal reactions<br>(reviewed in [106,135])<br>Staudinger ligation [121,122]<br>CuAAC [123-126] |

**Table 1:** Summary of the presented lipid modifications including relevant examples of each modification, some described functions and analytical methods reported for the study of these lipid modifications.

recent review by Hendrickson and co-workers [20]). As an example, GPI-anchored proteins could be detected after treatment with a phosphoinositol specific phospholipase C in order to release the GPIs units. The remaining proteins were then analysed by mass spectrometry (MS) for protein identification [21]. Alternatively, a LC-MS-based method was recently developed by Almeida and co-workers and applied for the structural characterization of the GPI units present in proteins. Using this approach, 90 GPI species could be identified of which 79 were novel [22]. Despite these advances, much work is still needed to elucidate the role of the GPI anchor and its involvement in cell function as well as in disease. The relationship between structural diversity and function is difficult to establish due to the lack of appropriate methods. Bertozzi´s group has reported important contributions in this area, studying the role of different component of the glycan core on GPI function [23,24]. Additionally, the structural differences detected in GPI-units from parasites in contrast to mammals have been also exploited for the generation of vaccines [25].

**Cholesterylation:** Another important lipidation is the C-terminal attachment of a cholesterol molecule detected in members of the mammalian Hedgehog family, important morphogens with a crucial role in regulating cellular proliferation and differentiation. The lipidation of Sonic Hedgehog (Shh) occurs during maturation in the ER and starts with the cleavage of a N-terminal recognition sequence present in the initial 45 kDa proprotein. This autocatalytic process originates a 19 kDa protein that is cholesterylated at the C-terminal glycine in a intein-like process [26] and S-palmitoylated at the N-terminal cysteine by the Hedgehog acyl transferase [27]. This S-acyl moiety migrates to the N-terminal amino group after an S-N acyl shift to form a permanent amide bond. Both lipidations are essential for correct function of the protein (Figure 2). Beachy and co-workers detected and confirmed this lipidation for the first time in 1996 by using tritiated cholesterol [28]. Recently, an azide-containing cholesterol was reported by Tate´s group and its use as substrate for the detection of cholesterylated proteins was investigated in cells overexpressing Shh [29]. In both studies other potentially cholesterylated proteins were detected suggesting that this modification may not be restricted to the members of the Hedgehog family. Despite these initial results, the identity of these other proteins has not yet been elucidated. Hence, it is envisaged that this azide-containing cholesterol or other tagged analogs could serve as an interesting tool for MS-based proteomics approaches to identify other cholesterylated proteins.

**N-Myristoylation:** The attachment of the saturated 14-carbon myristic acid to a N-terminal glycine via an amide bond, after cleavage of the initiating methionine, is known as N-myristoylation and is catalysed by a N-myristoyl transferase (NMT) [30] (for more information on this lipid modification the reader is referred to a recent excellent review by Berthiaume and co-workers on protein myristoylation [10]). NMT recognizes a general consensus sequence for myristoylation (Gly-X-X-X-(Ser/Thr/Cys)) containing a N-terminal glycine, 3 other amino acids and a serine, threonine or a cysteine in the fifth position. This modification, present in 0.5% of all eukaryotic proteins, [31] occurs mostly cotranslationally. However, it can also take place posttranslationally during apoptosis when an internal glycine residue gets exposed upon caspase cleavage. This posttranslational

myristoylation is essential for the correct function of the protein. For instance, the proapoptotic protein Bid needs to be myristoylated for targeting the mitochondria where it activates apoptotic signals [32]. Other proteins such as actin, p21-activated protein kinase (PAK) 2 and gelsolin are also cleaved and myristoylated [33,34]. In this later case, myristoylation of gelsolin is also produced after induction of apoptosis, however, this modification is not targeting the protein to the mitochondria but is involved in the anti-apoptotic effect of gelsolin [33].

Most of the mammalian species contain two isoforms of NMT which differ mainly in their N-terminal domain. It is not clear the real function of these two isoforms. Some preliminary *in vitro* and *in vivo* studies suggest that they may have distinct, albeit overlapping, substrate specificities or different cell localization [35]. Different roles in apoptosis have been also suggested for the two isoforms since knockdown of NMT1 decreases the proliferation of the cells around 27% whereas knockdown of NMT2 has no effect on cell division [36].

Many of the myristoylated proteins, such as the oncogen src, are involved in signal cascade regulation. Furthermore, virus and bacteria use the host NMT for myristoylation of essential proteins to increase infectivity or to achieve maximal virulence [37,38]. This is the case of the HIV viral protein Gag, whose myristoylation is critical for the formation of the viral capsid [39]. Similarly, the Nef protein, essential for viral replication, high virus load and progression to acquired immunodeficiency syndrome (AIDS), requires membrane association via myristoylation to exert its functions [40]. This together with the fact that myristoylation is misregulated in many other diseases (cancer, [41] neurological disorders [42]) has supported NMT as a potential therapeutical target.

**S-Isoprenylation:** The recognition motif for isoprenylation is a C-terminus sequence known as CAAX box, where C is a cysteine, A are aliphatic amino acids and X can be any amino acid. If X is Leucine, a 20-carbon isoprenoid geranylgeranyl is attached to this cysteine catalysed by Geranygeranyl Transferase I (GGTase I), in any other case, the 15-carbon farnesyl will be attached by the Farnesyl Transferase (FTase) [43]. The isoprenoid directs the prenylated proteins to the ER where the RCE1 (Ras converting enzyme 1) will cleave the last three amino acids and Icmt (Isoprenyl cysteine carboxymethyltransferase) will methylate the C-terminal isoprenylated cysteine (Figure 3) [44]. The isoprenylated and carboxymethylated cysteine mediates protein membrane association usually in combination with other membrane targeting motifs.

Another relevant example of isoprenylated proteins are the members of the Rab family. Rab proteins, who belong to the superfamily of Ras small GTPases, have a crucial role in controlling many steps of membrane traffic within the cell [45]. Most of the members of the Rab family lack the C-terminal CAAX box recognized by either FTase or GGTase. Instead of this, these proteins possess a CC or a CXC motif and are doubly geranylgeranylated by Geranylgeranyl Transferase II (GGTase II), also known as Rab Geranylgeranyl Transferase (RabGGTase). Prenylation takes place only when the Rab proteins are presented to the RabGGTase by a Rab escorting protein (REP). If

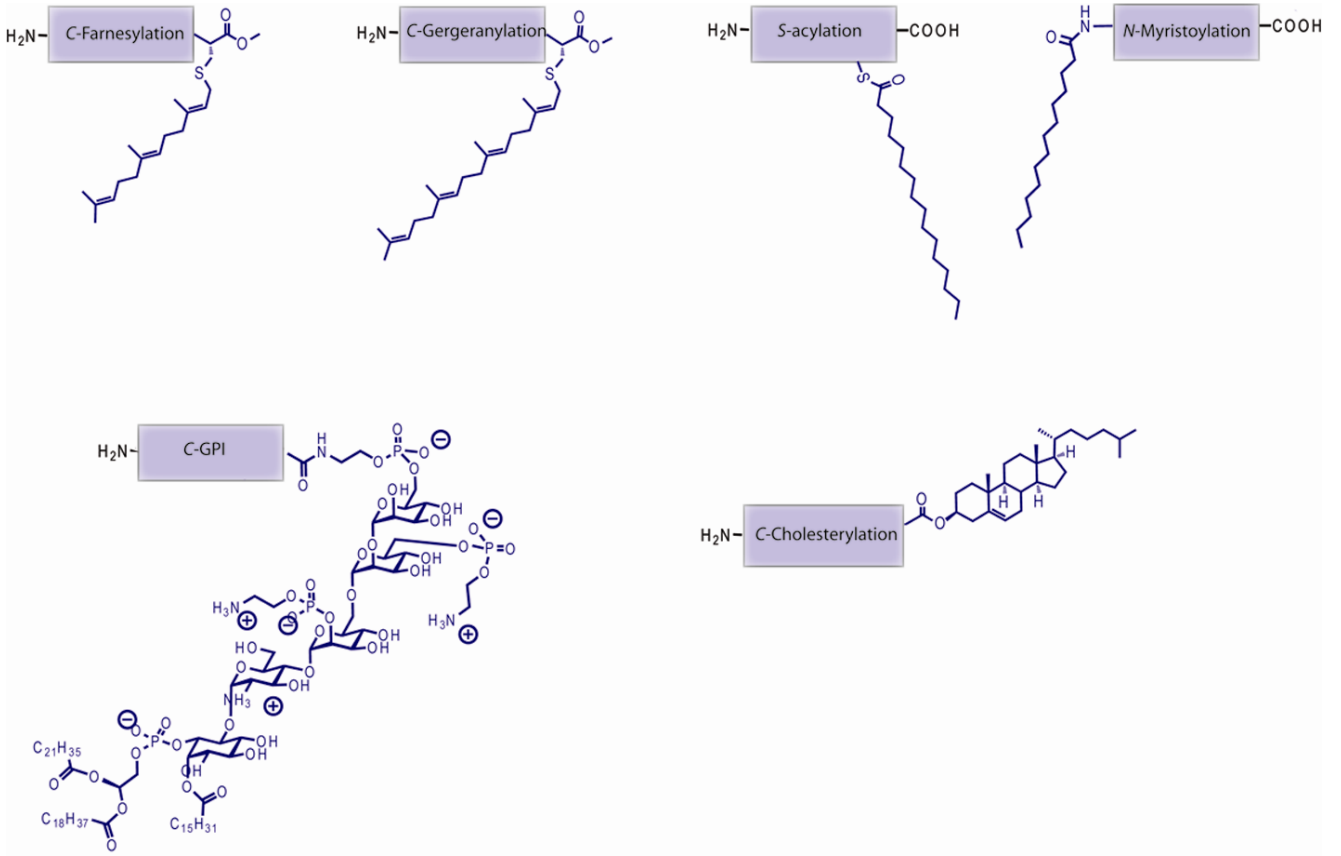


**Figure 1:** Most relevant lipid modifications in proteins. Isoprenylation of cysteines (either farnesylation or geranylgeranylation) occurs C-terminally, similarly to cholesterylation and the attachment of GPI anchors. Conversely, myristoylation occurs on a N-terminal glycine. The reversible S-acylation is restricted to cysteine residues, usually requiring the presence of a nearby myristoyl or isoprenyl moiety.

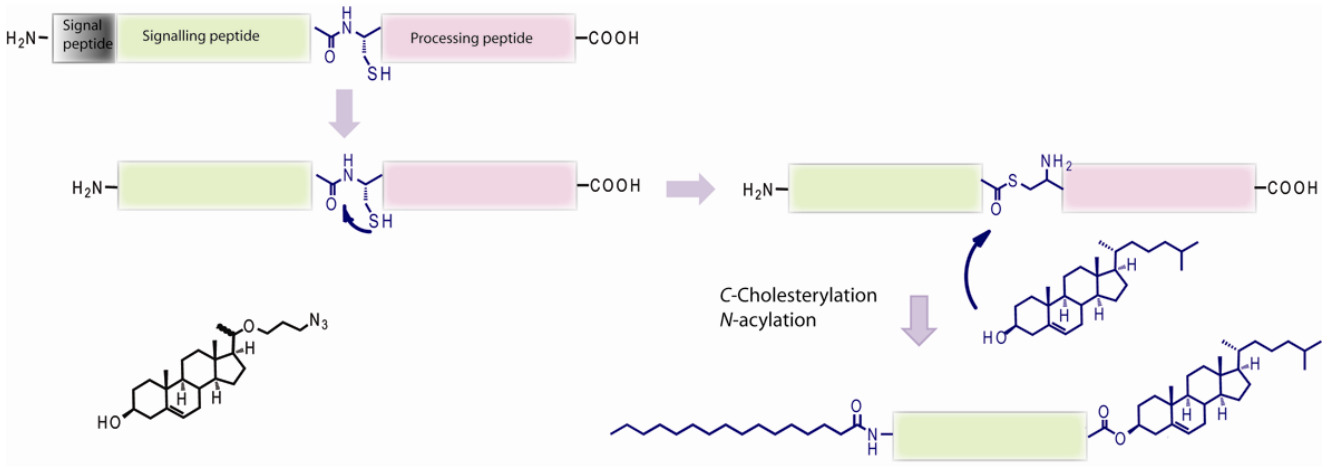


**Figure 2:** The secretory protein Sonic Hedgehog (Shh) is lipidated by C-terminal cholesterylation and N-terminal palmitoylation after intramolecular processing. Recently, an azide-containing cholesterol was developed by Tate and co-workers and it may be applied for the identification of cholesterylated proteins (structure in black) [29].

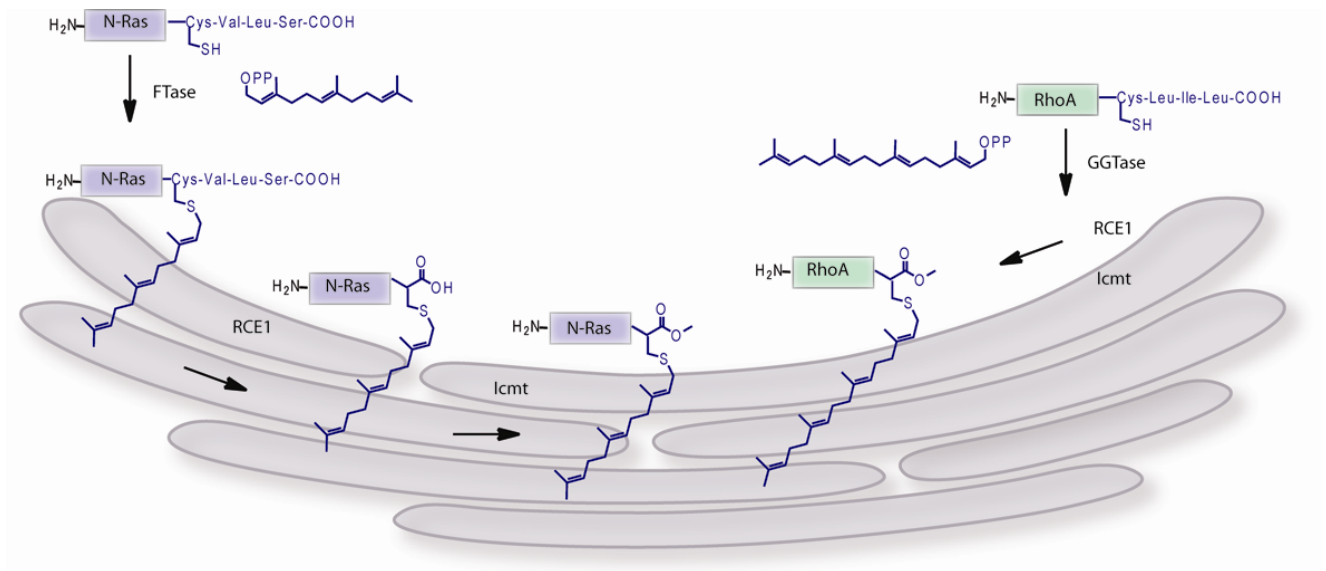


**Figure 3:** Proteins containing a C-terminal CAAX box are prenylated by GGTase (if X is Leu) or by FTase. The lipidated proteins are then directed to ER where the RCE1 cleaves the last three amino acids and Icmt methylates the resulting C-terminal cysteine. N-Ras and RhoA are depicted as examples of farnesylated and geranylgeranylated proteins.

the final sequence is CXC the proteins will also be methylated at the C-terminus, which does not occur in the case of a CC-ending sequence [46]. Methylation of isoprenylated proteins increases their membrane affinity and may have an important role in proper membrane association, especially in the case of the farnesylated proteins [47,48].

**Acylation:** Acylation is mainly limited to cysteine residues, thus forming a labile and reversible thioester linkage. The 16-carbon saturated palmitic acid is the most commonly attached lipid and S-acylation is therefore also known as S-palmitoylation. Occasionally, acylation of other amino acids as well as the presence of other fatty acids have been detected thereby suggesting that heterogeneity in the fatty acid residues could have an additional role in the regulation of protein localization and function. Briefly, N-palmitoylation of N-terminal cysteines has been reported for the $G\alpha_s$ subunit [49] or the Shh protein [27] and the Wnt protein has been found to be O-acylated in a serine residue with palmitoleic acid [50]. Similarly, other lipids such as stearic acid or arachidonic acids have been identified on cysteine residues [51].

Both soluble and integral membrane proteins can be acylated. In soluble proteins, acylation takes place on cysteine residues located near already lipidated N- or C-termini and this acylation step is essential for ensuring stable membrane association. Alternatively, integral membrane protein, such as G-protein coupled receptors or ion channels, can be acylated at the cytoplasmic part. The role of this acylation step in integral membrane proteins is still not clear. Generally, it has been suggested that it may be required for increasing the affinity of integral membrane proteins for raft-like domains [17]. However, additional functions for the palmitoyl group cannot be discarded. As example, in the particular case of ion channels acylation has been shown to be crucial for their regulation, maturation and trafficking (recently reviewed in [52]).

Importantly, due to the lability of the thioester linkage, there are two potential points of regulation i.e acylation and deacylation. Although some prediction tools have been described for the detection S-acylation sites, much discussion is out about their reliability and therefore no consensus sequence has been yet described [53-55]. In addition, there is still controversy if this step occurs spontaneously or catalysed by Palmitoyl Acyl Transferases (PAT). Supporting an enzymatic process, a S-palmitoyl transferase family of transmembrane proteins characterized by the presence of a DHHC (aspartate-histidine-histidine-cysteine) motif within a ~50 amino acid cysteine-rich-domain has been reported to be responsible for S-acylation. The high number of DHHC proteins (23 have been detected in mammals) and their different localization in cells, mostly in endomembranes but some of them are also detected in the plasma membrane (PM), [56] suggest a tighter regulation here than in the deacylation reaction. However, no clear substrate specificity has been detected for the members of the DHHC family to support this idea [57]. Deacylation in turn is carried out by Acyl Protein Thioesterases such as APT1 (recently suggested to be also palmitoylated itself) [58] or the lysosomal Protein Palmitoyl Thioesterase 1 (PPT1) thus generating the original non-acylated cysteine. Although some additional studies may be required to elucidate the factors regulating this palmitoylation/depalmitoylation machinery, it is clear that presents a broad substrate tolerance since it accepts different lipidation patterns and tolerates variation of amino acid sequence and configuration next to the palmitoylation site [57,59].

Altogether, acylation and deacylation determine the most important characteristic of S-acylation, which is its reversibility. An important consequence of this reversibility is that the acyl group half-life is shorter than the protein half-life, resulting in a palmitoylation/depalmitoylation cycle that controls the localization and function of acylated proteins. Briefly, after acylation, predominantly confined to Golgi apparatus [57], palmitoylated proteins enter the secretory

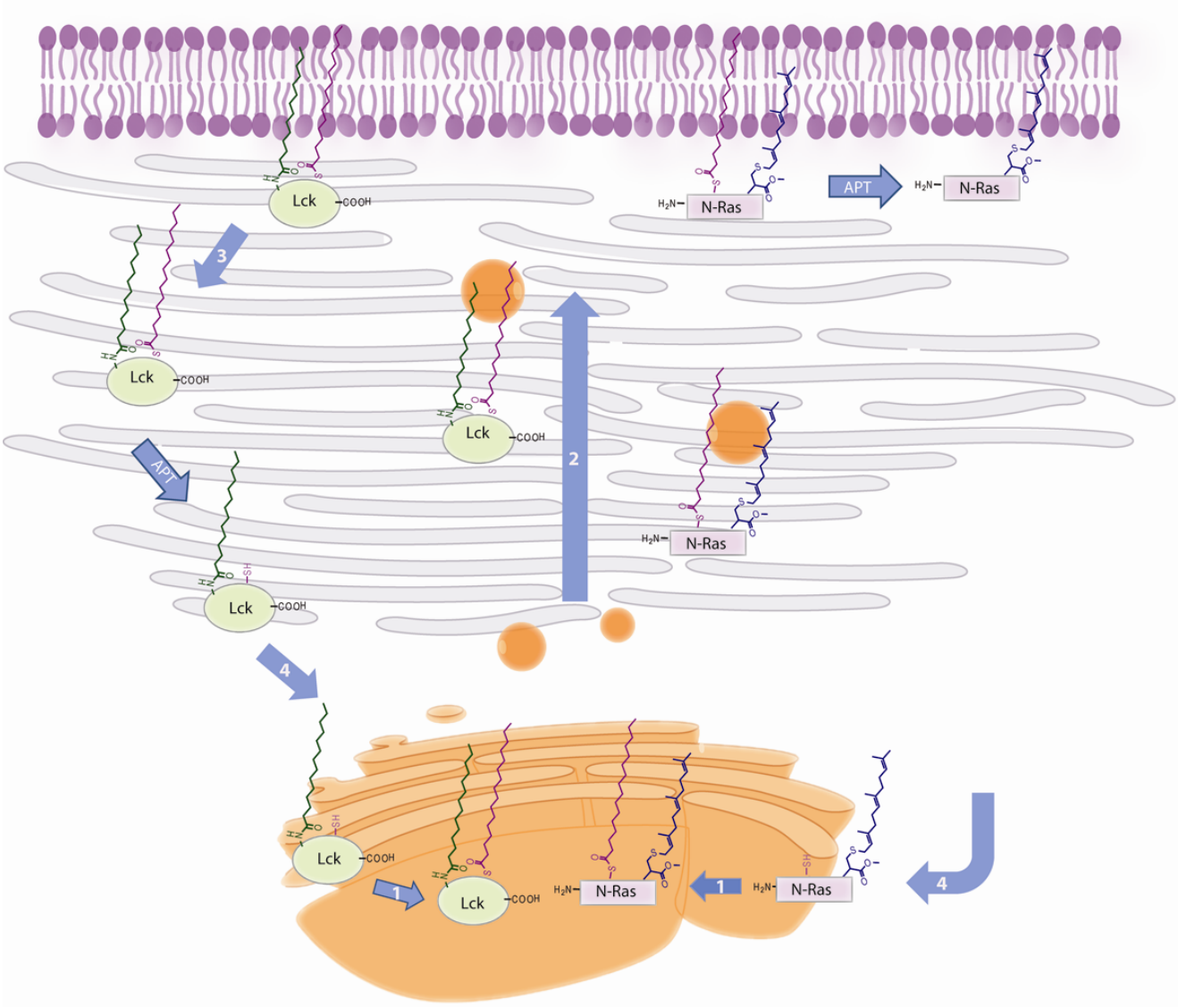


**Figure 4:** After palmitoylation in the Golgi (1), lipidated proteins enter the secretory pathway (2) and are mainly targeted to the plasma membrane, although some of them can also redistribute to all cellular membranes to reach equilibrium (3). Depalmitoylation catalysed by APT1 takes place all over the cell and the resulting deacylated proteins present are redirected to the Golgi for the next palmitoylation step by local kinetic trapping (4). Myristoylated Lck and Farnesylated N-Ras are depicted as example in this figure.

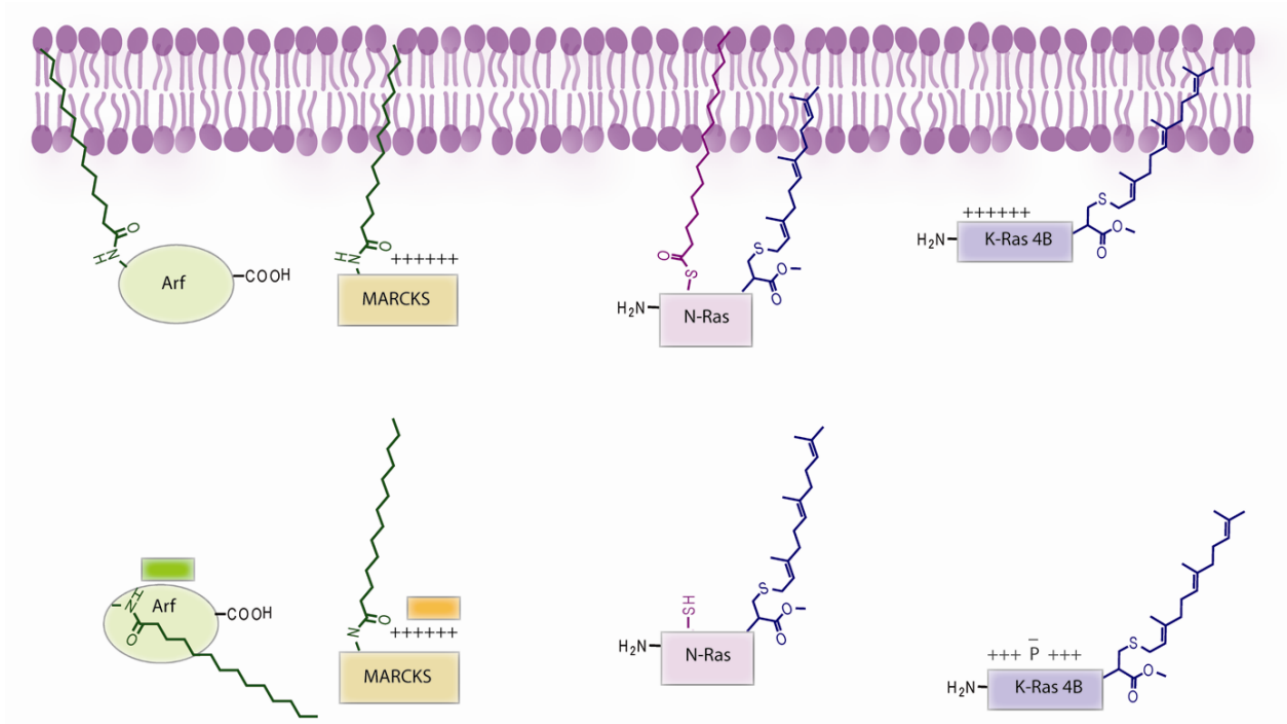


**Figure 5:** Reversible membrane association is usually achieved by the presence of different membrane targeting motifs. This is the case for isoprenylated and myristoylated proteins which require either another lipid (palmitoylation of N-Ras) or a polybasic stretch (K-Ras4B, MARCKS). Release from the membrane can be achieved by depalmitoylation (N-Ras), phosphorylation of residues near the polybasic stretch (K-Ras), or binding of ligands (MARCKS, Arf).

pathway and are first targeted to the PM. These palmitoylated proteins can remain in the PM or be redistributed to other cellular membranes. Depalmitoylation occurs everywhere in the cell and the deacylated proteins are directed to the Golgi where they will be kinetically trapped by palmitoylation in order the re-start the cycle (Figure 4) [57]. There are several prominent examples of this regulation of protein function and localization by the palmitoylation cycle. As a general example, acylation determines the spatial organization of palmitoylated peripheral membrane proteins [57] and regulates synaptic plasticity [13]. More specifically, acylation determines the intracellular distribution of SNAP-25 (a component of the neuronal/neuroendocrine SNARE complex that mediates exocytosis),[60] and controls protein trafficking and activity of signaling proteins such as Ras [59]. Recently, the interference with this palmitoylation/depalmitoylation reversibility by means of small molecules inhibitors of the depalmitoylating enzyme APT1 has been successfully used for characterizing Ras function and blocking its signalling [61].

**Membrane Targeting:** A short overview on the different lipid posttranslational modifications has been presented here. Some of these lipid modifications are enough to ensure stable membrane association of proteins. However, in some cases the presence of a lipid residue is not sufficient and other membrane targeting motifs are required. Some proteins such as SNAP-25, achieve this by solely S-acylation in multiple cysteine residues [62]. In most of the cases, reversible association is achieved with combined lipid residues or with lipidated residues together with a polybasic stretch that tightly interact with the negatively charged lipids present in the membrane. This is the case of myristoyl or isoprenyl groups, characterized by a low hydrophobicity which results in low affinity and short association times with the membrane [63]. As a result, several myristoylated proteins can also be found in the cytosol. Therefore, a second signal, either another lipid or a polybasic stretch, is required to ensure high affinity, longer association times and a reversible membrane association process. A second lipid motif is found in members of the Ras superfamily of GTPases such as N-Ras and H-Ras which apart from the C-terminal isoprenylated cysteine and a C-terminal methyl ester contain respectively one or two S-acylated cysteines near the C-terminus. Remarkably, the presence of one or two palmitoyl residues influences the resident time of these protein in the PM, resulting in longer dwell time for H-Ras in the PM compared to N-Ras and thus regulating its localization and signaling location [59]. Alternatively, members of the src family kinases [64] or Gα proteins [65] are N-terminal myristoylated and S-acylated. In this case S-acylation is usually taking place near residues at the N-terminus. The release from the membrane to the cytosol or the localization in internal membranes is achieved again via the reversibility of the S-acylation. Another level of regulation, known as myristoyl switch, has been detected in myristoylated proteins. In this case, upon ligand binding, the protein undergoes a conformational change and the myristoyl group is released from the membrane and hidden within a hydrophobic pocket of the protein, for example as occurs to Arf after binding of GTP [66]. Alternatively, the myristoyl or the farnesyl group can be present in the protein together with a polybasic stretch that specifically binds to the negatively charged phospholipids, localized preferentially in the inner leaflet of the plasma membrane. This is the case of myristoylated proteins such as MARCKS (myristoylated alanine-rich C kinase substrate), [67] HIV gag, [68] pp60c-src [69,70] or farnesylated proteins such as K-Ras4B. Reversibility of the binding is achieved by an electrostatic switch mediated by either phosphorylation of residues near the polybasic sequence to diminish the electrostatic interaction with the membrane (PKC dependent phosphorylation of K-Ras4B [71] and MARCKS), [72] or by binding of ions or ligands (binding of calcium to recoverin induces a conformational change

to allow the hidden myristoyl group to interact with the membrane [73] and binding of calmodulin to the C-terminal region of K-Ras4B induces the release from the plasma membrane and the binding to internal membranes [74-76]).

## Methods for detection of Protein Lipidation

Several bioinformatics methods have been developed for the prediction of GPI-anchored proteins, [77] N-myristoylated, [78,79] S-isoprenylated [80] or S-palmitoylated proteins [53,54]. However, as discussed the variable reliability of some of these prediction programs and the need to validate the predictions make it necessary the development of methods for the study of lipidated proteins. Hence, the detection and analysis of lipidated proteins is an emerging field. Improvements reported in the last years have already permitted significant advances in this area. However, due to the limited available methods, it remains challenging to study the specific function and mechanism of protein lipidation in physiology as well as in disease. In this section the different strategies for the detection of N-myristoylated, S-acylation and S-isoprenylated proteins will be discussed in more detail.

**Radioactivity:** The classical methods used for detection of lipidated proteins are based on the incorporation of radioactive precursors and subsequent detection of the proteins by fluorography. Although this method presents important drawbacks (long exposure times, purification of the protein before detection is required, radiolabeled precursors are costly and hazardous and does not allow quantification), it is still one of the methods of choice for confirming the presence of posttranslational modifications since it is not based on an indirect detection. Relevant examples of this approach are the use of $^{3}$H-myristic acid to show that actin [81] and gelsolin [33,34] are posttranslationally N-myristoylated upon caspase-mediated cleavage during apoptosis. Later, Resh and co-workers developed several $^{125}$I-iodinated fatty acids and successfully applied them to characterize proteins bearing a myristoyl group or both myristoyl and palmitoyl groups [82,83]. This method has overcome some of the limitations by reducing the exposure time for detection from days to hours. However, these precursors are not commercially available and need to be prepared prior use, thus requiring expertise not present in most cell biological labs. This together with the high toxicity of this γ-emitter radioisotope has hampered its use. Recently, Utsumi and co-workers have introduced a method based on the combination of cell-free protein synthesis derived from insect cells and metabolic labeling using the less harmful $^{3}$H-myristic acid. The optimized approach worked with short exposure times (10 h) without requiring any purification steps and could be applied to the study of cotranslational and posttranslational myristoylation [33] using a model protein and the cytotoxic apoptotic protein Bid [84]. Altogether, although radioactive labeling is still one of the preferred methods for validating or confirming the presence of lipids and thereby probing known or candidate proteins, this method is time-consuming when using tritiated precursors and expensive and hazardous in all the cases. Furthermore, it cannot provide quantitative information about the extent of acylation and it may not be useful for detecting proteins with low levels of expression. Another important limitation of radioactivity is that it is generally not applicable to global analysis considering all the mentioned aspects, it is clear that other approaches would definitely be preferred.

**Mass spectrometry:** Mass spectrometry (MS)-based methods have been extensively used for the mapping of posttranslational modifications. This technology presents important advantages. Apart from a high sensitivity, it can be applied to identify new posttranslational modifications and to determine the modification site [85]. Conversely, some drawbacks of this technique include the need for prior purification of the protein and a lack of compatibility between some existing MS-based methods and hydrophobic proteins.

The methods mostly used for protein analysis are ESI-MS and MALDI-MS, which differ from the system used for sample ionization, either electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) (reviewed in [86]). Subsequent mass analysis is usually performed using time-of-flight (TOF), quadrupole mass analyzer or ion trap methods including high-resolution methods like the orbitrap. Proteins or protein mixtures to be analysed are usually first digested into small peptides to facilitate the analysis. Briefly, after isolation or separation of the proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) or by two dimensional gel electrophoresis (2DE), they are subjected to in gel-digestion. The resulting peptide fragments are then analyzed by mass spectrometry (either ESI-MS or MALDI-MS) and identified by peptide-mass fingerprint. A classic example of this approach is the detection of the palmitoylated Shh protein using an HPLC method coupled to a triple quadruple mass spectrometer, followed by confirmation by sequencing of the lipidated N-terminal peptide by MALDI [88]. Similarly, the heterogenicity of the lipid groups present in the GAP-43 protein, which has two acylated cysteines near the C-terminus, was studied by MALDI-TOF and confirmed by Quadropole-TOF MS/MS indicating that not only palmitoyl but also stearic acid can be attached on these cysteines [51]. Recently, MALDI-MS was also used for the detection of purified myristoylated and isoprenylated proteins generated in an insect-cell free protein synthesis system [88-90]. However, one of the limitations of this approach is the high hydrophobicity of the lipidated samples, which usually results in low efficiency of peptide recovery during sample preparation as well as peptide detection. To solve this problem the addition of detergents compatible with MS or new sample preparations methods (such as liquid-liquid extraction) have been successfully applied to the detection of myristoylated [91] and palmitoylated peptides or proteins [92]. Alternatively, digested proteins may be analysed by HPLC-MS using the Multidimensional protein identification technology (MudPIT) developed by Yates group [93]. This approach is a non-gel technique based on a 2-dimensional chromatography separation of the peptides (usually a strong cation exchange column followed by a reverse phase chromatography column) prior to electrospray ionization. One of the great advantages of this technique is that it is compatible with hydrophobic or membrane proteins, which are generally excluded from gel-based analysis. MudPIT technology has been extensively used for detection of acylated proteins in combination with other methods such as the acyl-biotin exchange (ABE) or the metabolic labeling with lipid derivatives for bioorthogonal reactions (see below). Additional advantages of this approach are that it enables the identification of the lipidation-site when used in combination with tandem mass spectroscopy (MS/MS) and permits sample quantification. The latter can be performed thanks to the spectral count number, that considers both the number of different peptides belonging to a same protein and the redundant identification of the same peptide in different fractions [94]. Another method has recently been developed by Casey and co-workers to identify known and new novel sites of endogenous S-acylation [95]. This approach is a modified version of a previously reported method using resin-assisted capture (RAC) [96] coupled with MS-based proteomics to detect S-nitrosylation sites in proteins.

Not only the identification acylated proteins is of great interest, but also the characterization of the acyl groups present in these proteins in order to study the heterogeneity of the acyl moieties (palmitoyl,

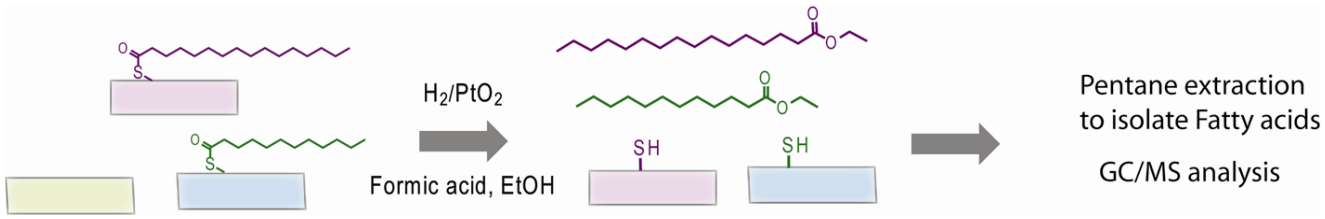


**Figure 6:** Method developed by Yalovsky and co-workers for the characterization of the acyl residues present in acylated proteins. The method consists on the release of S-acyl groups by hydrogenation and simultaneously conversion to ethyl esters. Subsequent analysis by GC/MS of the derivatized fatty acid allows their identification [97].

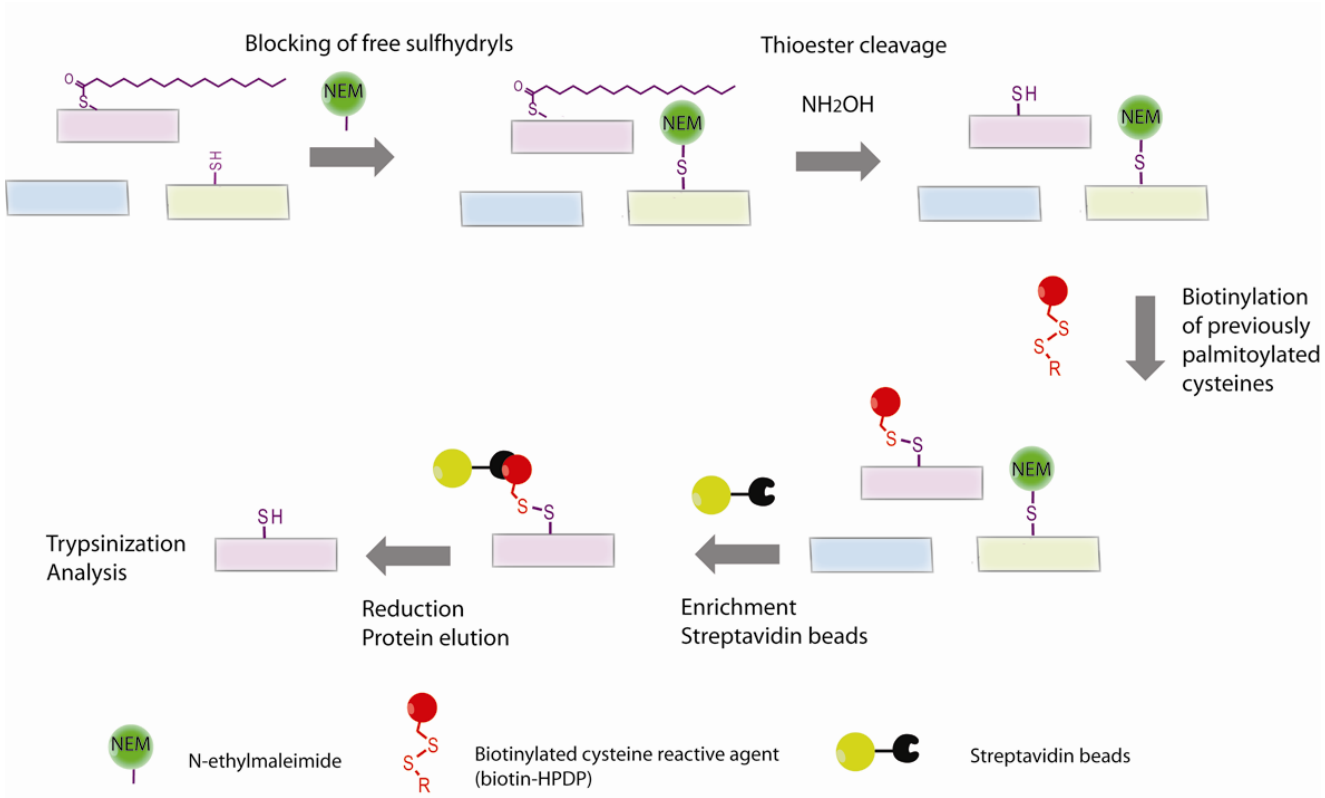


**Figure 7:** Identification of acylated proteins by MS-based proteomics using the acyl-biotin exchange method (ABE) established by Drisdel and Green [98].

stearoyl, oleoyl, etc.) present in acylated proteins. Recently, a method using gas chromatography coupled to mass spectrometry (GC/MS) was described by Yalovsky and co-workers for the identification of S-acyl groups in proteins (Figure 6) [97]. Mainly, the method is based on the cleavage of the thioesther group, thus releasing the fatty acid, by hydrogenation in presence of formic acid and ethanol, which results in acid esterification facilitating the analysis by GC-MS. Using this approach the authors could show that the Arabidopsis Rho related GTPase (ROP6) and the calcium sensor protein CBL1 were both modified by palmitic acid as well as by the 18-carbon stearic acid. However, this approach presents some disadvantages since it can only be used with purified protein and at least 1 µg is needed, which makes it not practicable as a high-throughput method or for proteins with low expression levels. Moreover, it only gives information about the acyl groups present in the protein but not about the acylation site. A LC/MS-based method would probably overcome these limitations, however as mentioned above, the high hydrophobicity of the lipidated samples has hampered a high-throughput analysis up to now.

**Acyl-biotin exchange:** Acyl-biotin exchange (ABE) was first developed by Drisdel and Green in 2004 as a non-radioactive method for the detection of protein palmitoylation [98]. This approach, which can be performed on protein samples or tissues, is based on the blocking of existent free sulfhydryl groups by N-ethylmaleimide (NEM), followed by the subsequent hydrolysis of the thioesters present in the sample by treatment with hydroxylamine. The resulting free sulfhydryls are then treated with cysteine reactive agents such as biotin-BMCC (1-biotinamido-4-[4′-maleimidomethyl)cyclohexanecarboxamido] butane) or biotin-HPDP (Biotin-HPDP-N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio) propionamide) and the labeled proteins can be then enriched or precipitated by streptavidin agarose [98-100] and identified by Western blotting or by MS-based proteomics (Figure 7).

The approach has been successfully applied together with different methods for MS-based proteomics, including the MudPIT technology, to identify numerous palmitoylated proteins and to illustrate their role in the cell. In one of the first examples, this technology was used to

analyse the palmitoyl-proteome of *Saccharomyces cerevisiae* leading to the identification of 12 known and 35 new palmitoylated proteins [101]. Similarly, this strategy was employed for the analysis of the rat neural palmitoyl-proteome detecting 68 known palmitoylated proteins and several unknown candidates for palmitoylation. At least 20 of these candidates could be confirmed in this work [102]. These results, together with additional experiments, pointed out that dynamic palmitoylation may have crucial role in controlling the morphological and functional changes occurring during synapsis. Later on, this strategy was also applied to gain an insight into the substrate specificity of members of the putative palmitoyl transferase family DHHC [103].

Acylated proteins have also an important role in controlling several crucial processes in parasites such as invasion or motility (reviewed in [104]). Thus, the analysis of the parasitic palmitoylome may shed light on elucidating the contribution of this lipidation in infectivity. A recent study offered some preliminary information in this direction by detecting around 120 known and unknown acylated proteins from *Trypanosoma bruzei*, the parasite causing the African sleeping sickness [105].

Due to all the significant improvements (avoids the use of radioactivity, can be used to detect proteins with low levels of expression and it does not require prior cell labeling), ABE has been widely applied in the last years. However, some important limitations have to be taken into account. Mainly, ABE is restricted to S-acylated proteins that will be hydrolysed after treatment with hydroxylamine and therefore it cannot be applied to covalent lipid modifications such isoprenylation or myristoylation. Moreover, all the steps taking place in this method, both the blocking of the already present sulfhydryl groups with NEM and the subsequent hydrolysis and labeling of the previous acylated thiols, should be quantitative to avoid false-positives. This requires the use of negative controls to discard false results. Apart from this, since it is based on an indirect detection, it usually needs confirmation of the lipidation by radiolabeling methods and it cannot be applied for cellular imaging of lipidated proteins. Furthermore, this method gives normally only information of previously acylated proteins without giving any information about the identity of the acyl residues or the acylation sites. To overcome this particular limitation, the PalmPISC approach (pamitoyl protein identification and site-characterization) was recently developed as a combination of ABE with a label-free spectral counting method [58]. Briefly, the localization site is here determined after trypsin digestion of the biotin-labeled proteins. Biotinylated fragments are then enriched by using Streptavidin beads and after removal of the biotin tag under reducing conditions, peptides can be analysed by nano LC-MS. This new approach led to the identification of 67 known and 331 novel S-acylated protein candidates and the localization of 25 known and 143 novel S-acylation candidate sites. Interestingly, this was carried out in lipid-raft enriched and non-raft membrane domains revealing that palmitoylated proteins were preferably found in non-raft fractions.

**Bioorthogonal probes:** Bioorthogonal probes and bioorthogonal chemical reactions, i.e. reactions compatible with biomolecules that allow their study in their native settings, have witnessed an enormous development in the last decade (recently reviewed by Bertozzi and Sletten [106]). Concomitantly, progress in this area has been successfully applied to increase the available methods for the study of posttranslational lipid modifications. Indeed, the use of bioorthogonal probes has greatly facilitated the research providing unique opportunities to analyse protein lipidation. This approach is essentially based on metabolic labeling with lipid analogs containing chemical groups not present in biological samples (mostly azide and alkyne modifications) that allow for bioorthogonal reactions with chemical tags. Two different bioorthogonal reactions have been mainly employed, the Staudinger ligation and the copper-catalyzed azide–alkyne 1,3-dipolar cycloaddition (CuAAC) of azides with terminal alkynes also referred to as Huisgen reaction or click chemistry.

The Staudinger ligation, a modification of the known Staudinger reduction, was first developed by Bertozzi and co-workers [107] and is based on the reaction of an azide with a modified triarylphosphine to yield an aza-ylide that will undergo an intramolecular attack to form a stable amide bond. Similarly, the CuAAC developed independently by the Sharpless [108] and Medal research groups [109] is also based on the reaction of azide but in this case with a terminal alkyne. Azides and alkynes are small and absent in all biological systems which makes them bioorthogonal to biomolecules. Both reactions present some advantages and some limitations. The CuAAC, in contrast to the Staudinger ligation, requires the use of copper as a catalyst which can result in denaturation of the proteins [110] or can cause toxicity in living systems thus restricting its use in cells [111]. However, higher reaction rates have been described for the CuAAC or Huisgen reaction which makes this approach more suitable for studying dynamic systems or for animal studies where metabolic clearance of the probes should be minimized. Nevertheless, a copper-free version of the CuAAC has been developed by Bertozzi´s group. This approach which involves the use of the strained, and therefore more reactive, cyclooctyne may overcome this limitation [112]. Regarding the use of bioorthogonal probes, the Huisgen reaction presents some advantages since it permits the use of both azido and alkyne tagged analogs while the Staudinger ligation requires the use of azido-tagged analogs and phosphine derivatives. This advantage relies on the fact that azido-tagged probes may suffer from reduction to amines in cellular conditions and additionally, phosphine derivatives can be oxidised by molecular oxygen thus decreasing its half-life in living systems. For all these reasons the use of alkyne-tagged probes is preferred for cell metabolic labeling. A schematic overview of the employment of these two bioorthogonal reactions for the analysis of protein lipidation is depicted in Figure 8.

**Staudinger ligation:** One of the first examples of the application of the Staudinger ligation was reported by Zhao and co-workers in 2004 [113]. The authors successfully used azido-containing farnesyl substrates for protein labeling in cells. After incubation of the cell lysate with a biotinylated phosphine-capture reagent, the resulting prenylated proteins were enriched using streptavidin beads, followed by digestion with trypsin to analyse the proteins by nano-HPLC/MS. The sample showed specificity for FTase modified proteins in front of GGTase. Several known farnesylated proteins were detected showing the applicability of the bioorthogonal probes for the study of lipid protein modifications.

Waldmann and Alexandrov also reported the synthesis of isoprenoid pyrophosphates containing azido and diene reacting groups. These derivatives could be used as a substrate for FTase mediated prenylation of Ypt7 as well as fluorescent proteins containing the required CAAX box as recognition motif. The resulting prenylated proteins could be further modified by the Staudinger ligation or Diels Alder reaction [114]. Later, the authors used a biotin-geranylgeranyl (GerGer) pyrophosphate analog and an engineered protein prenyltransferases to analyse the in vivo effects of protein prenyltransferase inhibitors by quantifying by MS the amount of biotin-GerGer-tagged RabGTPases after treatment with inhibitors [115]. Synthesis of additional farnesyl diphosphate analogs bearing alkyne or azide groups [116,117] as well as in vitro studies to characterize them as substrates for the corresponding prenyl transferases have also been described by other groups [118,119].

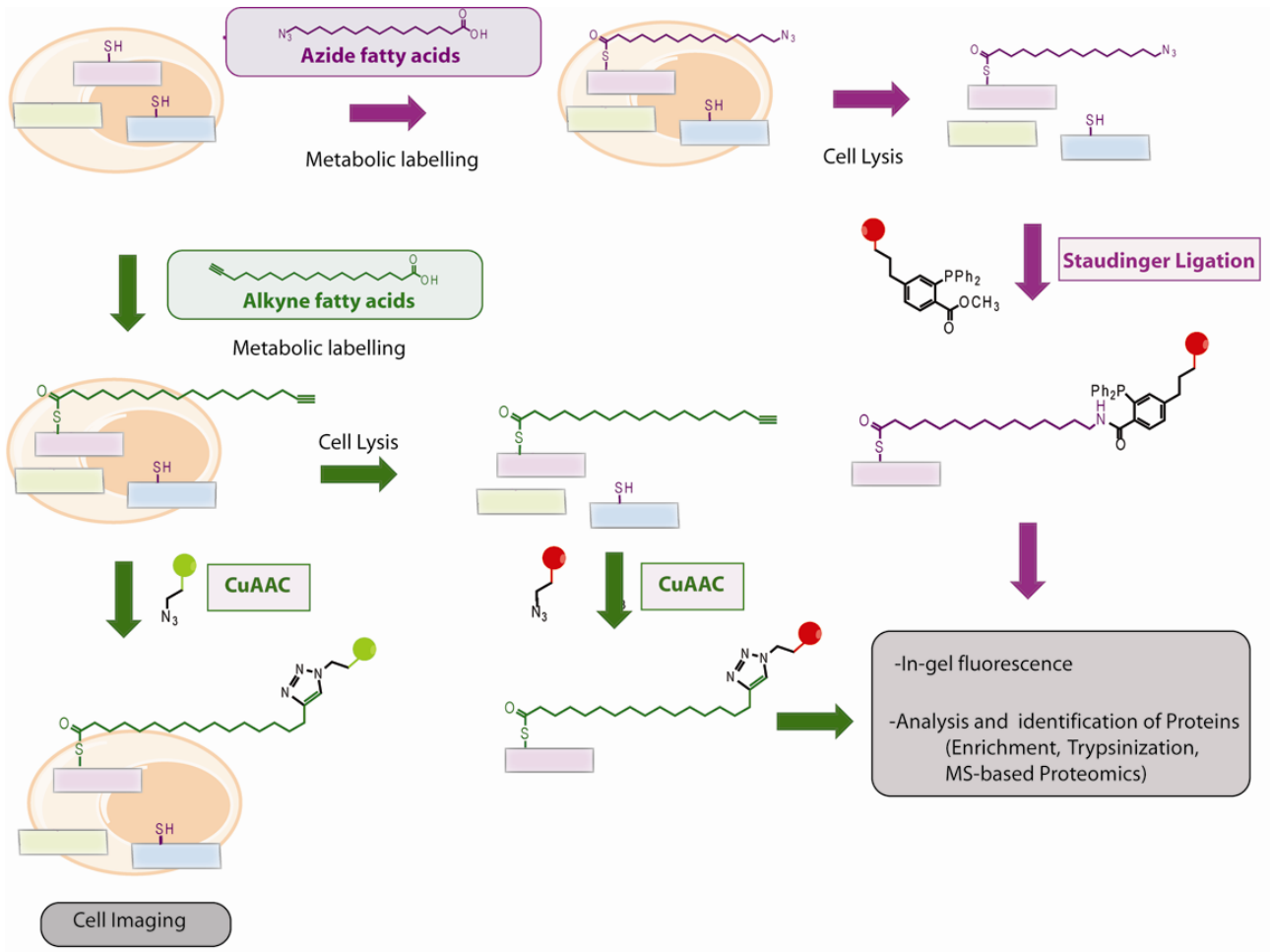


**Figure 8:** Lipid-modified proteins have been studied using bioorthogonal reactions. After metabolic labeling with alkyne (in green) or azide-containing lipids (in purple) and cell lysis, proteins were labeled by means of Staudinger ligation or CuAAC with biotinylated or fluorescent tags for further analysis and identification. Alternatively, these methods can be also used prior to cell lysis for cell imaging in fixed cells.

Recently, Tate and co-workers reported an azido-derived GerGer pyrophosphate that could serve as a substrate for both GGTase I and RabGGTase and be subsequently modified by CuAAC to incorporate a double labeled tag [120].

After the initial work characterizing isoprenylation, this approach was applied for the investigation of other lipid modifications. One of the first examples was described by Ploegh and co-workers who employed the Staudinger ligation to identify myristoylated and palmitoylated proteins [121]. The authors reported the synthesis of azido-fatty acids of different lengths (12-16-carbons) and showed that the 12-carbon length fatty acid selectively targeted N-myristoylation while the longer ones served as a substrate for S-palmitoylation. This was proven by labeling of a myristoylated and palmitoylated Lck, a src family member protein kinase essential for T-cell activation. Later Berthiaume´s group also exploited it for the identification of palmitoylated mitochondrial proteins [122].

**CuAAC:** Previous works combining azido-fatty acids together with Staudinger ligation have already allowed tremendous progresses in the detection of palmitoylated proteins. However, a great advance in this field was achieved by Martin and Cravatt who in 2009 reported the first profiling of protein palmitoylation using an alkyne derived palmitic acid [123]. Briefly, after metabolic labeling with this alkyne-fatty acid and reaction via CuAAC with a biotin-azide, proteins were enriched and subjected to proteomics analysis using the MudPIT technology leading to 125 predicted palmitoylated proteins. Furthermore, a straightforward gel-based validation of some of the predicted proteins could also be used with the same protein sample using rhodamine-azide, avoiding the use of immunoblotting which was applied in previous methods. In parallel, Hang and co-workers reported a detailed study on the use of alkyne and azide modified fatty acid and CuAAC to study palmitoylation [124]. The authors also noted that in-gel fluorescence detection of proteins gave better results than streptavidin blotting and noted as well that metabolic labeling with alkyne fatty acids seemed to give a better signal-to noise ratio than the use of azido-fatty acids. Later, Hannoush et al. showed that this technique could be also used for detection and imaging of protein fatty-acylation in cells by fluorescence microscopy. However, due to the toxicity of copper catalysis, live-cell imagining is not possible and cells need to be fixed before protein visualization [125]. Interestingly, this bioorthogonal reaction has been recently applied by Hang´s group for the simultaneously monitoring of dynamic S-acylation and protein turnover of the protein kinase Lck [126]. To achieve this, the authors took profit of the lipidation pattern of Lck, cotranslationally labeled with the irreversible bound myristic acid at the N-terminus and posttranslationally labeled with the reversible S-palmitoyl modification. Briefly, cells were metabolically labeled

using an alkyne-palmitic acid and an azido-myristic acid, thus enabling detection of both lipidation events in the same experiment. Subsequent incorporation of a fluorescent tag was performed via CuAAC with two different functionalized flurophors. The authors could prove with this approach that the palmitoyl turnover in Lck increases upon T-cell activation. Metabolic labeling with alkyne-containing fatty acids has also been used by other groups for the study the lipidated N-Ras and H-Ras, [127] for the characterization of myristoylated proteins [128] or for confirming the acylation of Histone H3 variants [129,130]. In addition, isoprenoid derivatives have also been reported and used for the analysis of the prenylome using CuAAC [116,117,131,132].

Although this method shows a substantial improvement and has been extensively employed for the study of palmitoylation, some limitations have to be considered. For example, as it requires metabolic labeling, it cannot be applied to sample tissues. Additionally, it could be a difficult method to use in cancer cells due to the overexpression and hyperactivity of fatty acid synthase, which decreases the intake of exogenous long chain fatty acids [133]. Apart from these limitations, altogether the combination of alkyne-fatty acid and CuAAC seems to be a rapid and robust method to characterize palmitoylation in cells. Furthermore, the method is suitable for performing dynamic measurements, as detection of palmitoylation in fixed cells at distinct time points following labelling, which will be of great importance for an in-depth characterization of this modification or to determine the extent of palmitoylation in diseases.

## Concluding Remarks

Important advances have been reported in the development of methods for analysis of protein lipidation. The establishment of the acyl-biotin exchange strategy or the application of bioorthogonal reactions for metabolic labeling followed by protein identification has enabled promising advances in this field. Additionally, as a key player in this area, the significant developments over the last years in MS-based methods have strongly contributed to our knowledge in the role of lipidation in regulating protein function and activity. However, despite all these methodological and technical advances, there is still demand for strategies as well as for analytical improvements to address unsolved problems. Major challenges are the detection of new lipid posttranslational modifications and the in-depth characterization of relatively unexplored topics such as the complex GPI anchors or the role of lipidation in autophagic proteins. Other interesting points to be addressed in the future are the monitoring of the dynamic S-acylation and the elucidation of its role in physiology as well as in disease and the establishment of label-free methods to study palmitoylation in cells or tissues. Moreover, the development of high-throughput methods to analyse lipid heterogeneity in S-acylated cysteines and to correlate them with changes on protein localization and functioning will definitely give us a better insight into this area. In summary, it has become clear in the last years that protein lipidation is not only a tool to confer membrane affinity but also possess crucial regulatory functions. The development of novel strategies to tackle these and other questions will certainly contribute to our understanding of this field.

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