Software

MYRbase: analysis of genome-wide glycine myristoylation enlarges the functional spectrum of eukaryotic myristoylated proteins

Sebastian Maurer-Stroh[*], Masaki Gouda[†], Maria Novatchkova[*], Alexander Schleiffer[*], Georg Schneider[*], Fernanda L Sirota[‡], Michael Wildpaner[*], Nobuhiro Hayashi[†] and Frank Eisenhaber[*]

Addresses: [*]IMP Research Institute of Molecular Pathology, Dr. Bohr-Gasse 7, A-1030 Vienna, Austria. [†]Division of Biomedical Polymer Science, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan. [‡]Service de Conformation de Macromolecules Biologiques et de Bioinformatique, Université Libre de Bruxelles, Boulevard du Triomphe - CP 263, 1050 Bruxelles, Belgium.

Correspondence: Sebastian Maurer-Stroh. E-mail: stroh@imp.univie.ac.at

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Abstract

We evaluated the evolutionary conservation of glycine myristoylation within eukaryotic sequences. Our large-scale cross-genome analyses, available as MYRbase, show that the functional spectrum of myristoylated proteins is currently largely underestimated. We give experimental evidence for *in vitro* myristoylation of selected predictions. Furthermore, we classify five membrane-attachment factors that occur most frequently in combination with, or even replacing, myristoyl anchors, as some protein family examples show.

Background

Myristoylation is an important lipid modification of a variety of eukaryotic and viral, and also a few bacterial, proteins [1-4] that can direct proteins to membranes. There, it can influence their submembrane localization (for example, in lipid raft or raft-like compartments [5]) as a function of additional factors (for example, subsequent palmitoylation or the possession of a polybasic region) [6,7]. Also, myristoyl anchors have been found to be involved in specific protein-protein interactions [8,9]. Complex conformational switches can trigger changes in accessibility of the lipid moiety [10-13]. The enzyme myristoyl-CoA:protein *N*-myristoyltransferase (NMT) [14] recognizes a motif [15] at the amino terminus of substrate proteins (which can also become amino-terminal after proteolytic cleavage) and attaches myristic acid, a saturated 14-carbon fatty acid, to the amino-terminal glycine. As bacteria and viruses, with the exception of two predicted NMTs in entomopoxviruses [15], lack the myristoylating enzyme, their proteins are modified by eukaryotic host NMT.

A sensitive prediction method using position-specific, redundancy-corrected profiles of known substrates in combination with physicochemical constraints on enzyme-substrate interactions has been developed [16] and is available from the web [17]. The sensitivity (cross-validation performance over the learning set of known substrates) is above 95% and the rate of false-positive prediction is estimated as <0.5% for the general eukaryotic, and <0.3% for the fungal, parameter set for proteins starting with glycine. The authors acknowledge that taxon-dependent enzyme-substrate specificities might influence prediction performances to a larger extent than included in the current implementation of the prediction algorithm. Large-scale application of this NMT predictor over GenBank (from the National Center for Biotechnology Information

Table 1

Numbers of analyzed sequences, experimentally verified myristoylated proteins plus their homologs and the set of additional new predictions

	Number in GenBank*	Number experimentally verified + homologs†	Number established NEW true predictions‡
Total	600,916	1,122	2,037
Leading methionine§	426,128	997	1,548

*Eukarya, non-identical sequences (nr100), February 2003. †Experimentally verified myristoylated proteins and their homologs with conserved myristoylation site (BLASTP E-value <0.005, plus manual curation). ‡Estimated NEW true predictions: (total number of predictions) minus (expected number of false-positive predictions) minus (number of experimentally verified + homologs) = number of true predictions not closely related to already experimentally verified examples (possibly additional new functions for myristoylated proteins?). §When a leading methionine is present in the sequence, it is less likely to represent a non-amino-terminal fragment in the database. However, this also excludes some true amino-terminal fragments, where a glycine becomes amino-terminal after proteolytic cleavage.

(NCBI)) produces lists of thousands of potential NMT substrates. The total number of analyzed sequences and expected number of true predictions after subtraction of potential false positives are given in Table 1.

To make these data more accessible and interpretable in terms of biological significance, we evaluated the evolutionary conservation of the predicted myristoylation motif among groups of homologous proteins. This approach ranks predicted myristoylated proteins according to the number of homologous sequences with a conserved motif for this common lipid modification. Although not an absolute requirement, the evolutionary conservation of a motif in large protein families can be used to postulate its functional importance. These results are accessible through MYRbase [18], an easily navigable, searchable, web-based collection of tables containing the multiply linked and annotated results of our large-scale predictions, ordered by their number of occurrences in clusters of closely related proteins.

A more detailed description of MYRbase is given in the next section and on the accompanying website [18]. The main part of this manuscript is dedicated to a comprehensive discussion of the results, which also include the *in vitro* experimental verification of predicted myristoylation for amino-terminal peptides derived from human homologs of several non-obvious substrates (for example, 47 kDa GTPase IIGP, ubiquitin hydrolase Ubq-M, lung cancer candidate FUS1, potassium channel Kir2.1 and potassium channel interacting protein KChIP1). From these results we suggest extending the previously known functional spectrum of myristoylated proteins, representing a first step towards a characterization of the entire set of myristoylated proteins.

MYRbase

We applied the NMT predictor for glycine myristoylation to taxonomic subsets of publicly available databases (SWISS-PROT, GenBank). The NMT prediction methodology is described in detail elsewhere [16]. Then, we first removed redundancy within our predictions using the program mcd-hit [19,20] with a 40% amino-acid identity threshold. This procedure already results in a dramatic reduction of the prediction lists to a much smaller set of representative sequences (for example, from approximately 4,400 sequences to approximately 2,000 for the eukaryotic subset). So as not to lose the information in the removed sequences, they were assigned to groups according to their clustering by mcd-hit. The conservative threshold of 40% amino-acid identity allows the interpretation that sequences within the clusters are homologous to their representative sequence but also results in the appearance of more remotely related sequences in separate clusters. The size of these clusters was used to rank the representative sequences in the tables in the database and is linked to view the full set of sub-tables listing all cluster members.

We have also estimated the taxonomic distribution of sequences within a cluster, as occurrence in a larger set of organisms might reflect the functional need for conservation of the motif during the aeons of evolution. Special emphasis is given to the known experimental status of myristoylation to distinguish new predictions. Therefore, we indicate the annotation of a cluster member for myristoylation in the SWISS-PROT [21] database below the cluster size and add further details to the sequence description. Besides this short description of the protein, the predictions are linked to their corresponding GenBank entries, an RPS-Blast with CD-search [22] to view the domain architecture and PSI-Blast [23] to collect the set of homologous sequences.

Then, the position in the sequence of the glycine that is predicted to be myristoylated (position 2 often implies prior cleavage of a leading methionine) and the full-length motif follow. We highlight cysteines that could be subject to palmitoylation in yellow and positive charges by a blue background. Both factors can not only influence membrane affinity but also induce changes in subcompartment-specific localizations [5]. Finally, the score assigned by the NMT predictor (yielding details of the single score components on mouse-over), the estimated probability of false-positive prediction and a simple prediction attribute are listed.

Entries in MYRbase can be accessed by simple browsing or entering the corresponding MYRbase identifier on the index page (MYRbase identifiers correspond to GenInfo numbers of entries from the latest database built). Alternatively, we recommended using our MYRbase-specific BLAST-engine (also

available from the index page) to be linked directly to the cluster of the best hit of the query protein with MYRbase entries.

Results and discussion

Conservation of glycine myristoylation in the evolution of eukaryotes

When analyzing single eukaryotic genomes of human, mouse, fly, worm, yeast and plant, we found a conserved relative occurrence (around 0.5-0.8% of all proteins in the genome) of predicted myristoylated proteins, with an elevated percentage for *Arabidopsis* [16] that is also supported by combined theoretical/experimental data for this organism [24]. In our genome-spanning analysis, we find that the most prominent and taxonomically widespread groups of myristoylated proteins comprise GTP-binding proteins (several alpha subunits of G proteins and ADP ribosylation factors), serine/threonine kinases, calcium-binding EF-hand proteins with diverse functions (for example, recoverin, calcineurin B, guanylate cyclase activators) and tyrosine kinases (see Table 2 for complete listing).

It is interesting that for proteins with conserved myristoylation there are a few families with a large number of members, and a large number of families with only a few members. An interpretation of this situation, which is reminiscent of a power-law distribution (see Figure 1 and legend), in evolutionary terms might be that the few larger families are the oldest cases of eukaryotic protein glycine myristoylation. The many smaller families, on the other hand, appear to be examples of functional specialization, as they are often confined to taxonomic subgroups. Most of the larger families with a conserved motif include proteins with experimentally verified myristoylation (Table 2). Hence, future research will focus on the role of the predicted lipid anchors for the specialized smaller groups.

The amino-terminal myristoylation motif is an exchangeable module in evolution

We do not only observe myristoylation of proteins specific to taxonomic subgroups but also examples of myristoylation of proteins from some species that have multiple orthologs in other species that lack the motif for this lipid modification. In several of these cases, the myristoyl anchor has been substituted by other factors that can facilitate membrane association, such as other lipid anchors (palmitoyl, farnesyl, geranylgeranyl), transmembrane regions or specific protein-lipid or protein-membrane protein interaction domains. Such substitutions can have occurred not only in orthologs and paralogs but even in isoforms of the same protein.

For example, several G_{α} subunits have either a myristoyl-, both myristoyl- and palmitoyl-, or only palmitoyl-anchors [25]. While the *Arabidopsis* Rab5 ortholog Ara7 is geranylgeranylated on carboxy-terminal cysteines just like Rab5 in other species, its closely related paralog Ara6 lacks the carboxy-terminal cysteines but has an experimentally verified amino-terminal myristoylation motif instead [26]. Furthermore, it has been shown that an artificially introduced transmembrane helix can substitute for acyl-mediated membrane targeting of eNOS [27]. While most phospholipase C-gamma's are expected to be targeted to membranes by amino-terminal pleckstrin homology domains (PH, which bind phospholipids), a phospholipase C in *Trypanosoma cruzi* utilizes a myristoyl-anchor instead [28]. Indeed, myristoylation was also able to substitute for PH domain-mediated membrane targeting of the scaffolding protein Gab1 [29].

Diverse shuffling, similar to that of globular domains, of several membrane-attachment factors (MAFs) within homologous proteins apparently occurred during evolution (by substitution, addition or deletion). It should be noted, however, that most of these changes also imply drift in targeting specificity or strength, and that the contributions and importance of single MAFs for membrane attachment might differ.

The notion of evolutionary modularity should not be misunderstood as a common ancestry for all sequence segments comprising myristoylation motifs. On the contrary, sequence-based comparisons appear to favor a model of multiple independent evolution (for example, for the emergence of those single motifs; data not shown). The modularity we describe here is that targeting specificity is achieved by combining different modules for membrane attachment in arrangements that are not fixed but can change dynamically during evolution. The evolutionary steps required for these changes from one targeting motif to the other are most likely to involve intermediates that contain several of the interchangeable factors.

Myristoyl anchors mediate targeting of proteins to many locations inside and outside of cells

Such evolution has apparently led to lipid-anchor-mediated targeting to a wide range of subcellular localizations. The role of glycine myristoylation is not limited to the classical attachment of proteins to the plasma membrane. There, several myristoylated proteins are found to target cholesterol and sphingolipid-enriched detergent-resistant compartments, designated as lipid rafts [30]. In addition, the myristoyl anchor is involved in targeting proteins to membranes throughout the cell, ranging from endoplasmic reticulum (ER), Golgi and mitochondrial membranes to the nuclear envelope and even to extracellular localization (see Table 2). Extracellular localization is the result of an alternative myristoylation- and palmitoylation-dependent export mechanism identified in *Leishmania* and possibly conserved to higher eukaryotes [31].

Specificity and strength of targeting can be achieved by optimizing lipid interactions by the type and number of lipid anchors (for example, acyl (myristoyl, palmitoyl) [6], prenyl (farnesyl, geranylgeranyl) [32,33] and GPI [34,35]), possibly

Table 2

Experimentally verified myristoylated proteins and their subcellular localizations

Experimentally verified protein type(s)	Total number in MYRbase	Subcellular localization	References
GTP-binding proteins	288		
G-protein alpha subunits (G_αi), G_αz), G_αo), G_αt))	201	Type-dependent (receptor-specificity); for example, cytosol/plasma membrane (lipid rafts)	[25,95]
ADP ribosylation factors (ARF) and ARF-like (ARL)	79	Type-dependent; cytosol, intracellular membranes, cytoskeleton, nucleus/nucleolus (for example, ARL4, ARL5)	[109,110]
Rab5-related (Ara6)	8	Cytosol, endosomes, ER, plasma membrane	[26]
S/T-kinases	206		
Ca^{2+}/calmodulin-dependent kinases	82	Type-dependent; for example, cytosol, plasma membrane, ER membrane	[111]
Fen, Pto and related S/T-kinases	60	Cytosol, plasma membrane (~)	[112]
cAMP-dependent kinases (PKA catalytic subunit)	51	(Nucleus)/cytosol/(plasma membrane over anchoring proteins)	[113,114]
cGMP-dependent kinase II	13	(Cytosol)/plasma membrane	[115]
Ca2+-binding EF-hand proteins	141		
Recoverin, neuronal calcium sensor, visinin, frequenin, neurocalcin, hippocalcin,	66	Type-dependent, triggered by Ca^{2+}/myristoyl switch; for example, cytosol, plasma membrane, Golgi, neurofilament-rich structures	[78,116]
Calcineurin B phosphatase subunit, p22	43	Cytosol, plasma membrane (phosphatidylserine-rich regions), microtubule cytoskeleton (p22)	[117,118]
Guanylate cyclase activators	21	Cytosol/rod outer segment membranes of photoreceptor cells	[119]
DNA-dependent kinase interacting protein KIP = calcium and integrin binding protein CIB = calmyrin	11	Cytosol, plasma membrane, ER, nuclear envelope, nucleoplasm, cytoskeleton	[120,121]
Other			
Tyrosine kinases (Abl, Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, Yes)	136	Type-dependent; for example, cytosol/plasma membrane (lipid rafts/caveolae)	[122,123]
t-Actin (15 kDa carboxy-terminal fragment of cytoskeletal actin after caspase cleavage)	89*	Cytosol, mitochondrial membrane	[16,124]
26S ATP/ubiquitin-dependent protease regulatory subunit 4	26	Nucleus, cytosol, microsomes	[16,62,125]
Endothelial nitric oxide synthase (eNOS)	18	Cytosol, Golgi, plasma membrane (lipid rafts/caveolae)	[126,127]
Golgi reassembly stacking protein 1 (65 kDa) and 2 (55 kDa)	17	Golgi membrane	[128,129]
Myristoylated alanine-rich carboxy-kinase substrate (MARCKS)	15	Cytosol, cytoskeleton, plasma membrane (lipid rafts)	[101]
Flagellar calcium-binding proteins in trypanosomes	14	Flagellar membrane	[130]
NADH cytochrome b-5 reductase (diaphorase)	14	Cytosol, ER membrane, mitochondrial outer membrane	[131]
43 kDa acetylcholine receptor-associated protein of the synapse (RAPSYN)	13	Cytosol, Golgi, plasma membrane (lipid rafts/caveolae)	[99]
S/T protein phosphatase + EF hand (PPEF)	12	Axons, dendrites, ciliar membranes	[132]
Hydrophilic acylated surface protein B (HASPB)	10	Extracellular (!) face of the plasma membrane	[31]
Erythrocyte membrane protein band 4.2 (Pallidin)	10	Plasma membrane, intracellular vesicles	[133]

Table 2 *(Continued)*

Experimentally verified myristoylated proteins and their subcellular localizations

Vps15 (phosphatidylinositol 3-kinase-associated p150)	10	Cytosol, intracellular membranes (late Golgi)	[134]
Neuronal axonal membrane protein NAP-22 (brain acid soluble protein 1 BASP1; CAP23)	9	Golgi, plasma membrane (lipid rafts)	[97]
SSeCKS (A-kinase anchor protein 12)	9	Cytosol, cytoskeleton, plasma membrane	[135]
Sip2, beta subunits of 5'-AMP-activated protein kinase	9	Cytosol, plasma membrane	[136]
T-lymphoma invasion and metastasis inducing protein 1 (TIAM1 protein)	9	Cytosol, plasma membrane	[137]
PSD-Zip70, FEZ1	9	Neuronal membranes (postsynaptic density, dendritic rafts), plasma membrane (lipid rafts)	[138]
S/T protein phosphatase Z	8	Nucleus	[139]
Annexin XIII	6	Cytosol, Golgi, plasma membrane (apical, lipid rafts)	[100]
Ezrin-binding partner PACE-1	6	Cytosol, Golgi, lamellipodia	[140]
BH3 interacting domain death agonist (BID)	5	Cytosol, mitochondrial membrane	[45]
Calpastatin (testis-specific isoform tCAST)	5	Cytosol, intracellular membranes	[141]
A-kinase anchor protein 200	5	Cytosol, plasma membrane	[142]
Golgi-associated PR-1 protein	5	Golgi, plasma membrane (lipid rafts/caveolae)	[143]
Vps20 (vacuolar protein sorting)	4	Cytosol, endosomal membranes	[144]
Adenylate kinase 1	4	Cytosol, plasma membrane	[145]
A-kinase anchor protein 7 (18)	3	Cytosol, plasma membrane	[114]
2'-5'-oligoadenylate synthetase splice form 2	3	Cytosol, rough ER, ribosomes, nucleus	[57]
NADH ubiquinone dehydrogenase B18	3	Mitochondrial membrane	[146]
Hisactophilin 1 and 2 (Histidine-rich actin binding protein)	2	Cytosol, plasma membrane, nucleus	[147]
Phosphoinositide-specific phospholipase C in *T. cruzi*	2	Cytosol, plasma membrane	[28]
Actin-myosin network maintenance protein Nullo	2	Plasma membrane (metaphase furrows during mitosis, cellularization front)	[148]
t-Gelsolin (fragment after caspase cleavage)	2*	Not determined for myristoylated form, cytoskeleton(?)	[124]
Igloo (growth associated protein)	1	Cytosol, neuronal membranes	[149]
Flagellar creatine kinase (ATP:guanido phosphotransferase)	1	Sperm tail membranes	[150]

*Not yet in MYRbase; cleavage motif is not automatically detected but is predicted when submitted as fragment to the predictor.

aided by additional features (such as clusters of positive charges [36]), but also by specific protein-lipid (for example, PIP_2 [37]) and protein-protein (for example, PDZ domain [38]) interactions.

Classification of five membrane-attachment factors frequently co-occurring with myristoyl anchors (coMAFs)

As several factors in addition to myristoylation contribute to subcellular localization and membrane attachment, we have analyzed their distribution within our sets of known and predicted myristoylated proteins. We distinguish five major classes that are defined in Table 3 and depicted in Figure 2. From our analysis, subsequent cysteine palmitoylation (class I) and clusters of positive charges (class II) seem to occur in comparable amounts in both known and predicted subsets, while there is a clear tendency for the remaining classes (class III-V) to be more common among the new predictions. The prediction of the myristoylation of eukaryotic transmembrane proteins (class IV), in particular, will have to be verified in the future, although it seems generally reasonable, as viral examples exist [39,40] and the very similar acyl modification with palmitoyl anchors is commonly found in combination with transmembrane regions [41].

We also expect that final targeting specificity will be determined by not one but a combination of several MAFs, and the subcellular localization will remain difficult to predict, especially in regard to the involvement of protein-protein interactions (class V). Reversibility of membrane attachment can be

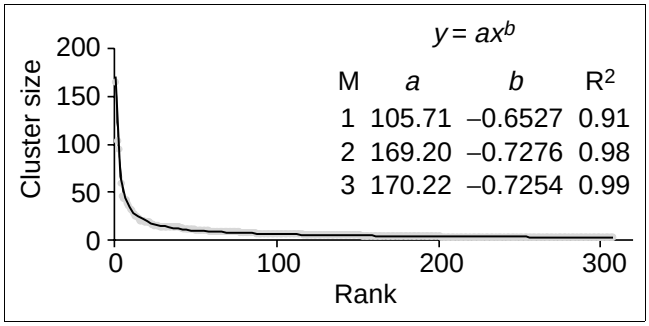

Figure 1
Power-law distribution of family clusters in MYRbase with maximal 40% sequence identity and a minimal size of 3 (to exclude false positives). The inset table gives the values and correlation coefficients for different minimal cluster sizes. The power function distribution (without shift along the argument axis *x*), the Pareto distribution and the Zipf's law (if the rank is approximated by the argument *x*) have the common analytical form $y = a \cdot x^b$. Such distributions generally occur as the limit distribution of a multiplicative stochastic process with a lower boundary constraint (here, minimal cluster size). The common phenomenological form does not imply a unified mechanism for generating samples obeying these distribution functions. We suggest interpreting cluster size in terms of time for evolutionary divergence within the cluster (see text).

regulated by depalmitoylation, disruption of the cluster of positive charges through introduction of negative charges (phosphorylation, deamidation of asparagine to aspartate), a pH-dependent switch when there are histidines within the positive charge clusters, or conformational changes due to proteolytic cleavage, binding of small molecules or altered protein-protein interactions.

The functional spectrum of myristoylated proteins is underestimated

From Table 2 we can see that the functional spectrum of experimentally verified myristoylated proteins is much more diverse than their well-known involvement in signaling. However, complete analysis of eukaryotic sequences in GenBank suggests that, after subtraction of known examples and their homologs, a significant number of proteins remain whose function has not been implicated with myristoylation so far (Table 1). To investigate the spectrum of these predictions with functions 'new' to myristoylated proteins, we have systematically analyzed the domain composition of a nonredundant (90% identity) subset of the respective proteins (HMMer [42] against PFAM [43]; E-value 0.01) and summarize the results in Table 4 (ranked by the number of occurrences of domains and corrected for repeated occurrence of the same domain in one entry). As a rule, the proteins associated with domain hits in Table 4 do not belong to a unique protein family but to several families that differ in their overall domain architecture. Consequently, we also analyzed the set of new predictions in respect of clearly defined protein families, and summarize them in Table 5. This list only includes families with at least 10 different proteins that, furthermore, begin with a methionine in their sequence (for exclusion of possible non-amino-terminal fragments). Not surprisingly, as myristoylation of remotely related family members has already been established, we find additional examples of kinases, phosphatases and phosphodiesterases. Several 'new' predictions will be discussed in the following sections. If not listed in Tables 5 or 6, a MYRbase identifier (MI) that equals the GenInfo number of the predicted entry in the analyzed database will be given to access the corresponding entries in MYRbase.

In vitro verification of selected predictions

We also picked a few interesting predictions from MYRbase and investigated the capacity of the corresponding amino-terminal peptides (Table 6 and Additional data file 1) to be myristoylated. Our *in vitro* experiments with synthetic peptides and bacterially expressed NMT (see Materials and methods) confirm the computational results for specific immunoglobulin μ-heavy and λ-light chains, 47 kDa GTPase IIGP, ubiquitin hydrolase Ubq-M, lung cancer candidate FUS1, potassium channel Kir2.1 and potassium channel interacting protein KChIP1. Hence, we show that our predictor recognizes the capability of substrates to productively interact with NMT. However, it is clear that *in vitro* myristoylation of synthetic peptides only gives limited information about the situation of the full-length protein *in vivo*. Some positively predicted substrates might be myristoylatable *in vitro*, but whether they come into contact with NMT *in vivo* depends on the cellular context and needs to be proven or at least shown to be plausible.

Immunoglobulins and possible non-amino-terminal fragments in MYRbase

For example, MYRbase also includes a series of specific immunoglobulin chains. By our *in vitro* myristoylation experiments, we show for a μ-heavy and a λ-light chain (Table 6, and Additional data file 1) that peptides derived from the amino termini of the corresponding database entries are in principal agreement with the physicochemical requirements for productive substrate-NMT interaction as modeled by our predictor.

It is unclear whether the predicted entries only represent non-amino-terminal fragments in the database. Differing sequence constructs for that region could be attributable to V(D)J recombination [44]. However, we could not find convincing expressed sequence tag (EST) evidence for variants with a starting methionine just before the predicted glycines. Proteolytic cleavage is an alternative possibility for glycines to become amino-terminal (for example, BID [45] and several viral proteins). Immunoglobulin chains are normally targeted to the ER via signal peptides and the existence of NMT activity in the ER has been proposed [2]. None of the predicted cleavage sites [46] of immunoglobulin precursors after the removal of the signal peptide appeared to result in an amino-terminal glycine. However, a different subcellular targeting mechanism, as well as alternative cleavage sites [47]

Table 3

The five classes of co-occurring membrane attachment factors (coMAFs)

coMAF class*	I	II	III	IV	V
	Palmitoylatable cysteine†	Amino-terminal cluster of positive charges‡	PIP_2-specific binding domain§	Transmembrane segments¶	Other/not attributable (for example, protein-protein interactions)¥
Experimentally verified + homolog	453	443/133/12	9	0	432
Additional NEW prediction	728	729/269/58	21	203	1,860

*Distribution of the five classes of coMAFs among the set of experimentally verified myristoylated proteins plus their homologs and the set of additional new predictions. Assignments are not necessarily unique, but can be combinations thereof. †At least one palmitoylatable cysteine within the first five residues (starting with the myristoylated glycine). ‡The content of positive charges (amino acids lysine (K), arginine (R) and histidine (H)) in the region of positions 6 to 35 is compared to the average composition in GenBank in the same region. The values correspond to hits occurring using threshold deviations of 1, 2 and 3σ from the GenBank average. §Significant HMMER-hit (below E-value of 0.01) with phosphatidylinositol-bisphosphate (PIP_2)-specific binding domains (PH, ENTH, FERM, PX or FYVE). ¶A very conservative method was used to detect putative transmembrane segments. Besides requiring the attribute 'trusted' by the sensitive method DAS-TMfilter [151], we only list the hits that have more than three predicted TM regions and that are additionally filtered for their overall polarity measured by the content of positive charges. ¥All other proteins that did not fulfill any of the above criteria.

and cleavage by other proteases, cannot be excluded. Myristoylation at the predicted sites may occur *in vivo* only if the sequences, starting with glycine as they appear in the database, can interact with NMT within the cell.

Early work exists describing *in vivo* incorporation of radiolabeled myristate into the same specific immunoglobulin chains as those predicted [48]. In this light, the possible alternative of myristoylation of an internal lysine (a theoretical suggestion of Pillai and Baltimore [48]) has to be addressed experimentally. Reports of internal lysine myristoylation are rare in the literature [49,50]. These potentially myristoylated immunoglobulin chains should have a yet-unknown cellular function (possibly, in a cell-type and cell-state-dependent manner) [48,51] in which the lipid anchor influences subcellular targeting.

Myristoylation and the innate immune response

Plant-specific disease-resistance proteins, with the nucleotide binding motif NB-ARC and leucine-rich repeat domains (for protein-protein interactions), are prominent in Table 5. While this manuscript was in preparation, octapeptides derived from the amino terminus of respective proteins were shown to be myristoylatable by plant NMT [24]. Myristoylation of those proteins that are involved in the innate immune response [52] fits well into the scheme that they act through interaction with avirulence proteins that are injected into the plant host cell through the type III secretion system of the parasitic bacteria [53]. The lipid anchor may facilitate co-localization since a series of type-III secreted bacterial proteins are also myristoylated by host plant NMT [4].

In the immune response of mammals to intracellular pathogens, expression of families of 65 kDa and 47 kDa GTPases is induced by interferon-gamma [54]. In this work, we show *in vitro* myristoylation (Table 6 and Additional data file 1) of the amino terminus of a human homolog of 47 kDa GTPase IIGP [55]. This protein seems to be the only family member, besides very close homologs, that has a myristoyl anchor. Lipid modifications are quite common within the large superfamily of GTP-binding proteins, for example, Ras and Ras-related as well as Rho and Rab small GTPases are most often either farnesylated or geranylgeranylated [32]. Also, members of the 65 kDa family of the interferon-inducible GTPases [54] share the CaaX box motif that most likely directs their geranylgeranylation. In the case of the 47 kDa GTPase IIGP, a myristoyl-anchor could be involved in localizing the protein to membranes of the ER and Golgi [55,56]. In the context of myristoylation and mammalian innate immune responses, it is noteworthy that interferon-inducible, double-stranded (ds) RNA-activated 2'-5'-oligoadenylate synthetase isoform 2 is also known to be myristoylated [57].

Myristoylation and ubiquitination

The amino terminus of the human ubiquitin-specific protease Ubp-M is myristoylated by NMT *in vitro* (Table 6, and Additional data file 1). This enzyme is suspected to be involved in the deubiquitination of histone 2A, cell-cycle regulation and caspase-dependent apoptosis [58,59]. However, the observations were made with a protein amino-terminally tagged with green fluorescent protein (GFP), which questions the functional importance of a putative amino-terminal myristoyl anchor. Interestingly, only a GFP-tagged mutated inactive version was localized to the nucleus, while the GFP-tagged protein lacking inactivating mutations was found in the cytoplasm [58]. This indicates that several factors influence its localization, and it cannot be ruled out that a myristoylated amino terminus would result in a different localization pattern, possibly also targeting the 'active' wild-type protein to its actual place of action.

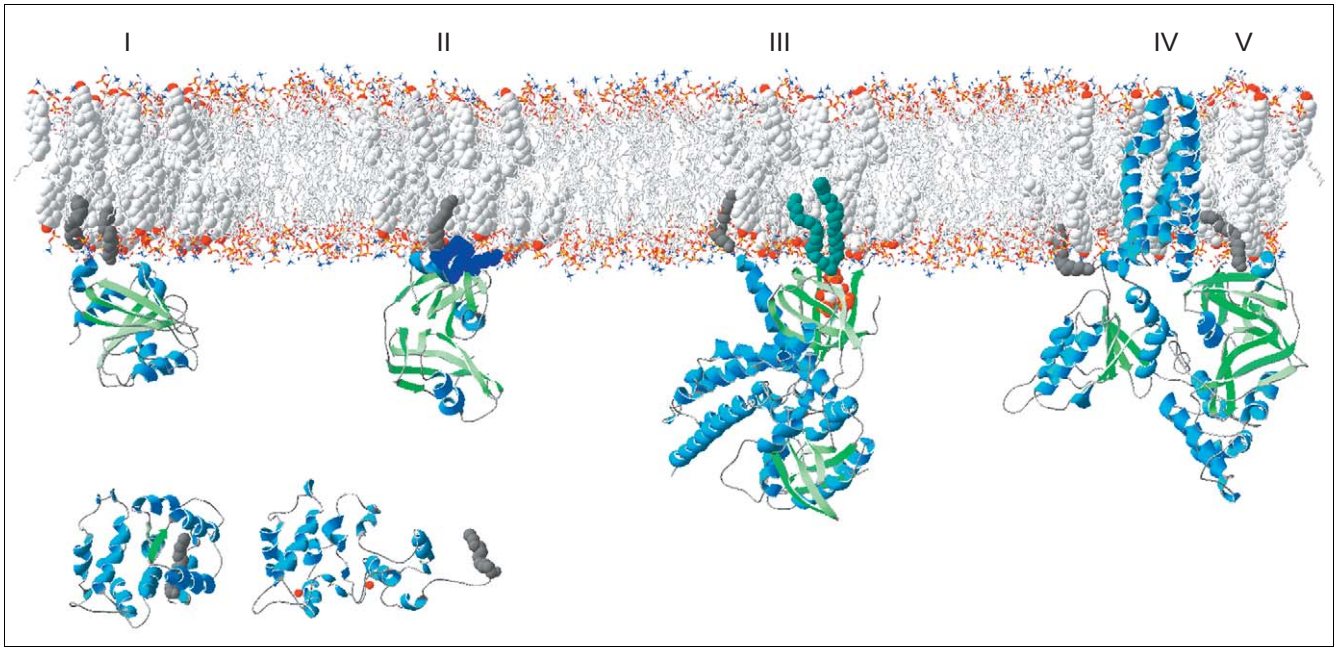

Figure 2
Schematic representation of the membrane attachment of proteins with a myristoyl anchor (dark gray, space-filling atomic representation) in combination with different co-occurring membrane-attachment factors (coMAFs). Class I, plus palmitoyl anchor (also dark gray, space-filling); class II, plus cluster of positive charges (dark blue, spacefill); class III, plus PIP_2-specific binding domain (PIP_2 in space-filling, alkyl tails in cyan); class IV, plus transmembrane segments; class V, plus a domain for specific protein-membrane protein interactions. White space-filling molecules in the model membrane represent cholesterol and symbolize targeting to specialized compartments. Two different states of the calcium/myristoyl-switch of recoverin are depicted in the lower left of the figure (calcium ions in red). Visualization is with SwissPdb-Viewer [108].

In addition to Ubq-M, we predict myristoylation for another group of ubiquitin-specific proteases conserved in rat, fly, worm and plants (for example, MI 27683149). These proteins are substantially shorter, lack additional domains (such as an amino-terminal zinc finger in Ubq-M), and most probably belong to the ubiquitin carboxy-terminal hydrolase subfamily [60]. Furthermore, we predict that the *Caenorhabditis elegans* cytokinesis defect protein 3 [61] has a myristoyl anchor followed by a calcium-binding EF-hand and an ubiquitin hydrolase homology domain (MI 16950531).

It might be interesting to check whether the predicted lipid modification for some of these ubiquitin-specific proteases could result in subcellular targeting similar to that of a 26S proteasome variant whose regulatory subunit 4 was predicted [16], and subsequently shown to be, myristoylated *in vivo* [62].

In this context, it is also interesting that we predict myristoylation for a group of proteins containing putative RING zinc finger domains (Tables 4 and 5) that are commonly found in ubiquitin ligases. Although RING finger domains do exist in some proteins known to be myristoylated (for example, rapsyn, see Table 2), most of the hits with this top-ranking domain in Table 4 seem to have 'new' functions in the context of myristoylated proteins. For example, a subgroup of the RING finger proteins are isoforms of the Notch pathway [63] protein neuralized [64], with a conserved myristoylation motif in human, mouse, frog, fly and worm (for example, MI 15128197). Another example of a RING domain-containing protein with conserved myristoylation motif in human, mouse, fly and worm is mahogunin (MI 27229238), which is involved in spongiform neurodegeneration [65]. Also, the myristoylation motif of the RING-containing peripheral nerve injury gene *nin283* (for example, MI 14150005) is conserved in human, mouse, fly and worm.

Furthermore, several F-box-containing proteins, parts of which could act as receptors for ubiquitination targets, are found within our set of proteins predicted to be myristoylated (for example, MI 6912466 and MI 7299840).

Myristoylation of mitochondrial proteins

As well as proteins involved in apoptosis or the mitochondrial respiratory chain that are known to be myristoylated (Table 2), we also predict amino-terminal myristoylation of a homolog of TOM40, a protein thought to be a central component of the mitochondrial import machinery [66], presumably acting as a pore-forming [67] sorting station [68]. Unexpectedly for a proposed all-beta integral membrane

Table 4

Domain composition of the set of new predictions

Number of entries	ID	Description
57	PF00097	Zinc finger, C3HC4 type (RING finger)
43	PF00069	Protein kinase domain
35	PF00481	Protein phosphatase 2C
26	PF00023	Ankyrin repeat
21	PF00931	NB-ARC domain
20	PF00646	F-box domain
18	PF04782	Protein of unknown function (DUF632)
18	PF04783	Protein of unknown function (DUF630)
16	PF00036	EF hand
14	PF00135	Carboxylesterase
14	PF00487	Fatty acid desaturase
13	PF00651	BTB/POZ domain
13	PF05049	Interferon-inducible GTPase (IIGP)
13	PF00233	3'5'-cyclic nucleotide phosphodiesterase
12	PF00443	Ubiquitin carboxyl-terminal hydrolase
12	PF00085	Thioredoxin
11	PF00001	7 transmembrane receptor (rhodopsin family)
11	PF00047	Immunoglobulin domain
9	PF00622	SPRY domain
9	PF00628	PHD-finger
9	PF00096	Zinc finger, C2H2 type
8	PF01459	Eukaryotic porin
8	PF03011	Plasmodium falciparum erythrocyte membrane protein (PFEMP)
8	PF00400	WD domain, G-beta repeat
8	PF00595	PDZ domain (Also known as DHR or GLGF)
8	PF00560	Leucine rich repeat
7	PF00520	Ion transport protein
7	PF01135	Protein-L-isoaspartate(D-aspartate) O-methyltransferase (PCMT)
7	PF00514	Armadillo/beta-catenin-like repeat
7	PF00989	PAS domain
7	PF00134	Cyclin, amino-terminal domain
7	PF00169	PH domain
6	PF00300	Phosphoglycerate Phos Phosphoglycerate mutase family
6	PF01145	SPFH domain / Band 7 family
6	PF01582	TIR domain
6	PF02174	PTB domain (IRS-1 type)
6	PF00462	Glutaredoxin
6	PF03193	Protein of unknown function, DUF258
6	PF00515	TPR domain
6	PF00240	Ubiquitin family
6	PF01265	Cytochrome c/c1 heme lyase
6	PF00702	haloacid dehalogenase-like hydrolase
6	PF04641	Protein of unknown function, DUF602
6	PF00271	Helicase conserved carboxy-terminal domain
5	PF00070	Pyridine nucleotide-disulphide oxidoreductase
5	PF01417	ENTH domain
5	PF00270	DEAD/DEAH box helicase
5	PF02230	Phospholipase/Carboxylesterase

Table 4 *(Continued)*

Domain composition of the set of new predictions		
5	PF00891	O-methyltransferase
5	PF00046	Homeobox domain
5	PF05003	Protein of unknown function (DUF668)
5	PF00010	Helix-loop-helix DNA-binding domain
5	PF00170	bZIP transcription factor
5	PF00018	SH3 domain
5	PF00255	Glutathione peroxidase
5	PF00017	SH2 domain
5	PF00089	Trypsin
5	PF00153	Mitochondrial carrier protein

protein of the outer mitochondrial membrane [69], sequences from human, mouse, rat, frog and fly reveal a conserved predicted myristoylation site at the amino terminus (Table 5).

Interestingly, the amino termini of cytochrome *c*-type heme lyase (CCHL) homologs in worm, fly, zebrafish, frog, mouse and human (for example, MI 7512486) also share a conserved predicted myristoylation motif. It should be noted that, in the human sequence, a proline at motif position 4 is rather unfavorable in terms of flexibility of the substrate to adopt the proper conformation in the NMT binding pocket. Fungal homologs also have amino-terminal glycines. Although possible myristoylation cannot be excluded, three large aromatic residues that are difficult to accommodate in the narrow NMT substrate-binding pocket follow these conserved glycines, which might also suggest a new, as yet unknown, role for the amino-terminal glycine conservation. CCHL catalyzes the conversion of apocytochrome *c* to holocytochrome *c* by attaching a heme group [70], which is an important step required for cytochrome *c* biogenesis and transport through the membranes [71]. Deletion of the mammalian gene encoding CCHL is associated with X-linked microphthalmia with linear skin defects syndrome [72].

Myristoylation of lung cancer candidate FUS1

We also show *in vitro* myristoylation of the amino terminus of human FUS1 (Table 6 and Additional data file 1), an apparent tumor suppressor. This protein is missing or carboxy-terminally truncated in lung-cancer cells [73,74]. The exact mechanism of inactivating its function as a tumor suppressor is unknown, but is most likely to be independent of the myristoylation status as the truncated protein form would still contain the predicted myristoylation motif. It is possible that CpG methylation downstream of the unmethylated 5' promoter region [73] could account for inactivation, as the CpG island appears to extend over the whole coding region (as tested with methods described in [75]). For example, in the case of the HIC1 tumor suppressor, CpG methylation was found to occur predominantly in introns and exons instead of the 5' promoter region. This methylation appears to be characteristic for cancer types and stages [76].

Myristoylation and potassium channels

Myristoylation has been predicted for specific modulatory proteins interacting with voltage-gated potassium channels [77]. In this work, we demonstrate *in vitro* myristoylation of the human representative of the KChIP1 subgroup of Kv4 channel-interacting proteins (Table 6 and Additional data file 1). The myristoylation motif is conserved in mouse, rat and human and, in support, there exists homology to hippocalcin, frequenin and neuronal calcium sensor proteins [77], whose myristoylation has already been shown experimentally to be of functional importance [78]. However, amino-terminal truncation experiments with KChIP1 suggest that, in the investigated system, the modulatory activity is sufficiently represented by the more conserved central domain containing EF-hand Ca^{2+}-binding motifs [79]. On the other hand, the role of a myristoyl lipid anchor for KChIP1 could also lie in subcellular targeting specificity. Voltage-gated potassium channel isoforms (for example, Kv2.1 and Kv1.5) can localize to distinct lipid-raft populations [80]. In the context of the similar targeting specificity of acyl chains [81], it is interesting that isoforms KChIP2 and KChIP3 that lack the predicted myristoylation motif are palmitoylated, and their lipid modification controls the plasma-membrane localization of the associated channels [82].

Furthermore, several mammalian and avian homologs of the strong inward rectifying potassium channel Kir2.1 are predicted to be myristoylated. The fact that the motif is evolutionarily retained over 11 organisms would rather support the functional importance of such conservation. The *in vitro* results show that the amino terminus of human Kir2.1 can productively interact with NMT (Table 6 and Additional data file 1). None of the other Kirs is predicted to be

Table 5

Examples of proteins predicted to be myristoylated

Protein(s)	Total number in MYRbase	MI in MYRbase (number in cluster)	Taxonomic range
*Disease-resistance proteins	32	25300600 (27) NB-ARC+LRR, 25453543 (5) TIR+NB-ARC+LRR	Plants
†S/T protein phosphatase 2C gamma (1G)	30	2130393 (6)(ptc3), 26450759 (6), 4505999 (5)(1G), 25411959 (4), 22326510 (3), 6728987 (3), 6319415 (3)(ptc2/3), 23172576 (3)Dm,23615237 (2)Pf, 22326564 (2), 7488279 (2), 15239565 (1), 23483487 (1)(1Gpf), 24417194 (1), 25341907 (1), 21626866 (1)(1Gdm), 6319601 (1)(ptc4)	Mammals, insects, fungi, plants, apicomplexa
S/T-kinases (Crk1(cyclin-dependent kinase cdc2-related)-related)	29	22327464 (11), 27817936 (10), 14532736 (4), 25402555 (3), 20805217 (1)	Plants
cGMP-specific 3',5'-cyclic phosphodiesterase 8A, 8B, 9A	23	6166014 (10) 9A, 27479159 (9) 8AB, 21626649 (2) Dm, 2706887 (2)	Mammals, insects
*Thioredoxin	20	15231958 (11), 1388078 (4), 28209505 (2), 28372832 (2), 28372834 (1)	Plants
Putative RING zinc finger proteins (ubiquitin ligases?)	17	20279471 (10), 27500282 (4) Hs Mm, 7292914 (1) Dm, 3874246 (1) Ce, 23488532 (1) Pf	Plants, mammals, insects, apicomplexa
Interferon-inducible GTPase; 47 kDa GTPase IIGP	16	25029534 (16)	Mammals
Mitochondrial import receptor subunit TOM40	16	12230369 (15), 6539563 (1)	Mammals, amphibians, insects, plants
‡Germ cell less GCL	15	21314704 (13), 7304006 (1), 6425507 (1)	Mammals, fish, insects, worms
Inward rectifier potassium channel Kir2.1	13	26336911 (13)	Mammals
Sphingolipid delta 4 desaturase DES1/DES2	12	27717299 (12)	Mammals, insects, worms
ARF GTPase-activating protein (PH, ArfGAP, ankyrin domains)	12	22051029 (12)	Mammals
Naked cuticle 1,2 homolog	12	22028145 (11), 27729789 (1)	Mammals
Protein-L-isoaspartate carboxylmethyltransferase	12	27882417 (10), 27713894 (1), 3133008 (1)	Mammals, amphibians, worms
Cyclin-box carrying protein 1	11	1078903 (11)	Mammals, insects, worms

Amino-terminal peptides of single representatives shown to be myristoylated in *[24] and †[152]. ‡Amino-terminal glycine to alanine mutation prevents nuclear envelope localization [153].

myristoylated, and a lipid anchor unique to Kir2.1 could be responsible for differing subcellular and submembrane localizations of isoforms, similar to those observed among other potassium channels [80,83].

Consequently, it is not surprising that artificial introduction of a myristoylation motif into the isolated cytosolic carboxy terminus of another Kir (Kir3.1) is sufficient to co-localize the fragment with intact channels [84]. After synthesis in the ER, carboxy-terminal signals direct the export of Kir2.1 to the Golgi complex [85,86]. Stockklausner *et al.* [87] showed that a cluster of positive charges in the amino terminus that are conserved with Kir1.2/4.1 can be held responsible for post-Golgi trafficking of Kir2.1 to the plasma membrane. There, Kir2.1 is localized to lipid rafts [87]. It was hypothesized that interaction with phosphoinositides (PIP_2) could be necessary for lipid-raft association but Stockklausner *et al.* [87] found that mutation of the corresponding residues had no effect on lipid-raft targeting. Other work suggests the involvement of PIP_2 interaction in regulating gating processes [88].

The functional role of the predicted myristoyl anchor for these channel proteins with respect to submembrane localization (lipid rafts), conformational flexibility of the amino terminus or protein-protein interactions remains to be investigated.

Myristoylation and targeting to lipid rafts

Many pieces of evidence suggest that not only the many different membranes in the cell, but also the microcompartments therein, can be distinguished by their lipid and protein compositions [89,90]. Proteomic analyses of detergent-

Table 6

Diverse amino termini of selected sequences shown to undergo *in vitro* myristoylation

Human protein	MYRbase identifier	Myristoylation motif
Immunoglobulin μ-heavy chain	27650590	GGTFSSYAISWV**R**QAPG
Immunoglobulin λ-light chain	4761381	GQTASIT*C*SGD**K**LGD**K**Y
47 kDa GTPase IIGP	23682869	GQLFSS**RR**SEDQDLSSS
Ubiquitin hydrolase Ubq-M	5454156	G**KKR**T**K**G**K**TVPIDDSSE
Lung cancer candidate FUS1	6005760	GASGS**K**A**R**GLWPFASAA
Potassium channel interacting protein KChIP1	7657247	GAVMGTFSSLQT**K**Q**RR**P
Potassium channel Kir2.1	2282068	GSV**R**TN**R**YSIVSSEEDG

Residues indicated in bold type are positively charged; The C denoted in italic type indicates a palmitoylatable cysteine.

resistant lipid-raft fractions from different cells [91-94] unveil partly overlapping but also cell-type specific populations of proteins. This includes several known myristoylated proteins that are involved in various signaling pathways (for example, G_{α} [95], Src-family tyrosine kinases [96], NAP-22 [97], endothelial nitric oxide synthase [98], rapsyn [99], annexin XIII [100], MARCKS [101], CAP23 [102]) and Nef [103] or participate in viral budding (for example, HIV Gag [104]).

In addition, we predict myristoylation for two more proteins of unknown function that co-localized with detergent-resistant membrane fractions. Each appears in a separate proteomic analysis of lipid rafts in Jurkat T-cells [91,94]. The first (161 amino acids, MI 8923579) protein has a conserved myristoylation site plus potentially palmitoylated cysteines in human, rat and mouse homologs. Interestingly, the second protein (227 amino acid, MI 12834233), which has a predicted central coiled coil region, was not only detected in detergent-resistant fractions of T cells in a dynamic, receptor-activation-dependent manner [94], but also in a chaotrope-resistant fraction derived from nuclei of neuroblastoma N2a cells that contains clusters of proteins localizing to the outer nuclear membrane [105]. It will be interesting to establish possible dynamic trafficking between distinct subcellular compartments of this protein (with highly conserved orthologs in human, mouse and frog, and more diverged in zebrafish and fly).

Existing proteomic analyses focus on the most abundant protein representatives within membrane fractions and can also only identify a temporally limited subset of dynamically targeted proteins. Therefore, it would not be surprising to find several more myristoylated proteins to participate in highly specific signaling events by reversible trafficking to and from membrane microdomains.

Targeting experiments suggest differences in submembrane localization that depend on the anchor type. For example, membrane partition clustering of non-dimerizing green fluorescent protein variants fused to peptides containing double acylation (for example, myristoyl+palmitoyl) or prenylation (for example, farnesyl, geranylgeranyl) consensus signals was shown to differentially depend on lipid raft disruption by cholesterol depletion [81]. However, targeting experiments with a few artificial constructs varying only in lipid anchor type cannot take into account the complexity of the interplay between different MAFs that coexist in real proteins. Hence, it is difficult to generally attribute targeting specificity purely to occurrences of particular lipid anchors. Nevertheless, a myristoyl anchor followed by a palmitoyl anchor seems to be a strong indication for targeting to the plasma membrane and also eventually to lipid rafts.

Conclusions

We have evaluated the evolutionary conservation of predicted glycine myristoylation within sequences of the eukaryotic section of GenBank. We find that a small number of very large families of myristoylated proteins seems to be opposed to a large number of very small protein families (Figure 1). Our approach allows the summarizing of the currently available knowledge of experimentally verified (Table 2) as well as newly predicted myristoylated eukaryotic proteins (examples in Table 5, throughout the text and in MYRbase).

We estimate that a substantial fraction of new predictions is associated with proteins whose function has not been known in the context of myristoylation (Tables 1, 4, 5) so far. By demonstrating *in vitro* myristoylation of amino termini derived from several interesting functionally diverse proteins (Table 6 and Additional data file 1), we strengthen the prediction results for this lipid modification. Nevertheless, we also want to emphasize that the actual *in vivo* modification of respective proteins remains to be established.

The myristoylation motif typically acts together with several other MAFs but can also be fully replaced by them within

protein families during evolution. The concerted effect of these MAFs defines subcellular localization and targeting specificity. We classify five coMAFs that most commonly co-occur with myristoyl lipid modifications (Table 3, Figure 2).

The results of this work are summarized and accessible through the web-based MYRbase [18] that aims to stimulate further experiments. A considerable amount of work will be necessary in the future to fully cover and understand the functional spectrum of the myristoylated proteins.

Materials and methods

In vitro myristoylation assay

The amino-terminal eight amino acids of selected predictions (Table 6) were synthesized with two minor modifications. To avoid racemization of the carboxy-terminal cysteine, one serine residue was added to the terminus of MI 4761381. Threonine and valine were added to the octapeptide of MI 5454156, as the dominance of positive charges was expected to result in difficulties in purification and handling. Peptides were purchased from Sigma Genosys (Japan). NMT was prepared as described previously [106] and the cDNA of yeast NMT was a gift from J. Gordon. Peptides were myristoylated *in vitro* essentially as described in [107] and analyzed by mass spectrometry. MALDI-TOF mass spectrometry was carried out on a Voyager DE Pro (PE Biosystems) and the matrix was 10 mg/ml alpha-cyano-4-hydroxycinnamic acid (Sigma) in 0.1% TFA-50% acetonitrile solution. The spectra were displayed and analyzed using the GRAMS-MS software and the spectra were calibrated using calibration mixture 1 or 2 (PE Biosystems). The spectra are available in portable document format (PDF) from the MYRbase homepage [18]. As positive controls, peptides of proteins known to be myristoylated *in vivo* have been analyzed, as well as corresponding peptides without modification by NMT as negative controls. Differences between theoretical and experimental masses for peptides ending in serine are most likely due to carboxy-terminal sodium salt formation. MYRbase identifiers can be found in the upper left corner of each page.

Additional data files

The mass spectra of the investigated peptides are available (Additional data file 1).

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