PHYLOGENY OF THE TRAF/MATH DOMAIN

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This chapter is dedicated to the memory of José Zapata Ruiz, my beloved father and strongest supporter.

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Abstract

The TNF-Receptor Associated Factor (TRAF) domain (TD), also known as the meprin and TRAF-C homology (MATH) domain is a fold of seven anti-parallel β -helices that participates in protein-protein interactions. This fold is broadly represented among eukaryotes, where it is found associated with a discrete set of protein-domains. Virtually all protein families encompassing a TRAF/MATH domain seem to be involved in the regulation of protein processing and ubiquitination, strongly suggesting a parallel evolution of the TRAF/MATH domain and certain proteolysis pathways in eukaryotes.

The restricted number of living organisms for which we have information of their genetic and protein make-up limits the scope and analysis of the MATH domain in evolution. However, the available information allows us to get a glimpse on the origins, distribution and evolution of the TRAF/MATH domain, which will be overviewed in this chapter.

Introduction.

TNF-Receptor Associated Factors are a family of proteins that were initially identified for their capability of interacting with and regulating different members of the TNFR family ¹⁻⁴. TRAFs are characterized by a C-terminal region encompassing about 180 aminoacids, forming a 7-8 anti-parallel β -sheets fold (TRAF-C domain) (Fig.1), which is preceded by a coiled coil (TRAF-N) domain ¹. Meprins, a family of extracellular metalloproteases, also contain a C-terminal domain with high sequence homology to the TRAF-C domain ⁵ which is predicted to form a similar anti-parallel β -sheets fold ⁶. Therefore, the TRAF-C domain is also known as the meprin and TRAF Homology (MATH) domain.

MATH domain (MATHd) encompassing proteins are found associated to a discrete set of other protein domains, including peptidases, filamin and RluA domains, broad-complex, tramtrack and bric a brac (BTB) domain, tripartite motif (TRIM), astacin domain and RING and Zinc finger domains (Fig. 2).

Remarkably, all different MATHd encompassing protein subfamilies seems to have a role in the regulation of protein processing. In this regard, the members of the MATHd ubiquitin proteases (UBPs) and meprins have intrinsic protease activity, while TRAF, TRIM/MATHd and BTB/MATHd proteins, as well as the MATHd structural homolog SIAH, appear to function as E3 ubiquitin ligases (Fig. 3).

The MATHd is found in a variety of proteins in humans ⁷, and blast searches using as bait the MATHd of TRAF2 readily produced hundreds of hits widely distributed among *eukaryota*. Thus, MATHd encompassing proteins are currently found in 72 species of eukaryotes and 3 iridoviruses (Fig. 4), but this number will grow as we gather more information of the genomes of new organisms. Interestingly, genes encoding MATHd proteins have been found in lower eukaryotes such as protozoa and unicellular fungi, but not in any of the *prokaryota* and *archaea* species for which the full genome sequence is known, suggesting that the MATHd might have appeared early in the evolution of eukaryotes.

Next we will overview the different types of MATHd encompassing proteins according to the other protein domains they are found associated to.

Ubiquitin Proteases (UBPs)

Ubiquitin proteases (UBPs), also known as deubiquitinating enzymes, are a family of cysteine proteases involved in the removal of ubiquitin from proteins. There are five UBPs subclasses, each of them encompassing a different type of deubiquitinase domain: 1) ubiquitin specific proteases (USP), 2) ubiquitin C-terminal hydrolases (UCH), 3) ovarian tumor proteases (OTU), 4) Machado-Joseph disease protein domain proteases (MJDP) and JAMM motif proteases ⁸. ⁹. Some UBPs are found associated to the proteasome where they remove the poly-ubiquitin chains from the proteins that are being degraded by the proteasome, allowing for ubiquitin recycling. In contrast, other UBPs remove the poly-ubiquitin chains from proteins to prevent their degradation by the proteasome ⁸. This is the case of ubiquitin specific proteases (USP), which account for the majority of UBPs found in mammalian genomes. USPs seem to counterbalance E3 ubiquitin ligases by removing ubiquitins from proteins, thus preventing their degradation, Indeed, a coevolution of both types of enzymes has been suggested ¹⁰.

MATHd encompassing UBPs are found in unicellular organisms, such as *Mycetozoa* (*Dictyostelium*), *Alveolata* (*Cryptosporidium*) and *Fungi* of the *Ascomycota* and *Basidiomycota* phyla. They are also found in plants (*Arabidopsis*, *Oryza*) and in *Metazoa* (Fig. 4). Interestingly, there is a remarkable conservation among organisms of both, the size of the protein, which contains over 1000 amino-acids, and its domain organization, with the MATHd located at the N-terminus of the protein followed by the ubiquitin protease domain. Theoretical phylogenetic analysis based on

the homologies of all MATHd protein sequences available showed that all UBP/MATHd proteins form a distinctive cluster, and that fungi, metazoa and plant UBP/MATHd proteins were distributed in three well defined subgroups (Fig. 5). One exception corresponds to the UBP/MATHds from *Cryptosporidium* (*Alveolata*), which according to the protein sequence comparison, contain the most divergent MATHds of all analyzed (Fig 6, bottom).

The analysis of the MATHd sequences shows a unique conservation among UBPs of the residues ExDWGF in the β -sheet 7 (Fig. 6), which correspond to residues ¹⁶²ENDWGF¹⁶⁷ of human USP7¹¹. Furthermore, in all cases the protease domain is a peptidase C19C (cd02659), a subgroup of the peptidase 19 group of ubiquitin carboxyl-terminal hydrolases that removes ubiquitin molecules from polyubiquitinated peptides by hydrolyzing bonds involving the carboxyl group of the C-terminal Gly residue of ubiquitin. It is also noteworthy that 35 out of 37 species included in our UBP/MATHd analysis contain only one UBP/MATHd. Only two species (*O. sativa* and *C. elegans*) contain two different UBP/MATHd genes. Altogether, these results illustrate the strong conservation of UBP/MATHd genes in eukaryotes, suggesting that UBP/MATHd proteins in different species might be orthologs.

The only UBP/MATHd protein found in the human and mouse genome is USP7. The crystal structure of the MATHd of USP7 has been recently solved¹¹. It is a fold of eight anti-parallel β -sheets very similar to the TRAF-C domain of TRAFs (Fig. 1). The USP7's MATHd has been implicated in substrate recognition. Indeed, USP7 was originally identified by its interaction with ICP0 protein from herpes simplex virus¹². USP7 also interacts with p53 and Mdm2¹³, and Epstein-Barr virus nuclear antigen 1 (EBNA1) protein¹¹.

Interestingly, USP7's MATHd accommodate the MATHd-binding-motifs of p53, Mdm2 and EBNA1 in a shallow surface groove in the middle of the β -sandwich which is much alike the TNFR-peptide binding crevice located across the edge of the β -strands of TRAFs¹⁴⁻¹⁷. However, the mode of peptide binding and the adopted conformation of the bound peptide differ significantly from previously observed TRAF-peptide interactions^{11, 13, 18}. Most interestingly, the key aminoacids in USP7's MATHd interacting with all these different substrates are ¹⁶⁴DWGF¹⁶⁷, which shape the peptide-binding pocket and, as indicated above, are distinctively conserved among UBPs. Other aminoacids participating in interaction account for the differences in affinity of the different

substrates, but interactions with the DWGF, in particular with Trp165, are critical for the specificity of the binding^{11, 13, 18}.

Structural and competition data support that Mdm2 has a higher affinity for binding USP7 than has p53^{13, 18}. Mdm2 is a ubiquitin ligase that regulates p53 activity, traffic and degradation¹⁹. It has been shown that USP7 might stabilize Mdm2 and promote p53 degradation^{20, 21}. However, functional²² and structural^{13, 18} data shows that USP7 also interacts with and deubiquitinates p53, thus preventing its degradation by the proteasome. Therefore, USP7 appears to play multiple roles in regulating the p53-Mdm2 pathway.

Interestingly, EBNA1 seems to have the higher affinity for USP7 of all known substrates, and functional studies have shown that interaction of EBNA1 with USP7 protects cells from apoptotic insults by preventing USP7-mediated deubiquitination of p53 *in vivo*, resulting in p53 degradation. Indeed, it has been proposed that this is the mechanism by which EBNA1 contributes to the survival of Epstein-Barr virus-infected cells¹¹. However, Mdm2 activity would also be affected by EBNA1 association to USP7, which as a result might prevent p53 degradation. Additional work is necessary to clarify the role of USP7 and their substrates in the control of p53-mediated activities.

It is noteworthy that p53, Mdm2 and related proteins are only found in vertebrates. In addition, herpes viruses only infect vertebrates. It seems likely that Epstein Barr virus EBNA1 and herpes simplex virus ICP0 have co-evolved along with p53 and Mdm2 to efficiently out-compete these two proteins from binding USP7, thus efficiently hijack-jacking p53-mediated pathways. Consequently, none of these proteins can be the original substrate(s) for USP7 and its putative orthologs in lower eukaryotes and plants. Considering the high conservation of MATHd/UBPs in evolution, it should be expected that this still unidentified primordial substrate(s) should be essential for all these organisms and should also have remained well conserved during evolution.

There is an additional set of MATHd sequences from *Arabidopsis thaliana* and *Dictyostelium discoideum* sharing a high homology with UBP/MATHd but lacking the ubiquitin protease domain. They still have the ExDWGF motif or close variations of it, further suggesting that they arose from a common ancestor (Fig 6).

RluA and Filament domains

MATHd encompassing proteins that also contain a pseudo-uridine synthase Rlu domain are found in *Alveolata*. One of these putative proteins is encoded by the genome of *Cryptosporidium parvum* (CAD98470), and the other one is found in *Plasmodium falciparum* (NP703459). The Rlu domain is involved in the conversion of uracil bases to pseudo-uridine²³.

The filament domain represents the N-terminal head region of intermediate filaments that bind DNA²⁴ and is found associated to MATHd in one putative protein from *Arabidopsis thaliana* (AAD23659). The function of this MATHd encompassing protein in *Arabidopsis* is not known.

BTB/POZ domains

The BTB (for broad-complex, tramtrack and bric a brac) domain, also known as POZ (for Pox virus and Zinc finger) domain is an evolutionarily conserved domain broadly distributed in eukaryotes^{25, 26}. The crystal structure of the POZ domain of the human promyelomonocytic leukemia zinc finger (PLZF) protein consists of a cluster of alpha-helices flanked by short beta-sheets at both the top and bottom of the molecule, that tightly homodimerizes by intertwining both domains producing an extensive hydrophobic interface^{26, 27}.

Proteins encompassing a MATH domain and a BTB/POZ domain are broadly represented among eukaryotes (Fig. 4). Thus, proteins of this group are found in lower eukaryotes, such as *Trypanosomatidae* (*Euglenozoa*), and *Coccidia* (*Alveolata*), plants (*Viridiplantae*) including both eudicotyledons and monocotyledons (*Liliopsidae*), and in *metazoa*. However, MATH/BTB proteins have not yet been found in *fungi*. The vast majority of BTB/MATHd proteins have an N-terminal MATHd and a C-terminal BTB domain, but there are also examples of genes encoding proteins with a N-terminal BTB domain and a C-terminal MATHd. Also, some proteins contain MATHd/BTB domains in tandem (Fig. 2).

A large cohort of putative genes encoding MATH/BTB proteins (about 49) are found in *Caenorhabditis elegans*. These genes would encode proteins with an N-terminal MATHd and a C-terminal BTB/POZ domain. One of these proteins, the maternal effect lethal (MEL)-26, has been shown to regulate the transition from meiotic to mitotic chromosome segregation²⁸. MEL-26 is part of the CULLIN (CUL)-3 ubiquitin ligase complex²⁹⁻³¹. CUL-3 is an E2 ubiquitin conjugating enzyme. MEL-26 interacts with CUL-3 through its BTB/POZ domain and acts as an E3 ubiquitin

ligase, which is required for ubiquitination and degradation of the meiosis defective (MEI)-1 protein^{29, 30}. Three other MATH/BTB proteins that interact with CUL-3 were identified in *C. elegans*²⁹

SPOP (also known as TEF2)^{7, 32} and its close relative TEF4³³ deserve special mention among the MATHd/BTB proteins. These two genes are strikingly conserved in metazoans, including *nematoda*, *arthropoda* and *chordata* (Fig. 7). SPOP (TEF2) has been shown to form an E3 ubiquitin ligase complex with CUL-3. SPOP (TEF2) regulates the ubiquitination and subsequent proteasome-mediated degradation of several proteins, including the polycomb group protein BMI1, which regulates survival and senescence of stem cells and cancer cells³⁴⁻³⁶, the variant histone MACROH2A, which is required for the silencing of one of the two X chromosomes in XX female somatic cells³⁷ and DAXX, a multifunctional protein involved in the regulation of cell death (³⁸ and references therein). These examples further extend the role of MATHd/BTB proteins as E3 ubiquitin ligases. It is worth noting, that the MATHds of SPOP and relatives are more conserved than their corresponding BTB domains. For instance, the percentage of identity between the BTB domains of *C.elegans* and *homo sapiens* is 56%, while the percentage of identity of their MATHds is 89%, thus suggesting a strong evolutionary pressure to maintain the MATHd of SPOP and relatives virtually unchanged from worms to humans.

It is worth mentioning that rice (*Oryza sativa*) contains the larger number of MATHd/BTB proteins (about 70) of all the species studied so far, including other monocotyledons (*liliopsidae*) such as wheat (*Triticum monococcum*) and sorghum (*Sorghum bicolor*). The vast majority of these rice's MATHd/BTB proteins have the MATHd at the N-terminus and the BTB/POZ domain at the C-terminus.

In summary, proteins combining the MATH domain with the BTB domain are found very early in the evolution of eukaryotes. The available evidence suggests that they might link specific protein substrates to ubiquitin ligase complexes. Additional research will be required to identify these putative substrates and to determine whether all the MATHd/BTB proteins found in diverse organisms have this function, particularly in rice and *C.elegans* that contain the larger number of *MATHd/BTB* genes identified so far. These studies will also help to elucidate whether each of these proteins is specific for a particular substrate or whether a functional redundancy exists, with different MATHd/BTB proteins targeting the same substrates.

MATH domain-only proteins.

There is a large number of hypothetical proteins containing one or multiple MATH-domains in tandem and lacking of any other distinguishable associated protein domain (Fig. 3). These proteins are found in plants (*Arabidopsis*, *Medicago*, *Oryza*, and *Sorghum*), lower *eukaryota* (*Trypanosoma*, *Dyctiostelium*, *Theileria* and *Plasmodium*), and in lower *metazoa* such as nematodes (*Caenorhabditis* sp) (Fig. 4). Many of these proteins are identified by conceptual translation of the genome and therefore some of them could be part of larger genes that might encompass other protein domains. However, it is noteworthy that these MATHd-only sequences represent the majority of the MATHd-encompassing proteins identified in *Arabidopsis thaliana* and *Caenorhabditis* sp, but are not very common in other species, including some whose genome have been fully sequenced. The MATHd of these MATHd-only proteins is in general more similar to that of MATHd/BTB proteins (Fig 7), but some of them have more similarity to the MATHd of UBP proteins (see above and Fig 6). The function of these MATHd-encompassing proteins as well as the significance of their elevated number in *Arabidopsis* and *Caenorhabditis* are not yet known.

It is noteworthy that TRAF1 could be considered as a MATH-domain only protein. However, TRAF1 is readily identified as a member of the TRAF family by the high homology of its MATH domain to those of other members of the TRAF family.

TRIM37

A group of MATHd encompassing proteins appears associated to a combination of protein domains known as the tripartite motif (TRIM). The tripartite motif is composed by a RING finger domain, followed by a special type of zinc finger domain coined the ZF-B box and a coiled coil (Fig 2). In humans, there are 37 genes encoding proteins encompassing a tripartite domain ³⁹ of which only one (*trim37*) contains a MATHd ⁷.

Mutations in the *Trim37* gene in humans are causative of Mulibrey Nanism, an autosomal recessive growth disorder that affects several tissues of mesodermal origin ⁴⁰. Mulibrey Nanism is characterized by severe growth failure of prenatal onset, constrictive pericardium with consequent hepatomegaly, hypoplasia of several endocrine glands, fibrodysplasia of bones and muscle and sterility ⁴¹⁻⁴³. Similar to other members of the MATHd and TRIM ⁴⁴ families, TRIM37 seems to function as a E3 ubiquitin ligase, although its physiological substrate(s) is still unknown ⁴⁵.

TRIM/MATHd proteins form a distinctive cluster in the tree (Fig. 5) and have a high degree of conservation (Fig.7). Overall, the TRIM's MATHd is more similar to those of BTB/MATHd proteins than to any other MATHd-encompassing protein subclass. Orthologs of TRIM37 are only found in *Coelomata* (*Metazoa*) (Fig 4). Indeed, TRIM37 is found in some insects such as bees (*Apis mellifera*, *hymenoptera*) but not in others such as *Drosophila* or *Anopheles* (*both Diptera*). However, there are two genes encoding a MATHd-only protein in *Anopheles gambiae* that have high similarities to the MATHds of the TRIM37 orthologs. TRIM37 seems to be very well conserved in *deuterostomia*, including the echinoderm *Strongylocentrotus purpuratus* (sea urchin) (XP791708; not shown) and *vertebrata*, including mammals, birds and fishes. Interestingly, although TRIM37 is found in zebra fish (*Danio rerio*) and spotted green pufferfish (*Tetraodon nigroviridis*), the latter seems to contain a *trim37* gene with a truncated MATHd. It is interesting to mention the high level of conservation between different TRIM37 orthologs. For instance, the MATHd of *Apis mellifera* TRIM37 is 69% identical to rodent's TRIM37, 74% to chicken's, 71% to zebra fish's and 74% to human's. Future research will determine the function of TRIM37 in insects and non-mammalian vertebrates and whether it also has a role in development, as it does in humans.

Meprins

Meprins are a family of extracellular metalloproteases, which are anchored to the plasmatic membrane and are involved in cleaving growth factors, extracellular matrix proteins, and biologically active peptides^{46, 47}. Meprins have two subunits, α and β , that form hetero- and homo-dimers covalently linked by disulfide bridges. Two of these dimers associate as a tetramer forming the functional meprin complex⁴⁶. The MATHd of meprins is located at the C-terminus and the catalytic astacin-like protease domain at the N-terminus (Fig.2). This MATHd seems to be involved in the oligomeric association of meprin subunits, similar to other MATHds⁶, but it might be also implicated in the recognition, folding and activation of zymogens. The MATHd of meprins form a distinctive cluster in the phylogenetic tree (Fig. 5), and seems to be closely related to the MATHd of TRAFs.

MATHd encompassing meprins appeared late in evolution, as they are only found in vertebrates, including fishes, *amphibia*, birds and mammals.

TRAFs

TNF-Receptors associated Factors (TRAFs) constitute a family of adapter proteins that were initially identified in humans and rodents by their association with different members of the TNF-Receptor (TNFR) family. Humans and other mammals contain seven TRAFs (TRAF1 to 7). The TRAF domain (TD) of TRAFs is always located at the C-terminus (Fig. 2). The crystal structures of the TD of human TRAF2 and TRAF3 showed that it is composed by the 7-8 anti-parallel β -sheet fold (the MATHd, also known as TRAF-C domain) (Fig. 1), followed by a coiled coil (TRAF-N domain). These crystal structures also showed that mammalian TRAFs associate in trimers, with the intertwining coiled coils helping to stabilize the complex¹. TRAF family-members, with the sole exception of TRAF1, also have a distinctive N-terminal RING finger domain followed by a variable number of zinc finger domains (Fig. 2)^{1-4, 48}. Although TRAF7 does not contain a TRAF domain, it was arguably included in the TRAF family because it contains a RING finger domain and zinc finger domains that are similar to those found in other TRAF family members and because it seems to participate in the control of TNF-family signaling as do other members of the TRAF family⁴⁹.

Similarly to the other members of the MATHd superfamily, TRAFs have been implicated in the control of proteolysis. Indeed, TRAF2 seems to function as a E3 ubiquitin ligase that regulates TRAF2 and TRAF3 ubiquitination and proteolysis^{50, 51}. TRAF3 has been shown to regulate NIK proteolysis⁵². However, TRAFs (in particular TRAF2 and TRAF6), working in conjunction with the E2 ligase complex Ubc13/Uev1A, have the ability to self-ubiquitinate or ubiquitinate other proteins with K63-linked poly-ubiquitin chains (Fig. 3). Contrary to K48 ubiquitination, K63 ubiquitination does not target TRAFs or other proteins for degradation, but instead confers TRAF2 and TRAF6 the ability to activate other components of the pathway⁵³.

TRAFs are key components of the Toll Receptor (TLR) family and Tumor Necrosis Receptor (TNFR) family signal transduction. TRAFs regulate the recruitment of kinases and other effector proteins to the activated receptor and other signaling complexes. TRAFs also mediate the activation of downstream components of these pathways, control the subcellular relocalization of the receptor-ligand complexes, and modulate the extent of the response by controlling the degradation of key proteins in the pathway.

Distribution of TRAFs

TRAFs are broadly represented in metazoans (Fig. 4). The older members of the family seem to be TRAF4 and TRAF6. Indeed, in a theoretical phylogenetic tree based on MATHd similarities (Fig. 5), all TRAF6 and TRAF4 orthologs form two distinctive clusters. Interestingly, a putative TRAF4 ortholog is already found in *Hydractinia echinata* (snail fur), a member of the phylum *cnidaria* that includes corals and jellyfishes. Other TRAF4 orthologs are found in *nematoda* (*C. elegans*), insects, such as the fruit fly *Drosophila* (where is known as DTRAF1^{54, 55}), mosquito (*Anopheles gambiae*) and bee (*Apis mellifera*), as well as in sea urchin (*Strongylocentrotus purpuratus*), early chordates, such as *Ciona intestinalis* (BAE93281, not shown in Fig. 4) and vertebrates.

TRAF6 orthologs have been found so far in insects, such as *Drosophila* (where is known as DTRAF2)⁵⁶, mosquitoes and bees, as well as in vertebrates.

As suggested by the analyses shown in Fig. 5, TRAF1, TRAF2, TRAF3 and TRAF5 seem to be the members of the TRAF family that appeared later in evolution. These analyses also suggest that these TRAF family members might have originally diversified from a common TRAF4 ancestor. However, there is a TRAF family member (CAH04636) in the sponge *Suberites domuncula*, a member of the older metazoan taxon still extant⁵⁷. Interestingly, this sponge TRAF shows closer similarities to TRAF3 than to any other TRAF family member when the sequence comparison is performed with the complete protein sequence. Indeed, this TRAF contains a RING and zinc finger domains in its N-terminus and the MATHd at the C-terminus. When only the MATHd is compared, this TRAF still shows higher similarities (about 30% identities) to both TRAF2 and TRAF3 than to any other member of the TRAF family (Fig. 8). Although it is unclear whether this *Suberites domuncula* TRAF is a *bona fide* TRAF3 ortholog, this evidence suggests that TRAF3 might have arisen very early in the evolution of metazoans. TRAF3 is also found in *Drosophila*⁵⁸, where it is known as DTRAF3⁵⁸, and in vertebrates.

In contrast, TRAF1, TRAF2 and TRAF5 have been so far only found in vertebrates. Furthermore, TRAF1 and TRAF2's MATHds share a high percentage of homology, suggesting that they arose not long ago from a common ancestor, probably by gene duplication. A similar scenario might have taken place between TRAF3 and TRAF5, which are closely related to each other. Considering also that TRAF1, TRAF2 and TRAF5 are functionally closer to TRAF3 (see below), it seems likely that these TRAFs diversified from a common TRAF3 ancestor early in vertebrate

evolution. In contrast TRAF3 might have split from TRAF4 much earlier in metazoan evolution. However, a much larger representation of TRAF sequences from early metazoans would be necessary to obtain a clearer picture of the phylogeny of TRAFs in this *phylum*.

Interestingly, several MATHd encompassing genes are encoded by the genome of the slime mold *Dictyostelium discoideum* (Mycetozoa) and among those, some of these proteins have striking similarities to TRAFs (see below). *Dictyostelium* is an unicellular amoebae that grows as independent, separate cells that aggregate under adverse conditions, forming colonies of up to 100,000 cells. These colonies are a true multicellular organism, organizing different tissues composed by differentiated cell types and having the ability of regulating its proportions and morphogenesis⁵⁹. Although the mechanism of formation of the multicellular organism is radically different to that of metazoans, many of the underlying molecular and cellular processes, such as differential cell sorting, pattern formation, stimulus-dependent gene expression, and cell-type regulation seem to be common to *Dictyostelium* and metazoans, thus suggesting that they have arisen from common primitive precursor cells⁶⁰. Genome and proteome based phylogeny analysis supported that *mycetozoa* are a true sister group of the *fungi/metazoa phyla*⁵⁹.

Remarkably, a subset of *Dictyostelium*'s MATHd encompassing proteins contains a N-terminal RING and zinc finger domains similar to those found in TRAFs. Furthermore, the number of aminoacids encoded by these TRAF-like genes is also strikingly similar to that of TRAFs. However, based on the homology of their MATHd, *Dictyostelium* TRAF-like genes are more similar to MATHd/BTB (the case, for instance of DG17, EA61916, EAL61981) and MATHd/TRIM (EAS66947) proteins (Fig. 7). Although still merely speculative, these results suggest that the association of MATHd from BTB proteins to TRAF-like RING (and zinc finger) domains might have been the starting point in TRAFs evolution, an event that might have happened after the branch leading to plants separated from the branch leading to *mycetozoa* and *metazoa*⁵⁹.

Evolutionary aspects of TRAFs function

The analyses on the function of TRAF family members also has provided valuable insights into the evolutionary pathways followed by this protein family.

The older member of the TRAF family for which we have functional information is the cnidarian *Hydractinia echinata* HyTRAF1⁶¹. HyTRAF1 has a N-terminal RING finger and 5 zinc finger domains and its TD is more similar to TRAF4 than to any other TRAF. One isoform of this

TRAF family member lacking the RING and one of the zinc finger domains is exclusively found at the larval and early metamorphic stages, and seems to regulate apoptosis mediated by c-jun N-terminal kinase (JNK) signaling. Additional functional information is provided by *Drosophila*'s DTRAF1 (TRAF4 ortholog), which has 7 zinc finger domains but no RING finger domain^{54, 55}. DTRAF1 seems to control apoptosis and imaginal discs and photosensory neurons development by regulating the activation of JNK and its upstream kinases Hep and DTAK1⁶². The role of TRAF4 in development is also supported by studies in zebra fish (*Danio rerio*)⁶³ and mouse⁶⁴, where it has been shown to participate in neurulation *in vivo*. TRAF4 involvement in JNK activation has been also demonstrated in mammals^{65, 66}. Indeed, TRAF4 seems to regulate JNK activation by binding to MEKK4 and promoting MEKK4 oligomerization⁶⁶. Most interestingly, mice deficient in MEKK4 develop strikingly similar neurulation and skeletal patterning defects to those observed in TRAF4 deficient mice^{67, 68}.

TRAF6 is also considered one of the older members of the TRAF family. *Drosophila*'s, TRAF6 ortholog, DTRAF2 participates in the control of gene expression by regulating the activation of the NFκB pathways^{56, 62, 69}. Indeed, DTRAF2 is necessary for the expression of the anti-microbial peptides diptericin, diptericin-like protein and drosomycin in response to fungal and gram-positive bacteria infection, thus supporting a role for DTRAF2 (TRAF6) in innate immunity⁶².

The involvement of TRAF6 in the control of innate immune responses has been also demonstrated in mammals. TRAF6 is a common mediator of the Toll-like Receptors (TLR)/Interleukin-1 Receptor (IL1R) superfamily. Eleven TLR family members have been identified in mice and ten in humans, which function as receptors for pathogen-associated molecular patterns (PAMPs) controlling host defense responses as part of innate immunity⁷⁰⁻⁷³. Nine members of this family are already present in *Drosophila melanogaster*⁷⁴ and 11 in *Anopheles gambiae* (mosquito)⁷⁵. However, only 4 *Drosophila* Toll family members have unambiguous orthologs in *Anopheles*, thus reflecting a very dynamic evolution of this family. The majority of the Toll family members in *Drosophila* are involved in the control of developmental patterning, and only two of them are also involved in the control of innate immune responses to fungal and gram-positive bacteria infections. However, these *Drosophila* Toll Receptors do not recognize PAMPs, as do their mammalian counterparts^{76, 77}, thus strongly suggesting that the Toll-Receptor family evolved to recognize specific types of PAMPs after the diversification of *protostomia* and *deutorostomia*.

TRAF6 does not directly interact with the members of the TLR/IL1R family. Instead, receptor activation recruits different adaptors, such as the myeloid differentiation factor 88 (MyD88), TIRAP, and TRAM. Receptor activated MyD88 will induce the formation of complexes containing TRAF6, IRAK family proteins, and IRF-5. In contrast, receptor activated TRAM will induce the formation of complexes containing TRIF, TRAF6 and RIP-1. In both types of complexes, TRAF6 will then associate with the ubiquitin conjugating enzymes Ubc13 and Uev1A. TRAF6 functions as a ubiquitin ligase (E3) that catalyzes the assembly of K63-linked polyubiquitin chains on itself and on IKK γ /NF κ B essential modulator (NEMO) ⁷⁸ (Fig. 3). K63-ubiquitinated-TRAF6 is required for the activation of the MAP3K transforming growth factor β activated kinase-1 (TAK-1), and TAK-1 binding proteins (TAB)-1 and 2 ^{79, 80}. Activated TAK1 will phosphorylate IKK and MKK6, leading to the activation of NF κ B, JNK, p38 and ERK (reviewed in ⁷³).

As indicated above, *Drosophila*'s response to fungal and gram-positive bacteria infections is mediated by Toll Receptors that recruit MyD88 upon activation. Similar to what observed with their mammalian counterparts, MyD88 recruitment of DTRAF2 (TRAF6) to the complex is essential for the response ⁷⁷. However, *Drosophila*'s response to gram-negative bacteria infection involves the protein IMD, the *Drosophila* RIP-1 ortholog, and requires the *Drosophila* E2 ubiquitin ligase Bendless (Uev1A) that catalyzes K63-ubiquitination (⁸¹ and references therein).

Interestingly, RIP-1 is required for TRIF-mediated NF κ B induction in response to TLR-3 and -4 activation in mammalian cells ⁸². In this regard, two recent reports have shown that TRIF utilizes TRAF3 for signaling ^{83, 84}. Indeed, similar to TRAF6, TLRs also recruit TRAF3 through MyD88 and IRAK1 and 4, but rather than activating MAP3K and IKK, which induce pro-inflammatory cytokines, TRAF3 engages TRIF-dependent signaling pathways leading to activation of TBK-1 and IKK- ϵ , inducing the expression of type I interferons and the anti-inflammatory IL-10 ^{83, 84}. Thus, TRAF3 may play important roles in interferon-dependent responses to viral pathogens, as well as in down-regulating innate immune responses *via* its effects on IL-10 production. The function of DTRAF3, the *Drosophila* TRAF3 ortholog ⁵⁸ is still unknown. It will be specially interesting to determine whether *Drosophila* TRAF3 plays also a role in pathogen protection.

As mentioned above, TRAFs were initially identified by their ability to interact with and regulate TNFRs. There are two subclasses of TNFRs. The first subclass contains a cytosolic death domain (DD) and does not normally engage TRAFs for signaling, with the exception of TNFR1, that

recruits TRAF1 and TRAF2 through their interaction with TRADD. These DD-encompassing TNFRs seem to be the older members of the family. Indeed, the older *bona fide* TNFR currently on record is Eiger, a *Drosophila* TNFR family member which encompasses a death domain (DD). Eiger regulates apoptosis by engaging similar signal transduction pathways than those used by other mammalian DD-encompassing TNFRs⁸⁵. The second subclass of TNFRs does not encompass any recognizable protein domain in the cytosolic tail, but its members contain peptide sequences that support the specific interaction of TRAFs. All available information suggests that this TNFR subclass has appeared during vertebrate evolution.

A total of 20 mammalian TNFR family members that utilize TRAFs for signaling have been described so far². TRAF6 regulates some of these TNFRs by specifically interacting with the hexapeptide motif PxExx(Ar/Ac), where the last amino-acid residue is either aromatic or acidic, in the cytosolic tail of TNFRs⁸⁶. In contrast, TRAF1, TRAF2, TRAF3 and TRAF5 recognize the tetrapeptide sequence (P/S/A/T)x(Q/E)E. TRAF4 has been proposed to interact with and regulate the Nerve Growth Factor Receptor (NGFR)^{87,88}, which seems to be one of the older members of the TNFR family, and the glucocorticoid-induced TNFR (GITR)⁸⁹, but its role as a TNFR regulator remains controversial.

The fact that TRAF1, TRAF2, TRAF3 and TRAF5 recognize the same peptide motif in the cytosolic tail of various TNFR family members further support the idea that these TRAFs have diverged recently, most likely from a common TRAF3 ancestor (see above). Interestingly, TRAF1 and TRAF2 are closer to each other than to any other TRAF family member, and functional evidence suggests that TRAF1 regulates TRAF2 activities⁹⁰⁻⁹². Similarly, TRAF3 and TRAF5 are more similar to each other than to any other TRAF. However, TRAF5 is able to activate NFκB, whereas TRAF3 has an inhibitory role on the activation of NFκB mediated by TRAF2 and TRAF5⁹³. Altogether, these results suggest that TRAF2 might have split from TRAF3 first, and later TRAF1 and TRAF5 diverged from TRAF2 and TRAF3, respectively. Interestingly, TRAF1, TRAF2 and TRAF5 are only found in vertebrates. Given the fast diversification of the TNFR family during vertebrate evolution, these members of the TRAF family might have emerged to increase the functional versatility of the members of the TNFR family.

The role of TRAFs in regulating TNFRs function has been more studied in the immune system, where they regulate differentiation, proliferation, and apoptosis, and are essential for

adaptive immune responses. However, there are also some developmental functions associated to TRAFs that seem to be also related to their role in the regulation of TNFR activities. TRAF6 has been shown to be involved in the control of bone formation and hair follicular development through its role in regulating RANK and XEDAR⁹⁴⁻⁹⁸. TRAF4 is also involved in development by controlling neurulation⁶⁴, although whether it is through the regulation of a TNFR family member is not known.

In summary, the available evidence suggests that TRAF4 and TRAF6 are the older members of the TRAF family, with TRAF3 probably diversifying from TRAF4 early in metazoan evolution. TRAF2, TRAF1 and TRAF5 appeared later during vertebrate evolution, most likely splitting from a TRAF3 ancestor. However, as new information on new genomes is gathered, the phylogenetic relations between the different TRAF family members should become more evident.

Viral immune evasion strategies interfering with MATH-dependent pathways

Protein sequence comparison analyses also identified the existence of 3 TRAF proteins in fish infecting iridoviruses. These viral TRAFs form a distinctive cluster in the tree shown in Fig. 5. These viral TRAFs seem to be more related to TRAF2, sharing a 56% homology with zebra fish TRAF2 and 43% homology with human TRAF2. Two of these genes also encode a zinc finger domain at the N-terminus of the molecule (AAL98835 and BAD98248), while the other one (YP249563) is a MATHd-only protein. The function of these iridovirus TRAFs is yet not known, but they are likely to interfere with the signaling of the host TRAF family proteins.

Other viral proteins have been described to interfere with the function of various MATHd proteins of the host, although they are not MATHd proteins themselves. Two of these proteins (LMP-1 and EBNA-1) are encoded by the genome of Epstein-Barr virus (EBV), a member of the herpes virus family that infects over 90% of the world adult population and can cause several immunological disorders and cancer in immunosuppressed individuals⁹⁹. The latent membrane protein (LMP)-1 mimics an activated CD40 and recruits various members of the TRAF family for signaling, but in a seemingly deregulated manner, leading to amplified and sustained B cell activation¹⁰⁰⁻¹⁰⁴. The Epstein-Barr nuclear antigen 1 (EBNA1) protein interacts with USP7 and contributes to the survival of the virus-infected cells by out-competing p53 from binding to USP7 and enforcing p53 degradation¹¹.

Herpes simplex virus type 1 immediate-early regulatory protein ICP0 also interfere with USP7 function. ICP0 stimulates lytic infection and reactivation from latency. ICP0 functions as a ubiquitin E3 ligase that ubiquitinate itself and is subsequently degraded by the proteasome. USP7 removes the ubiquitin chains from ICP0, thus preventing its degradation and preserving its function.^{12, 105}

Kaposi's sarcoma herpesvirus¹⁰⁶ and human molluscipox virus¹⁰⁷ encode homologs of the Flice/caspase 8 inhibitory protein (v-Flip) that interact with TRAFs to interfere with the pro-apoptotic pathways of the host cell.

SIAH

Drosophila seven in absentia (sina) protein and several orthologous genes in other species deserve special mention. Sina proteins are found already in plants and have remain very well conserved in evolution. Humans have two members of this family known as *seven in absentia* homologous (SIAH)-1 and 2. SIAHs are E3 ubiquitin ligases, acting either as single proteins or as part of a multiprotein complex that is analogous to the Skp1-cullin-1-F-box (SCF) complex¹⁰⁸. SIAH and TRAFs have only 10% sequence homology in their C-terminal domains based on a three-dimensional structure-based sequence alignment. However, the crystal structure of SIAH1a shows that its C-terminal domain forms an eight-stranded antiparallel β -sandwich, a fold that is virtually identical to a MATH domain (Fig. 1)¹⁰⁹. SIAH also have a N-terminal RING finger domains followed by 2 Zinc finger domains, besides the C-terminal TD-like domain¹⁰⁹, which further extents the similarities between SIAHs and TRAFs. However, the RING and zinc finger domains found in SIAH and in TRAFs are of different subtypes, further suggesting that these two protein families are not related

Similar to TRAFs and other MATHd encompassing proteins, SIAHs are E3 ubiquitin ligases that regulate ubiquitination and degradation of proteins implicated in a variety of physiological processes, including cell growth, differentiation, angiogenesis, oncogenesis, inflammation and stress (¹¹⁰ and references therein). Interestingly, one of these proteins targeted by SIAH is TRAF2. Indeed, SIAH interacts with TRAF2 through its TD and catalyzes its ubiquitination and degradation under stress conditions¹¹¹.

There are two possibilities to explain the similarities between sina C-terminus fold and the MATHd. First, it would be possible that Sina and MATHd genes were originally related, sharing a common ancestor very early in eukaryotes evolution, but have since then diversified at sequence level beyond recognition while preserving the 8 anti-parallel β -sheet fold characteristic of the MATHd. A second possibility would be that these genes are an unparallel example of convergent evolution, having evolved to develop similar protein structure and function. The answer to this question awaits further research.

Concluding remarks

In this chapter we have overviewed different aspects of the evolution of the MATHd. This fold appeared early in the evolution of eukaryotes and has remained very well preserved. Indeed, MATHd encompassing proteins from distant species can still associate both physically and functionally⁵⁵. Furthermore, MATHd encompassing proteins from different families can also interact with each other *in vitro*⁷. Functional diversification has been achieved by association of the MATHd with other protein domains. However, the overall function of the vast majority of MATHd encompassing protein families involves protein processing by participating in different aspects of protein ubiquitination and protein degradation.

Current efforts on the sequencing of genomes from different organisms, including low eukaryotes with biomedical relevance, will provide new information on the origins of the MATHd. Furthermore, in addition to the large pool of functional data already available on TRAFs, recent reports have provided the first insights into the function of other MATHd encompassing protein families, such as UBPs (USP7) and MATHd/BTB (SPOP, MEL26) proteins. These studies illustrate the seminal roles of MATHd proteins in different aspects of physiology and predict new exciting discoveries to come.

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Figure Legends

Figure 1. Models of the MATHd of human TRAF2 (1CZY), USP7 (2FOP) and SIAH (1K2F) were modeled using protein explorer. The MATHd of DTRAF-1, TRIM37, SPOP, Meprin and examples of Dictyostelium, Arabidopsis and C.elegans MATHds were constructed by threading on the structure of the MATHd of TRAF2 ^{16, 17}, using FFAS and MODELER ^{112, 113}.

Figure 2. Proteins encompassing MATH domains and their association with other protein domains were identified using the Conserved Domain Search service (CD-Search) ¹¹⁴, the Conserved Domain Database for protein classification (CDD) ¹¹⁵ and the Conserved Domain Architecture Retrieval Tool (CDART) ¹¹⁶, three NCBI web-based tools for detection of structural and functional domains in protein sequences. **A.** MATHd encompassing ubiquitin proteases (USP7 family), **B.** MATHd/Rlu proteins. **C.** MATHd-only proteins. **D.** MATHd encompassing broad-complex, tramtrack and bric a brac (BTB) domain protein family. **E.** MATHd/filament protein. **F.** TRAF family. **G.** TRIM37 family. **H.** Meprin family.

MATH: meprin and TRAF homologous domain (cd00121); UBP: ubiquitin protease domain(peptidase C19C, cd02659); RING: Really Interesting New Gene finger domain (cd00162); Rlu; pseudo-uridine synthase Rlu (cd02869); POZ/BTB Pox virus and Zinc finger/broad-complex, tramtrack and bric a brac domain (smart00225); Filament: intermediate filament protein domain (pfam00038); ZF: TRAF-type zinc finger domain (pfam02176); CC: coiled coil, TRAF-N domain; ZF-Box: B box zinc finger (pfam00643); BBC: B-box C-terminal domain, coiled coil (smart00502); Astacin: astacin (peptidase family M12A) domain (pfam01400); MAM: Meprin and mu domain (smart00137); EGF: epidermal growth factor-like domain (smart00181).

Figure 3. MATHd proteins regulate protein processing. The selective degradation of many proteins in eukaryotic cells is carried out by the ubiquitin system ¹¹⁷. In this pathway, proteins are targeted for degradation by covalent ligation at lysine (K)48 to ubiquitin, one of the most phylogenetically well-conserved proteins in eukaryota ¹¹⁸. Ubiquitin-mediated protein degradation by the proteasome is key for the control of numerous biological processes, including the control of cell-cycle, transcriptional regulation, and signal transduction, to mention just a few (reviewed in ¹¹⁷). A different type of ubiquitination (K63) does not result in degradation of the targeted proteins, but instead is necessary for the activation of and signaling from these proteins ⁵³. UBP (USP) can counteract the effect of E3 ligases by removing ubiquitin chains from proteins. Meprins are a family

of extracellular metalloproteases that are involved in cleaving growth factors, extracellular matrix proteins, and biologically active peptides^{46, 47}.

Figure 4. Phylogenetic tree of MATHd in living organisms and viruses. The nomenclature and phylogenetic relations of the NCBI Taxonomy browser (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Taxonomy>) were followed. Only those branches with species containing MATHd encompassing gene(s) are represented. The letters refer to the different subclasses of MATHd encompassing protein families described in Figure 2.

Figure 5. Rooted tree representing the theoretical phylogenetic relations of MATHd encompassing proteins. Sequence similarities are determined using MUSCLE^{119, 120}, and a BLOSUM62 matrix that is based on the likelihood method estimating the occurrence of each possible pairwise substitution¹²¹. Species listed (by alphabetical order): A.f. *Aspergillus fumigatus*; A.g. *Anopheles gambiae*; A.m. *Apis mellifera*; A.t. *Arabidopsis thaliana*; C.a. *Candida albicans*; C.b. *Caenorhabditis briggsae*; C.e. *Caenorhabditis elegans*; C.f. *Canis familiaris*; C.g. *Candida glabrata*; C.h. *Cryptosporidium hominis*; C.n. *Cryptococcus neoformans*; C.p. *Cryptosporidium parvum*; D.d. *Dictyostelium discoideum*; D.m. *Drosophila melanogaster*; D.r. *Danio rerio*; G.g. *Gallus gallus*; G.z. *Gibberella zeae*; H.e. *Hydractinia echinata*; H.s. *Homo sapiens*; K.l. *Kluyveromyces lactis*; L.m. *Leishmania major*; M.g. *Magnaporthe grisea*; M.m. *Mus musculus*; N.c. *Neurospora crassa*; O.m. *Oncorhynchus mykiss*; O.s. *Oryza sativa*; P.b. *Plasmodium berghei*; P.f. *Plasmodium falciparum*; R.n. *Rattus norvegicus*; S.b. *Sorghum bicolor*; S.c. *Saccharomyces cerevisiae*; S.d. *Suberites domuncula*; S.p. *Schizosaccharomyces pombe*; St.p. *Strongylocentrotus purpuratus*; T.a. *Theileria annulata*; T.b. *Trypanosoma brucei*; T.c. *Trypanosoma cruzi*; T.m. *Triticum monococcum*; T.n. *Tetraodon nigroviridis*; T.p. *Theileria parva*; U.m. *Ustilago maydis*; X.l. *Xenopus laevis*; X.p. *Xenopus tropicales*; Y.l. *Yarrowia lipolytica*.

Figure 6 Protein sequence alignment of the MATHd of UBPs.

Multiple alignment was initially performed using all available MATHd sequences, to obtain the same order shown in the tree shown in Fig. 5. Then, only MATHd containing UBP sequences were selected and realigned.

Figure 7. Protein sequence alignment of the MATHd of TRIM and BTB proteins. Multiple alignment was initially performed using all available MATHd sequences, to obtain the same order shown in the tree shown in Fig. 5. Then, MATHd sequences of members of the TRIM, BTB/POZ and MATHd only families were selected and realigned.

Figure 8. Protein sequence alignment of the MATHd of TRAFs and Meprins. Multiple alignment was initially performed using all available MATHd sequences, to obtain the same order shown in the tree shown in Fig. 5. Then, only MATHd sequences of TRAFs and Meprins were selected and realigned..

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Fig. 1

Fig. 2

Fig. 3

Fig. 4

Figure 5

Figure 5, cont

Fig.6

Fig. 7

Fig. 7 (cont)

Fig. 8

00	110	120	130	140	150	160	170	180	190	200
RLSATICVRDQ	..	AANPAEAVPIYKPI	..	..	VAKVKG	..	QRDKNQHY-VYVPHDQLYS	RG	..	YVKKDTLFLFVVFV
RLQAEIIRDQ	..	CTNVADAEDVYTKI	..	..	FVRKKS	..	DDFIQSNQYFHI.PHKVITS	RN	..	YLRNDSMFIEVRVLK
RHKVTFMLLDQ	..	NNREHIDAFAFRPD	..	LASAS	FQRPVN	..	DMNVASGCPPTFLPLTKLQS	..	..	..
EHKVSMILVDQ	..	SHRKHVVTGTFKPT	..	LESSS	FQQRPS	..	DMNVASGCPQFSKLSIFND	DS	..	YCKEDVMYIKCIVDT
RNKVTFMLLDQ	..	NNREHIDAFAFRPD	..	LSSAS	FQRPQS	..	ETNVASGCPFLFPLSKLQSPKHA	..	..	YVKDGTMLFKCIVDT
RNKVTFMLLDQ	..	NNREHIDAFAFRPD	..	LSSAS	FQRPQS	..	ETNVASGCPFLFPLSKLQSPKHA	..	..	YVKDGTMLFKCIVET
NQKVTLMLLDQ	..	NNREHIDAFAFRPD	..	ISSSS	FQRPVS	..	DMNIASGCPFLFCPLSKLDS	KNS	..	YIRDDTIFIKAIIVDT
NQKVTLMLLDQ	..	NNREHIDAFAFRPD	..	VTSSS	FQRPVS	..	EMNIASGCPFLFCPLSKLDG	KNS	..	YIRDDTIFIKAIIVDL
NQKVTLMLLDH	..	NNREHIDAFAFRPD	..	VTSSS	FQRPVS	..	DMNIASGCPFLFCPVSKMEA	KNS	..	YVRDDAIFIKAIIVDL
NQKVTLMLLDQ	..	NNREHIDAFAFRPD	..	VTSSS	FQRPVN	..	DMNIASGCPFLFCPVSKMEA	KNS	..	YVRDDAIFIKAIIVDL
SQKVTLMLLDQ	..	NNREHIDAFAFRPD	..	VTSSS	FQRPIS	..	EMNIASGCPFLFCPLAKLAG	KSP	..	YLKDDTIFIKAIIVDL
SQKVTLMLLDQ	..	NNREHIDAFAFRPD	..	VISTS	FLRPNR	..	EMNIASGCPFLFCPLAKLAG	KSS	..	YLRDDTIFIKAIIVDL
KQKVTLMLMDQ	..	GPKKKHLGDAFKPD	..	PNSSS	FRRPVV	..	EMNIASGCPFLFVSQSVLET	GS	..	YIKDDTIFIKV
KQKVTLMLMDQ	..	GSSRRHLGDAFKPD	..	PNSSS	FKKPTG	..	EMNIASGCPVFVFAQTVLEN	GT	..	YIKDDTIFIKVIVDT
KQKVTLMLMDQ	..	GSSRRHLGDAFKPD	..	PNSSS	FKKPTG	..	EMNIASGCPVFVFAQTVLEN	GT	..	YIKDDTIFIKVIVDT
KQKVTLMLMDQ	..	GSSRRHLGDAFKPD	..	PNSSS	FKKPTG	..	EMNIASGCPVFVFAQTVLEN	GT	..	YIKDDTIFIKVIVDT
KQKVTLMLMDQ	..	GPARKHLGDAFKPD	..	PNSSS	FRRPTA	..	EMNIASGCPFLFVAQTVLEN	GT	..	YIKDDTIFIKVIVDT
KQKVTLMLMDQ	..	GPARKHLGDAFKPD	..	PNSSS	FRRPTG	..	EMNIASGCPFLFVAQTVLEN	GT	..	YIKDDTIFIKVIVDT
RQKVTLMLLDQ	..	GKKKNIMETTFKPD	..	PNSSS	FKKRPG	..	EMNIASGCPRFVASHVLENAKNA	..	..	YIKDDTFLFKVAVDL
RQKVTLMLLDQ	..	GKKKNHIVETTFKAD	..	PNSSS	FKKRPG	..	EMNIASGCPRFVASHVLENAKNA	..	..	YIKDDTFLFKVAVDL
KQKVTLMLLDQ	..	GKKKNHIVETTFKAD	..	PNSSS	FKKRPG	..	EMNIASGCPRFVASHVLENAKNA	..	..	YIKDDTFLFKVAVDL
KHKVTFLLDQ	..	NQREHVIDAFAFRPD	..	LSSMS	FHQPVV	..	DMNVASGCPFLFPLKKLRSPKHA	..	..	YCKDDTIYIKCVDDT
RQNIITLLDQ	..	SGTRNHMSHTFIPD	..	VNSDS	FHRPTS	..	DANVATGTRFISHCDLEN	..	..	PRNAVFRDETFLFKVIVDT
NQKITFYLLDQ	..	GEDRKHKIHOLSPNRSL	..	PNVRA	VFNKPA	..	EENLGIGNPSFVSHEAIDA	GE	..	FVKEDTIFMKCEVEP
SHSITFTLFEQGA	..	QSGGGVAESFVDP	..	PTWEN	FQRPNS	..	EPDQLGFGFPFISHHELLHS	RP	..	FIKGDTVFLRVKIVDT
SHSVTFTLFEQGLSG	..	QSGGGVAESFVDP	..	PTWEN	FQRPST	..	EPDALGFGFPFISHHELLNR	RP	..	FVRDDTFLRVKIVDT
SHSVSFTTIFDQTVV	..	AEKACNIVESFIPD	..	PTWKN	FQRPST	..	EPDSLGFGFPFVSHEMVKK	RH	..	FVKDNTMFLRVKIVDT
ARRVTFSLLDQSDPG	..	LAKPOHVTETTFHPD	..	PNWKN	FQKPGT	..	WRGSLDESSLGFGYKPFISHQDIRK	RN	..	YVRDDAVFIRASVEL
ARRVTFSLLDQSDPG	..	LAKPOHVTETTFHPD	..	PNWKN	FQKPGT	..	WRGSLDESSLGFGYKPFISHQDIRK	RN	..	YVRDDAVFIRAAVEL
SYRVTFSLLDQSDPG	..	LAKPONITETTFNPD	..	PNWKN	FQKPGG	..	SRNSLDESTLGFYKPFISHQDIRK	RN	..	YVRDIAFLRASVEI
SYKVTFSLLDQSDPG	..	LAKPOHITETTFNPD	..	PNWKN	FQKPNV	..	SRNSLDESTLGFYKPFISHQDIRK	RN	..	YVRDIAFLRASVEI
SYKVTFSLLDQSDPG	..	LAKPOHITETTFNPD	..	PNWKN	FQKPCS	..	SRNSLDESTLGFYKPFISHQDIRK	RN	..	YVRDIAFLRASVEI
SHRITFTLIDQCD	..	PTARRNISYNIKPN	..	POKEN	KAF	..	ERNASFGTQKFLSHENMIKT	LD	..	YIRDDTIFIKVQVD
HRAIKISLLDQNR	..	PEDRVNITYVIDPR	..	KLKAN	EKFLARPG	..	ERNAAGFS			