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Review Article

Mapping the myristoylome through a complete understanding of protein myristoylation biochemistry

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Running title: Genome-wide overview of protein myristoylation

ABSTRACT

Protein myristoylation is a C14 fatty acid modification found in all living organisms. Myristoylation tags either the N-terminal alpha groups of cysteine or glycine residues through amide bonds or lysine and cysteine side chains directly or indirectly via glycerol thioester and ester linkages. Before transfer to proteins, myristate must be activated into myristoyl coenzyme A in eukaryotes or, in bacteria, to derivatives like phosphatidylethanolamine. Myristate originates through *de novo* biosynthesis (e.g., plants), from external uptake (e.g., human tissues), or from mixed origins (e.g., unicellular organisms). Myristate usually serves as a molecular anchor, allowing tagged proteins to be targeted to membranes and travel across endomembrane networks in eukaryotes. In this review, we describe and discuss the metabolic origins of protein-bound myristate. We review strategies for *in vivo* protein labeling that take advantage of click-chemistry with reactive analogs, and we discuss new approaches to the proteome-wide discovery of myristate-containing proteins. The machineries of myristoylation are described, along with how protein targets can be generated directly from translating precursors or from processed proteins. Few myristoylation catalysts are currently described, with only N-myristoyltransferase described to date in eukaryotes. Finally, we describe how viruses and bacteria hijack and exploit myristoylation for their pathogenicity.

Keywords: *N-terminus, cysteine, glycine, lysine, compartments, acylation*

HIGHLIGHTS

- The metabolic origins and regulatory role of myristate donors for protein myristoylation are described for each living organism including prokaryotes.
- *In vivo* protein labeling strategies that take advantage of click chemistry with reactive alkyl or azido analogs are described.
- We discuss new approaches to the proteome-wide discovery of myristate-containing proteins.
- We update the catalogue of the main myristoylated proteins together with how they shuttle in different compartments.
- We discuss how viruses and bacteria exploit myristoylation for their pathogenicity.
- New catalysts for myristoylation must be discovered soon to advance the field.

ABBREVIATIONS

AcCoA, acetyl-coenzyme A
ACP, acyl carrier protein
(L)ACS, (long-chain) acyl CoA synthase
ARF, ADP-ribosylation factor
ARL, ARF-like
AMPK, AMP-activated protein kinase
cBID, cleaved BH3-interacting domain death agonist
CBL, calcineurin B-like
CDPK, calcium-dependent protein kinases
CoA, coenzyme A
ER, endoplasmic reticulum
FA, fatty acid
FAE, fatty acid elongase
FAS, fatty acid synthase
GNAT, GCN5-related N-acetyltransferase
GPI, glycosylphosphatidylinositol
HBV, hepatitis B virus
Lgt, phosphatidylglycerol:prolipoprotein diacylglyceryl transferase
Lnt, (apo)lipoprotein N-acyl transferase
LPA, lysophosphatidic acid
MyrCoA, myristoyl-coenzyme A
NEFA, non-esterified fatty acids
NMT, N-myristoyltransferase
PBL, PBS1-like RLCK
PA, phosphatidic acid
PE, phosphatidylethanolamine
PEG, polyethylene glycol
PG, phosphatidylglycerol
PM, plasma membrane
RLCK, receptor-like cytoplasmic kinase
RTX, Repeat in ToXin class
TAAT, toxin-activating acyl-transferase
TAG, triacylglycerol
TAMRA, tetramethylrhodamine
TE, thioesterase
TEV, tobacco etch virus
TNF, tumor necrosis factor

TABLE OF CONTENTS

ABSTRACT	2
HIGHLIGHTS.....	2
ABBREVIATIONS.....	3
TABLE OF CONTENTS.....	4
1. Introduction: membranes, lipids, proteins, and fatty acylated proteins.....	7
2. Myristoylation sites in proteins in different organisms	8
2.1. Myristoylation in bacteria	8
2.1.1. Myristoylation of membrane proteins occurs at N-terminal cysteine residues.....	8
2.1.2. Lysine myristoylation of secreted virulence factors	11
2.2. Myristoylation occurs at various sites in eukaryotes.....	13
2.2.1. Residues modified by myristoylation in eukaryotes and their viruses.....	13
2.2.2. How myristoylation-reactive N-terminal Gly are generated <i>in vivo</i>	15
2.2.3. Palmitoylation sites may also incorporate C14 chains	15
3. Origins, distribution, and regulatory roles of myristate donors for protein myristoylation	16
3.1. Overview of myristate synthesis in living organisms	17
3.1.1. Fatty acid synthases (FAS).....	17
3.1.2. Fatty acid elongases.....	20
3.1.3. Regulation of chain extension by FAS.....	21
3.2. Origin of the cytosolic MyrCoA pool in eukaryotes	25
3.2.1. FA availability in biological fluids	25
3.2.2. FAS and LACS.....	27
3.3. Non-cytosolic subcellular pools of C14 FA and their impact on myristoylation	28
3.3.1. FA and β -oxidation in lysosomes and mitochondria	28
3.3.2. AcylCoA derivatives and protein acylation in the ER.....	29
3.4. Myristate in plastid-containing organisms.....	30
3.5. FA and the regulation of myristoylation in eukaryotes	31
4. Myristoylation in eukaryotes and subcellular shuttling.....	32
4.1. Main structural and biochemical features of NMTs	33
4.2. NMT subtypes in eukaryotes recapitulate the tree of Life	35

4.3.	Main myristoylated proteins, membrane targeting, and cell compartmentalization	38
4.3.1.	Myristoylated proteins shuttle between membrane compartments in eukaryotes	38
4.3.2.	Myristoyl switches facilitate compartment shifts	39
4.3.3.	Endosomes and lysosomes as newly recognized hubs exploiting myristoylation	42
4.4.	The myristoylome as the sub-proteome targeted by NMT	44
4.5.	Peptide libraries	44
4.5.1.	Labeling	45
4.5.2.	Medium-throughput approaches	46
5.	Defining myristoylation at the proteome scale	47
5.1.	Validating chemical probes for click chemistry	47
5.1.1.	Click chemistry, bioorthogonal labeling, and FA analogs	47
5.1.2.	Probes for myristoylation labeling	48
5.2.	Click chemistry enrichment approaches	51
5.3.	Combining subcellular enrichment and high-resolution peptide separation	54
5.4.	Towards complete view of myristoylomes in eukaryotes	57
5.4.1.	Co-translational myristoylation	57
5.4.2.	Post-translational myristoylation	59
5.4.3.	Fungi display a reduced percentage of targets in their G-myristoylome	59
5.4.4.	Plants display higher levels of G-myristoylated targets featuring many sensors	60
5.5.	Protein myristoylomes of bacteria	65
6.	The biological function of NMT-dependent myristoylation of viral and bacterial proteins	65
6.1.	Myristoylation and viruses	65
6.1.1.	General features	65
6.1.2.	Myristoylation-dependent membrane-to-plastid re-targeting of viral plant proteins	67
6.2.	Myristoylation of bacterial virulence factors within the host	68
7.	Conclusions and perspectives: new myristoylation catalysts need to be defined	70
7.1.	Glycine acyltransferases in bacteria	70

7.2.	Evolutionary considerations on the origin of myristoylation	71
7.2.1.	Protein acylation and FA derivatives in Archaea	71
7.2.2.	FAS/NMT links.....	72
7.3.	Myristoylation and the reactivity of protein amino groups.....	73
7.4.	Peptide myristoylation in drug discovery	75
	ACKNOWLEDGEMENTS	76
	COMPETING INTERESTS	76
	REFERENCES	78

ITEMS

Figure 1	10
Figure 2	17
Figure 3	18
Figure 4	20
Figure 5	22
Figure 6	24
Figure 7	26
Figure 8	36
Figure 9	39
Figure 10	41
Figure 11	50
Figure 12	56
Figure 13	58
Figure 14	63
Figure 15	64
Table 1	77

1. Introduction: membranes, lipids, proteins, and fatty acylated proteins

Organisms rely not only on duplicating and interpreting their genetic code but also on elaborating a dedicated container - the cell - to maintain their contents for much longer periods than individual components. To achieve this, the plasma membrane (PM) is an essential substructure that acts as the interface between the outside and the inside of the cell to control nutrition, replace damaged components, and sense the microenvironment of neighboring cells, symbionts, pathogens, and abiotic events. Membranes are mostly formed from lipids, which sustain the overall cellular architecture, and proteins, which act as the main sensors. The composition and type of membrane lipids are crucial for membrane organization into microdomains, so as well as acting as a physical barrier, the PM also contributes to sensing. In addition to transmembrane proteins, membranes display proteins with covalently linked lipids, usually fatty acids (FAs) or isoprenoids, but also sterols [1, 2]. This organization allows lipids to penetrate the membrane while making the protein fully available inside or outside the cell for interactions with other proteins, sensing, and signal transduction. Unlike prokaryotes, eukaryotes have progressively evolved refined internal membrane networks, or endomembranes, to compartmentalize many diverse intracellular functions such as DNA replication within the nucleus. This evolution therefore serves several different needs. Among the major protein FA additions, (i) amide bond acylation such as myristoylation, (ii) thioester bond-mediated events such as palmitoylation and isoprenylation/farnesylation, and (iii) mixed ester/amide bonds with glycosylphosphatidylinositol (GPI) anchors are particularly noteworthy [3]. Nevertheless, despite their importance in cellular transduction, the identification of the entire set of proteins

undergoing lipidation in each proteome has been challenging due to the poor handling characteristics of these amphiphilic, chimeric macromolecules.

Here we review a lipid modification often occurring at protein N-termini, protein myristoylation. Myristoylation describes the addition of a C14:0 fatty acid linked to the free side chains of cysteines and lysines or N-termini glycines and cysteines of proteins. Recent approaches exploiting cutting-edge technologies have provided an unprecedented description of the glycine myristoylome (G-myristoylome), which describes the entire set of proteins undergoing myristoylation on an N-terminal glycine. The G-myristoylome is not only the first modified proteome described in such detail and to near completion but also the first to do so in a range of organisms recapitulating evolutionary history [4, 5]. These and other studies have also revealed some of the functions of G-myristoylated proteins. We therefore discuss the many processes related to myristoylation in various prokaryotes and eukaryotes to highlight their relationship with disease pathogenesis and toxicity. With respect to eukaryotes, we also address the involvement of myristoylation in the endosome/lysosome compartment, where myristoylation has recently been shown to play major roles.

2. Myristoylation sites in proteins in different organisms

Myristoylation has now been described in most living organisms, including viruses, the exception being Archaea (for further discussion, see Section 7.2).

2.1. Myristoylation in bacteria

2.1.1. Myristoylation of membrane proteins occurs at N-terminal cysteine residues

In bacteria, protein N α -myristoylation and the addition of myristate to N-terminal cysteine (Cys) residues (S-myristoylation) have been described for both inner and outer membrane proteins. This mechanism can involve a triacylation. Glycerol thioether-mediated, ester-linked substitutions, and acylations (referred to as R₁ and R₂, **Fig. 1A,B**) on the side chain of Cys and amide-linked acylation (R₃, **Fig. 1C**) on the N-terminus have all been described. By virtue of both the thioester and ester bonds, the R₁ and R₂ modifications could theoretically be reversible; this is in contrast with the irreversible character of the R₃ modification, which involves an amide bond arising from leader peptide proteolytic cleavage. A survey of different modified groups has revealed only rare myristoylation (i.e., at R₃): 6-10% fatty acylation on proteins and the others saturated or mono-unsaturated C15, C16 (palmitate), C17, and C18 (stearate) acylations [6-8]. In *Corynebacterium glutamicum*, the purified MusE protein harbors only C16 [9], as does *Neisseria meningitidis* MetQ overexpressed in *Escherichia coli* [10]. The relative extent of bacterial myristoylation might vary depending on the phylum or the physiological conditions and has been shown to occur in proteins transported to the cytoplasmic membrane by the secretory pathway involving the SecYEG translocon and the twin-arginine transport protein Tat.

The triacylated N-terminal cysteine residue first arises by transfer of a diacylglyceryl group onto the prolipoprotein by phosphatidylglycerol:prolipoprotein diacylglyceryl transferase (Lgt, **Fig. 1A**). In triacylation, this first alkylation of the Cys side chain is followed by cleavage of the upstream signaling peptide by a prolipoprotein signal peptidase (Lsp, **Fig. 1B**). N-acylation, including myristoylation of diacylglyceride-cysteine, is catalyzed by (apo)lipoprotein N-acyl transferases (Lnt, EC:2.3.1.269, **Fig. 1C**), which use phosphatidylethanolamine (PE), phosphatidylglycerol (PG), or cardiolipin as lipid donors [11-14].

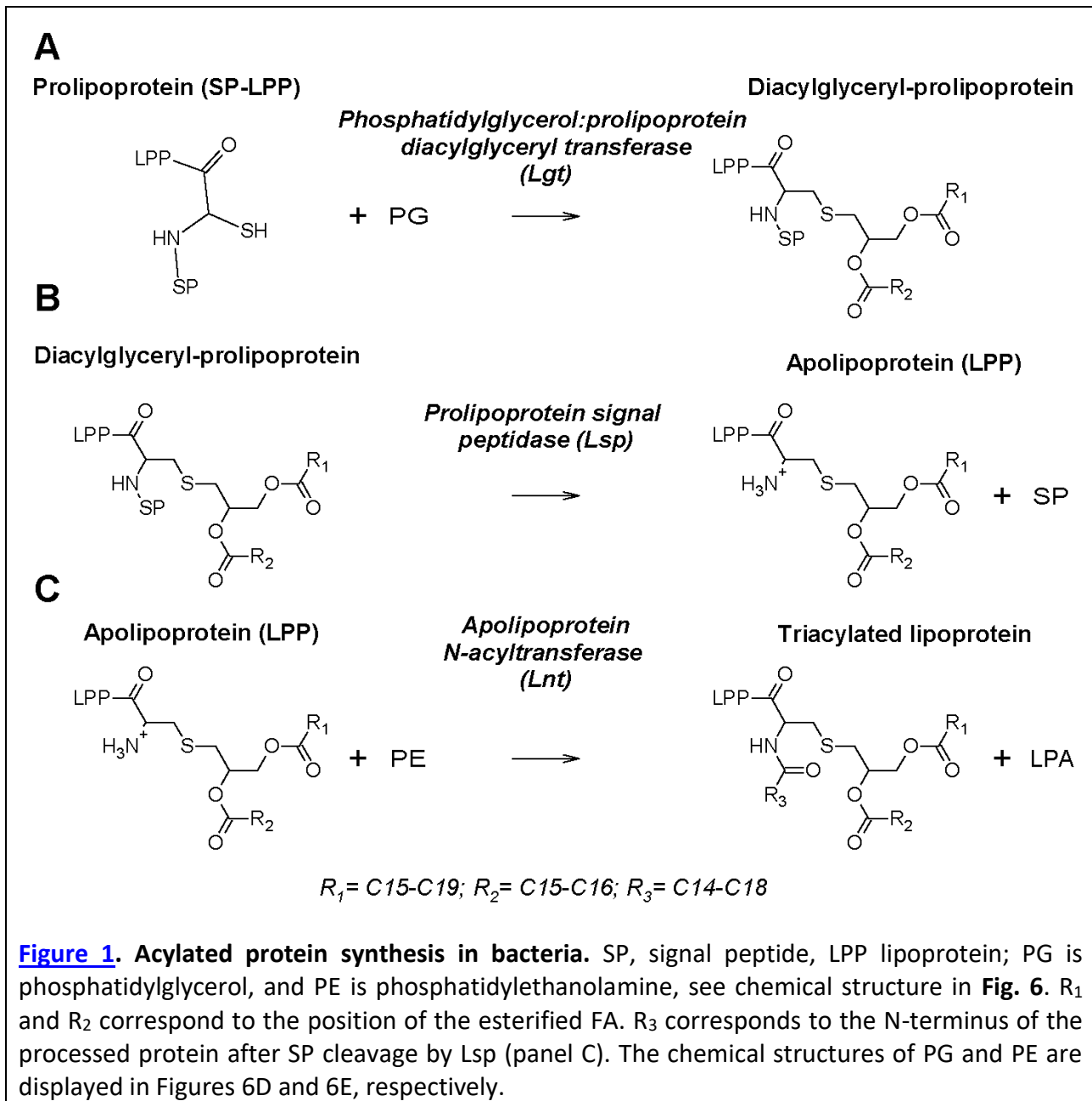


Figure 1. Acylated protein synthesis in bacteria. SP, signal peptide, LPP lipoprotein; PG is phosphatidylglycerol, and PE is phosphatidylethanolamine, see chemical structure in Fig. 6. R_1 and R_2 correspond to the position of the esterified FA. R_3 corresponds to the N-terminus of the processed protein after SP cleavage by Lsp (panel C). The chemical structures of PG and PE are displayed in Figures 6D and 6E, respectively.

Alternative N-acyl modifications including N-acetylation have been observed in lipoproteins from low-GC Gram-positive bacteria [8]. The 3D structure of *E. coli* Lnt (512 aa) reveals eight transmembrane helices that bind to the inner membrane [13]. The acyltransferase domain is 250 amino acids long and located at the C-terminus before the last transmembrane helix. The purified protein reveals a variety of bound lipids of various chain lengths by mass spectrometry (see

SupFig.4 in [13]), with palmitate (C16, 238 Da) the major form. The periplasmic hydrolase domain of Lnt contains a Cys–Glu–Lys catalytic triad, where Cys mediates the nucleophilic attack on the phospholipid carbonyl group and Lys stabilizes the oxyanion of the tetrahedral intermediate. A Trp residue is involved in the recognition of the phosphate head of the phospholipid acyl donor and a Phe in acyl-enzyme intermediate stabilization. Lnt belongs to the large nitrilase family, which includes amide-condensing enzymes, all featuring the aforementioned Cys-Glu-Lys triad [15].

The third acylation at R₃ on the N α strongly anchors the target proteins to the inner membrane towards the periplasmic space. After tri-acylation, a dedicated machinery (Lol) translocates mature (triacylated) lipoproteins from the inner cytoplasmic membrane through the periplasm to the outer membrane, where the acylated lipoprotein inserts [16].

2.1.2. Lysine myristoylation of secreted virulence factors

Several Gram-negative pathogens express Repeat in ToXin class (RTX) toxins, which are glycine-rich virulence factors exported from the cell via the type I secretion system [17] in a single step directly from the cytosol to the extracellular space. Once bound to the host membrane, RTX toxins form pores in host immune system cells to impair their function [17]. All RTX toxins (including RtxA in *Vibrio*, CyaA in *Bordetella*, HlyC in uropathogenic *Escherichia*, and LtkA in *Pasteurella*) share acylation of the ϵ -amino group of two lysines. This acylation involves an amide bond, while the donor is the acylated acyl carrier protein (ACP, see below), where the acyl group is linked through a thioester bond [18]. It is currently unknown whether this process is reversible. Bacterial cells are acylated by a large family of dedicated lysine acyltransferases (e.g., HlyC, RtxC),

compact enzymes of ~160 aa in the COG2994 superfamily (EC:2.3.1.-) of ACP:hemolysin acyltransferases [19]. High-resolution structural analysis of one family member, ApxC (a toxin-activating acyltransferase (TAAT) from pathogenic *Actinobacillus pleuropneumoniae*) revealed that RTX are members of the GCN5-related N-acetyltransferase (GNAT) superfamily [20]. GNATs cover different types of enzymes catalyzing acyl transfer to a variety of donors [21, 22]. RTX shows higher structural homology to FeeM, a tyrosine transacylase that uses ACP-C11 as acyl donor [23]. There are two main types of GNATs, those relying on the CoA donor and those relying on other donors such as ACP. Asp93 acts as a general base to deprotonate the receptor lysine of the protoxin. His24 then serves as a general acid, protonating the ACP thiolate. This extends the already large GNAT family to catalysts not using acyl or CoA derivatives as donors but the alternative cellular acyl donor ACP. As detailed below, holoACP displays the pantetheine group of CoA as a prosthetic group to react with the acyl donor.

Both lysines must be acylated for RTX-driven toxicity [24]. The nature of the fatty acyl groups appears to vary, but it frequently corresponds to a C14:0 (myristate), although saturated C15, C16, and C17 also occur [25, 26]. As a result, TAAT does not display a strong preference for a given C12-C16 FA. As already discussed for Lnt, myristate incorporation is likely to be host, metabolic pathway, and environment dependent. The associated toxicity will also rely on the target mechanism and competition with host processes. RtxA toxicity relies on myristoylation alone, while other RTXs also tolerate longer chains to achieve toxicity, and others like CyA require palmitoylation [27]. Each of the two lysines (separated by >100 aa) appear to be acylated independently [24], suggesting the presence of local recognition elements. The acylation sites are poorly conserved, although all lysines are preceded by a conserved glycine residue [28].

2.2. Myristoylation occurs at various sites in eukaryotes

2.2.1. Residues modified by myristoylation in eukaryotes and their viruses

Myristoylation was discovered in eukaryotes in the early 1980s as a modification targeting major proteins such as the catalytic subunit of human cAMP-dependent protein kinase (AMPK) [29] and the bovine Ca^{2+} -binding non-catalytic subunit of protein-phosphatase 2B (calcineurin B) [30]. Further discoveries included myristoylation of cytochrome b_5 reductase [31], MuLV, and HIV p15^{gag} and p27^{nef} or p60^{src} together with other non-receptor tyrosine kinases of the c-src family, the so-called SFKs. However, it took time for myristoylation to be appreciated as a relatively frequent modification (see [32, 33]). A recent large-scale analysis of protein modifications ranked myristoylation 546th of 4,278 modifications occurring in the human proteome i.e., in the top 13% [34]. Myristoylation occurs throughout most of the eukaryote lineage.

In eukaryotes, myristoylation is mainly associated with the modification of N-terminal (α) glycines (G-myristoylation). Lys-N- ϵ (K-myristoylation) or Cys-S-myristoylation (S-myristoylation) have also been detected, although to a lesser extent than G-myristoylation [34, 35]. For decades, K-myristoylation was thought to occur on the ϵ -amino group of only a couple of internal lysines in mammalian proteins including tumor necrosis factor- α [36], interleukin- α 1 precursor [37], and serine hydroxymethyltransferase (SHMT2) [38]. K-myristoylation of specific lysines on other proteins including subunit 1 of cytochrome *c* oxidase in *Neurospora crassa* fungus has also been described [17, 39]. A large survey of many protein modifications including K-myristoylation (see Supplementary Table 10 in [34]) indicates abundant proteins such as actin, myosin, and serum albumin display K-myristoylation marks. Together, these data suggest the presence of distinct

catalysts, unless the acylation is non-enzymatic [40], which could occur for serum albumin due to its natural avidity for myristate (see Section 3.2). Most recently, K-myristoylation has been described *in vitro* involving at least Lys3 of the small G-protein ARF6 *in vivo* [41-44]. The mechanism and catalyst are like those of G-myristoylation. However, the catalyst, N-myristoyltransferase (NMT, see Section 4), is not predicted to myristoylate most internal lysine residues [44]. Nevertheless, a lysine myristoyltransferase activity - still uncharacterized - specifically recognizes small peptides of 14 aa around target lysine residues as a substrate and uses MyrCoA as the myristate donor [37] (**Table 1**). In cells cultured in the presence of radioactive (9,10) ³H-myristate, three Lys and three Arg of the poxviral vaccinia virus A-type inclusion protein were found to be myristoylated [45, 46]. However, myristoylation of an average of two out of six sites corresponds to sub-stoichiometric labeling, so it is not unlikely that such myristoylation involves non-enzymatic, chemical labeling [47]. Vaccinia virus, like other poxviridae, rely on myristoylation since they contain at least four additional fully-labeled proteins starting with N-terminal Gly [48].

With respect to S-myristoylation, there is only one robust report of its existence in eukaryotes [35]. Investigations involving heterologous or analog metabolic incorporation (see Sections 2.2.3 and 5.1) indicate that Cys-S-acylation with C14 (myristoylation) instead of C16 (palmitoylation) sites is most likely to occur [49]. As a significant part of the human proteome is likely to be Cys-S-acylated, this provides a rational explanation for the frequent Cys-S-myristoylation observed (see Section 2.2.3). Finally, a large survey reported additional tags associated to myristoylation (210 Da; C₁₄H₂₆O) to other amino acids including Trp, His, Asp, Glu, or Thr. Such myristoylation tags are retrieved on abundant proteins such as hemoglobin or collagen in addition to the expected Gly

and Lys targets [34]. Whether these myristoylation events on unusual residues arise from enzyme catalysis is not yet known.

2.2.2. How myristoylation-reactive N-terminal Gly are generated *in vivo*

Early research showed that the majority of N-Gly residues are not myristoylated, as many were N-acetylated or unblocked [50, 51]. In addition, N-Gly residues usually appear to originate through initiator Met removal (Met1) via methionine aminopeptidase cleavage. Early data related to the picornaviral protein VP4 from foot-and-mouth disease virus suggested that the N-terminal Gly could also originate from internal cleavage following autocatalytic cleavage and removal from the precursor of the N-terminal leader protease [52, 53]. However, in some picornaviridae like poliovirus, myristoylation of VP4 homologs can also arise directly from methionine cleavage [54]. Since then, caspase-mediated proteolytic cleavage during apoptosis was shown to frequently unmask N-terminal Gly if preceded by a DEXD (where X is any amino acid) motif [55, 56].

2.2.3. Palmitoylation sites may also incorporate C14 chains

There is a dedicated enzymatic machinery for palmitoylation of Cys residues - the DHHC family of palmitoyl S-transferases [57, 58] - at membranes; mostly in the endoplasmic reticulum (ER), but also in lysosomes and the PM. DHHC are characterized by the presence of a 50-residue-long domain called the DHHC domain (named according to its highly conserved DHHC signature tetrapeptide Asp-His-His-Cys). DHHC enzymes modify Cys residue side chains, provided that they are located next to the membrane-binding region at the cytosolic side. DHHCs may tag any protein target to membranes by various mechanisms including the occurrence of TM domains and acylation events featuring prenylation or myristoylation. Palmitoylation can be removed by

acylprotein thioesterases, which consequently control PM shuttling (see Section 4.3). Palmitoylation could involve as much as 10% of the proteome [59]. Interestingly, base hydrolysis analysis of S-acetylated proteins indicates that, while C16 is the major acyl chain, C18 and C14 may also be incorporated at those sites [60]. The C16 preference likely translates to the overall relative cellular composition, with C16 and C18 being more abundant than C14 [60]. Most DHHCs do not display a strict cutoff for C12-16 acyl chains and may also incorporate C14 as S-linked fatty acids with almost similar selectivity [49, 61-63], for example with DHHC2/3/4/5/7/11/15 [64]. As a result, a low stoichiometry ratio of myristoylation to palmitoylation sites is expected, its extent varying on the relative availability of C14 in the cell (**Table 1**).

Palmitoylation has also been described close to myristoylation sites, i.e., at residues 3-8 [65]. It is now understood that such palmitoylation is only feasible if preceded by myristoylation acting as membrane anchor binding site; any substitution preventing myristoylation also alters palmitoylation, and other structural features on the N-terminal side might be relevant for specificity [66]. In other words, when retrieved together with myristoylation on close Cys residues, palmitoylation is a myristoylation-conditional modification. Myristoylation-dependent palmitoylation involves as many as 50% of myristoylated proteins in plants and 20% in humans [67]. Under such circumstances, double-myristoylated proteins are therefore possible when DHHCs incorporate a C14 instead of a C16 acyl chain.

3. Origins, distribution, and regulatory roles of myristate donors for protein myristoylation

In this section, we describe the origins of the myristate added to proteins and the potential relationship between the bound and free pools. As described above, the myristate donor in

bacteria corresponds to ACP-myristate, phosphatidylglycerol (PG), or phosphatidylethanolamine (PE) (**Table 1**). Conversely, the only known myristate donor for eukaryotic proteins identified to date is myristoyl-CoA (MyrCoA, **Fig. 2A; Table 1**) [68].

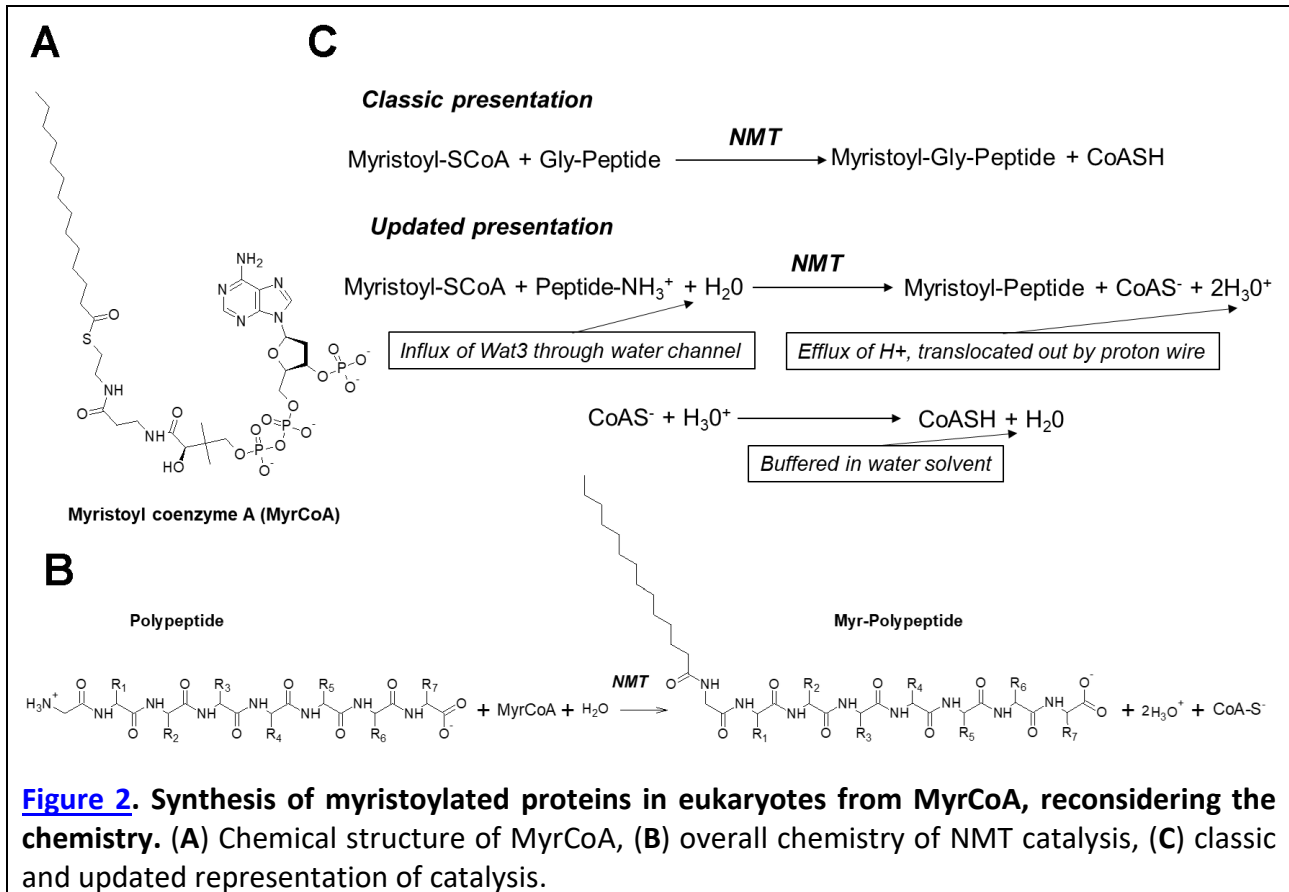
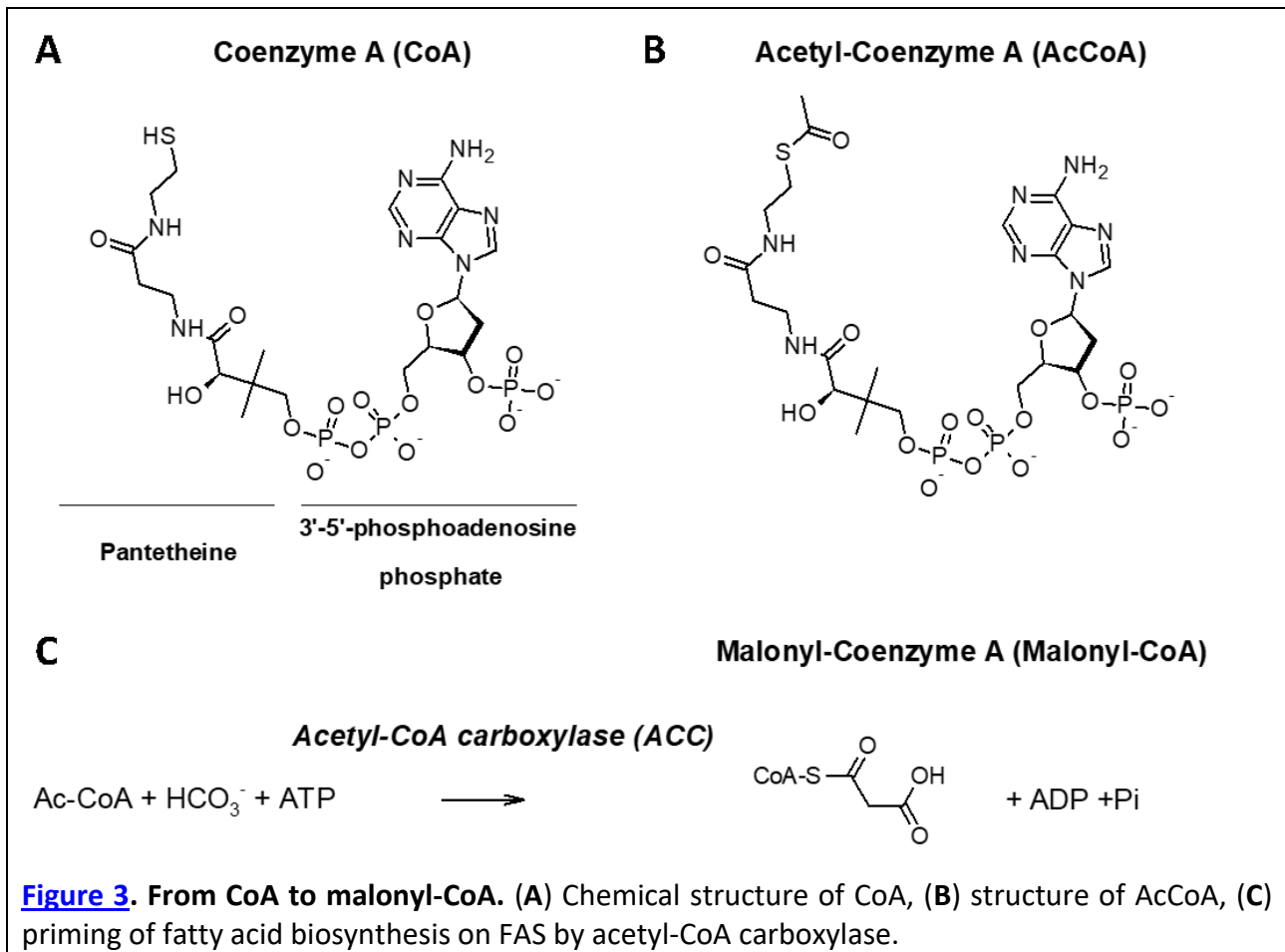


Figure 2. Synthesis of myristoylated proteins in eukaryotes from MyrCoA, reconsidering the chemistry. (A) Chemical structure of MyrCoA, (B) overall chemistry of NMT catalysis, (C) classic and updated representation of catalysis.

3.1. Overview of myristate synthesis in living organisms

3.1.1. Fatty acid synthases (FAS)

Myristate is a product of the classic, medium-chain FA biosynthesis pathway, which produces even C12-C20 chains. This biosynthesis is ensured by a large enzyme system known as FA synthase (FAS; EC 2.3.1.85) via a multistep reaction pathway [69]. The reaction involves acetyl-CoA (AcCoA) as a key carbon donor and an early carboxylation reaction to give malonyl-CoA (**Fig. 3**).



There are two main FAS systems in living organisms, the major difference being their organization. FAS1 corresponds to multifunctional enzyme complexes, whereas FAS2 is expressed as individual enzymes from a dozen separate genes, each corresponding to a distinct domain of FAS1 [70]. Both fungi and animals use a cytosolic FAS1. The fungal system (FAS1a) comprises two multifunctional protein chains, which are 210 kDa (α -chain) and 230 kDa (β -chain) chains carrying four and five functional domains, respectively, to produce a 2.6 MDa $\alpha_6\beta_6$ complex. The animal system (FAS1b) integrates all functional domains into a single chain to produce a functional 540 kDa homodimeric complex.

Bacteria use FAS2, except for the mycolic acid-producing subgroup (a C50-C60 FA) Actinomycetales, which display multifunctional organization of FAS similar to that found in animals [69]. Organelles like mitochondria and plastids also display FAS2. Mitochondrial FAS2 appears to mostly support synthesis of the C8 precursor of lipoic acid and small to large acyl forms [71]. Kinetoplastid parasites like *Trypanosoma* and *Leishmania* spp. use this pathway to produce long chain FAs, including C14 [72, 73]. Whatever the organism, mitochondrial FAS2 is, however, unlikely to contribute to the cytosolic C14 pool. This contrasts with the plastid FAS2, which is the major provider of FAs to all plant cell compartments. Disruption of plastid FAS2 activity through knockout of the enoyl-acyl carrier protein gene indeed leads to premature cell death associated with a dramatic decrease in lipid content [74]. The classic plastid-synthesized FA pathway involves direct transfer to the ER by a dedicated machinery. From the ER, FAs are then distributed to various compartments and membrane networks of plastid-containing cells.

FA chains are elongated directly on the ACP domain/subunit of FAS. A serine side chain of the ACP is first activated by addition of a phosphopantetheine prosthetic group from CoA (holo ACP, **Fig. 4A**). The final thiol of the prosthetic group corresponds to the acceptor of the acyl side chain, which grows as a covalent intermediate (**Fig. 4B**). Acyl biosynthesis occurs after a preliminary cycle of initiation involving malonyl-CoA formation from both AcCoA and acetate to produce a malonyl-S-ACP early C3 intermediate [75]. Extension involves AcCoA as the C2 donor (**Fig. 4**) to produce C-chains from C8 to C20. β -ketoacyl-ACP synthases (EC 2.3.1.179) KASI and III are involved in elongation up to C16, including myristate, while KASII mediates further elongation [76].

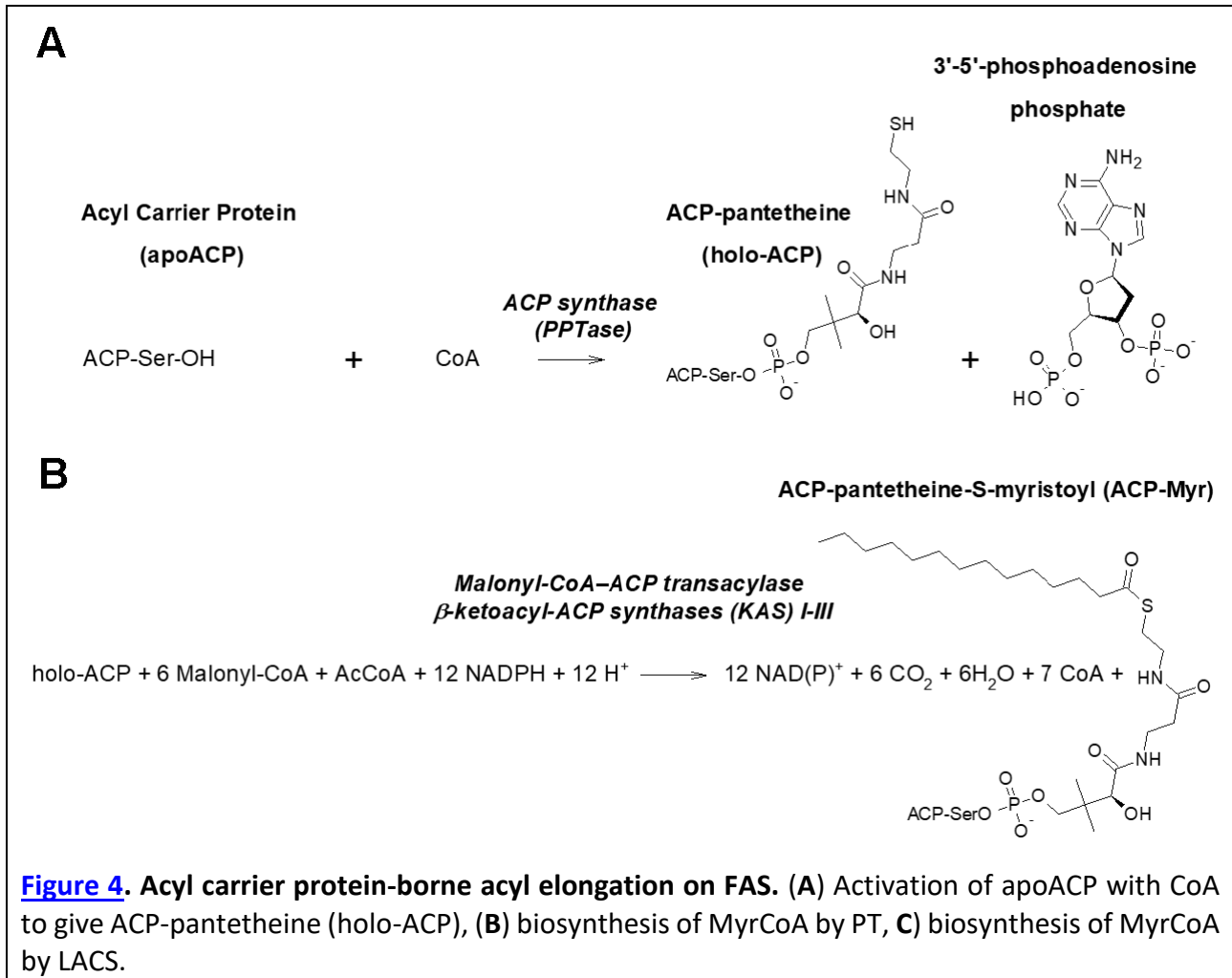


Figure 4. Acyl carrier protein-borne acyl elongation on FAS. (A) Activation of apoACP with CoA to give ACP-pantetheine (holo-ACP), (B) biosynthesis of MyrCoA by PT, (C) biosynthesis of MyrCoA by LACS.

3.1.2. Fatty acid elongases

ER membrane-located fatty acid elongases (FAE) of kinetoplastids are unique, as they can elongate long chain acylCoAs from butyrate (C4) to C14 [73]. This is in contrast to most organisms - including Apicomplexa - which need long chain acyl derivatives (usually C16) as primers of very long chain FA (VLCFA) to produce C22 or longer FAs [77]. Whether and how the ER AcylCoA pool is available for cytosolic MyrCoA is unknown, even though this pool is clearly important for the GPI-myristate anchor membrane pool of surface antigens occurring in bloodstream forms. Note

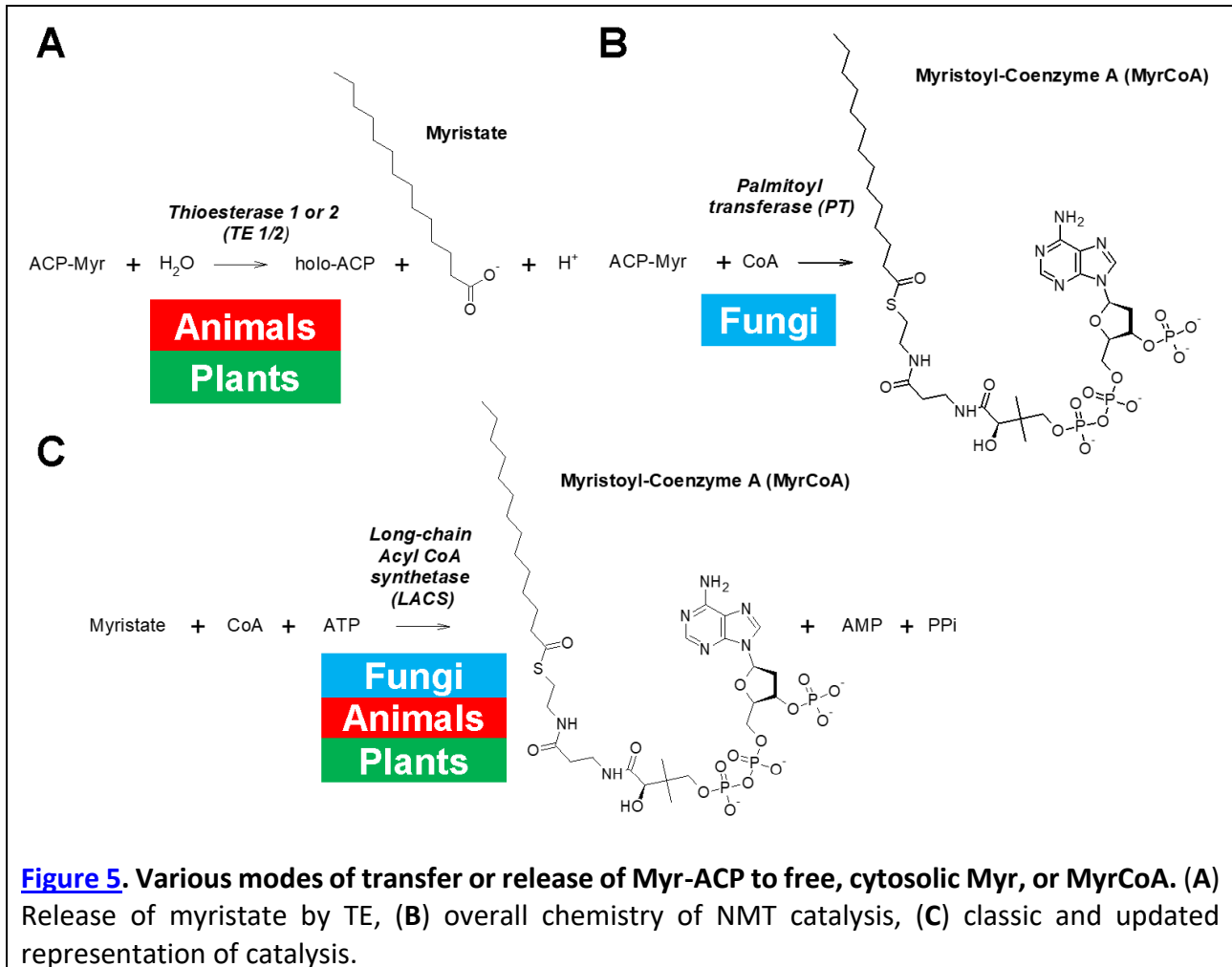
that, similar to other parasites, kinetoplastids may also obtain FAs including C14 by importing them from the host serum or cells [73].

3.1.3. Regulation of chain extension by FAS

The size of the main FAs changes depending on organism, with C16 predominating in animals and C16 and C18 balanced in fungi and plants. Myristate is usually a minor FA. For instance, in mammalian cells, C14 represents <10% of the CoA ester pool, far less than the C16 (palmitate), C18, and C20 pools [60]. Nevertheless, some tissues like mammary glands [78, 79], the oil glands of birds [80], or palm fruits including palm kernels and coconuts [81] produce significant amounts of C12 and C14 medium chain length FAs. One challenge is to understand how FAS controls the chain length growth and impacts the C14 pool.

Apart from the organization of each FAS, another important difference in FAS systems is in how chain length is regulated and how FAs are released from the ACP to ensure metabolic utilization of such compounds by the cell [82]. Two FAS activities may contribute to this, thioesterase (TE) and palmitoyltransferase (PT) activities (see Figure 4 in [70]). Animals and plants use thioesterase 1 (TE1) activity to release a free acyl chain, which can then be activated into an AcylCoA derivative by acylCoA synthase (**Fig. 5A**). Mammary gland epithelial cells of non-ruminant mammals use thioesterase 2 (TE2) [79, 82]. Thioesterases determine the selectivity for the released acyl chain using a channel that acts as a ruler. Interestingly, TE2 shifts the specificity from C16-C18 for TE1 to C14-C12. This feature alters the composition of breast milk in different species. In ruminants, the PT activity of the FAS1 complex appears to be involved in producing small chain FAs like C6/C8/C10-CoA derivatives [82], but this does not impact the C14 pool.

Fungal FAS1 does not have TE activity and instead uses PT activity, which directly transfers the acyl derivative from ACP to MyrCoA in the cytosol (**Fig. 5B**).



Bacteria directly transfer the acyl group from the ACP-acyl intermediate to glycerol 3-phosphate to produce lysophosphatidic acid (LPA, **Fig. 6A**). This encompasses two different mechanisms in two to three steps involving the *plsXY* and/or *plsB* gene products [83]. Bacteria lacking *plsB*, such as some Gram-positive bacteria, use only the PlsXY pathway. The lysophosphatidic acid (LPA) product is then converted to phosphatidic acid (PA, **Fig. 6B**) by PlsC, then to CDP-diacylglycerol (DAG, **Fig. 6C**), and finally to phosphatidylglycerol (PG, **Fig. 6D**) or phosphatidylethanolamine (PE, **Fig. 6E**). Each of these derivatives may be used as final products,

for example in membrane structures or as acyl donors. Bacteria can also use exogenous FAs by using the FadL transporter and the FadD acyl CoA ligase [84, 85], with maximal activity observed with C12-C14 FAs. The bacterial MyrCoA pool can be used for TAG or wax ester synthesis [26] and is also available for any transgenic acyltransferase such as NMT to mediate myristoylation of chosen targets (see, for instance [86, 87]).

There are two plant plastid TEs, FATA and FATB [88]. Each appears to generate different chain lengths, with FATB producing the C6-C14 species, which leads to the cytosolic AcylCoA pool including MyrCoA. The *FATB* knockout in *Arabidopsis* is not lethal but leads to severe growth retardation and reproduction defects that accompany modifications to lipid composition [89]. In the *Arabidopsis* knockout mutant background, a pool of shorter chains appears to be maintained by degradation of longer acyl chains by β -oxidation [90], highlighting the robust homeostatic control of FA composition in plants through turnover from longer to shorter forms and vice versa. Long chain acyl CoA synthetases are involved in plant FA activation into AcylCoA derivatives and transport from the plastid to the ER or cytosol [91]. It is unclear, however, whether the cytosolic MyrCoA pool arises from direct export from the plastid, whether it first transits via the ER, or whether both pathways are possible [92].

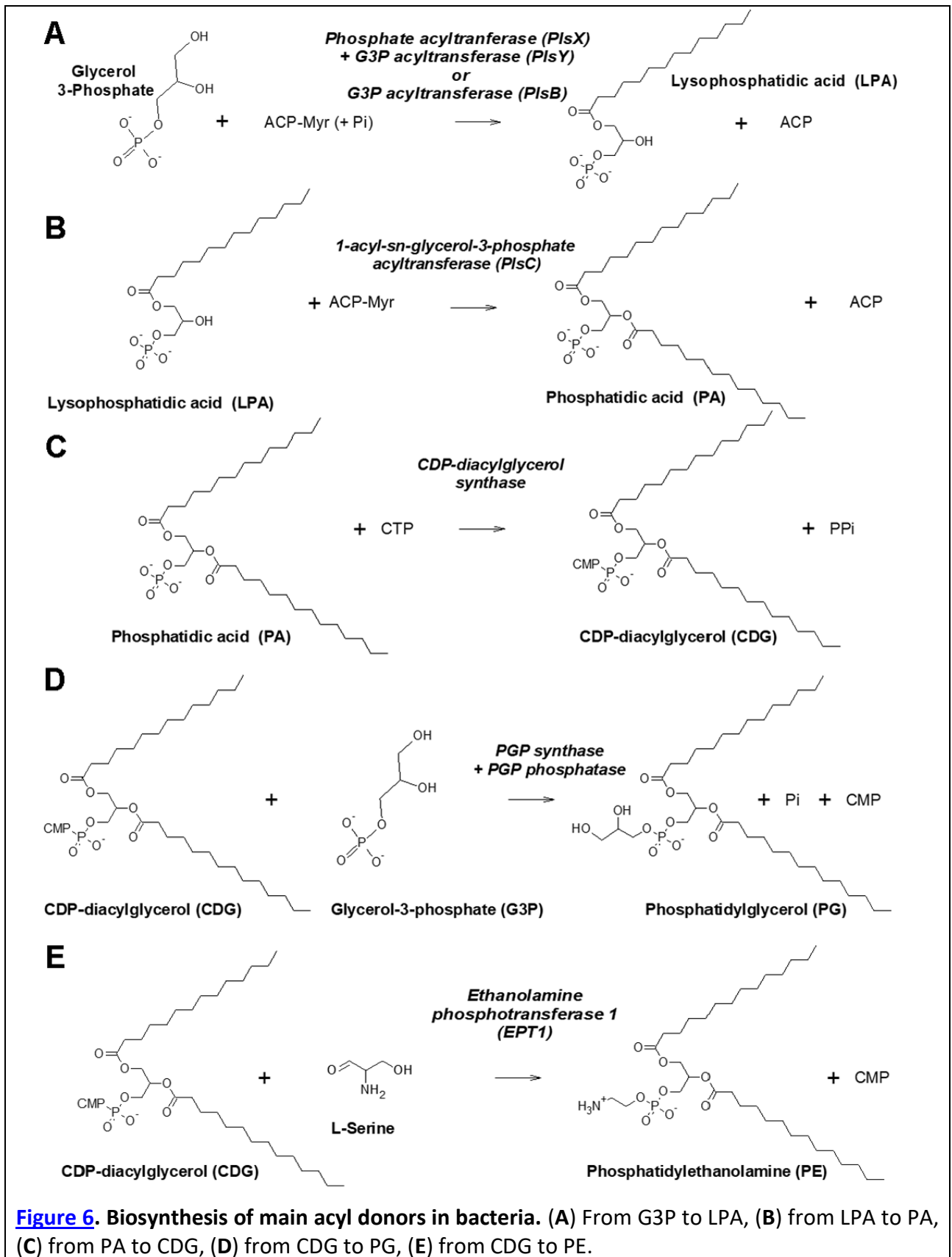


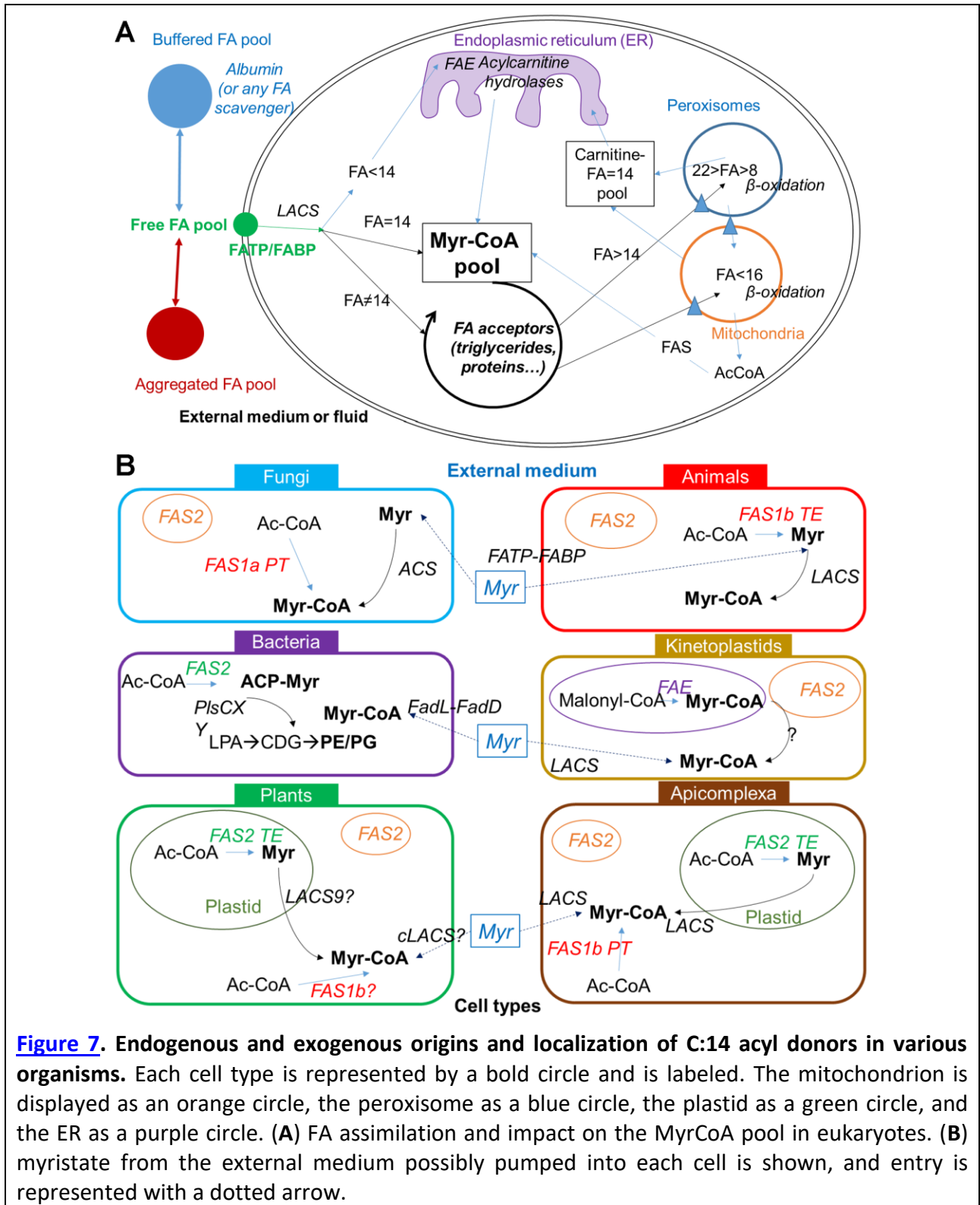
Figure 6. Biosynthesis of main acyl donors in bacteria. (A) From G3P to LPA, (B) from LPA to PA, (C) from PA to CDG, (D) from CDG to PG, (E) from CDG to PE.

3.2. Origin of the cytosolic MyrCoA pool in eukaryotes

3.2.1. FA availability in biological fluids

Saturated fatty acids including myristate display low solubility at physiological pH (20-30 μM). FAs in solution tend to aggregate even at low concentrations, and effective solubility might be even lower than 1 μM for FA $>\text{C}_{14}$ [93]. This solubility limit is overcome in biological fluids provided that a FA-biological scavenger, like albumin, is present (**Fig. 7A**). Albumin displays seven myristate binding sites each with high affinity ($K_d=0.1 \mu\text{M}$) [94, 95]. Albumin's high solubility and concentration - 0.6 mM in the blood - together with its seven FA binding sites per monomer, accommodate an average of 1-2 mM FAs [96]. Even though non-esterified fatty acid (NEFA) concentrations are low, if cells can internalize and metabolize FA from the external medium, albumin buffers FA and can use them as a nearly inexhaustible stock. It was long thought that FA could only cross membranes by free diffusion, but the so-called FA-transport and FA-binding proteins (FATP and FABP) exist at the membrane and in the cytosol, respectively [97]. These FA binders cooperate to facilitate cellular penetration of FAs (**Fig. 7A**). This is true for both animal and fungi, while plants have developed a sophisticated FA transport system across intracellular membranes including the ER [98]. Finally, free intracellular FA-CoA cytosolic concentrations available for protein incorporation range from 5 to 200 nM in animals [99, 100]. Most of the total pool (20-95%) is sequestered in organelles or associated with FA binders [100].

Finally, it is important to note that exogenously supplied FA influences the type of fatty acylation [101].



3.2.2. FAS and LACS

FAS expression is under hormonal control in mammals. Glucocorticoids appear to increase FAS mRNA stability, which reduces the activity of FAS in most tissues and increases it in glucocorticoid-sensitive cells [102]. In human adults in the industrialized world, saturated FAs including myristate are thought to originate from the diet [103] except for in the liver, adipose tissue, and the lactating breast or in pathological conditions such as alcohol abuse or cancer [104]. In mammals, fasting induces adipose tissue lipolysis and FA release into the blood [105].

As FAs almost exclusively originate from food in adult animal cells, it is not a surprise that cultured mammalian cells also depend on external FAs. FAs - including myristate - are present in the albumin in fetal calf serum used to supplement growth media [106]. Acyl-CoA synthetases (ACSs; EC 6.2.1.x) are responsible for activating C2-C30 FAs into CoA derivatives in two steps. This pool of AcylCoA derivatives is available for all cellular metabolic fates of FA including protein acylation. Long-chain ACS isoforms (LACS; 13 out of 26 ACS in humans [107]) can activate medium-sized FAs (**Fig. 5C**) [108]. LACS activity may be directly coupled to the FA transporter FATP. LACS prefer saturated C10-C18 chains including myristate and display optimal activities for C12-C14 chains [109]. LACS contribute to the assimilation of exogenous myristate (**Fig. 7**). The energy required for FA activation is much lower than that required for *de novo* FA synthesis. When mammalian cells are incubated in the presence of radiolabeled myristate, 40-80% of the label is retrieved as amide derivatives (protein pool) and 9-24% as esters (lipid pool). In the amide fraction, 70-80% is found in myristoylated G peptides [110, 111]. Clearly, the N-terminal glycines are a major but non-exclusive target of diet-derived myristate associated with proteins.

3.3. Non-cytosolic subcellular pools of C14 FA and their impact on myristoylation

AcylCoA derivatives cannot directly cross biological membranes [112]. The aim of this section is to introduce the other non-cytosolic acyl pools putatively available for protein acylation, including myristoylation, in cellular compartments other than the cytosol.

3.3.1. FA and β -oxidation in lysosomes and mitochondria

β -oxidation describes the intracellular pathway leading to progressive FA trimming to shorter chains [113]. β -oxidation occurs in peroxisomes for C22 to C8 and in mitochondria for C16 to AcCoA (**Fig. 7A**). Carnitine-based transporters known as carnitine palmitoyltransferases (CPT1 and CPT2) for long chains, carnitine octanoyltransferases for C6-10 (CrOT), and carnitine acetyltransferases (CrAT) for short chains (C2-10) mediate the exchange of acyl groups from the cytosol before release into some compartments as acyl-CoA conjugates. CPTs are membrane proteins, with only CPT1 giving direct access to the cytosolic pool of acylCoA. Mitochondria display two CPT1s, CPT1A and CPT1B. CPT2 localizes to the mitochondrial inner membrane, and CrOT to the peroxisomal matrix. CrATs are found in the peroxisome, the mitochondrial matrix, and the ER. AcylCoA compounds are used in progressive degradation in lysosomes and the mitochondrial matrix. The shorter chains arising from peroxisomes can be directly imported into mitochondria for further degradation up to C2 (AcCoA). As a result, NEFA produced by β -oxidation can eventually contribute to the cytosolic MyrCoA pool. For example, in humans, C14 can be produced from C16 or C18 [114], and plants also display similar features as discussed below (Section 3.4).

Finally, AcylCoA derivatives may be reactivated and oxidized by the pathway. Overload of the system can occur under some pathological conditions including diabetes, obesity, or a high-fat acid diet [115].

3.3.2. AcylCoA derivatives and protein acylation in the ER

FACoA derivatives are also found in the ER and are required for acylation in the lumen by dedicated acyltransferases (Porcupine, Hhat, or MBOAT) of secreted acylated proteins including Hedgehog, Wnt, or Ghrelin (for a review, see [3]). Long chain FAs including C16 translocate from the cytosol to the ER [116]. To achieve this transport from the cytosol to the lumen, a dedicated carnitine palmitoyltransferase (CPT1C) was shown to localize in the ER in neurons and to be induced in cancer cells [117]. CPT1C appears to have significantly weaker activity *in vitro* than its mitochondrial counterparts, and its substrate specificity (including for C14) is unknown [118]. Acylcarnitine hydrolases are present in the ER lumen [119]. It was also demonstrated that membrane-bound O-acyl transferase (MBOAT) multispinning membrane proteins such as Hhat facilitate FACoA transport across the ER membrane [3, 120]. Finally, CrAT was also retrieved in the ER lumen [121]. Coupled with FAE, CrAT may also contribute to provide the ER with C14 reactive derivatives for protein myristoylation.

There is currently no evidence of myristoylation activity in the ER. MBOAT members acting on the luminal side of the ER appear to exclusively use C16, although Hhat, for instance, binds C10-C14 with higher affinity than C16-C20 [122]. The predominance of C16 FAs on acylated ER proteins appears to be driven by their higher availability compared with C14. Little is known about the relative availability of the various types of AcylCoA derivatives in the ER lumen and how much Hhat, CPT1C, or any other yet uncharacterized catalyst contributes to the pool (**Table 1**). Finally,

a model of TNF secretion through the ER suggests that the myristoylated domain is always located on the cytosolic side, suggesting cytosolic rather than ER lumen-mediated myristoylation [123].

3.4. Myristate in plastid-containing organisms

Unlike animals, plastid-containing organisms including green algae and land plants do not rely on external FAs. The plastid uses a type II FAS to produce acyl-ACPs. These are then hydrolyzed and acyl-CoAs produced. The plastid uses the essential FAS2 [73, 124]. Plant plastids are expected to produce most FAs, and there is no cytosolic FAS isoform. Due to FAS-dependent synthesis and plant autotrophy, FA metabolism in plants was originally mainly studied by taking advantage of precursors including acetate, CO₂, and NEFA, with C14 and C16 being the most active precursors [125, 126].

In Apicomplexan parasites, host apicoplast and scavenged myristate are substrates for parasite NMT (**Fig. 7B**). Apicomplexa like *Toxoplasma*, *Cryptosporidium*, *Bestnoitia*, and *Neospora* possess a FAS1 with 21 domains and a long chain reductase domain [127]. The TE domain of FAS1 appears to be missing, but the PT domain exists. BLAST analysis with human FAS1 shows that some plant genomes including the *A. thaliana* Landsberg *erecta* (Ler) ecotype [128] or *Quercus suber* (cork oak) display a FAS1 open reading frame resembling the apicomplexan multienzyme FAS1b (ATUG03410/A0A178U5S5). The chain is large (8120 amino acids) but also has no TE domain; MyrCoA could therefore be directly produced via PT activity without the need for cytosolic LACS. Nonetheless, the reductase domain - which replaces the TE domain in this FAS1b homolog - could lead to fatty alcohols rather than MyrCoA. No FAS1 complex has been identified

in diatom genomes. Clearly, the origin of cytosolic MyrCoA in plastid-containing organisms is still an open field (**Fig. 7B**).

Unlike Apicomplexa, photosynthetic algae and plants are usually considered to mostly rely on the plastid, FAS2-dependent FA pool to provide MyrCoA. Plants can nevertheless assimilate scavenged myristate like all other organisms. Indeed, treating tobacco leaves with various C14 and C16 analogs leads to incorporation of the analog into proteins [129]. Incubation of *Arabidopsis* with C12 radiolabeled precursors also produced higher-order FAs, including C14 retrieved as triacyl glycerol or phosphatidylcholine derivatives [130]. Long-chain acyl-CoA synthetases LACS1-3 from *A. thaliana* appear to possess exogenous FA uptake activity, as they can rescue *fat1*-deficient yeast mutants [131]. Whether this scavenging ability is local or involves the whole plant through inter-cell circulation is unknown. It is also unknown whether plant roots can extract FAs from the soil to contribute to lipid metabolism. Nonetheless, of 63 members [132], nine LACS have already been characterized in *A. thaliana*; seven appear to be localized to the ER and LACS6 is localized in the peroxisome, the second most common cellular location of β -oxidation together with mitochondria, and LACS9 is localized on the plastid envelope [88]. LACS9 might be involved in the plastid-cytosol export of MyrCoA. Many membrane contact sites centered on the ER are expected to drive lipid trafficking in plants [133], which might also contribute to the cytosolic MyrCoA pool.

3.5. FA and the regulation of myristoylation in eukaryotes

FAs play a major role in regulating myristoylation, most likely by sensing an essential myristoylated protein in yeast [134, 135]. For worms, in which FA levels regulate sex

determination, the AcylCoA synthetase ACS4 plays a key role in refueling the myristoyl-CoA pool from food [136]. In *acs4* mutants, this decreases the levels of myristoylated proteins, as assessed by reduced intensity of the labeled proteins on gels. A mitogen-activated protein kinase (MAPK) pathway, possibly involving a myristoylated phosphatase 2, senses the balance and is essential for germline sex determination [136], and the depletion can be rescued by adding myristate to the growth medium. This effect can be mimicked in yeast, as a poorly active N-myristoyltransferase (NMT) variant can be rescued by adding myristate to the growth medium [137]. In prostate cancer cells, ACS4 is overexpressed and induces Src myristoylation, likely via an increase in MyrCoA synthesis [138]. These data suggest that heterologous levels of myristoylated proteins can occur in animals by varying FA availability from the diet and fat storage. It has been observed that myristate supplementation appears to increase the activity of one myristoylated protein [139, 140]. Interestingly, reactivation of *de novo* FA synthesis is associated with cancer [141]. In addition, a high-fat diet in mice increased tumor progression mediated by the myristoylated oncogene Src SFK [142]. Reciprocally, blocking Src myristoylation inhibited its kinase activity and suppressed cancer progression [143]. Finally, growth media containing FAs from serum albumin have a direct impact on intracellular FA composition, favoring shorter chains in fungi and FAS1-containing bacteria [144, 145].

4. Myristoylation in eukaryotes and subcellular shuttling

The dominant eukaryotic myristoylation catalyst is N-myristoyltransferase (NMT), which remains the only one characterized to date (**Table 1**).

4.1. Main structural and biochemical features of NMTs

NMT was identified, purified, and characterized in the early 1980s in *Saccharomyces cerevisiae* as a monomeric 55 kDa protein [146, 147]. It was further characterized in various organisms including other fungi, humans, plants, apicomplexa, and kinetoplastids. NMT uses MyrCoA as myristate donor and releases CoA (see above for the biological origin of myristate). Heterogeneous acylation of NMT targets by C12:0, C14:1, or C14:2 or even C16 and C18 has been observed *in cellulo* and appears to be tissue specific or depend on the availability of dietary FA [60, 148-150]. MyrCoA analogs can be obtained *in vitro* from free CoA and any FA derivative using either chemical or enzymatic approaches [151-153]. From studies of such compounds, it appears that NMT can also efficiently accommodate C10 to C15 chains [154, 155] and appears to tolerate variations at the extreme side of the acyl chain beyond C5/6 [156-158]. Nevertheless, C14:0 is considered the major acyl form used by NMT. NMT displays one order of magnitude lower catalytic efficiency for Palmitoyl-CoA than MyrCoA due to the limiting size of its acyl-binding pocket [159, 160]. However, this only partially explains why NMT favors C14 over C16, as C16 CoA derivatives are much more abundant than C14. AcylCoA binding domain-containing proteins were suggested to contribute to the high selectivity of NMT for C14 [161]. The *K_m* of NMT for MyrCoA is of the order of 1 μ M [159, 162], much higher than for the actual free MyrCoA value (see Section 3.2.2), allowing NMT to adapt its activity to the available acyl substrate. This suggests that myristoylation might be partial under low myristate concentration conditions and may be significantly enhanced if higher myristate concentrations are available. Together, these pioneering studies paved the way for taking advantage of reactive myristate analogs to characterize myristoylation mechanisms (see Section 5).

MyrCoA features an NMT-binding constant in the low micromolar range and needs to bind to NMT before the peptide substrate. The protein substrate may be mimicked *in vitro* by peptides as short as octapeptides starting with Gly2 to aa9 [147], since these residues are sufficient *in vivo* to specifically trigger myristoylation [163, 164]. Strong binding of a peptide ligand to NMT is ensured if the amino terminal moiety is progressively depeptidized down to a minimal decanoyl chain, whose length corresponds to the first four amino acids [165, 166]. Progressive C-terminal truncation to six residues diminishes myristoylation efficiency *in vitro*, while truncation to five residues precludes myristoylation [165]. This sensing of a very short N-terminal peptide sequence is consistent with myristoylation being mainly an early co-translational event [167, 168]. NMT was originally classified as a glycylopeptide N-tetradecanoyltransferase (EC 2.3.1.97, NMT), but because NMT was recently demonstrated to catalyze myristoylation of lysine side chains, we consider this nomenclature misleading and now prefer N-myristoyltransferase (NMT) instead. Recent characterization of the atomic G-myristoylation mechanism revisited the chemical balance of the reaction, which involves protons and water together with a positively charged aminoacyl acceptor (**Fig. 2B,C**).

cDNA or gene cloning established that NMT is 500 ± 65 amino acids long, with size divergence mostly due to the N-terminal variable domain involved in ribosome binding. Indeed, binding of NMT to the ribosomal subcellular fraction involves the first hundred residues of the N-terminal NMT extension [169]. This extension, which is poorly conserved in both length and sequence, has a basic character that might favor ribosomal binding. These residues are dispensable for NMT activity, and a 59 N-terminal-deleted form is still active *in vivo* in yeast [86]. In mammals, two splice variants produce a long NMT1 isoform with an extension (NMT1_L) and a short form arising

from an alternative downstream start codon without the extension (NMT1_S) [169-171], the latter displaying full activity *in vitro* [172]. Alternative isoforms from the same gene do not occur in plants [171]. The second isoform retrieved in mammals, NMT2, appears to also produce only one isoform corresponding to NMT2_L [173].

Crystal structures usually need to use protein constructs devoid of the long N-terminal extension of NMT1/2_L, which is likely disordered in solution. The 400 amino acid-long catalytic core of NMT is very homogeneous and displays a remarkably well conserved 3D structure as demonstrated by the many high-resolution crystal structures now available [74 to date from *Saccharomyces cerevisiae*, *Aspergillus fumigatus*, *Candida albicans*, *Plasmodium vivax*, *Trypanosoma brucei*, *Leishmania major*, and *Homo sapiens* (HsNMT1 and HsNMT2; see <http://www.rcsb.org/pdb/results/results.do?tabtoShow=Current&grid=4C582535>)].

NMTs belong to the GNAT superfamily [21, 22]. NMTs are atypical GNAT members featuring two adjacent GNAT domains with reduced sequence homology [174]. The N-terminal GNAT domain contains the active site, while the C-terminal domain has mostly evolved to accommodate the binding pockets of the peptide. NMT's C-terminus carboxylate groups hydrogen bond with the protonated amino terminus of the peptide substrate. The yeast enzyme has an optimal pH of 7.5-8.0 *in vitro*, consistent with the necessity for a cationic N-terminal protein substrate (pK_a=7.7) for catalysis [41, 43].

4.2. NMT subtypes in eukaryotes recapitulate the tree of Life

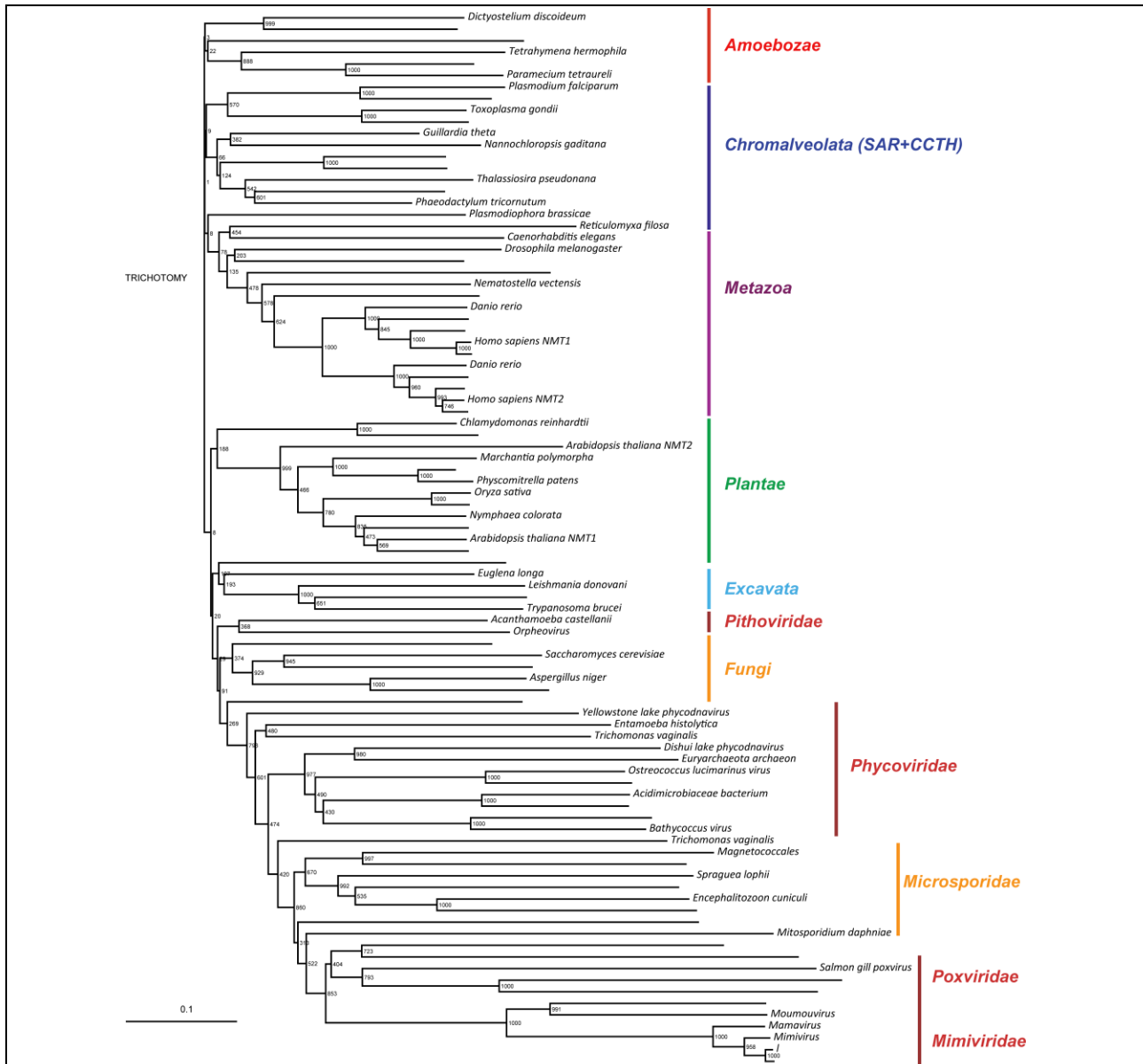


Figure 8. NMT families. 91 sequences were selected to represent NMT sequence diversity among eukaryotes. The sequences were aligned, and the bootstrap tree was constructed as previously described [360]. Internal values labeled on each node record the stability of the branch over the 1,000 bootstrap replicates. The list includes the 69 recently assembled sequences (see SupFigure in [43]) and was completed through the inclusion of further sequences from available genomes to better sample and cover the evolutionary landscape. CCTH refers to Cryptophyta, Centrohelida, Telonemia, and Haptophyta. SAR stands for Stramenopiles, Alveolata and Rhizaria. The classification was according to [175]. All sequences are available upon request. For the sake of easy reading, only representative names of the organism genome from which the NMT sequence was extracted are shown.

An updated sequence comparison shows that NMTs display an average of 50% identity. Phylogenetic clustering (**Fig. 8**) reveals that NMTs scatter into specific classes: Plantae, Metazoa, Chromalveolata, Amoeboezae, Excavata, and Microsporidiae, in keeping with the evolutionary tree of life [175]. Every available eukaryotic genome displays at least one NMT homolog. Land plants and vertebrates have two NMT gene copies. Vertebrates have two distinct NMT types, NMT1 and NMT2, as established early on in humans [173]. Nevertheless, vertebrate NMT1 and NMT2 display identical substrate specificity [67, 176]. Upon caspase-mediated cleavage of the amino terminal non-essential extension during apoptosis, NMT2 relocates to membranes while NMT1 remains cytosolic [177]. These different localizations might be the functional reason for the two NMT lineages in vertebrates. It has also been observed that while NMT2 is overexpressed in various solid tumor lines like brain and kidney cancer, expression was significantly reduced in hematological cancers [178].

NMT-dependent myristoylation is essential in all organisms, with strong phenotypes associated with (i) cell survival in fungi [179, 180] or (ii) very early developmental stages in multicellular organisms, as demonstrated through single gene substitutions and interference experiments in organisms with a single NMT gene, such as in malaria parasites, worms, or flies [181-183]. In vertebrates, the NMT1 isoform appears to predominate in most tissues, contributing up to 95% of activity in early embryos and explaining why NMT1 is essential in early mouse development [184]. Inhibition of myristoylation leads to apoptosis in human cancer cell cultures [185]. The plant model *Arabidopsis thaliana* is the only land plant described to date that features only one functional NMT (AtNMT1), essential in early development, with the second NMT gene in *A. thaliana* (AtNMT2) poorly expressed and inactive as an NMT [171]. The cDNAs encoding

human NMT1 (HsNMT1) or AtNMT1 complement a *S. cerevisiae* mutant (*nmt-181*) featuring a temperature-sensitive NMT [172, 186]. Unlike HsNMT1 and HsNMT2, *S. cerevisiae* NMT (ScNMT) cannot complement an NMT-deficient *A. thaliana* plant line [171], perhaps due to the slightly narrower substrate specificity of unicellular NMTs, as discussed below.

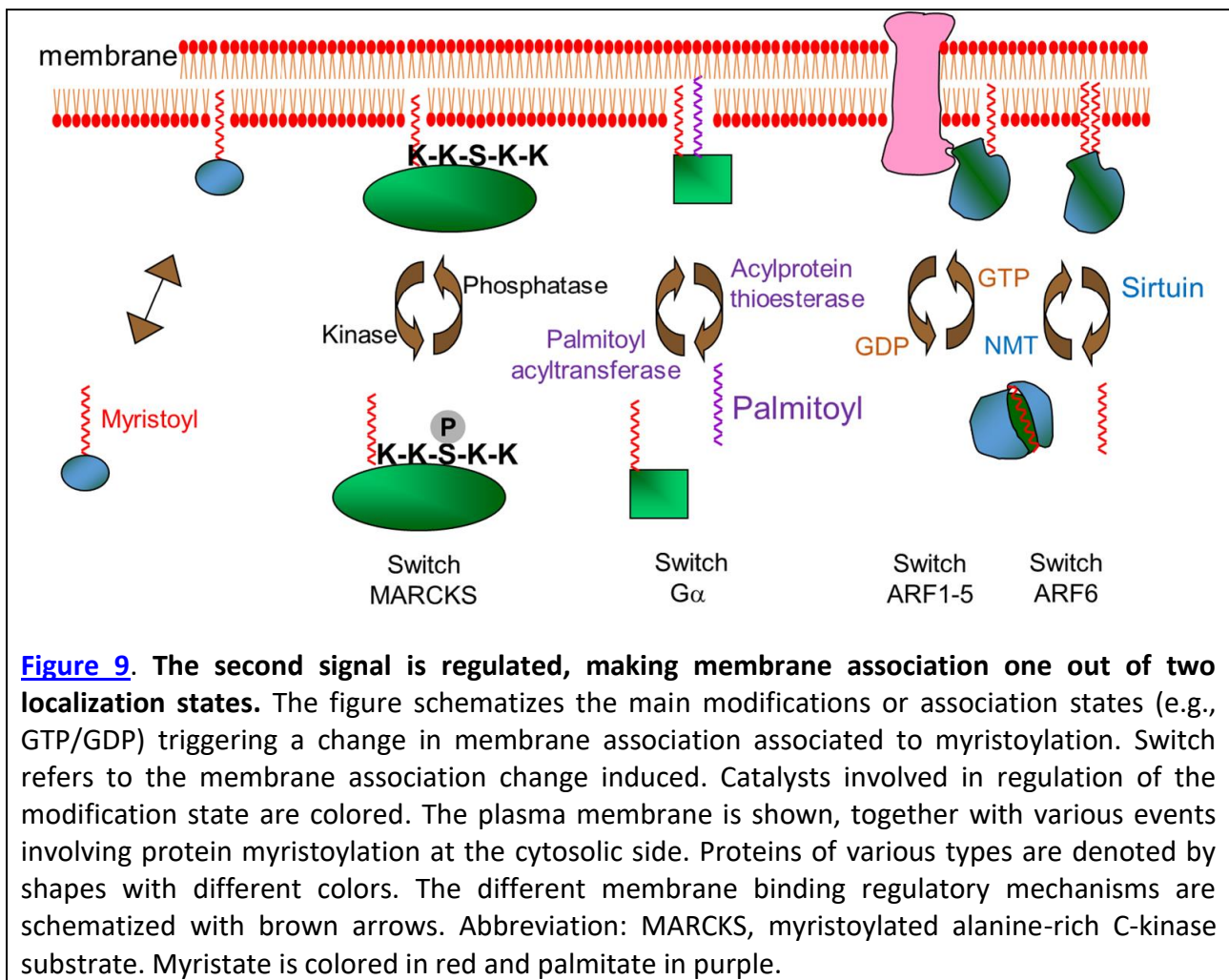
4.3. Main myristoylated proteins, membrane targeting, and cell compartmentalization

4.3.1. Myristoylated proteins shuttle between membrane compartments in eukaryotes

Seventy proteins are predicted to be myristoylation targets in yeast (i.e., ~1.1% of the proteome), and a dozen of the corresponding G-myristoylated target genes have been shown to be associated with deleterious phenotypes when inactivated [135, 187]. An initial screen included two of the three ADP ribosylation factor (ARF) genes (Arf1 and Arf2), the Ptc2 type 2C protein phosphatase [188], the Sip2 subunit of the Snf1 kinase (homologous to the aforementioned myristoylated human AMPK, one of the three possible β subunits of the complex and one of two G-myristoylated with Sip1), the Vps20 subunit of the ESRCT-III complex, the Rpt2 subunit of the 26S proteasome, the Gpa1 subunit of the heterotrimeric G protein, and an EF hand calcium-binding protein of the recoverin family. In *A. thaliana*, three major essential targets were retrieved, the SnRK1 β -subunit (homologous to Sip2 in yeast), Arf, and a calcium-dependent protein kinase [171, 189, 190], each a major protein at different developmental stages. In yeast, only inhibition of Rpt2 G-myristoylation induced a major phenotype [191]. Finally, in humans, Mazzanti syndrome is caused by a point mutation promoting G-myristoylation of SHOC2 [192]. These data illustrate the important functional impacts of G-myristoylation on target proteins and its ability to override other targeting signals, if present [193].

4.3.2. Myristoyl switches facilitate compartment shifts

All myristoylated targets, whatever the binding site, shuttle between the cytosol, the endoplasmic reticulum, and various compartments including the nucleus (Rpt2/Sip2/Ptc2 in yeast), the plasma membrane (Gpa1), the endosome (Arf1/Arf2/Vps20), mitochondria or lipid droplets [43, 194]. Both the hydrophobic character of the myristate group and also its relative position at the N-terminus are crucial for compartmentalization. For instance, a C-terminal prenylation site introduced into a non-myristoylated variant of ARF6 cannot compensate for proper endosomal targeting and biological activity of ARF6 [195].



The involvement of myristoylation in inter-compartmental shuttling was recognized early on, along with its regulation via additional features. These include GTP switching for ARF [196] or further protein modifications including phosphorylation or palmitoylation of vicinal Ser or Cys residues to trigger so-called electrostatic and palmitoylation switches, respectively (for reviews, see [3, 197]; **Fig. 9**), or even Ca^{2+} sensing for EF-hand calcium-binding proteins [198, 199]. Such myristoyl switches subtly control the impact of myristoylation on subcellular trafficking. As a result, each myristoylated protein can be distributed in one or another compartment or both. An unusual observation was made for mammalian protein kinase A, which distributes from the nucleus to the cytoplasm due to significant deamination of Asn3 in muscles caused by an electronegative charge induced by the new Asp3 [200]. Recent data have shown that the conserved net neutral charge plays a crucial role in ensuring PM localization in the absence of additional binding domains other than myristoylation [201]. The wide cellular destinations of myristoylated proteins were noted in early works, including, for example, the PM, Golgi, cytosolic, and nuclear distribution of SFK. Several such targets are illustrated in **Fig. 10A** (left). The PM localization involves palmitoylation of Cys residue(s) in the immediate vicinity of the myristoylation site, as initially discovered for yeast Gpa1 [202] and confirmed with Vac8 [66]. Yeast only harbor three such proteins including Gpa2 [203]. It remains to be confirmed whether the DHHC palmitoyltransferase Pfa3 also acylates both Gpa isoforms in addition to Vac8.

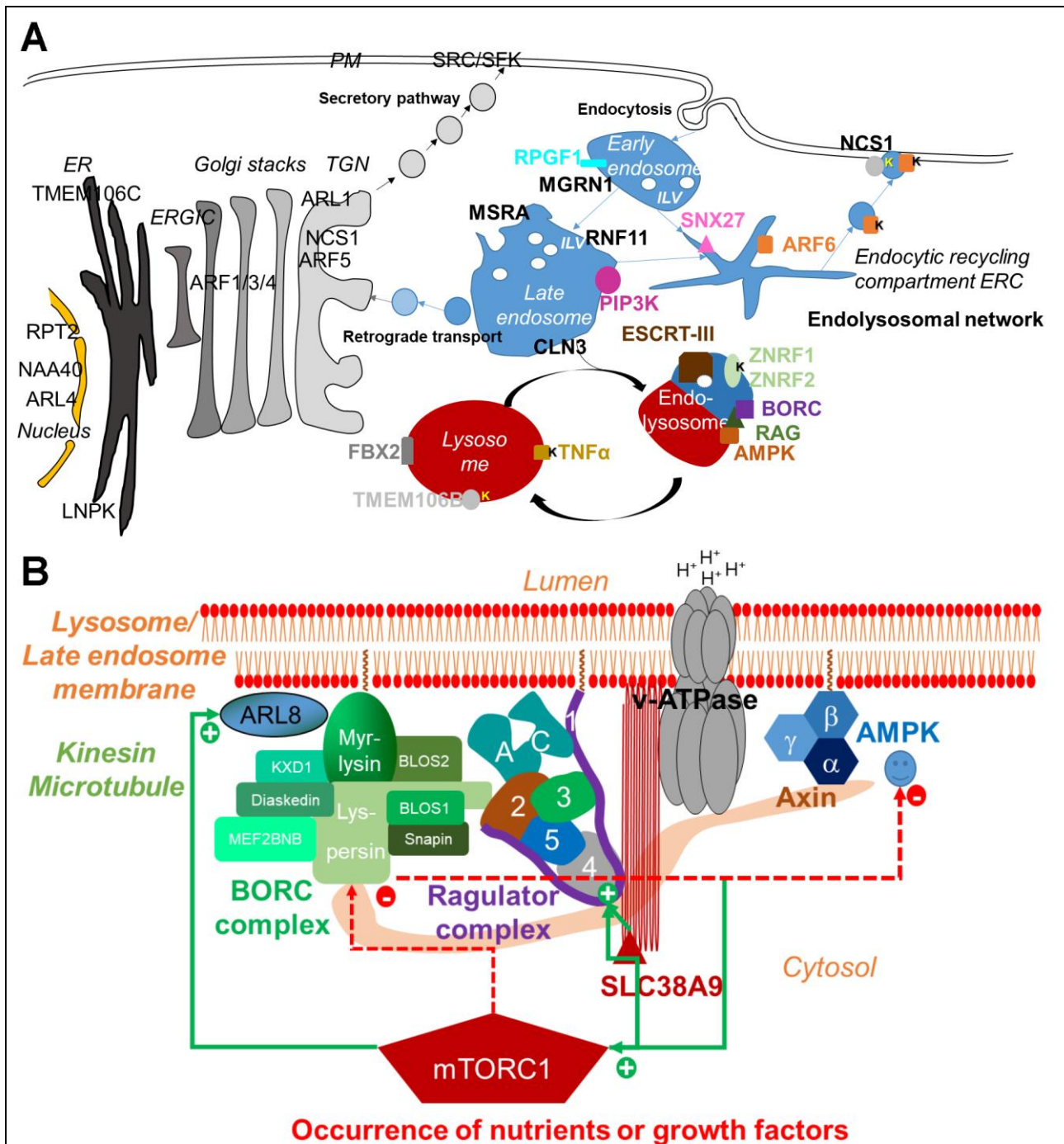


Figure 10. The endolysosomal network is a rich membrane recipient of myristoylated proteins. **(A)** Overview of both the secretory and endolysosomal networks, highlighting major proteins targeting either compartment. ERGIC is the ER–Golgi intermediate compartment. Intraluminal vesicles (ILVs) are small, cargo-enriched vesicles within the lumens of maturing late endosomes. Abbreviations: TGN, trans-golgi network; BORG, BLOC-1-related complex (see panel B); CLN3, battenin; ESCRT-III, endosomal sorting required for transport complex III; FBX27, F-box only protein 27; NCS1, neuronal calcium sensor 1; LNPK, endoplasmic reticulum junction formation

protein lunapark; MGRN1, E3 ubiquitin-protein ligase MGRN1; MSRA, peptide methionine sulfoxide reductase (cytosolic isoform); NAA40, N-alpha-acetyltransferase 40; PIP3K, phosphoinositide-3-kinase; RAG, Ragulator complex (see panel B); RNF11, RING finger protein 11; RPGF1, Rap guanine nucleotide exchange factor 1; RPT2, 26S proteasome regulatory subunit 4; SNX27, sorting nexin-27, TMEM, transmembrane protein; TNF α , tumor necrosis factor-alpha; ZNRF, E3 ubiquitin-protein ligase ZNRF.

(B) Myristoylated components of a giant multi-complex organization network anchored at the lysosome/late endosome membrane allowing major metabolic regulation. The figure highlights the crucial involvement of 3 myristoylated components of the 3 major complexes, BLOC-1 related complex (BORC), Ragulator, and AMP-activated protein kinase (AMPK). The cartoon shows the normal situation in the presence of nutrients, with AMPK active. The impact on AMPK inactivation and mammalian target of rapamycin mTORC1 activation upon nutrient or growth factor starvation is illustrated. The figure was constructed based on data from [209, 210, 361]. Abbreviations: BLOS, biogenesis of lysosome-related organelles complex 1 subunit; KXD1, KxDL motif-containing protein 1; MEF2BNB, BLOC-1 related complex subunit 8 (also known as BORCS8); SLC38A9, solute carrier family 38 member 9.

In the case of lysine myristoylation, TNF- α is targeted and retained in the lysosome via this modification [123]. It appears that this internal myristoylation is one of the few not catalyzed by NMT. By removing myristoylation, sirtuin 6 favors TNF- α secretion. The specificity of such lysosome targeting might involve other determinants. For instance, the ARF-like (ARL) Arl8 targets lysosomes through N-acetylase NatC-dependent N- α -acetylation of the Met-Leu N-terminus [204]. The yeast Arl3 instead targets the Golgi via a similar NatC acetylation. This is unlike the very closely related ARF1-5 GTPases, which target the Golgi via G-myristoylation. ARF6 is directed to the endocytic recycling compartment via G-myristoylation, while it also undergoes a second myristoylation at Lys 3, which is major for PM binding and membrane recycling ([42]; **Figs. 9,10**).

4.3.3. Endosomes and lysosomes as newly recognized hubs exploiting myristoylation

Secretory pathways have long been acknowledged as major targets of myristoylated proteins. Recent data on myristoylated proteins in various organisms (see Section 5) have shown that, in addition to the ER-Golgi network and the PM, which are systematically retrieved as targets of

myristoylated proteins, several other unexpected compartments are similarly targeted (**Fig. 10A**), including mitochondria, cilia (see Refs in [43]), and nuclei [205-208].

The endolysosomal network also contains many key myristoylated components and is still very poorly acknowledged as a rich site of myristoylated proteins (see **Fig. 10A**, right hand side, in bold). Lysosomes orchestrate the degradation of molecules arising from various pathways including endocytosis. The endocytic pathway starts with the creation of endocytic vesicles from the PM, where the small GTPase RAB5 is a main marker. While moving along microtubules, RAB5 is progressively replaced with RAB7 (Rab switch), and early endosomes mature into late endosomes. En route, they integrate with components from the trans-Golgi network of the secretory pathway, including hydrolase and membrane components. Late endosomes eventually fuse to form lysosomes. This entire pathway is now known to involve many key myristoylated proteins, many of which play important roles in GTP/GDP exchange either directly (such as ARF6 or GEF) or indirectly. This includes Lamtor1 subunit of Ragulator, which is a GEF of Rag; and myrlysin of BLOC1-related complex BORC, which is a GEF of Arl8 (**Fig. 10B**). Ragulator activates myristoylated AMPK/catabolic liver kinase B1 signaling via the axin scaffold protein upon nutrient starvation [209] and directly interacts with BORC, which promotes lysosome dispersal [210]. When amino acids are supplied, membrane-bound SLC38A9 tightly binds Ragulator and Rag GTPases, activates RagA/B, recruits mTORC1, and allows BORC and Arl8 to recruit kinesins 1B and 5B (**Fig. 10B**). The myristoylation activity of NMT1 has been shown to be required for both mTORC1 activation through Lamtor1 and AMPK activation [211, 212], two main kinase complexes acting as master regulators of cell metabolism. Finally, ARF6, in concert with NCS1, plays a role in endocytic recycling (**Fig. 10A**).

4.4. The myristoylome as the sub-proteome targeted by NMT

NMT has strikingly high specificity for N-terminal Gly residues, and replacement with Ala or other amino acids established that a single substitution of Gly2 made N-terminal myristoylation impossible [213]. When a vicinal Lys occurs however, such change may promote Lys-myristoylation [42, 44]. The search for NMT targets started with the early observation that single substitutions in known substrates resulted in a dramatic loss of myristoylation; for instance, the single substitution K7N [214] in c-Src precludes myristoylation. In addition, early studies indicated that a wide range of sequences around the first eight amino acids contributed to myristoylation, with Gly2 the only common residue (Table 1 in [32] and Table IV in [110]). It was also observed that the same protein target, while retaining its myristoylation status, could display significant variations in its amino terminal octapeptide sequence [33]. Nevertheless, all NMTs appear to display close substrate specificity [215, 216], the most distinctive features involving the occurrence of negatively-charged residues at positions 7 or 8 of the substrate preventing recognition by fungal NMTs [110, 216, 217].

4.5. Peptide libraries

The sequence space created by seven amino acids next to the common Gly is huge ($20^7 = 1.28 \times 10^9$) and cannot be explored easily without computer-assisted approaches [43]. Gordon *et al.* first used small peptide libraries to investigate the myristoylation space. A variety of sequences and changes at the N-terminus and the use of substitute amino acids led to the early discovery of the major factors influencing myristoylation, including the crucial role of Ser6, which significantly lowers the K_m , although most searches were based on the GNAAA₆RR peptide of human G α ,

which does not display this residue. The analysis led to a rough consensus sequence (reviewed in [32]). The PS00008 PROSITE G[^DEFHKPRWY]XX[ACGNST][^P] (where ^ means exclusion of the following residue) derived from these discoveries was used to screen and identify the aforementioned 70 NMT targets in 6,220 proteins in yeast [187]. Searches in other proteomes with this motif led to many false negatives and positives [186, 217]. Alanine scanning experiments [165] starting with peptide GLYASKLS were also performed, but this peptide displays the large Leu3 residue, which is known to disfavor recognition (see below and **Fig. 1B**), which although compensated for by the presence of two very favorable peptide residues Ser6-Lys7, is likely to have biased the conclusions.

4.5.1. Labeling

An early step in the discovery of myristoylated proteins was the identification of an N-terminal Met-Gly context followed by assays using protein extracts grown in the presence of a radioactive tritiated precursor myristate (obtained as described in [151]), which the cell converts into MyrCoA with long chain fatty acyl-CoA synthetase (LACS, see Section 3). Such approaches required the availability of specific antibodies against the target protein. Frequent mobility shifting of the modified protein in 2D and even 1D gels was observed due to cancellation of the positive charge at the N-terminus and the addition of a very hydrophobic modification as large as two amino acids [218]. Some authors also provided evidence of myristoylation in reconstituted co-expression of both NMT and the target protein in *E. coli*. Confirmatory experiments were then needed, including the use of a Gly2Ala variant, which would be expected to inhibit myristoylation and induce N-alpha acetylation (NTA) with a close negatively charged residue. Finally, changes in the subcellular localization profile because of G2A substitution of a recombinant overexpressed protein fused to

GFP was used as a proof of the functional relevance of the modification. The G2A substitution usually involved PM or ER re-localization to the cytosol. All these approaches, which often need to be combined, are suited to the interrogation of the biological impact of myristoylation but introduce bias due to protein overexpression, radioactive labelling (which often precludes quantification), and easy quantification of myristoylation of the target. Overall, these approaches impeded the discovery of a global view of the modification.

In the early nineties, the global view was mainly predictive and made use of computational tools trained using only a low number of positive myristoylated targets including annotation by similarity alone [186, 219-221]. The most useful software used at the time also suffered from a lack of knowledge of non-myristoylated proteins starting with Gly. This knowledge gap has only been filled over the last decade, with targeted approaches by the Gevaert, Arnesen, and Overall groups aiming to discover N-acetylated proteins [222]. For instance, a comparison of human and *A. thaliana* N-acetylation systems showed similar features of the cytosolic machineries. In the context of these studies it was, however, clear that such approaches were not well suited to the discovery of myristoylated proteins, as only a few could be identified [223].

4.5.2. Medium-throughput approaches

Progress in cloning, genome annotation, and insect cell expression with fusion to a reporter protein (gelsolin) of peptides derived from human proteins deduced from 2,000 cDNAs allowed the screening of 141 Gly-starting proteins using radioactive metabolic labeling. With this approach, 27 new G-myristoylated targets were added to the G-myristoylome [224]. *In vitro* assays combined with *in vivo* studies of proteins or their N-termini fused to GFP for a dozen *A.*

thaliana peptides [225-228], together with confirmation of the impact of G2A substitution on subcellular fractionation and radioactive myristate labeling, represented first efforts towards high-throughput analysis of myristoylated proteins in a single organism.

Significant progress towards large-scale characterization of the G-myristoylome was first made in *S. cerevisiae*. Fifteen myristoylated targets were discovered through a pioneering *in vitro* analysis and *in silico* searches performed with the complete proteome [135, 187]. A recent survey indicated that, often individually, 70 myristoylated proteins in humans [67] and 43 in *A. thaliana* [229] were experimentally identified and verified by the early 2010s. Predictions at the time indicated that over 300 targets should be expected in complex proteomes [186, 221, 230]. Clearly, new approaches were needed to further and fully describe the modification and obtain knowledge at the genome-wide scale.

5. Defining myristoylation at the proteome scale

The advent of new large-scale, mass spectrometry-based technologies dedicated to protein modifications including lipids, and specifically myristoylation, has significantly modified our approach and understanding of myristoylated targets. Because of the very recent discovery of NMT-dependent lysine myristoylation (K-myristoylation), at the time of writing no large-scale approaches have been described to identify K-myristoylated targets *in vivo*. Therefore, this section describes the approaches that have taken advantage of improvements in mass spectrometry to discover G-myristoylation targets.

5.1. Validating chemical probes for click chemistry

5.1.1. Click chemistry, bioorthogonal labeling, and FA analogs

As discussed in Section 3, most if not all organisms are expected to be able to utilize FAs such as C14 present in the external medium as a precursor of MyrCoA (**Fig. 7**). Click chemistry refers to chemical reactions that are rapid, selective, and high yield. The development of click chemistry for highly selective biomolecular labeling is based on precursors not reacting with groups occurring in biological molecules. This defines bioorthogonal labeling reactions, which were first introduced in 2003 for O-glycosylation [231]. Such approaches quickly became popular in chemical biology for introducing many synthetic groups, including FA, within proteins [232, 233]. The Staudinger ligation, which describes engineering phosphines ligation with azide derivatives, was the first example of bioorthogonal chemistry, of which the azide-alkyne cycloaddition is another prototype [234, 235]. Major progress in the field was made with the development of reactive biomimetic FA compounds acting as precursors of CoA derivatives accommodated by NMT *in vivo* after free diffusion from the growth medium to the cytosol [153, 236-240]. Such reactive reporters of myristate correspond to azide (az)- or alkyne (Alk)-modified FAs including az-11, az-12, Alk-C13, or Alk-C14 (**Fig. 11**). We refer to all of these approaches as click chemistry from hereon in.

5.1.2. Probes for myristoylation labeling

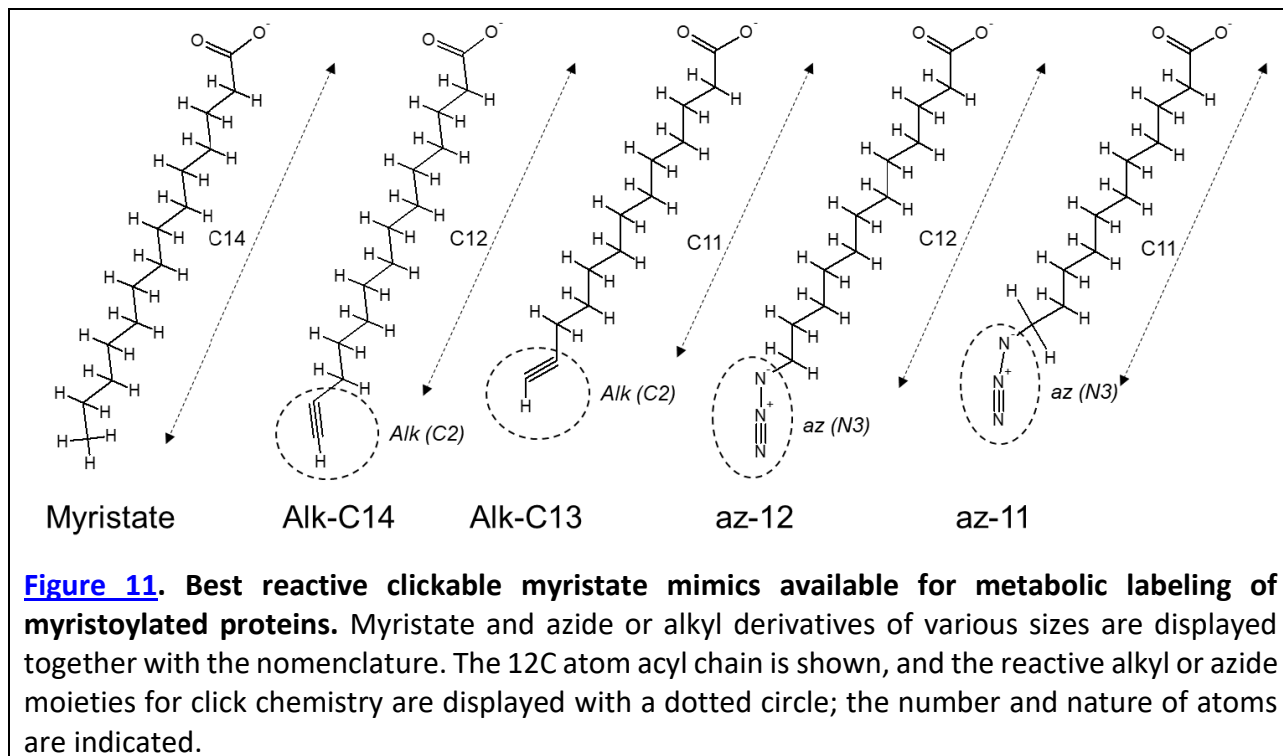
Metabolic labeling with a tagged FA like myristate implies that the tagged protein pool corresponds to only the myristoylated fraction. Nevertheless, both β -oxidation and LACS activities may produce shorter or longer AcylCoA derivatives (see Section 2.2).

It was quickly noted, unexpectedly, that FA-derived reactive compounds may also undergo *in cellulo* chain extension by LACS. Lipidated protein labeling by shorter compounds was also likely due to the size tolerance of their AcylCoA binding pocket to shorter chains. For instance, an Alk-

C16 (alk-14 in the study) derivative labelled proteins in both their myristoylated and/or palmitoylated sites [241, 242]. Moreover, larger reactive compounds like Alk-C19 (alk-17) were incorporated at both Cys-palmitoylated and G-myristoylated sites [243]. The difference between the two modifications can be distinguished after hydroxylamine treatment, in which amide bonds are resistant to dissimilar thioester bonds; this suggested that β -oxidation is a major route for FA probe degradation [243]. Together, this leads to false positives arising from other longer fatty acylated proteins. Finally, Alk-C14 - also referred as ω -alkynyl myristate, tetradec-13-ynoic acid, YnMyr, or alk-12 in various works (see **Fig. 11**) - was found to be more suited to gel or cellular fluorescence visualization and has since been preferred over the azide derivative az-12 [241, 244]. Fatty azide profiles of various azide derivatives followed by quantification revealed C16 azide retrieved in the az-C12 sample [64]. This indicates that C14 analog labeling may result in C16 incorporation and false positives. It is actually difficult to distinguish such false positives from real incorporation of a C14 derivative at an unusual site, such as a Cys palmitoylation site, even though this is described as feasible (Sections 2.2 and 3). It requires determination of the mass of the FA tag, implying that the tagged peptide itself - rather than an associated peptide of the tagged protein - can be identified.

In plants, Alk-C14 was also preferred over az-12, as it appears to be 10-fold less toxic in passive infiltration studies (see Fig.S1 in [129]). Nevertheless, az-12 was successfully used in vacuum infiltration studies of thioredoxin-h2 [245]. A crystal structure of *Plasmodium vivax* NMT was determined in complex with the CoA derivative of Alk-C14 (PDB 2YNC). The alkyne terminal group of the compound accommodated the binding pocket without any steric clashes [246]. Measurement of the kinetic parameters showed a K_m similar to MyrCoA (2 μ M) and a slightly (4-

fold) reduced k_{cat} value (see Sup Fig. 2 in [246]). Together, the data validated Alk-C14 as a highly valuable biomimetic analog of myristate in the context of NMT biology. In this context, G-myristoylation also needs to be acknowledged as one of the shortest and most resistant to chemical release *in vitro* [29]. This facilitates the analysis, as use of a C14 derivative of the same size almost prevents incorporation of the compounds in less than 14-carbon acylated proteins, while incorporation of larger chains can be prevented in other ways (see below). Finally, as expected (see Section 3.2 and **Fig. 7**), serum deprivation of FAs with charcoal-stripped fetal serum albumin [247] supplemented with Alk-C14 significantly improved the detection limit of the resulting myristoylated proteins from mammalian cell cultures [240, 248]. While not strictly required for metabolic labeling to occur [249], this strategy likely favors incorporation of C14 on unusual or low C14 stoichiometry protein targets (**Table 1**).



5.2. Click chemistry enrichment approaches

From 2007 [243, 250, 251], new click chemistry tools significantly enhanced the discovery of novel substrates while progressively also taking advantage of the availability of (i) new generation mass spectrometry instruments with improved sensitivity and accuracy, and (ii) strongly improved and curated databases that made mass spectrometry-based discovery far more reliable for myristoylation. All approaches that use labeling and protein extracts are based on four main steps: (i) bioorthogonal labeling of biotin-labeled azido or alkyne derivatives; (ii) affinity enrichment of reactants on streptavidin beads; (iii) proteolytic cleavage, typically with trypsin; and (iv) shotgun proteomics. To date, the following alternative approaches have been successfully used to identify 30-90 myristoylated proteins in various proteomes.

Tate and coworkers have significantly contributed to bioorthogonal/click chemistry-based discovery of NMT substrates over the past decade. Metabolic labeling approaches using FA analogs as precursors of MyrCoA bearing the bioorthogonal alkyne tag Alk-C14 (**Fig. 11**), coupled with copper-catalyzed click chemistry (CuAAC) with a trifunctional compound bearing an azide function, a biotin for streptavidin beads pull-down equipped with a polyethylene glycol (PEG) spacer (**Fig. 12A,B**), and/or a fluorophore like TAMRA as a reporter tag, have been successfully used to identify several dozen G-myristoylated proteins in several organisms including humans and parasites [241, 246, 249, 252]. Although powerful, this technique has limitations. First, Alk-C14 labeling is not entirely specific for NMT-directed myristoylation as it also involves other targets, mainly GPI-anchored proteins but also a few other unknown proteins including palmitoylated proteins which incorporate the myristate analog. Unless a specific NMT inhibitor (such as the commercially available IMP-1088) and normalization approaches making use of

differences between proteins trapped in the presence and absence of inhibitor are used, it is difficult to accurately identify relevant targets [253]. Note that each G-myristoylated protein responds specifically to the inhibitor depending on, for example, its turnover. Finally, for several reasons, metabolic labeling approaches can only be performed with single cells, tissues, or organoid cultures grown in the presence of a predefined growth medium in which both metabolic precursor and inhibitor are provided.

Using this approach, 76 G-myristoylated proteins (including 35 new proteins) were retrieved from human cells in one shot [253]. Most interestingly, the approach allowed the identification of another 40 proteins post-translationally G-myristoylated following caspase cleavage during apoptosis (see SupData4 in [253]). Although the physiological impact of this post-translational myristoylation modification is unknown, it is clearly required for at least relocalization of fragments of BID [254], actin [255], or PAK2 [256]. The switch in BID contributes to enhancing release of cytochrome *c* and cell death [254]. A fragment of Huntingtin was also shown to relocalize to the autophagosome as a result of myristoylation [257], while the G-myristoylated gelsolin fragment is cytosolic [258].

To recover the captured protein and better characterize its amino terminus status (myristoylated or not), the capture reagent coupled with biotin and a fluorophore was next equipped with a trypsin-cleavable linker [259] (**Fig. 12C**). This improvement aimed to overcome the relative lack of specificity of the reactive FA derivative towards a variety of acylated proteins. Such capture reagents facilitate pull-down of labeled peptides and peptide ionization, but the modification status of the N-terminal peptide must be confirmed by mass spectrometry. This is not the case for a majority of identifications and explains why the use of other controls including

inhibitors is still useful to confirm effective myristoylation of the target. One capture reagent allowed both quantification and confirmation of myristoylation [259]. With this approach, 83 myristoylated proteins were confirmed in humans. This also indicated that there is room for improvement of discoveries made with the powerful click chemistry-based approaches if the overall chemistry is further improved. For instance, the pulldown needs several replicates and leads to many false positives that need to be discarded through replication, measurement of the impact of an NMT inhibitor, and/or filtering protein entries starting with MG. This general click-chemistry strategy has been applied to organisms other than humans for the global analysis of protein myristoylation such as in the developing zebrafish *Danio rerio* (56 identifications, [259]) and in the parasite *Trypanosoma brucei* (46 NMT G-starting substrates, many new [260]).

To distinguish myristoylated from GPI-anchored substrates, new approaches apply base treatment prior to streptavidin-based pulldown; for this, NaOH can be added to a final concentration of 0.2 M prior to neutralization with HCl. Another method takes advantage of a capture reagent featuring a tobacco etch virus (TEV) protease-cleavable linker sequence (ENLYFQS↓GG) to delay the release of the myristoylated peptide from streptavidin beads after trypsin cleavage [261]. This ensures the release and further identification of myristoylated peptides only after TEV protease cleavage. With this approach, 76 targets were identified in *T. gondii* [261], including 31 N-terminal G-myristoylated peptides identified by either trypsin- or TEV protease-cleavable capture reagents. The approach also identified 102 human G-starting proteins that co-purified in the extracts (see SuppFile 2 to Fig2; [261]). Though it confirmed 84 already known human targets, this list displayed 20% outliers, i.e., unexpected targets not fitting the known substrate selectivity of NMT. To unambiguously identify G-myristoylated targets, proteins

displaying strong Alk-C14/myristate enrichment, the presence of an MG motif, and insensitivity to base treatment were filtered out. Fifty-six proteins met these criteria in *T. gondii*. Finally, with the additional use of an NMT inhibitor as an additional cross-validation step, the authors retained 65 targets in *T. gondii* classified into two confidence categories depending on identification by either or both approaches [261]. With cross-validation and the identification of a significant number of G-myristoylated peptides, this approach represents the most advanced click-based strategy in the field.

An alternative approach took advantage of Alk-C14 coupled to the transpeptidase activity of the *Staphylococcus aureus* sortase, which specificity relies on peptides with a free N-Gly and that has been used in various biotechnological applications [262]. Using a biotin-labeled ALPET-Haa depsipeptide substrate of sortase and a mutated sortase [263], N-Gly-free proteins can be pulled down. Together with a specific NMT inhibitor *in cellulo*, it is feasible to identify peptides sensitive to the inhibitor and thus likely to be myristoylated [264]. The advantage of the approach is that it simplifies trapping, but a major drawback is that it still relies on NMT inhibitors, as the method is only specific for N-free Gly residues. The approach revealed less than a dozen new myristoylated proteins compared to previous studies from the same group.

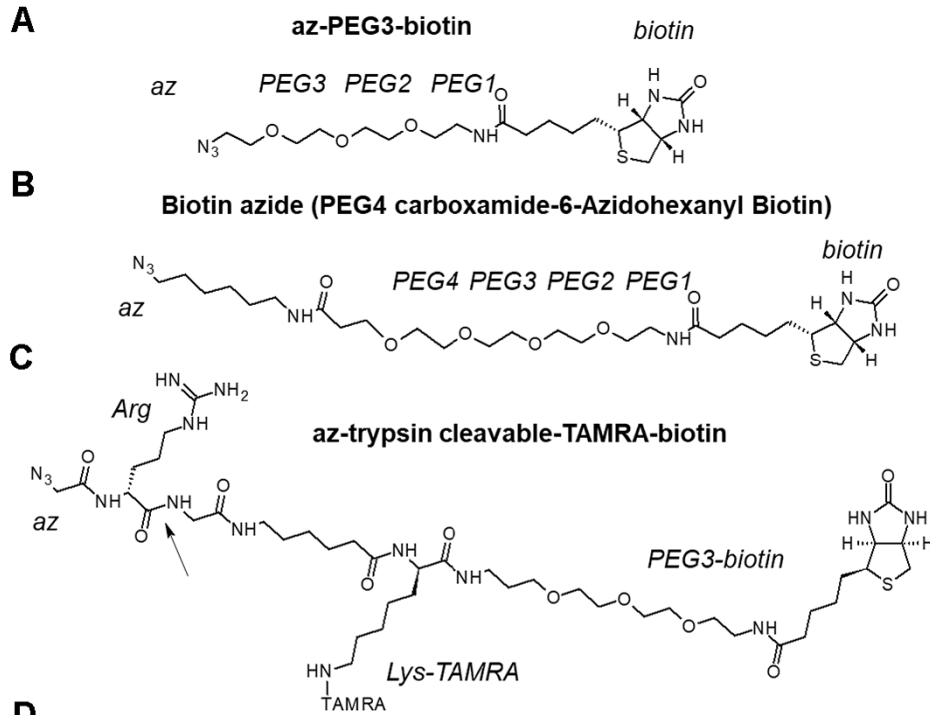
5.3. Combining subcellular enrichment and high-resolution peptide separation

A combination of subcellular fractionation, high-resolution separation of hydrophobic peptides by mass spectrometry-coupled liquid chromatography, and newly developed mass spectrometry data-dependent acquisition [67, 229] recently produced the largest *in vivo* dataset of 72 non-redundant *Arabidopsis thaliana* G-myristoylated proteins and 69 human myristoylated

proteins. The direct characterization of myristoylation by mass spectrometry involved the identification of the b_n series as marker ions of myristoylation starting with the 268 Da b_1 fragment (see pipeline summarized in **Fig. 12D**). The neutral loss of 210 Da associated to myristoylation is low-intensity by collision mass spectrometry using various ionization sources [229, 265, 266].

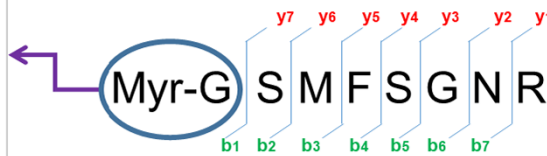
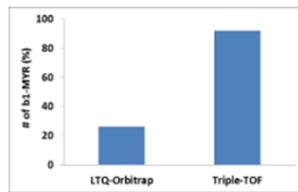
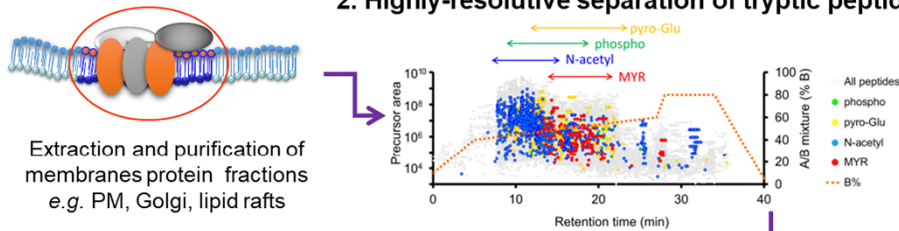
The difficulties in identifying the entire set of G-myristoylated proteins have led to improvements in predictive software over the last five years. Specifically, integrated tools involving structural biology, peptide macroarrays, proteomics, and bioinformatics have suggested that the complete myristoylome [67] comprises ~600 G-myristoylated proteoforms in humans or in *A. thaliana*. We performed a new annotation with the latest Araport11 reannotated version of the *A. thaliana* proteome, which resulted in 935 G-myristoylated candidates, i.e., 1.9% of the entire proteome (**Table 1**), a significant rise from 1.7 to 1.9% of myristoylated proteins relative to the number of isoforms. A main feature of the *A. thaliana* G-myristoylome is that all octapeptide derivatives were assayed *in vitro* [67]. *In vivo* approaches to confirm these mass spectrometry data resulted in 100% accuracy, indicating high reliability of the database [67]. Therefore, inhibitors are no longer required to ascertain the myristoylation state provided any new retrieved protein is on this list.

Finally, we still are awaiting information of the relative modification state of those proteins where the same proteins might or might not undergo various N-terminal modifications, including myristoylation and NTA, which will require the development of new approaches. In addition, the absolute myristoylation level of a protein needs to be clarified and quantified, as only relative increases or decreases of myristoylation are deduced in the published data (**Table 1**).



1. Extensive fractionation of plant extracts

2. Highly-resolutive separation of tryptic peptides



4. Data processing and analysis; Label free quantification

Figure 12. Strategies for capture or protein enrichment to evidence myristoylated proteins at the proteomic scale. (A) Structure of azide-PEG3-biotin conjugate; (B) Structure of commercially available biotin azide, PEG4 carboxamide-6-azido-hexanyl biotin; (C) Structure of trypsin-cleavable, TAMRA-labeled azide biotin conjugate - the trypsin cleavage site is indicated with an arrow; (D) Strategy to enrich for myristoylated proteins based on membrane protein fractionation and data-dependent mass spectrometry acquisition. The approach was developed in plants [229]. Abbreviations: PEG, polyethylene glycol; TAMRA, tetramethylrhodamine; az, azide (N₃).

5.4. Towards complete view of myristoylomes in eukaryotes

5.4.1. Co-translational myristoylation

The approaches described above have led to the unambiguous identification *in vivo* of hundreds of myristoylated Gly-starting proteins in various proteomes. In **Fig. 13A-C**, we compile the weblogs of these data according to the various classes of NMTs except plants, which are displayed in **Fig. 13D-F**. The data show the strong conservation of the motif in different species and throughout evolution. The data also reveal the occurrence of frequent Cys residues next to Gly2, which have been shown not to be directly involved in NMT recognition but rather contribute to palmitoylation, as discussed above. This feature is even more prominent in plants due to the high number of both myristoylated and palmitoylated proteins (50% in plants vs. 20% in humans or 5% in fungi) [67]. The data also highlight complexities in the pattern recognized by NMT and how difficult recognition of a myristoylated protein might be. To overcome this challenge, nearly all Gly-starting peptides originating from *A. thaliana* have been tested using an *in vitro* myristoylation assay, which indicates that the 834 myristoylated-positive G-starting octapeptides show a pattern similar to the one originating from the 108 identified G-myristoylated substrates, but also amino acids that disfavor myristoylation (**Fig. 13E** and **Fig. 13F**). Clearly, charged residues between positions 3 and 6 are disfavored, and a positive role is revealed for bulky hydrophobic residues at position 5 [186]. These trends allow for the easy identification of whether an unknown Gly-starting protein is myristoylated or not. A complete study of the positive sequence from humans and *A. thaliana* is now available [67]. Although these data are robust enough to translate the data from one proteome to another in closely-related species (**Fig. 13**, [267]), predictive tools require further refinement to obtain new insights into different proteomes. An unresolved issue

is why G-myristoylation does not necessarily use nearly identical recognition motifs with a few strictly required residues like most proteases. There appear to be two major routes of recognition, one featuring Asn3 and the other Ser6. One interesting and recently proposed hypothesis in the case of a feline virus protein was that the availability and folding of the N-terminal myristoylated end would differ between motifs [268], which might explain why the two poorly overlapping motifs have persisted.

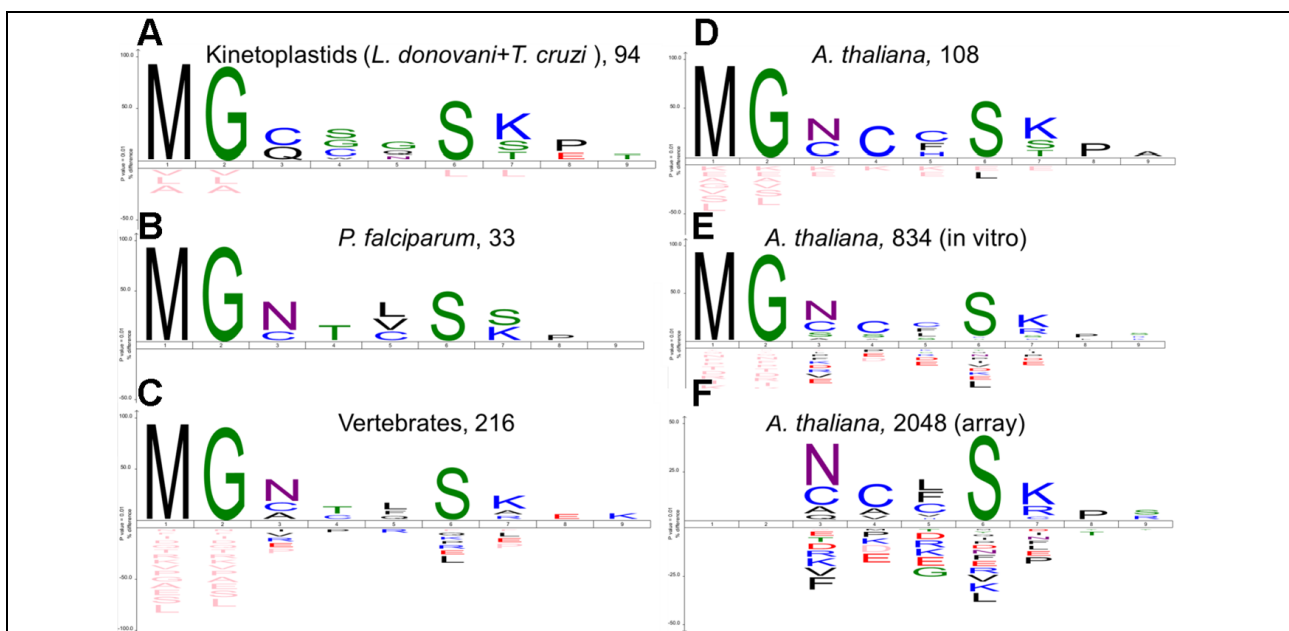


Figure 13. N-terminal motifs emerging from proteome-wide analysis of myristoylated proteins. The 9 first N-terminal residues of the sequences retrieved as myristoylated, mostly from metabolic labeling studies in various organisms, were aligned and displayed with iceLogo [362]. A P-value of 0.01 was chosen for the display. In the representation, the size of each letter is proportional to its frequency. To construct the dataset, all positive substrates were compared to the negative set. The number of instances (corpus) taken into account is indicated after the species name. The colors denote the following: green, class of small hydrophilic uncharged residues including S, T, G; red, acidic residues including D or E; blue, positively-charged residues including K, R, and H; purple, N or Q; black, hydrophobic residues (A, I, M, V, L, W, Y, F, P). (A) Kinetoplastids; (B) *P. falciparum*; (C) Vertebrates; (D) *A. thaliana*. E and F show the distribution from approaches arising from peptide libraries either screened against the whole protein data library of the organism (E) or against the complete set of MG-starting proteins assayed for myristoylation (array) in [67] in (F); this explains why MG is not visible in panel F, as the whole set shares this starting motif.

5.4.2. Post-translational myristoylation

Post-translational G-myristoylation as a result of caspase cleavage has a much vaguer consensus sequence than that arising from the N-terminus (see Fig. 1D in [43] and [253]). Cleavage occurs at a conserved DEXD/G₂ dipeptide, where the last D is strictly conserved at P1. Most features associated with myristoylation can be retrieved, including Ser6. Surprisingly though, a positively charged residue has never been retrieved at position 7, despite it strongly boosting myristoylation. Finally, there is no evidence of Cys residues, in keeping with relocalization events observed upon myristoylation not involving the PM and palmitoylation as a result. The corpus of sequences undergoing post-translational myristoylation currently amounts to only a few dozen, very few compared to the 36,330 sequences containing a D/G dipeptide motif predicted to be cleaved by caspase in humans (CaspDB; <http://caspdb.sanfordburnham.org>), of which 6,365 show possibly favorable residues at position 3 and 6. This suggests that further targets have yet to be identified.

Finally, it is interesting that NMT inhibition in cancer cell lines causes ER stress and apoptosis. Apoptosis requires cleaved BH3-interacting domain death agonist (cBID) for proapoptotic Bcl-2-family protein Bax to oligomerize at the outer mitochondrial membrane [269]. As cBID needs to be myristoylated to trigger cytochrome *c* release and cell death, NMT inhibition-induced apoptosis probably relies on another pathway.

5.4.3. Fungi display a reduced percentage of targets in their G-myristoylome

Recent comparisons of various G-myristoylomes have disclosed well-conserved G-myristoylated proteins corresponding to $1.5 \pm 0.5\%$ of proteoforms [43]. 0.7% of these, i.e.,

46/6,700, include 15 verified G-myristoylated targets, although the *S. cerevisiae* myristoylome was an outlier with a lower content (for a compilation, see [43]), perhaps due to its unique adaptive evolution shaped by human domestication [270]. Recent click analysis of the wheat fungal pathogen *Zyoseptoria tritici* [271] revealed 0.5% G-myristoylated targets, even lower than that of *S. cerevisiae*. Although it is possible that the quality of gene annotation accounts for this observation, only 5% of the ORFs await N-terminal annotation, so this is an unlikely explanation. Most G-myristoylated targets identified have homologs in *S. cerevisiae* [271] that are also myristoylated [43]. It therefore appears that fungi – not only *S. cerevisiae* – might have reduced G-myristoylomes, which might be due to genome compaction in this branch of Unikonta [272].

5.4.4. Plants display higher levels of G-myristoylated targets featuring many sensors

Unlike fungi, plants display a particularly rich G-myristoylated content [67]. Detailed proteomic analyses of the myristoylome and its localization in various compartments in *Arabidopsis*, together with the identifications described above, have identified 101 myristoylated targets (see Fig. 8 in [229]). The majority of myristoylated proteins localize to the PM and are involved in calcium signaling and pathogen responses [273]. This very abundant localization to the PM cannot be explained by a specific machinery, since there are as many DHHC palmitoyltransferases in plants (24) as in humans [274]. Instead, it is most likely due to the fact that myristoylated *Arabidopsis* targets have stronger enrichment of Cys residues within the myristoylation consensus (compare **Fig. 13D-F** to **Fig. 13A-E**) leading to palmitoylation and PM localization [67].

This enrichment in calcium-dependent functions first encompasses a four out of 10 EF hand motif containing calcineurin B-like (CBL) sensors [275], where myristoylation is conserved in crops including tomato, rice, and maize. CBL belongs to the same family as neuronal calcium sensors present in mammalian brains and retinas and which are also myristoylated and transduce calcium signals [67, 198]. CBL4/SOS3 is involved in salt tolerance [276]. The *Arabidopsis* G-myristoylome displays another set of 4 myristoylated calcium sensors related to CBLs [67]. Myristoylation of such EF-hand calcium-binding proteins appears to be remarkably well conserved in many organisms [43].

The high enrichment of G-myristoylated proteins in plants appears to be tightly linked to the remarkably rich G-myristoylated kinase content, which represent 23% of targets. Unlike animals, plants do not possess the G-myristoylated Src family tyrosine kinases, only Ser/Thr kinases. Within the set of G-myristoylated kinases in *Arabidopsis*, it is remarkable that three quarters belong to only four subgroups of tightly-related kinases. Calcium-dependent and calcium-related kinases correspond to one class of calcium-sensing kinases localized at the PM; such calcium-responsive kinases belong to the calcium-dependent protein kinase (CDPK) family and display an N-terminal kinase domain fused to a C-terminal calcium-binding sensor in the aforementioned calmodulin family featuring EF hand Ca^{2+} -binding sites [277]. Most plant CDPKs are both myristoylated and palmitoylated (**Fig. 14A**) and have multiple functions in plants [277, 278]. CDPKs represent half of the 107 myristoylated kinases retrieved from plants. Another group of kinases closely related to CDPKs are the IBS family, which features a remarkably conserved double myristoylation and palmitoylation motif (**Fig. 14B**). Such kinases appear to be involved in plant signaling for protection from bacterial pathogens or salt stress [279]. It is interesting that both the occurrence

of CDPK and their myristoylation and palmitoylation features are conserved in all plastid-containing organisms, including Apicomplexa [280, 281].

Receptor-like kinases from plants represent a large family of transmembrane proteins accounting for about 2% of plant proteomes - including 610 members in *Arabidopsis* - all involved in biotic and abiotic stress signaling [282]. Of these, receptor-like cytoplasmic kinases (RLCK) are a group of 149 members in *Arabidopsis*, all featuring a cytoplasmic-oriented kinase domain and lacking both the extracellular so-called ectodomain and the single-pass transmembrane domain [283]. Plant RLCKs are subdivided into 17 subgroups and many feature N-terminal myristoylation and palmitoylation motifs ensuring PM attachment [284], which is mandatory for their association with the corresponding receptor kinases sensing the signal, while RLCK transduce it. There are two main myristoylated RLCK subfamilies. The first corresponds to members of subgroup VII, which comprise Botrytis-induced kinase 1 (BSK1), PBS1 (the AvrPphB receptor, see Section 5.2), and various PBS1-like (PBL) kinases [285-287]. PBLs are thought to contribute to the plant innate immune system by transducing pathogen-derived molecular signals received by pattern recognition receptors of the receptor-like family [288, 289]. Most PBLs feature a myristoylation/palmitoylation double motif to ensure PM attachment (**Fig. 15A**). The second RLCK family with myristoylation features is the brassinosteroid signal transduction (BSK) family, all of which display a C-terminal tetratricopeptide repeat. Like PBL, BSK members display a G-myristoylation/palmitoylation motif (**Fig. 15B**) [290]. BSKs include SSP (BSK12), which is involved in fertilization [189], BSK3, involved in steroid hormone signaling for plant growth and development [291], and BSK5, implicated in immune responses [292].

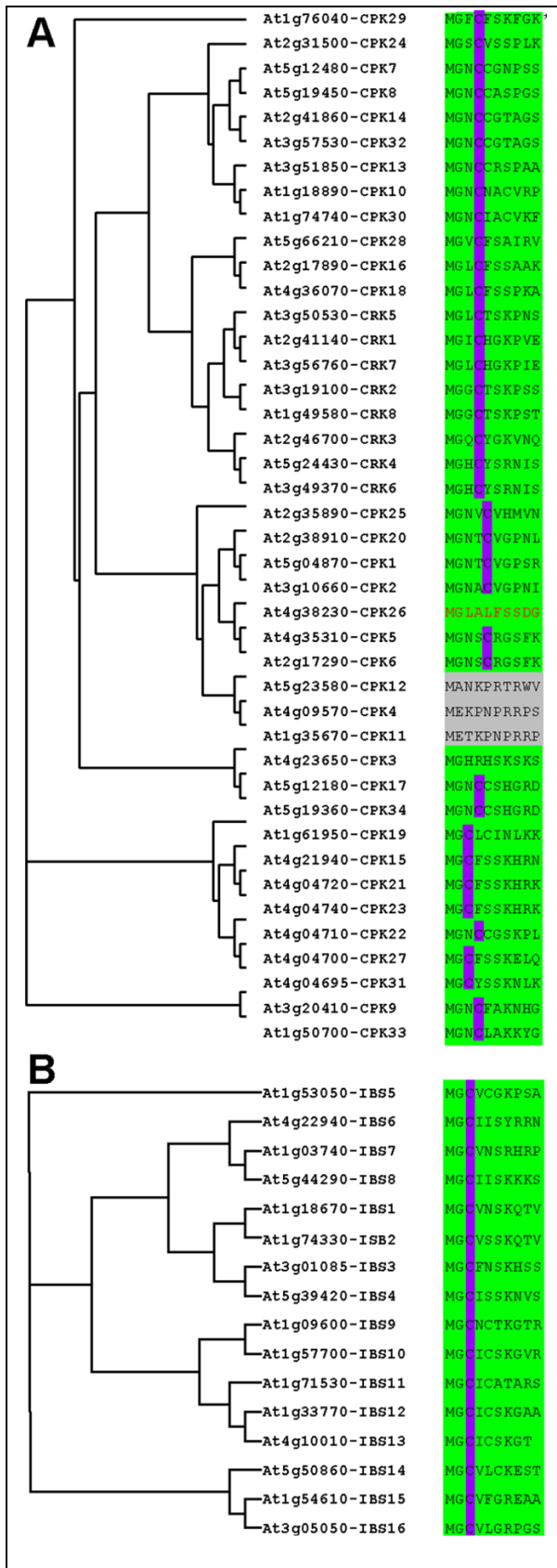


Figure 14. Calcium-dependent protein kinases from *A. thaliana* and myristoylation. The calcium-dependent protein kinases (CDPK; [277, 278]) and CDC2-like kinases (IBS, acronym derived from BABA-induced sterility; [279]) from *A. thaliana* were aligned in **A** and **B** respectively, as described in **Fig. 8**. The entry number and short names are indicated together with the 10 first residues of the ORF to display the entire myristoylated motif. All myristoylated sequences are highlighted in green, and when a palmitoylation site is predictable it is colored in purple. When a sequence was not myristoylated the gene name are in italics. If this feature is because it does not start with MG, it is indicated in grey, and if it is because of a sequence starting with MG which was not demonstrated to be myristoylated, then it is colored in red. The data are from [67]. The asterisk on CPK29 indicates the second Met (Met28) of the annotated ORF, which features an unusually long extension compared to all other family members.

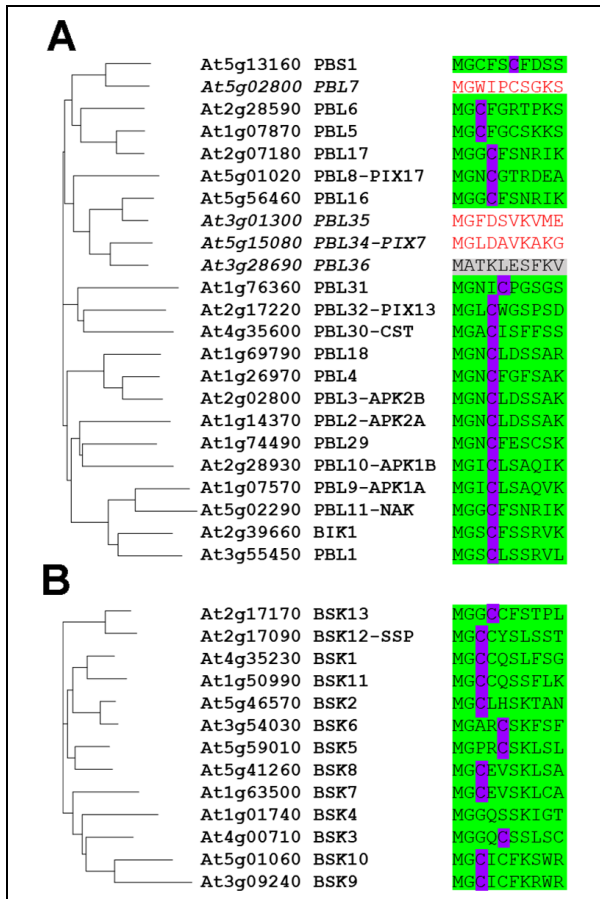


Figure 15. Receptor-like kinases from *A. thaliana* and their myristoylation site. Receptor-like cytoplasmic kinases (RLCK) of subgroup XI (family 1.6.1) and subgroup VII (family 1.2.2) [282, 283] were aligned and displayed as described in Fig. 14. (A) The PBS1-like kinase (PBL; [363]) family is displayed. Alternative names corresponding to some members of the family are indicated in brackets. PBL12-15 – which are closest neighbors in the VII RLCK family [289] - were omitted. (B) Brassinosteroid-signaling kinases (BSK [290]) include SSP [189].

The *Arabidopsis* G-myristoylome reveals a further set of disease resistance proteins in the aforementioned NBL-LRR family including RPS2, RPS5, and RPP1, which sense avirulence (Avr, see Section 6.2) proteins [67, 186, 229, 293]. It was hypothesized that competition at the myristoylation binding site with myristoylated Avr proteins might contribute to sensing [186]. Taken together, it appears that plants have acquired a highly sophisticated system to sense any external perturbation including biotic and abiotic stresses in which G-myristoylation and its associated palmitoylation play a crucial role by linking intracellular responses, the PM, and stress receptors.

5.5. Protein myristoylomes of bacteria

An internal Cys located after the so-called lipobox ([LVI][ASTVI] [GAS]/Cys₊₁) is the modified residue of acylated proteins in bacteria. This motif suggests that about 40-200 protein targets occur in Gram-negative bacteria (i.e., 1-3% of the entire proteome, similar to eukaryotic NMT-driven G-myristoylation, see previous section; **Table 1**) [294, 295]. A proteomic analysis using the reactive precursor alkyne-reactive myristate (Alk-C14) as a lipid probe identified lipoproteins in *Clostridium difficile* [296]. 187 proteins were enriched, of which 56 were reduced in the presence of excess non-reactive myristate in the growth medium in a “chase” experiment. Forty-six were predicted to be lipoproteins *in silico*.

6. The biological function of NMT-dependent myristoylation of viral and bacterial proteins

6.1. Myristoylation and viruses

6.1.1. General features

Myristoylation of many viral proteins has been reported, including in animal viruses such as arena-, hepadna- lenti-, mimi-, picorna-, pox-, polyoma- retro- and rota-viriridae ([297, 298]) but also plant viruses including geminiviridae (e.g., tomato yellow leaf curl virus) [299]. In all cases, one, two in HIV (see before), three in vaccinia [48], and up to eleven G-myristoylated viral proteins (herpes simplex, see [300]) were detected. G-myristoylation appears an obligate modification for viral pathogenicity [301]. Upon G-myristoylation, lipid-based partitioning-driven oligomerization of Gag was observed for budding of HIV particles [302], while Nef can dimerize at the membrane

[303]. G-myristoylation of protein Z of the Lassa hemorrhagic fever virus – a member of the Arenaviridae family - was similarly proposed to be involved in viral budding through binding to lipid membranes [304].

Upon viral infection, the intracellular pattern of the G-myristoylated protein also changes in animal or plant viruses, such as with turnip mosaic virus PCaP [305]. Click chemistry approaches have also been applied to cells infected with HSV or HIV to identify host proteins G-myristoylated upon infection (see Section 6.1). Interestingly, G-myristoylation of host inner mitochondrial membrane proteins appears to be particularly involved [306].

Overall, host NMT appears to mediate viral protein myristoylation. Interestingly, many viral genomes harbor an open reading frame with significant homology to an NMT (**Fig. 8**), but to date there has been no functional characterization of these proteins. These NMT homologs are more related to fungi and microsporidae (fungal parasites), and viruses with NMT homologs include nucleocytoplasmic large DNA viruses (NCLDV) such as pox-, phycodna-, pitho-, and mimiviridae [307]. If these ORFs do indeed have NMT activity, their relevance to pathogenicity must be addressed. For instance, it could be interesting to determine whether they are located in dedicated compartments such as membraneless organelle viral factories formed from liquid-liquid phase separation, including aggresomes, which correspond to perinuclear inclusions formed by large DNA viruses including mimiviridae [308, 309]. These NMT homologs detected in viral genomes predict the presence of viral target proteins, such as is the case of virion A protein from mimivirus, a highly conserved protein in mimiviridae.

Recently, the importance of myristoylation in viral progression has been exploited, with specific NMT inhibitors being shown to have value against picornaviridae [310], paving the way for new inhibitors with efficacy against a variety of picornaviridae without host toxicity [311]. These inhibitors were shown to block capsid entry viral protein VP0 of rhinovirus, which causes the common cold, by preventing assembly of intact, infectious virions.

6.1.2. Myristoylation-dependent membrane-to-plastid re-targeting of viral plant proteins

Myristoylation and chloroplast-targeting signals can co-exist in plants, for instance in various calcium-dependent protein kinases (see Section 5.4.4) including CPK16 [193]. Myristoylation is stronger than targeting signals, and the proteins appear to bind the PM under normal conditions as a result. Abolishing myristoylation on these proteins leads to plastid re-localization, while adding myristoylation inhibits their intended subcellular localization. For example, in the tomato leaf curl Yunnan virus (TLCYNV), altering myristoylation of the C4 protein [299] decreased infectivity [312]. Recent data indicate that C4 may re-localize from the PM to plastids upon activation of defense responses and, as a result, interfere with chloroplast antiviral salicylic acid synthesis [313]. CPK16 also appears to re-localize upon bacterial elicitor peptide stimulation but, unlike C4, instead enhances anti-pathogenic responses. This plant pathogen defense mechanism involves host-pathogen co-evolution and reveals how pathogens take advantage of the plant host using its own defense mechanisms [314].

6.2. Myristoylation of bacterial virulence factors within the host

Some pathogenic bacteria are equipped with a K-myristoylation machinery designed to hijack the eukaryotic host cell (see Section 2.2). Nevertheless, bacteria do not have an intrinsic, specific myristoylation similar to that catalyzed by NMT in eukaryotes. Pathogenic bacteria may take advantage and hijack crucial NMT-based mechanisms to promote their own interactions with their eukaryotic hosts. The exclusive use of type III secretion systems (T3SS) involves mostly detrimental/pathogenic but also beneficial/symbiotic interactions. T3SS relies on the elaboration of a complex nanomachine that allows direct injection of virulence factors from the bacterium into the host cell [315, 316]. Along with other modulations, myristoylation targeting appears to be a common route of host hijacking (reviewed in [317]).

The plant innate immune system uses disease resistance (R) proteins to recognize type III effectors called avirulence proteins (Avr). Avr proteins promote pathogenesis in R-deficient plants through direct secretion into the plant host. A number of Avr bacterial proteins are myristoylated [318]. Once secreted and internalized into the host cell, Avr proteins may be myristoylated (i) directly, due to N-terminal methionine excision and absence of acetylation, or (ii) upon autoproteolytic cleavage, which reveals a new N-terminus with a cryptic myristoylation sequence. The internal proteolytic cleavage is due to the cysteine protease activity of Avr [319, 320]. This plant-specific processing is highly reminiscent of the caspase-dependent post-translational process occurring in animal cells during apoptosis (Section 5.4.2). However, one distinction is at the cleavage site, as plant caspases display a GDK motif upstream of the cleavage site instead of

the DEXD motif in animal caspases, and a second distinction is in the targeted subcellular compartment, usually the PM. Unlike caspase myristoylated targets, Avr proteins usually undergo palmitoylation as a result of Cys residues embedded into the myristoylation consensus site [297, 320]. This is typical of the plant system, which overuses Cys residues in the immediate vicinity and palmitoylation linked to myristoylation (**Fig. 13D-F**). An interesting feature is that PM targeting of Cys proteases like AvrPphB/HopAR1 leads to specific cleavage of protein kinase PBS1 [319], a myristoylated protein located at the PM [321]. Together with other kinases, PBS1 (BIK19 in **Fig. 15B**) is a member of the aforementioned RLCK family (**Fig. 15B**). In *Xanthomonas* and *Pseudomonas* spp., several type III effectors such as XopJ have been proposed as acetyltransferases [322]. Nevertheless, XopJ behaves as a protease that degrades the proteasome both in the nucleus and the cytosol, which contain myristoylated proteasomes (see above and [323]). Finally, symbiotic bacteria of the *Rhizobium* family appear to use a similar pathogen-like system to induce nodulation in legumes, i.e., the nitrogen-fixing organ resulting from symbiosis [324]. NopT effectors also display cysteine protease activity [297, 320], cleaving at a PDK/MG junction and requiring further removal of the N-terminal unmasked Met residue, most likely by host methionine aminopeptidase, to reveal the Gly-starting peptide and allow further myristoylation [325]. This mechanism integrates the two major proteolytic mechanisms revealing myristoylation sites, i.e., Cys protease and methionine aminopeptidase activity.

IpaJ is another cysteine protease secreted by *Shigella flexneri*, which causes dysentery. Unlike the aforementioned cysteine proteases, IpaJ displays demyristoylation activity, removing Myr-Gly moieties from myristoylated proteins [326]. IpaJ acts as human virulence factor targeting

myristoylated ARFs *in vivo* [327]. Click chemistry approaches have been used to study IpaJ targets [326]. Metabolic labeling with Alk-C14 followed by click chemistry approaches have identified many HsNMT targets (see Fig. 4C in [327]). There was a significant decrease in myristoylated proteins upon IpaJ treatment on streptavidin affinity columns, suggesting that IpaJ was specific *in vitro* for any Myr-Gly peptide independent of the rest of the sequence (see their Fig. 3).

7. Conclusions and perspectives: new myristoylation catalysts need to be defined

As mentioned throughout this review, several catalysts involved in myristoylation have yet to be identified, but their discovery would not only facilitate research in this area but also help to explain evolution of myristoylation. In these conclusions and perspectives, we provide a few insights related to this need.

7.1. Glycine acyltransferases in bacteria

Recent data have shown that N-terminal glycine monoacylation by palmitate (C16) of the CexE lipoprotein is ensured by the AatD subunit of the Aat secretion system [328, 329]. AatD is a close relative of Lnt, part of the nitrilase family. AatD is about 400 residues in length and, like Lnt, AatD displays four N-terminal alpha helical TM domains. Whether AatD - like Lnt (**Fig. 1C**) - can also ensure myristoylation in specific organisms or depending on growth conditions remains an open question.

It is also interesting to highlight that glycine lipidation with C14, C16, and C18 derivatives and the associated acyl transferase GlsB occurs in *Bacteroides*, a Gram-negative, obligate anaerobic

bacterium [330, 331]. Mono glycine lipids are membrane components important for growth [331]. However, the bacterial transferases appear to be more closely related to the lysophospholipid acyltransferase superfamily (pfam13444/cl17185), which include various lipid acceptors different from polypeptides and including polysaccharides such as in lipid A [332].

Finally, NMT homologs of the phytoviridae class have been identified in various marine bacterial genomes (**Fig. 8**, bottom; [333, 334], which requires further validation along with identification of the associated substrates and functions.

7.2. Evolutionary considerations on the origin of myristoylation

7.2.1. Protein acylation and FA derivatives in Archaea

The advent of high-throughput approaches has allowed the identification of myristoylation in most living kingdoms. Myristoylation has been also reported in Euryarcheota [335]. Nevertheless, to our knowledge, no protein acylation has yet been reported in Archaea, which is required to confirm that myristoylation is conserved in all living cells. This would make sense, as de-myristoylation acylases of the sirtuin family have been described in this kingdom [336].

Protein acylation is therefore a missing link in the evolution of Archaea to eukaryotes. An obstacle to filling this gap is the availability of myristate donors in Archaea. Many archaeal genomes have extensive sets of typical bacterial-like genes encoding the FAS2 complex, but there is always an absence of genes encoding acyl-carrier protein (ACP) or ACP synthase [337]. For instance, *Sulfolobus acidocaldarius* harbors a 30 kb cluster consisting of genes encoding a dozen enzymes involved in FA metabolism [338]. These genes might have arisen from horizontal gene

transfer from bacteria to Archaea. In fact, Archaea do not insert FAs in their membranes, as they rely on methyl-branched isoprenoid diether (rather than acyl ester) derivatives for the lipid membrane pool [4]. The small amount of FAs produced in Archaea is tightly regulated [338]. It is postulated that this pool might be involved in fatty acylation or stabilization of membrane-bound energy-conversion proteins such as rhodopsin or cytochromes. Finally, some Archaea such as *Archaeoglobus fulgidus* can grow on a range of FAs from C4-C18 as a carbon source [339], and many possess LACS. Archaea may also produce MyrCoA via β -oxidation of larger FAs. We conclude that a MyrCoA pool is likely to be available in Archaea for further transfer to actual protein targets via as yet unidentified dedicated acyltransferases. This needs to be clarified through the identification of myristoylation catalysts and associated protein targets. To date, while some NMT homologs related to the viral branch of the tree have been identified in some prokaryotes including Archaea (**Fig. 8**, bottom), it remains uncertain whether they originate from lateral gene transfer in some species or from contaminating DNA leading to erroneous annotation of their genomes. This branch of the NMT tree appears to be highly mobile and mixed between tiny primitive eukaryotes such as amoeba or microsporidia. Lateral gene transfer might involve large DNA viruses, as suggested for 185 genes including NMT [340]. In any case, if myristoylation does exist in Archaea, the actual role of myristoylation in relation to membrane targeting will need to be established in this kingdom. In this context, it is also interesting to note that Lnt homologs have been identified in metagenomes of several Euryarchaeta.

7.2.2. FAS/NMT links

Cytosolic FAS1 in eukaryotes might originate from the early engulfment and further endosymbiosis of a bacterial ancestor into a proto-eukaryotic cell, which led to mitochondria. Gene migration from the bacterium to the nucleus and further evolution, including gene fusions of ancestral FAS2, could have progressively led to the multifunctional FAS1a/b in current eukaryotes. Absence of FAS1 in plants, kinetoplastids, or diatoms might arise from early divergence between the various kingdoms or later exclusion due to the redundant function of FAS1 in the presence of efficient plastid FAS2. Nevertheless, the myristoylation mechanism in eukaryotes is very different to that in bacteria. This indicates the requirement for parallel adaptation of both the myristoylation catalyst and protein target substrates for this protein modification to work in eukaryotes. Whether this also occurs in Archaea or bacteria is unknown. NMTs are believed to be derived from acetyltransferases by gene duplication of a yet unidentified ancestor [43, 187].

7.3. Myristoylation and the reactivity of protein amino groups

The alpha amino terminal group of proteins ($N\alpha$) is the most frequent site for protein modifications, including myristoylation. It is also the most diverse, with 82 distinct modifications described so far from -89 Da to +738 Da [34]. This is not only because it is the earliest common reactive group emerging from the ribosomal tunnel during protein synthesis but also because N-termini are >80% solvent exposed [341] and because the neutral pK of the alpha amino group (7.7 ± 0.5 ; [342]) is close to intracellular physiological pH (7.2 ± 0.2). Facile enzyme-mediated deprotonation makes the amino group behave as a nucleophile and react with a variety of donors,

and various bond types may be made. The protonability and acidobasic character of the primary amine are lost upon amide bond creation by formylation, acetylation, or any fatty acylation. The pK of tertiary amines, as modified for instance by methylation, is higher than that of secondary amines, which in turn is higher than that of primary amines (so-called N α). Due to the low pK value, non-enzymatic N-acylation - including long chains such as myristoylation - must also be considered *in vivo* [40]. Finally, the lower pKa of the alpha amino groups than the epsilon of Lys residues (10.5 \pm 1.1; [342]) allows selective reactivity of this group *in vitro* with chemically-reactive compounds such as TMPP at pH 8.2 [343]. Enzyme-mediated N α modification may also involve sortase A, butelase, or subtiligase (for a review, see [341]). Such properties may be exploited to deal with myristoylation at the proteomic level. myristoylation remains a challenging modification.

In bacteria and in eukaryotic organelles, the remarkable reactivity of the N-terminal group needs to be revealed early through the immediate removal of the N-formyl group by peptide deformylases. N-formylation otherwise blocks the initiator methionine residue (Met1) [344]. Co- or post-translational proteolytic mechanisms contribute to unmask the penultimate residue 2 (N-terminal methionine excision) or larger signal/transit peptide cleavage (20-100 aa long) [345]. This generates newly reactive amino termini. In this review, we show that myristoylation arises from these many opportunities to tag protein N-termini both in bacteria and eukaryotes via the smallest natural amino acids, usually Gly but also Cys.

Compared to N-terminal myristoylation, the current data indicate that K-myristoylation is scarce, reversible due to de-myristoylation catalysts, and less efficient [44]. The associated catalysts need further characterization. Interestingly, both K-myristoylation enzymes reported to date, i.e., bacterial RTX and eukaryote NMTs, are members of the GNAT family, a large acyltransferase family including N-acetyltransferases [21, 346]. It is therefore expected that yet unknown K-myristoylation catalysts also belong to the GNAT family. Such catalysts, as for the K-myristoylation catalyst of TNF- α in humans (see Section 4.3), need to be identified (**Table 1**).

7.4. Peptide myristoylation in drug discovery

A number of therapeutic strategies take advantage of myristoylation as a means to improve delivery via improved uptake of small compounds including polypeptides into cells [347]. Myristoylation can improve stability and bioavailability by binding to albumin [348, 349].

Several myristoylated polypeptides have been now market approved. One remarkable example is bulevirtide (Hepcludex[®]), formerly MyrB or Myrcludex-B. The design of buleverdine started with the observation that hepatitis B virus (HBV) large surface protein L is essential for infectivity [350]. N-myristoylation of the L protein is conserved amongst Hepadnaviridae and is required for infectivity [350-352]. Infection entirely relies on the N-terminal pre-S1 domain of 77 residues, which is located at the external side of the virion [353]. Potent inhibition of HBV infection *in vitro* was initially observed with N-acylated pre-S1 domains of 47 residues starting with Gly2, the myristoylated residue [354, 355]. The effect was confirmed *in vivo* in monkey HBV infection, providing essential preclinical data for the subsequent clinical development of these

inhibitors [356]. Bulevirtide works by attaching to and blocking the HBV sodium taurocholate cotransporting polypeptide NTCP cellular receptor [357]. Bulevirtide was approved for clinical use in the European Union in July 2020 under the brand name Hepcludex® as an antiviral medication for the treatment of chronic hepatitis delta virus (HDV) infection [358].

Acylation strategies can take advantage of NMT-driven heterologous myristoylation in bacteria [359]. Further understanding the specific features of myristoylation enzymes including NMT and the discovery of new myristoylation catalysts will be important to help adding myristoylation independently of *in vitro* peptide synthesis, especially for the largest polypeptides for which *de novo* sequences are difficult to attain.

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COMPETING INTERESTS

The authors declare no competing interests.

Table 1: Myristoylomes and the associated catalysts in living Kingdoms

Kingdom ^a	Enzyme	Iso-forms	Mechanism ^b	C14 donor	Proteolytic cleavage (>1 aa)	Modified group in protein targets				Myristoylome ^d	Myristoylation stoichiometry on target ^e	Competing acyl chains	Cellular localization of acylated protein
						Gly N α	Lys N ϵ	Cys N α	Cys S				
P	Lgt	1-2	P	PE, PG, cardiolipin	N				X	60-200	0.1	C16>C18	Inner membrane, periplasm side
	Lnt	1-2	P		Y			X		60-200	0.1	C16>C18	Outer membrane
	RtxC	1	P	ACP	Y		X			1	0.9	C15, C16, C17	Secreted
E	NMT	1-3	C	MyrCoA	N ^c	X				50-935	0.9	C12>C16-C10	Internal membranes, cytosol side
			P		Y	X			5-100				
			P		N		X		1-15				
	DHHC	8-24	P		N				X	35-2339	0.1	C16>C18	Internal membranes
	KAT	?	P		?		X			4	0.9	?	Secreted
	Hhat	1	P		Y				X	1	0.1	C16>C18, C12, C10	Secreted

^a P, Prokaryotes; E, Eukaryotes

^b P, post-translational; C, co-translational

^c previous co-translational Met cleavage is mandatory to unmask the reactive N-terminus on Gly2

^d number varies from one organism to another; lower values in unicellular organisms compared to pluricellular eukaryotes

^e This ratio assesses roughly the mean stoichiometry of myristoylation with respect to all other acylations (i.e., C14/C2 \neq 14) on a given protein target, considering chain length preference of the catalyst(s) and FA availability in the cell; it may vary depending on organism, cell type, and nutrient supply including FA availability (see text)

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