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Review

Post-translational myristoylation: Fat matters in cellular life and death

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ABSTRACT

Myristoylation corresponds to the irreversible covalent linkage of the 14-carbon saturated fatty acid, myristic acid, to the N-terminal glycine of many eukaryotic and viral proteins. It is catalyzed by N-myristoyltransferase. Typically, the myristate moiety participates in protein subcellular localization by facilitating protein-membrane interactions as well as protein–protein interactions. Myristoylated proteins are crucial components of a wide variety of functions, which include many signalling pathways, oncogenesis or viral replication. Initially, myristoylation was described as a co-translational reaction that occurs after the removal of the initiator methionine residue. However, it is now well established that myristoylation can also occur post-translationally in apoptotic cells. Indeed, during apoptosis hundreds of proteins are cleaved by caspases and in many cases this cleavage exposes an N-terminal glycine within a cryptic myristoylation consensus sequence, which can be myristoylated. The principal objective of this review is to provide an overview on the implication of myristoylation in health and disease with a special emphasis on post-translational myristoylation. In addition, new advancements in the detection and identification of myristoylated proteins are also briefly reviewed.

1. Protein lipidation: an overview

Lipid modification of proteins plays many roles inside and outside of the cell including directing proteins to various cellular membranes, sub-membrane domains and promoting protein–protein interactions. Thus, protein lipidation adds another level of functionality to many proteins. Proteins can be co- or post-translationally modified with a wide variety of lipids. These covalent lipid modifications of proteins are characterized by the identity of the lipid moiety attached, the nature of the covalent bond, the attachment site of the lipid on the protein and the enzymes catalyzing the reactions involved. Four major types of lipid modification are known; cholesteroylation, prenylation, glypiation and fatty acylation [1]. In cholesteroylation, a cholesterol molecule is esterified to a C-terminal glycine residue in an autocatalytic process [2]. Prenylation involves the formation of a thioether bond between either of the farnesyl or geranylgeranyl isoprenoids and one or two C-terminally located cysteine residue(s) by a variety of prenyl transferases [3]. In glypiation, a glycosylphosphatidylinositol (GPI) anchor is added to the C-terminus of a protein by a transaminidase in the lumen of the endoplasmic reticulum [4]. Finally, fatty acylation mainly consists of the covalent addition of palmitic or myristic fatty acids to proteins. In S-acylation, saturated and unsaturated long chain fatty acids of various carbon chain lengths can be attached to cysteine residues of a protein by a variety of protein fatty acyl transferases (PATs) belonging either to the zDHHC-PATs family or the membrane bound O-acyl transferases (MBOATs) family [5,6]. Because palmitic acid is the most abundant fatty acid, S-acylation typically corresponds to the reversible attachment of the 16-carbon saturated fatty acid palmitate to a cysteine residue via a thioester bond and is thus typically referred to as palmitoylation [7]. In some cases, the palmitoyl moiety is linked to proteins via an amide bond on N-terminal cysteine or glycine residues [8,9]. Palmitoylation also occurs non-enzymatically (spontaneously) on several mitochondrial proteins [10,11]. In myristoylation, various saturated and unsaturated 14-carbon fatty acids are also found on the N-terminal glycine of proteins [12], but, typically, myristoylation consists of the

Abbreviations: ARF, ADP ribosylation factor; Bim, Bcl-2 interacting mediator; CAD, caspase activated DNAse; Caspase, cysteinyl-aspartyl protease; CD-IC2A, cytoplasmic dynein intermediate chain 2A; DED, death effector domain; DES, dihydroceramide delta4 desaturase; DISC, death inducing signalling complex; GCL, glutamate cysteine ligase; GFP, green fluorescence protein; GOAT, ghrelin-O-acyl-transferase; GPI, glycosylphosphatidylinositol; HIV, human immunodeficiency virus; HSC70, heat shock protein 71; LPS, lipopolysaccharide; MARCKS, myristoylated alanine-rich C kinase substrate; MBOAT, membrane bound O-acyl transferase; NIP71, NMT inhibitor protein 71; NMT, N-myristoyltransferase; PAK2, p21-activated kinase 2; PAT, protein fatty acyl transferase; PKCε, protein kinase C ε; ROS, reactive oxygen species; TRAM, TRIF-related adaptor molecule; VDAC, voltage-dependent anion channel.

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covalent addition of the 14-carbon saturated fatty acid myristate to the N-terminal glycine residue through a stable amide bond [13,14]. The reaction is catalyzed by myristoyl-CoA:protein N-myristoyltransferase (NMT) [15]. In some rare cases of myristoylation, including the insulin receptor [16] and interleukin 1 α and β [17], myristic acid is also attached to an internal lysine via an amide bond. Alternative forms of fatty acylation have also been reported. The appetite-stimulating peptide hormone ghrelin is known to be acylated on serine 3 with octanoate [18], a reaction catalyzed by Ghrelin-O-Acyltransferase (GOAT), a member of the MBOAT family [19]. GOAT appears to be regulated by dietary lipids [20]. Since octanoylation is essential for ghrelin's physiological activity [21], inhibitors of GOAT could represent attractive new appetite suppressants helpful in the treatment of the metabolic syndrome. Furthermore, the signalling protein Wnt is not only known to be fatty acylated with palmitic acid on Cys77 [22], but also modified with unsaturated palmitoleic acid on a highly conserved Ser209. The acylation at both sites is catalyzed by the MBOAT Porcupine [23]. Recently, Aquaporin 0 was demonstrated to be acylated with oleic acid on a lysine residue via an amide bond, which may contribute to target the protein to lipid rafts [24].

The objective of this review is to provide insights on the roles of myristoylation in health and disease states with a special emphasis on post-translational myristoylation in cell death.

2. Myristoylation: a matter of fat

Historically, N-myristoylation was initially described as an unusual blocking group, which prevented the direct use of Edman degradation at the N-terminus of the catalytic subunit of cyclic AMP-dependent protein kinase [25] and calcineurin B [26]. It was later determined by mass spectrometry that the blocking group was myristic acid. N-myristoylation was previously described as a co-translational modification [27,28] that occurs on the nascent polypeptide following the removal of the initiator methionine residue (Fig. 1A). Now it is well established that myristoylation can also occur post-translationally on an internal glycine within a cryptic myristoylation consensus sequence exposed by the action of caspases in apoptotic cells [29–34] (discussed in detail in the following sections below) (Fig. 1B). Interestingly, removal of the initiator methionine is a common modification of proteins, occurring in up to 80% of total proteins. The reaction is catalyzed by methionine aminopeptidase [35] and is even more efficient when the amino acid residue following the initiator methionine is a glycine residue [36], yet only selected proteins are myristoylated due to the selectivity of NMTs. Similarly, analysis of the caspase-cleaved proteome revealed that there is a high propensity to have a glycine residue after the various caspase-cleavage sites [37,38], suggesting that there might be numerous cases of post-translational myristoylation yet to be uncovered.

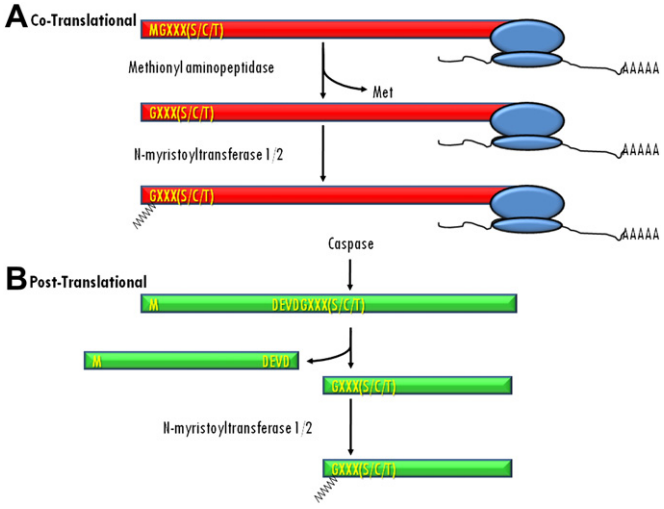

Fig. 1. Co- and post-translational attachment of myristate to proteins. Myristoylation involves the covalent addition of the saturated 14-carbon fatty acid myristate to the N-terminal glycine residues of (A) a nascent polypeptide following removal of the initiator Met (co-translational myristoylation) or (B) a cryptic myristoylation site following exposure by caspase cleavage (post-translational myristoylation).

2.1. How myristoylation affects proteins

Many myristoylated proteins play key roles in cellular signalling pathways [e.g. non-receptor protein tyrosine kinases, heterotrimeric G_{α} proteins, MARCKS and calcium binding proteins (e.g. Recoverin, Neurocalcin) reviewed in [6]]. For many of these proteins, the myristoyl moiety has been shown to mediate subcellular targeting, protein–protein and protein-membrane interactions required for the activities of these proteins [39]. Although myristoylation is required for membrane binding, it is not sufficient and must be augmented by downstream interactions in order to provide stable membrane anchoring [40]. This is usually achieved by an adjacent or distant polybasic domain, by one or two nearby palmitoylated cysteine residues or by a protein–lipid binding domain [41]. Myristoylation can also participate in differential targeting to membranes and sub-membrane domains known as lipid rafts [42,43].

Although myristate is irreversibly bound to proteins it allows the acylated protein to reversibly sample membranes because of its weak hydrophobic nature. Indeed, the half-life of a membrane bound myristoylated peptide is in the order of minutes in comparison to hours for palmitoylated or dually acylated (myristoylated and palmitoylated) peptides [44]. Again, although myristoylation is an irreversible modification, the anchorage of myristoylated proteins to membranes can also be dynamically regulated via various "myristoyl switches". One of these switches relies on a "ligand"-dependent conformational change of the protein leading to exposure of the myristoyl moiety previously sequestered in a hydrophobic pocket to the cytosol [45]. This concept is exemplified by the binding of GTP to the ADP ribosylation factor (ARF) proteins or calcium to recoverin that allow the subsequent exposure of the myristoyl moiety and targeting of these proteins to membranes [46,47]. Another variation of this mode of regulation is referred to as the myristoyl electrostatic switch. For instance, protein kinase C dependant phosphorylation of serine residues of the polybasic domain of MARCKS protein releases the protein from the membrane by reducing the positive charge of the polybasic domain that contributes to the membrane binding [48]. A variation on the above, linking acylation and phosphorylation, is exemplified by c-Abl, which is known to be regulated by a "myristoyl/phosphotyrosine" switch [49]. In that case, the myristoyl moiety binds to a hydrophobic pocket and induces a conformational modification of the protein allowing the docking of the SH2 and SH3 domain onto the kinase domain and auto-inhibition of the enzyme [50]. Interestingly, a similar pocket has recently been predicted to exist in the c-Src kinase, but in that case, myristoylation acted in a different manner and enhanced c-Src activity [51]. This suggests that myristoylation of many other non-receptor tyrosine kinases (e.g. Blk, Lyn, Fyn, Fgr) might not only be required for the regulation of proper localization but also of their activity.

2.2. N-myristoyltransferases

In vertebrates, N-myristoylation is catalyzed by two NMTs, NMT1 and NMT2, which are members of the GCN5 acetyltransferase

superfamily [52]. NMTs have been purified and cloned from many organisms [53–57]. To date, an NCBI database search for NMT annotated entries revealed the presence of the gene encoding for NMT in 53 organisms from taxa spanning animals, plants, apicomplexan parasites (e.g. *Plasmodium falciparum*), fungi and yeast. Of these 53 organisms, 16 contained both NMT1 and NMT2. The NMT orthologues from *Saccharomyces cerevisiae* is the most extensively characterized and has provided the majority of information regarding the enzymology of the reaction [58,59]. NMTs perform their catalysis via an ordered Bi Bi reaction mechanism with myristoyl-CoA binding first, peptide binding second followed by a direct nucleophilic addition–elimination reaction and the sequential release of CoA and the myristoyl-peptide [60]. The two genes encoding for the two isoforms of NMT were identified in humans and share about 76% amino acid sequence identity. The two NMTs have unique as well as overlapping substrate specificities [56,61]. In organisms where there is only one isoform of NMT, it has been shown to be essential for survival (e.g. *S. cerevisiae* [53] and *Drosophila* [62]). NMT1$^{-/-}$ mice also die during embryogenesis suggesting that NMT2 is not able to rescue N-myristoylation of proteins for the proper development of the mice embryos. This clearly indicates that both NMTs must have their own subsets of unique substrates and that NMT1, or some proteins perhaps strictly myristoylated by NMT1, play some crucial roles in development [61].

NMTs are highly selective for the substrate myristoyl-CoA in *in vitro* enzymatic assays [63,64]. This selectivity was in part explained by looking at the 3D structure of NMT, which revealed a bent active site cavity only large enough to optimally accommodate a myristoyl moiety [65]. However, different *in vitro* studies have shown that NMT can catalyze the linkage of a synthetic peptide that mimics the N-terminal region of a protein to different fatty acids, such as lauric, tridecanoic, pentadecanoic and palmitic acid [66,67]. In the retina, several proteins are heterogeneously myristoylated with the unsaturated fatty acids C14:1 n-9 and C14:2 n-6 (reviewed in [12]), but no retinal isoform specific of NMT able to use various fatty acyl-CoA substrates has been identified so far [68].

Cellular concentrations of myristoyl-CoA available for N-myristoylation are very low; approximately 5 nM in animal cells [69]. In rat hepatocytes, only 0.05% of radiolabeled myristic acid added to the cells is dedicated to the N-myristoylation of proteins [70]. Because NMT can use palmitic acid as a substrate *in vitro*, albeit at a reduced efficiency compared to myristate [67,71,72], and the fact that cellular palmitic acid concentrations are significantly higher than myristic acid suggest that the access of cytosolic NMTs to a specific pool of myristoyl-CoA is of critical importance [39]. For instance, it has recently been suggested that myristic acid captured via the CD36 receptor may be specifically dedicated to the myristoylation of tyrosine kinase pp59^{fyn} [73].

The general consensus peptide sequence recognized by NMTs is Gly-X_3-X_4-X_5-(Ser/Thr/Cys)$_6$ where X represents most amino acids, except for proline, aromatic or charged residues in position X_3. The amino-terminal Gly residue is absolutely required for myristoylation to occur and substitution of this residue to any other abrogates myristoylation. While Ser, Thr, Cys are preferred at position X_6, other amino acids can be tolerated at this position (e.g. Ala found in Annexin XIII and Gly found in c-Abl tyrosine kinase) [74,75]. Moreover, the combination of amino acids at position X_3, X_6 and X_7 play important roles for the N-myristoylation of the candidate protein [76]. For instance, a Lys residue at position 7 reduces the stringency for certain amino acids at position 3 and is also important in conferring membrane binding properties [77]. These studies and others have formed the basis for the development of myristoylation prediction models [78–81]. Using computer analysis, the 17 N-terminal residues following the initial methionine were demonstrated to be required for the prediction of the myristoylation status of the protein [82]. Using these computer algorithms, it is estimated that 0.5–3% of the mammalian and plant proteomes consist of myristoylated proteins [78–81].

2.3. Regulation of myristoylation in health and disease

Little is known about the regulation of NMT activity and it is presumed that levels of NMT activity are necessary and sufficient to efficiently acylate endogenous proteins, but it was also suggested that NMT activity might actually be limited by the scarce levels of myristoyl-CoA itself [83,84]. In that context, the fact that NMT enzyme levels are elevated in several types of tumours is puzzling [85].

It has also been suggested that NMT has endogenous inhibitors in the cell. An NMT inhibitor named NMT inhibitor protein 71 (NIP71) was isolated from bovine brain using an *in vitro* NMT assay [86]. NIP71 shares 43% identity with the heat shock cognate protein 70 (HSC70). In addition, the same group also demonstrated that the glycolytic enzyme enolase is able to inhibit N-myristoylation *in vitro* [87]. Whether these inhibitors play a significant role in cells was not investigated.

2.3.1. Crosstalk between myristoylation and phosphorylation

Phosphorylation of hNMT1 was also shown to regulate the activity of the enzyme. A Y100F mutation in NMT1 resulted in a 98% decrease of NMT activity [88]. However, the extent and the site(s) of phosphorylation were not fully characterized since this mutant was still partially phosphorylated (17% as compared to wild type). Interestingly, NMT is phosphorylated by Src family tyrosine kinase members of Lyn and Fyn [88] that are, in turn, myristoylated [85]. These results underline a possible complex regulation of the phosphorylated NMT by myristoylated tyrosine kinases. It is well established that those myristoylated tyrosine kinases (pp60^{src}, pp60^{yes}, pp56^{lck}, pp59$^{fyn/syn}$ and c-Abl) [89] display elevated activity in many human cancers [90]. In addition, increased protein and mRNA levels of pp60^{src} were shown in human colon carcinoma, and many human cancer cell lines such as HT29, COLO 201 and COLO 205 [91,92]. These data suggested that the regulation of pp60^{src} happens at the level of transcription with two distinct promoters (SRC1A and SRC1α) [93]. Inhibition of pp60^{src} myristoylation decreased colony formation, cell proliferation and targeting to the membrane demonstrating the importance of pp60^{src} myristoylation for tumorigenicity [94]. Moreover, myristoylation seems to be critical for the dephosphorylation of pp60^{src} and its subsequent kinase activity [95]. Myristoylation increases pp60^{src} kinase activity but also regulates the stability of the protein. Recently, it was shown that myristoylation-dependant membrane targeting of pp60^{src} regulates the degradation and ubiquitination of the protein [51] and the myristoyl moiety was recently proposed to possibly reside in a hydrophobic pocket like it does in c-Abl [49], but would stimulate rather than inhibit kinase activity [51].

Also linking phosphorylation and myristoylation is the fact that the N-terminal domain of hNMT1 interacts with the Lyn tyrosine kinase in a phosphorylation dependant manner [85]. Because, NMT1 and NMT2 have their major amino acid sequence differences at their N-termini [56], this suggests that differential phosphorylation might allow for the fine tuning of the two NMT activities inside the cell. Interestingly, although the N-terminal region is not required for the activity of NMTs and because it exhibits significant sequence differences between the two enzymes, it has been postulated that the N-terminus allows the differential targeting of NMTs to various cellular compartments, as well as provide access to

cellular myristoyl-CoA pools [96] and the differential binding to ribosomes [14].

2.3.2. Myristoylation and cancer

Magnuson et al. [97] were the first to demonstrate that NMT activity was increased in rat colonic tumours, human colorectal tumours, adenocarcinomas [98] and stage B1 tumours, suggesting that NMT is important in the early stages of colonic carcinogenesis [97]. This increase is correlated with higher expression levels of NMT [98], and particularly the second isoform NMT2 [99]. Increased expression of NMT was also found in gallbladder cancer [100], breast cancer [101] and brain tumours [102]. It is easy to speculate that the overexpression of the myristoylated oncogenic kinases may require increased NMT activity for their proper acylation and, ultimately, function.

2.3.3. Spurious myristoylation in disease

Recently, spurious myristoylation of the SHOC2 protein containing a S2G mutation was documented and corresponds to a disease phenotype. Cordeddu et al. [103] identified an aberrant myristoylation of the SHOC2 protein in the development of the Noonan-like syndrome with loose anagen hair in twenty-five patients. This syndrome is a rare developmental disorder resulting in reduced growth, facial dysmorphism, cognitive deficits and malignancies [104,105]. The authors found that the $SHOC2^{S2G}$ mutant incorporated [^{3}H]-myristic acid and was targeted to the cell membrane instead of the nucleus leading to increased activation of Ras and the MAPK pathway. This is the first demonstration of the direct involvement of myristoylation in the development of a human disease and the first example of a spontaneous genetic mutation leading to aberrant myristoylation.

2.3.4. Myristoylation in infectious diseases

The covalent attachment of myristate to the N-terminal glycine residue occurs on many mammalian [78] in addition to viral and even secreted bacterial [106] proteins, and, is essential to these proteins' ultimate function [13,14]. Therefore, NMTs represent attractive therapeutical drug targets. The NMT protein substrate specificity has been well characterized in *S. cerevisiae* using synthetic peptides corresponding to the N-terminus of protein [15,107,108]. Using this system for mammalian NMT1, the first ten amino acids were shown to be necessary for effective myristoyltransferase activity. Interestingly, human and yeast NMTs have different yet overlapping substrate specificities [109]. The differences were significant enough to allow for the development of selective inhibitors of yNMT [110] paving the way to the design of NMT inhibitors against pathogenic yeasts and fungi such as *Candida albicans*, *Cryptococcus neoformans* and *Histoplasma capsulatum* [54,111]. The fact that the NMT activities appear to be critical for the virulence of *C. albicans* and *C. neoformans* in an immunosuppressed model, again highlights the potential of NMT as a useful therapeutic target. Of note, several parasitic protozoa such as *Leishmania major* and *Leishmania donovani* (leishmaniasis desease), *Trypanosoma brucei* (African sleeping sickness disease) and *P. falciparum* (malaria) also possess their own NMT that appears to be essential for their survival [112–115]. Investigations are now focusing on the validation of inhibitors of protozoan NMTs. Different strategies have been used towards the development of these inhibitors and were based on the use of either peptidomimetics able to bind to the NMT active sites or small molecule inhibitors of NMTs. Recently, a pyrazole sulphonamide specific inhibitor of the *T. brucei* NMT has been identified [116]. Its selectivity over human NMT is 100-fold and a tight correlation between NMT activity and *T. brucei* proliferation is observed suggesting that NMT activity is the target of this compound. Pyrazole sulphonamide competes with the peptide binding site of NMT. This compound rapidly kills *T. brucei* and eliminates the bloodstream form of *T. brucei*. Moreover, this compound not only works *in vitro*, but also cured all animals in a mouse model of human African trypanosomiasis. Therefore, it shows promising therapeutic properties to treat sleeping sickness.

Of note, myristoylation is required for assembly and replication of viruses such as lentiviruses (e.g. HIV) or picornaviridae [106]. In the case of HIV replication, both the structural proteins Gag and Nef are myristoylated by the host cell NMT. The Gag protein consists of a polyprotein precursor containing several domains, including a matrix domain, which is myristoylated and directs the protein to the plasma membrane in combination with its polybasic domain [117]. Subsequently, this anchoring is critical for the downstream cleavage of the polyprotein and the release of the mature myristoylated matrix domain to the cytosol [118]. These crucial steps are required for the formation of the viral capsid, as inhibition of myristoylation causes the accumulation of the Gag protein precursor in the cytosol and blocks formation of a competent viral progeny [119]. Recent studies suggest that NMT1 and NMT2 display different affinity for Gag and Nef, allowing the possible development of selective inhibitors [120,121]. In addition, the competition for the myristoyl-CoA substrate pool appeared to be critical in this pathophysiological situation. It is estimated that the concentration of myristoyl-CoA necessary for the maturation of viral particles is about 1 μM [13], while the endogenous cellular concentration is about 5 nM [69]. This suggests that the production of myristoyl-CoA might be a rate-limiting step and that the size of the myristoyl-CoA pool might be regulated during viral infection [122].

Certain types of bacteria are known to inject proteins (via type III secretion) containing a myristoylatable N-terminal glycine residue directly into the cytoplasm of the host cell. The injected protein is then myristoylated by the host NMT and relocalizes to the plasma membrane. Myristoylation of the protein is essential for maximum virulence [106,123]. This type of myristoylation of type III secreted bacterial proteins was demonstrated in *Pseudomonas syringae* [123] and predicted for *Shigella sonnei* [124]. Drugs targeting eukaryotic acylation of bacterial proteins may represent an interesting strategy to fight plant and animal infections.

Finally, the examples listed above highlight the importance of understanding and characterizing myristoylation under normal and pathological conditions. For instance, because apoptosis ultimately results in caspase activation and is often deregulated in cancer, the importance of characterizing post-translational myristoylation of proteins cleaved by caspases during apoptosis is undeniable, may be critical to understand the development and progression of cancer and will be discussed in the next section.

3. Post-translational myristoylation: different means to an end

3.1. Post-translational myristoylation during apoptosis

Until about 10 years ago, myristoylation was thought to be exclusively a co-translational process, wherein myristate is added to the amino-terminal glycine via an amide bond following the removal of the initiator methionine from the nascent polypeptide by a methionyl aminopeptidase (Fig. 1A) [13,14]. However, the pro-apoptotic protein Bid was shown to be post-translationally myristoylated on an internal glycine residue exposed after caspase cleavage during apoptosis [33]. Since then, we and others have identified several proteins post-translationally myristoylated during apoptosis and now it is well established that myristoylation can also occur post-translationally upon caspase cleavage and exposure of an internal glycine within a cryptic myristoylation consensus sequence (Fig. 1B) [30–32,34].

Apoptosis, or programmed cell death, is critical for the maintenance of organisms and tissue homeostasis and dysregulation of this process can have detrimental consequences [125,126]. For instance, unwanted upregulation of apoptosis can lead to tissue and organ damage (e.g. brain in Alzheimer's disease) while downregulation of apoptosis can promote a cancerous state [127–129]. Apoptosis is physically characterized by cell shrinkage, condensation of DNA and membrane blebbing [125,126]. During the onset of apoptosis, hundreds of proteins are specifically cleaved by cysteinyl-aspartyl proteases (caspases) [130]. Caspase cleavage leads to the activation of pro-apoptotic regulators or the inactivation of many proteins including anti-apoptotic regulators and, in many cases, can lead to changes in localization of the cleaved proteins [130,131]. Apoptosis can be triggered through two main pathways that are intricately linked and referred to as the intrinsic and extrinsic pathways (Fig. 2). The intrinsic pathway is activated through cell stresses such as DNA damage, hypoxia and growth factor deprivation, and is mediated through the mitochondria [132]. In contrast, extrinsically activated apoptosis is receptor mediated. Typically, binding of a death ligand to its receptor (such as Fas ligand to the Fas Receptor in Fig. 2) leads to trimerization of the receptor and recruitment of an adaptor protein to its intracellular domain [133]. The adaptor protein, in turn, recruits inactive pro-caspase-8, an initiator caspase. This complex leads to the cleavage and activation of caspase-8 and formation of the death inducing signalling complex (DISC). Subsequently, active caspase-8 will cleave and activate the executioner caspase, caspase-3. Another important substrate of the DISC is the BH3-interacting-domain death agonist (Bid) [132,133].

The cleavage of 21 kDa Bid by caspase-8 results in 7 kDa and 15 kDa N-terminal and C-terminal fragments, respectively. As a result of the cleavage, an internal myristoylation motif becomes exposed at the newly exposed amino terminus of the C-terminal fragment (ctBid) allowing it to be post-translationally myristoylated by NMT [33,134]. Because the N-terminal fragment of Bid remains associated with the ctBid, myristoylation of ctBid results in the translocation of the complex to mitochondria, promotes the release of cytochrome c, and, ultimately, cell death [33]. When the glycine residue essential for myristoylation was substituted to an alanine (G60A) in ctBid, mitochondrial association of mutant ctBid was drastically reduced to about 30% of wild type levels. Myr-ctBid was also shown to have a higher affinity for mitochondrial membranes and was approximately 350 times more effective than non-myristoylated G60A-ctBid at promoting the maximal release of cytochrome c from purified mitochondria.

Cytosolic cytochrome c is important for recruiting and oligomerizing with apaf-1 and another executioner caspase, caspase-9, to form the apoptosome, which, in turn, can also activate caspase-3. As an executioner caspase, caspase-3 has many important substrates, such as the inhibitor of caspase activated DNAse (ICAD) [135]. Cleavage of ICAD releases it from CAD, leading to DNA fragmentation, a key hallmark of apoptosis [136]. Therefore, the cleavage and subsequent myristoylation of ctBid plays a crucial role in linking the extrinsic and intrinsic cell death pathways.

Additional substrates of caspase-3 include three proteins that were found to be post-translationally myristoylated in separate studies; actin, gelsolin [31,32] and p21-activated kinase 2 (PAK2) [34]. First, Utsumi et al. [32] appended the first nine amino acids immediately following the caspase cleavage sites of six known substrates that exposed a new N-terminal Gly to the TNFα protein, which was used as a reporter protein. COS-1 cells transiently expressing the TNFα chimeras were metabolically labelled with [^{3}H]-myristate. Incorporation of the radiolabel into both ctActin- and ctGelsolin-TNFα was detected by autoradiography.

Actin is one of the key structural components of the cytoskeleton and plays a crucial role in cell structure, motility, division and organelle movement. Actin can be found in two main forms; globular (G-actin), or monomeric, and filamentous (F-actin), or polymerised [137]. Gelsolin is an actin filament-severing protein that regulates actin assembly and disassembly [138]. Interestingly,

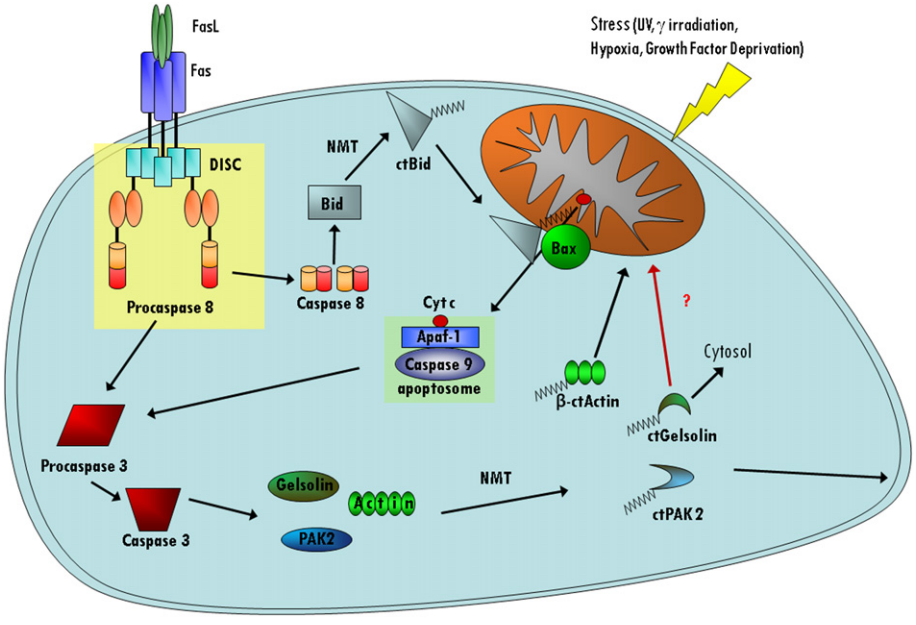

Fig. 2. Post-translational myristoylation of proteins during apoptosis. Activation of the extrinsic pathway begins with binding of a death ligand [e.g. Fas ligand (FasL)] to its corresponding death receptor (e.g. Fas). Subsequent binding of adaptor proteins leads to the formation of the death inducing signalling complex (DISC) and activation of caspase-8. Caspase-8 cleaves the pro-apoptotic protein BID, which is then post-translationally myristoylated by NMT at an N-terminally exposed glycine of the C-terminal fragment, which is essential for ctBid's translocation to the mitochondria and progression of apoptosis by the release of cytochrome c. β-Actin, Gelsolin and p21-activated kinase 2 (PAK2) are all cleaved by caspase-3 to yield caspase-truncated (ct) products: ctActin, ctGelsolin and ctPAK2, which are subsequently post-translationally myristoylated. The post-translationally myristoylated caspase-truncated products translocate to their new respective membrane locales to affect apoptosis.

myr-ctActin–FLAG co-localized with mitochondria, like myr-ctBID, but it was not found to have any effect on cellular morphology, actin networks, nor was any effect on apoptosis established [31,32].

In a follow up study by the same authors, myr-ctGelsolin–HA was shown to be primarily cytosolic. Indeed, cell fractionation studies showed ctGelsolin–HA predominantly in the cytosolic fraction regardless if it was myristoylated or not. In addition, myr-ctGelsolin was also concluded to be cytosolic based on indirect immunofluorescence data [31]. Regardless, cells expressing myr-ctGelsolin had an increased protection from apoptosis induced by the topoisomerase inhibitor etoposide, when compared to non-myr-ctGelsolin or vector alone. Once again, this demonstrates the importance of post-translational myristoylation in regulating the function of caspase-cleaved proteins during apoptosis. Interestingly, previous studies have shown that both the full-length form and ctGelsolin were anti-apoptotic through a mechanism that is thought to involve the voltage-dependent anion channel (VDAC) at the mitochondrial membrane [139,140]. In that context, it would be interesting to compare the potency of full-length versus caspase-cleaved myristoylated gelsolin in order to assess whether myristoylation augments the ability of gelsolin to suppress apoptosis. Interestingly, ctBid is also thought to promote apoptosis by associating with VDAC and promoting an open pore conformation [141]. Although it should be noted that in that study, post-translational myristoylation was blocked by the presence of an N-terminal epitope tag on ctBid. It may be of interest to determine whether myr-ctActin, which translocates to the mitochondria, also interacts with the VDAC. Of note, it has been suggested that destabilization of the interaction between cardiolipin and the mitochondrial permeability transition pore, of which VDAC is a main component, promotes an open conformation of the pore during apoptosis to allow the release of cytochrome c [142]. In the absence of cardiolipin, Bid does not localize to the mitochondria [142]. These results suggest that post-translationally myristoylated proteins may be directed to VDAC in a cardiolipin dependent manner to regulate the open/closed formation of the pore complex.

In addition to ctBid, ctActin and ctGelsolin, our laboratory has identified another post-translationally myristoylated protein: PAK2 [34]. PAK2 is a Ser/Thr kinase whose activity is regulated by Rac and Cdc42 [143]. In dividing cells, PAK2 activity stimulates cell growth, cell motility and cell survival [144]. In apoptotic cells, PAK2 is cleaved by caspase-3 resulting in the release of the highly pro-apoptotic constitutively active C-terminal kinase domain from the N-terminal regulatory domain [145]. We have shown that the newly exposed N-terminal glycine of the caspase-cleaved PAK2 kinase domain (ctPAK2) is post-translationally myristoylated [34]. It was observed that myr-ctPAK2 was a more potent activator of apoptosis than its non-myristoylated counterpart. Indeed, after 12 h of transient transfection of ctPAK2-Myc, more than 50% of myr-ctPAK2-Myc expressing cells were apoptotic compared to only 23% in both the vector control and G213A-ctPAK2-Myc transfected cells. While WTctPAK-Myc induced 50% of cell death within 12 h, the non-myristoylated G2A mutant reached a maximal cell death of 50% after 24 h. In addition, a concomitant 5-fold and 3.5-fold increase in phosphorylation of the stress-activated signalling kinase JNK was observed in cells expressing the myristoylated- and non-myristoylated ctPAK2 respectively, over vector alone. Conversely, the myristoylated form of ctPAK2 was found to be hypophosphorylated compared to its non-myristoylated counterpart suggesting that loss of myristoylation lead to a dysregulation of PAK2 phosphorylation. In addition, myr-ctPAK2 was found to localize to plasma membrane ruffles and early endosomes while the non-myristoylated ctPAK2 remained primarily cytosolic, thus suggesting once more that myristoylation leads to proper localization and enhanced biological activity. Interestingly, and excitingly, cellular death induced by expression of myr-ctPAK2 bypassed several key hallmarks of apoptosis. Indeed, cells undergoing myr-ctPAK2 mediated cell death did not release cytochrome c from the mitochondria nor lose mitochondrial potential. In addition, these cells did not expose phosphatidylserine at the cell surface during the cell death process. These observations suggest that myr-ctPAK2 mediates its actions downstream of the mitochondrial "step".

Because post-translational myristoylation potentiates the pro-apoptotic activity of ctBid and ctPAK2, post-translational myristoylation of caspase-cleaved proteins could represent an elegant means developed by the cell to expand the functionality of its encoded proteins.

3.2. Potential roles for co-translational myristoylation and myristate in the regulation of apoptosis

Myristoylation may also play an important role in the metabolism of sphingolipids and ceramides, two types of molecules known to be involved in the regulation of apoptosis. For instance, several proteins involved in these pathways contain N-terminal glycines that may be myristoylated, such as ceramide kinase [146] and neutral sphingomyelinase [147]. In fact, dihydroceramide delta4 desaturase (DES) has been shown to be myristoylated and its activity is even upregulated when "free" myristate is added to cells [148,149]. Myristoylation of DES targets the enzyme to mitochondria where sphingolipid metabolism may be altered [150]. Furthermore, this acylation induces ceramide production, cytochrome c release from mitochondria and, consequently, apoptosis in COS-7 cells. It is well known that ceramides induce apoptosis by targeting mitochondrial metabolism via the formation of channels in the outer mitochondrial membrane and release of cytochrome c [151]. Ceramides also interact with key components of the electron transport chain [152] and induce the production of ROS species [153] leading to the induction of apoptosis. We can also hypothesize that changes in myristate concentration may alter the outer mitochondrial membrane composition in phospholipids and cardiolipins by structurally altering their fatty acid constituents and, therefore, change the mitochondrial membrane permeability.

Overall, it thus appears both co- and post-translational myristoylated proteins play important roles in the regulation of mitochondrial integrity and the generation of pro-death or pro-survival signals. Altogether these are key components of the cell's ability to make life and death decisions.

4. Detection of myristoylation: click chemistry and the solution to long film exposures

4.1. Detection using radioactive fatty acids

The key roles played by co- and post-translationally myristoylated proteins in the generation of pro-survival or pro-death signals warrant the identification of the co- and post-translational myristoylated protein proteomes or "myristoylomes". Indeed, in addition to the previously identified post-translationally myristoylated proteins, we have observed the presence of nine other post-translationally myristoylated proteins using metabolic labeling of apoptotic Jurkat cell lysates with [^{3}H]-myristic acid [30]. However, the use of radioactive [^{3}H]-myristic acid has a low sensitivity, is very time consuming, expensive and represents a health hazard. Overall, progress in the identification and characterization of myristoylated proteins has been impeded by the long exposure times required to monitor incorporation of radioactive myristate into proteins (typically 1–3 months). Still using [^{3}H]-myristic acid as a label, the Utsumi laboratory recently developed cell free assays that utilize rabbit reticulocyte lysates or insect cell lysates for the synthesis and labeling of potentially myristoylated proteins [154–156]. In their

paper, using these cell free systems myristoylation detection was reduced to days/weeks film exposure using fluorography. This allowed for the identification of 27 myristoylated proteins, of which 18 were new myristoylated proteins [155]. Despite identifying a number of novel myristoylated proteins, this approach was rather time consuming and still relied on metabolic labeling techniques linked to rather lengthy fluorographic film exposures and an exhaustive mass spectrometric/proteomic approach.

To alleviate the lengthy fluorographic exposures inherent to the use of [^{3}H]-myristic acid to label cells, an [^{125}I]-iodomyristate has also been synthesized and used to reduce exposure time to days or less, but this requires the handling of large quantities (mCi) of the hazardous high energy ^{125}I radioisotope [157,158]. Unfortunately, this compound is not commercially available and must be generated in the laboratory [158].

4.2. Chemical biology and click chemistry

In order to circumvent this and to bypass the use of radioactivity, we and other laboratories have developed a number of non-radioactive techniques for the labeling of myristoylated proteins taking advantage of bio-orthogonal analogs of fatty acids that can be incorporated into proteins and selectively reacted with probes facilitating their detection [29,30,159–164]. These new techniques could ultimately be used for affinity purification and identification of co- and post-translationally myristoylated proteins. Initially, the very specific reaction between an alkyl-azide and a phosphine using the Staudinger ligation (Fig. 3A) was exploited to detect various fatty acylated proteins [30,159]. In this method, the isosteric and bio-orthogonal analog of myristate, azido-myristate (12 carbon with an ω-azido group; Az-C12), can be specifically incorporated co- or post-translationally into proteins at the N-terminal glycine residues of various proteins. Subsequently, the azido moiety was chemoselectively ligated to triarylphosphines bearing FLAG or biotin and detected by Western blotting with short exposure times to film [30,159]. For instance, detection of azido-myristoylated ctPAK2 in apoptotic Jurkat cell lysates was reduced from 2 months using [^{3}H]-myristic acid to 1 s after ligation and detection of the biotin-phosphine probe [30]. This represents over a million-fold signal amplification in comparison to using radioactive labeling methods. This novel use of a myristate analogue that can be chemically ligated to probes was shown to be specific for N-terminal glycine residues of several proteins [30,159] and to rely on N-myristoyltransferases for their incorporation [30].

In order to discover new post-translationally myristoylated proteins, we identified 48 protein candidates, from a review listing 280 caspase substrates that exposed an N-terminal glycine after their caspase cleavage sites [131]. The 48 amino acid sequences downstream of the cleavage sites were subjected to myristoylation prediction analysis using Myr Predictor and Myristoylator and 9 proteins were predicted to be myristoylatable [165,166]. Following an initiator methionine, the first ten amino acids of the C-terminal fragment of these 9 potential substrates were appended to the N-terminus of EGFP. Using metabolic labeling of COS-7 cells transiently expressing the chimeric GFPs with the Az-C12 analog, the incorporation of the Az-C12 into proteins was monitored by chemical labeling with a biotinylated triarylphosphine, as described in [30,159]. Doing so, we identified 5 new post-translationally myristoylatable proteins [Protein Kinase C ε (PKCε), cytoplasmic dynein intermediate chain 2A (CD-IC2A), the pro-apoptotic protein Bap31, mammalian Ste20-like kinase (MST3), and the catalytic subunit of glutamate cysteine ligase (GCLC)] [30]. Because myristoylation is typically necessary and sufficient to exclude GFP from the nucleus [42], we used this exclusion criteria to assess whether our chimeric EGFPs were *bona fide* myristoylated proteins. We demonstrated that chimeric EGFP bearing N-terminal sequences of caspase-cleaved fragments of PAK2, PKCε and CD-IC2A were extensively excluded from the nucleus while chimeras of Bap31, MST3 and GCLC led to little to no nuclear exclusion of EGFP. Therefore, PAK2, PKCε and CD-IC2A chimeric EGFPs were assessed as strong candidates for myristoylation based on our criteria while the Bap31, MST3 and GCLC chimeric EGFPs may not be efficiently myristoylated nor strong candidates for myristoylation. Critically, in our approach we originally used an expression system relying on co-translational myristoylation to investigate and assess the post-translational myristoylation potential of caspase-cleaved protein sequences. Some of the observed discrepancies may thus be attributed, in part, to the substrate specificity of methionine aminopeptidase and its ability to remove the initiator methionine in these artificial substrates. Alternatively, post-translational myristoylation may rely selectively on one of the two NMT isoforms. Since NMT1 associates with ribosomes [14] and thus may play a more predominant role in co-translational myristoylation, NMT2 might be the key NMT required for post-translational myristoylation. Furthermore, immunoprecipitation studies have shown that NMT2 interacts with caspase-3 [85]. This finding may support the idea that NMT2 may play a greater role in post-translational myristoylation during apoptosis than NMT1. Therefore, the myristoylation of a protein normally post-translationally myristoylated by NMT2 might be difficult to assess using a co-translational expression system possibly relying preferentially on NMT1.

Subsequently, we and others developed a chemical ligation technique that relies on the Cu (I) − catalyzed [3 + 2] Huisgen cycloaddition reaction (commonly referred to as Click chemistry) that uses an ω-alkynyl-myristate analog (Alk-C14) and various azido-probes (Fig. 3B) [29,162–164]. Like Az-C12, Alk-C14 is readily taken up by cells and incorporated into proteins. Subsequently, the alkynyl moiety of the fatty acid analog can be covalently linked to an azido-probe using Click chemistry (Fig. 3B). The probe may contain biotin for Western blot detection [29,162,164] and/or affinity purification (as shown for palmitoylation) [164], a fluorescent rhodamine [163,167] or a fluorogenic coumarin [29] for in-gel fluorescence detection. Use of the fluorescent azido-rhodamine and fluorogenic azido-coumarin allows flatbed in-gel detection of fatty acylated proteins separated by SDS-PAGE, thereby bypassing Western blot analysis and facilitating detection and excision of fatty acylated proteins for identification by mass spectrometry.

The development of an assay that can assess post-translational myristoylation will be required to establish the post-translational myristoylation of cancer cells and evaluate the fatty acylation of the newly discovered myristoylated proteins. Interestingly, we also demonstrated the existence of at least 15 post-translationally myristoylated proteins in apoptotic Jurkat cell lysates using the Staudinger ligation approach [30], an observation we also corroborated using click chemistry [29]. Thus, the presence of numerous post-translationally myristoylated proteins in apoptotic cells suggests an underappreciated role for post-translational myristoylation in the regulation of cell death. Altogether these new techniques at the chemical biology forefront will accelerate the data acquisition process not only for myristoylated proteins but also for NMTs as well as other types of acylated and acylating proteins (please see [168] for a complete review).

5. Discussion

5.1. Potential roles of recently identified putative post-translationally myristoylated proteins in apoptosis

The previous screen of Martin et al. [30] identified five proteins that were predicted to be cleaved by caspases to reveal an

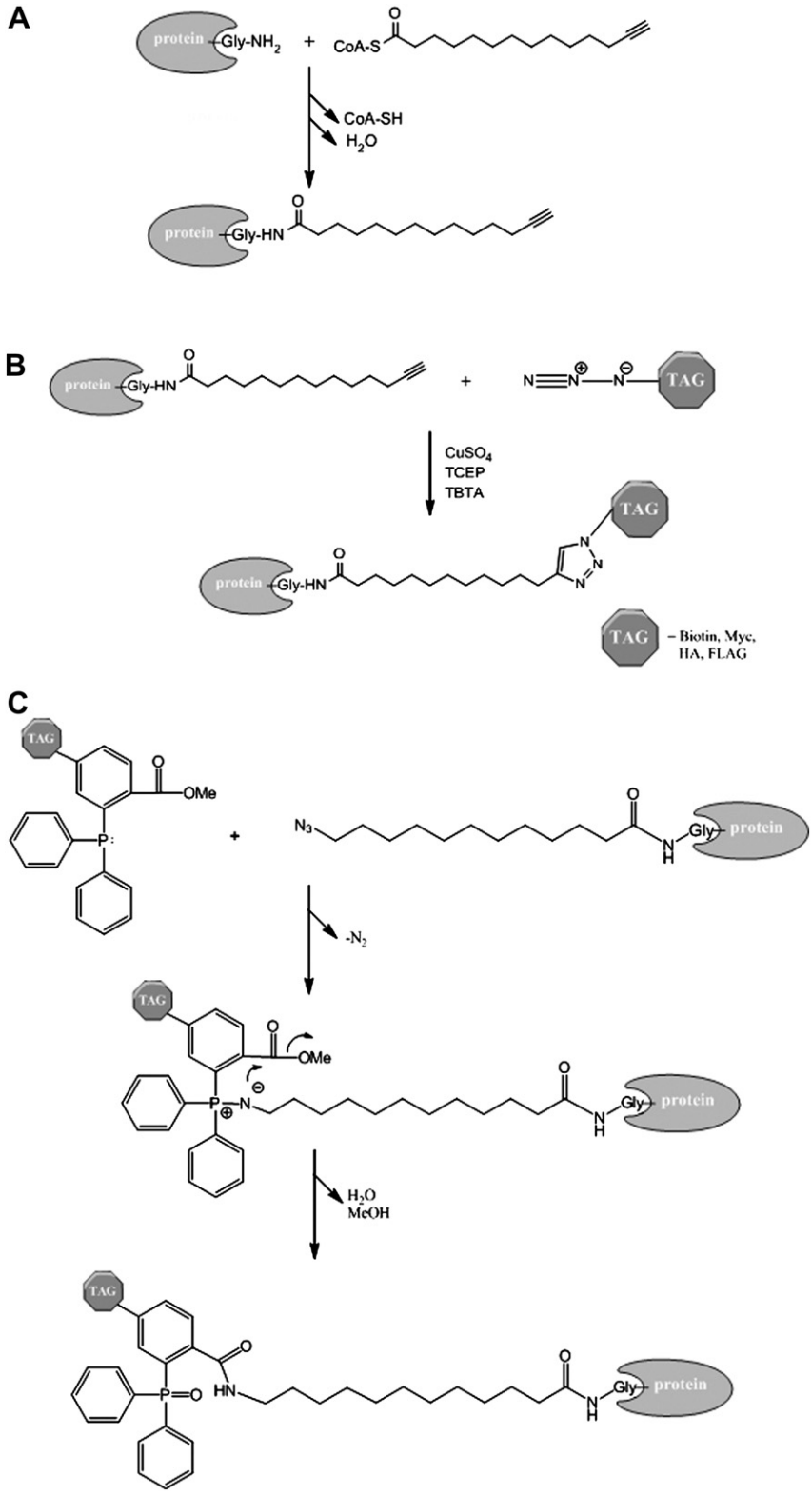

Fig. 3. Schematic of the Copper (I)-catalyzed Azide-Alkyne cycloaddition and the Staudinger ligation used to detect myristoylation of proteins. (A) ω-alkynyl-myristate is added to the cells, which leads to its activation to the CoA ester and its NMT dependent addition to the N-terminal glycine residue of a protein. ω-alkynyl-myristoylated proteins are reacted to a variety of tags using the Copper (I)-catalyzed Azide-Alkyne cycloaddition commonly referred to as "Click" reaction [29] (B). Similarly an azido-myristate analog (ω-azido-dodecanoate, Az-C12) can be incorporated into cellular proteins and ligated to a variety of tagged-phosphine probes by the Staudinger ligation (C).

N-terminal glycine on their C-terminal cleavage fragment. As mentioned before, these included two kinases, PKCε and MST3, the apoptotic regulator Bap31, the structural component CD-IC2A and finally an enzyme involved in glutathione metabolism, GCLC. A novel assay aimed at strictly detecting post-translational myristoylation was recently developed in our laboratory (Martin et al., manuscript in preparation). Using this assay, it appears that the first 10 amino acids of ctPKCε and ctCD-IC2A remain strong candidates for post-translational myristoylation, whereas Bap31, GCLC and MST3 are not (unpublished results). Using this assay, we also revealed several new candidates for post-translational myristoylation, including Mcl-1 (see below).

Like ctPAK2, caspase cleavage of PKCε at D383 separates the N-terminal regulatory domain from the C-terminal kinase domain, which becomes constitutively active [169]. PKCε contains a second caspase cleavage sites at D451 that leads to an inactive form of the kinase domain. The former cleavage site appears to be the primary site of cleavage and is located within the hinge domain, between the regulatory and kinase domains, whereas cleavage at the second site is delayed and is found within the catalytic domain and, presumably, inactivates the kinase. Full-length PKCε binds diacylglyceride during its activation. Therefore, loss of the diacylglyceride-binding domain in the N-terminal region may be substituted by post-translational myristoylation of the constitutively active ctPKCε to provide membrane binding during apoptosis.

The CD-IC proteins are required for the interaction between cytoplasmic dynein and dynactin through binding of $p150^{Glued}$, via their N-termini. Dynein and dynactin regulate the secretory and endocytic pathways, microtubule organization at interphase, and the ER serves as cargo for cytoplasmic dynein in *Xenopus* egg extracts. During apoptosis, CD-IC2 is cleaved within the $p150^{Glued}$ binding domain, but remains associated with the heavy and intermediate light chains of dynein, probably via its overlapping WD-40 repeats [170]. Very interestingly, light chain 8 of dynein associates with the pro-apoptotic protein Bcl-2 interacting mediator of cell death (Bim) in normal cells and during apoptosis this complex translocates to membranes of various organelles, including mitochondria, where it binds and inhibits BCL-2 or functional homologs [171]. Alternatively, during apoptosis, molecular motors promote the paranuclear clustering of mitochondria, which increases the apoptotic effect [172]. It is possible that myr-ctCD-IC2 may be involved in either of these processes to direct the progression of apoptosis.

5.2. On the prevalence of post-translationally myristoylated proteins

Since the identification of the new post-translationally myristoylatable substrates described above [30], the number of identified caspase substrates with known caspase cleavage sites has increased from 280 [131] to well over 400 [37,38,173]. Likewise, the number of cleaved protein products with N-terminally exposed glycines has increased from 48 to 72 and this accounts for ~17% of all the known caspase substrates, suggesting that many more potential substrates for post-translational myristoylation may exist. In addition, a recent study looking at preferred caspase cleavage sites demonstrated that glycine was the most favoured amino acid adjacent to the aspartate residue (DXXDG) further strengthening the idea that more post-translationally myristoylated substrates exist [38]. Unfortunately, in the same study [38] the technique used for the labeling, affinity purification and identification of caspase-cleaved proteins by mass spectrometry relied on subtiligase, a modified form of the bacterial protease subtilisin BPN, which can perform ligation of peptides. Unfortunately, subtiligase requires a free N-terminus for its activity and, therefore, myristoylation would abrogate the addition of an affinity purification tag by subtiligase and prevent the identification of post-translationally myristoylated proteins by this method. Nonetheless, using the information obtained in that study and others [37,173], the newly identified caspase substrates were investigated for their potential myristoylation as we did before using a combination of myristoylation prediction programs (e.g. Myr Predictor and Myristoylator) [165,166]. By doing so, we list over a dozen potential substrates for post-translational myristoylation in Table 1. Of particular interest, one of the predicted putative post-translationally myristoylatable proteins includes the anti-apoptotic protein Mcl-1. Once cleaved, the C-terminus of Mcl-1 becomes pro-apoptotic and, like ctBid, it promotes an open confirmation of the pore complex [141,174]. In fact, the new post-translational myristoylation assay used to confirm the post-translational myristoylation of the first ten amino acids of ctPKCε and ctCD-IC2A suggests that ctMcl-1 is a substrate for post-translational myristoylation as well (unpublished results). If Mcl-1 is a *bona fide* post-translationally myristoylated protein, this would suggest, once again, that post-translational myristoylation may promote the translocation of various apoptotic regulators to mitochondria to promote pore complex assembly and promote apoptotic progression. The eventual combination of a post-translational myristoylation detection assay and click chemistry with azido-probes will lead to the identification and characterization of many more post-translationally myristoylated proteins.

5.3. New regulatory roles for myristoylation during cell death and development

The fact that some post-translationally myristoylated proteins promote apoptosis (myr-ctBid and myr-ctPAK2) [33,34] while others prevent it (myr-ctGelsolin) [31] is paradoxical and suggests antagonistic roles for post-translational myristoylation. This again suggests a potential regulatory role for post-translational myristoylation in cell death/survival. The final outcome may be decided by the identity and function of newly post-translationally myristoylated proteins. In addition, the fact that caspases are active and essential for cell proliferation and differentiation in various cell lines may suggest a potential involvement of post-translationally myristoylated proteins in the developmental context as well [175–178]. For example, caspase-8 activity was shown to be required for the differentiation of monocytes into macrophages and PAK2 has been proven to be cleaved in a caspase-dependent manner, at what appears to be at very low levels in those cells during this process [179]. Alkyne-myristate labeling of differentiating U937 cells suggests that ctPAK2 is in fact post-translationally myristoylated under these conditions (unpublished results). Furthermore, gelsolin is also cleaved during the differentiation of megakaryocytes [180]. It seems likely that these proteins would be post-translationally myristoylated in these contexts. Again, myr-ctGelsolin may aid in cell survival while myr-ctPAK2 may promote cytoskeletal or morphological changes required for both apoptosis and differentiation, depending on the level of cleavage or activation of myr-ctPAK2. Differentiation and proliferation may provide yet another potential pool of post-translationally myristoylated proteins to discover and another level of cellular regulation by NMTs and myristoylation.

NMT was also shown to play a role in the regulation of immune function and differentiation [181,182]. When embryonic stem cells from homozygous NMT1 (−/−) were stimulated to undergo differentiation with M-CSF a drastic decrease in macrophage production was observed [182]. These results led the authors to conclude that NMT1 is essential for proper monocytic differentiation. Furthermore, if NMT activity was reduced in inflamed bovine lungs, total NMT1 activity and expression were significantly increased in neutrophils and macrophages exposed to LPS *in vitro* [181]. Finally, NMT1 knockdown increases the cell death of normal

Table 1
List of proven, putative and predicted post-translationally myristoylated proteins. Group I – Proven post-translationally myristoylated proteins [31–34]. Group II – Putative post-translationally myristoylated proteins identified in Martin et al. [30]. Group III – Caspase-cleaved proteins from CASBAH [173] and Dix et al. [37] predicted to be post-translationally myristoylated by NMT predictor [166] and Myristoylator [165].

Substrate	Physiological role	Cleavage effect	Cleavage sequence (caspase/myristoylation)	Prediction of myristoylation
Group I – Proven				
β-actin	Cytoskeleton	Inactivated	ELPD(244)/GQVITIGNER	Twilight zone (166)
Bid	Apoptosis activator	Activated	LQTD(59)/GNRSSHSRLG	Medium confidence (165) reliable (166)
Gelsolin	Severs & nucleates actin	Inactivated	DQTD(403)/GLGLSYLSSH	Medium confidence (165) reliable (166)
p21-activated kinase 2 (PAK2)	Cytoskeletal dynamics, activated by Rac & Cdc42	Activated	SHVD(212)/GAAKSLDKQK	High confidence (165) twilight zone (166)
Group II – Putatative				
B-cell receptor-associated protein 31 (p28 Bap31)	Bcl-2 adaptor at the ER, originally identified as B-cell receptor-associated protein	Inactivated	AAVD(163)/GGKLDVGNAE	Low confidence (165)
Cytoplasmic dynein intermediate chain (CD-IC2A)	Mediates dynein/dynactin interaction	Inactivated	DSGD(99)/GAVGSRTLHW	Medium confidence (165) reliable (166)
Glutamate-L-cysteine ligase (GCL)	Rate-limiting enzyme in glutathione synthesis	Inactivated	AAVD(499)*/GCGKAQNSTE	Reliable (166)
Mammalian STE20-like protein kinase (MST3)	Stress signalling	Activated	AETD(325)*/GQASGGSDSG	Twilight zone (166)
Protein Kinase C epsilon (PKCε)	Cell signalling (various)	Activated	SSPD(383)/GQLMSPGENG	High confidence (165)
Group III – Predicted				
Cell division cycle 6 homolog (CDC6)	Required for prereplicative complex formation	Inactivated	SEVD(442)*/GNRMTLSQEG	High confidence (165)
Golgi resident protein GCP60	Involved in the maintenance of Golgi structure by interacting with giantin	Unknown	VSVD(15)/GLTLSPDPEE	High confidence (165)
Guanine nucleotide binding protein like 1 (GNL1)	Part of MHC1 involved in heat shock response	Unknown	DTSD(52)/GESVTHHIRR	High confidence (165)
Highly Similar to 14-3-3 protein epsilon	Unknown	Unknown	MQGD(216)/GEEQNKEALQ	High confidence (165)
Heterochromatin protein 1 homolog beta (HP1β)	Required for the formation of heterochromatin	Decreased binding to protein 4.2	EQGD(205)/GGTEGHSPSG	Twilight zone (166)
Huntingtin (Htt)	Polyglutamine tract protein defective in Huntington's disease	Aggregates	DLND(552)/GTQASSPISD	High confidence (165) reliable (166)
Isoform 1 of YTH domain family protein 2 (YTHDF2)	Unknown	Unknown	NGVD(367)/GNGVGQSQAG	Medium confidence (165) reliable (166)
Induced myeloid leukemia cell differentiation protein (MCL-1)	Bcl-2-related protein, anti-apoptotic	Pro-apoptotic	TSTD(157)GSL PSTPPPA	High confidence (165) reliable (166)
Lens epithelium derived growth factor (LEDGF)	Transcriptional coactivator	Inactivated	DAQD(486)/GNQPQHNGES	High confidence (165)
Microtuble-actin crosslinking factor 1 (MACF1)	Cytoskeletal linker protein that associates with both actin and microtubules	Unknown	DAPD(3022)*/GSDASQLLHQ	High confidence (165) reliable (166)
Myc-associated factor X (Max)	Basic helix-loop-helix/leucine zipper protein that plays a role in the activity of c-Myc	Inactivated	SAFD(135)/GGSDSSSESE	High confidence (165) twilight zone (166)
Proteasome activator subunit 3 (PA28 gamma)	Proteasome activator	Unknown	DGLD(80)/GPTYKKRRLD	Low confidence (165)
Proteasome subunit p58 (PSD3)	26S proteasome non-ATPase regulatory subunit 3	Unknown	GEAD(58)/GKTAAAAAEH	Reliable (166)
RAD51 homolog protein isoform 1	Involved in homologous recombination and DNA repair	Inactivated	AQVD(187)/GAAMFAADPK	Twilight zone (166)
RNA-binding protein FUS (RBPF)	Oncogene FUS, oncogene TLS, translocated in liposarcoma protein, DNA-pairing protein	Unknown	DWFD(355)/GKEFSGNPIK	High confidence (165)
Zinc finger protein multitype 1 (GATA-1)	Erythropoeitic transcription factor	Inactivated	EDLD(125)/GKGSTSFLET	Twilight zone (166)

*Denotes different caspase cleavage positions identified in PubMed from those listed in CASBAH [173].

or LPS-treated neutrophils. This may be explained by the myristoylation of the TRIF-related adaptor molecule (TRAM) that is part of the Toll-like receptor signalling pathway. TRAM myristoylation allows targeting to the plasma membrane and is critical for macrophage LPS responses [183].

Altogether, these studies strongly demonstrate the importance of not only co-translational myristoylation, but potentially post-translational myristoylation, in differentiation and proliferation. As such, both co- and post-translational myristoylation appear to play an important role in several aspects of the cellular lifespan.

6. Conclusion

Myristoylation has been known to play an important role in a myriad of processes within the cell and, as reviewed herein, several new aspects of myristoylation have emerged within the last decade. For instance, blocking myristoylation in trypanosomes has proven to be a promising new approach to the treatment of African Sleeping Sickness and may prove to be a target in other trypanosome-related diseases [116]. In Noonan-like Syndrome, we saw the first example of a mutation leading to an aberrant gain of myristoylation that contributed to a disease state, which suggests that it may be possible for other non-myristoylated proteins to become myristoylated to acquire altered functions that may have deleterious effects on health [103]. However, we think the most rapidly expanding area of myristoylation in the upcoming years will involve the identification of post-translationally myristoylated proteins following caspase cleavage during apoptosis, proliferation or development [29–34]. These proteins are proving to have important roles in apoptosis that are intricately linked to their post-translational myristoylation. So

far, apoptosis induced by myr-ctBid and myr-ctPAK2 can be significantly reduced by abrogating myristoylation of these proteins [33,34]. Paradoxically, the anti-apoptotic effect of myr-ctGelsolin is also linked to its myristoylation status [31]. This suggests that there may be dual roles for post-translationally myristoylated proteins. It is possible that some proteins promote cell survival until the cell has reached an apoptotic threshold and has committed to cell death. Alternatively, some of the pro-survival factors may have a greater role in cell differentiation and proliferation where caspase cleavage is essential and at least PAK2 and gelsolin are targets for caspase cleavage and most likely post-translationally myristoylated [179,180]. With PAK2, it appears that the level of cleavage is not as great in differentiating cells as in apoptosis, which may suggest that lower levels of constitutively active myr-ctPAK2 in differentiating cells may not be toxic or is tightly regulated to prevent apoptosis [34,179]. As such, it may provide a structural role in differentiation and proliferation while myr-ctGelsolin would act as a pro-survival factor in this scenario.

Several new findings suggest that there remains many more, as of yet, unidentified co- and post-translationally myristoylated proteins. For instance, in the cell free assay described above, Utsumi's group identified 1.4% of the proteins as myristoylated of the 1929 proteins in the query proteome they investigated [155]. Therefore, this may suggest that the myristoylome is larger than previously predicted (0.5%) [78,82]. Interestingly, and of particular note, they found that the two commonly used prediction analysis programs used for myristoylation were strong predictors of myristoylation. However, while Myr Predictor [166] had more false-negative predictions, Myristoylator [165] had more false-positive predictions, and both programs missed two proteins that were shown to be myristoylated [155]. This demonstrates the importance of testing for myristoylation *in vitro* and *in vivo*.

The possible existence of other post-translationally myristoylated proteins is supported by the fact that we have identified 16 new proteins that are predicted to be post-translationally myristoylatable (Table 1). Furthermore, we also showed that there is a clear difference in the cellular contents of co- and post-translationally myristoylated proteins in non-apoptotic and apoptotic Jurkat cells and the identification of these myristoylated proteins awaits [29,30]. Moreover, the fact that several studies have demonstrated that the preferred amino acid following caspase cleavage sites is glycine [37,38], further suggests the existence of more potential sites for post-translational myristoylation. Furthermore, cryptic myristoylation sites might also be found downstream of other proteolytic sites (e.g. calpain, granzyme B, cathepsins, etc.). Using chemical biology as a means to speed up detection and isolation of post-translationally myristoylated proteins, the first proteomic analyses of the post-translational "myristoylomes" of normal and metabolically compromised cells should emerge in the near future. Because post-translational myristoylation occurs in apoptotic cells and apoptosis is often deregulated in cancer and neurodegenerative diseases, the importance of identifying and characterizing post-translationally myristoylated proteins is undeniable. A better understanding of novel molecular components involved in apoptosis will not only offer insights into pathogenesis but could also allow for the development of diagnostic, prognostic and therapeutic tools.

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