Minireview

# The mitochondrial inner membrane AAA metalloprotease family in metazoans

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**Abstract Three metalloproteases belonging to the AAA superfamily (Yme1p, Afg3p and Rca1p) are involved in protein turnover and respiratory chain complex assembly in the yeast inner mitochondrial membrane. Analysis of the completed genome sequences of *Caenorhabditis elegans* and *Drosophila melanogaster* indicates that this gene family typically comprises 3–4 members in metazoans. Phylogenetic analysis reveals three main branches represented, respectively, by *Saccharomyces cerevisiae YME1*, human *SPG7* (paraplegin) and *S. cerevisiae AFG3* and *RCA1*. mt-AAA metalloproteases are weak candidates for several previously studied *Drosophila* mutants. A full elucidation of the cellular and physiological roles of mt-AAA metalloproteases in metazoans will require the creation of targeted mutations.** 

*Key words:* Metalloprotease; AAA protein; Chaperone; Mitochondrion

## 1. Introduction

The AAA superfamily (**A**TPases **a**ssociated with diverse cellular **a**ctivities) includes proteins involved in a variety of cellular processes, including the regulation of cell division, protein turnover, vesicle transport and organelle biogenesis [1–3]. They are characterized by a conserved region of approximately 220 amino acids, which includes an ATP binding site [4]. Eubacteria typically contain only one or a few AAA proteins, but eukaryotes contain many. In yeast, a subset of them (Afg3p, Yme1p and Rca1p, also designated, respectively, Yta10p, Yta11p and Yta12p) show a clear phylogenetic relationship with the eubacterial family of AAA metalloproteases, such as *Escherichia coli* FtsH [3,5,6]. All three are found in mitochondria [5,7–9] and are located in the inner mitochondrial membrane, where they appear to be involved in the turnover of unincorporated or mistranslated mitochondrial translation products and in promoting the assembly of the respiratory chain complexes [3,10,11]. One of them, Yme1p, is oriented towards the inter-membrane space, where it forms a homo-oligomeric complex designated i-AAA [10]. The two others, Rca1p and Afg3p, form a hetero-oligomeric complex oriented towards the matrix (m-AAA). Null mutants in any of the genes encoding these proteins show defects in respiration [5,8,9] as well as additional phenotypic properties that are incompletely understood. For example, disruption of *YME1* causes petite lethality [12], greatly increased escape of mtDNA to the nucleus and additionally a cold-sensitive growth phenotype on rich glucose medium [8].

Both the ATPase and protease activities of the mitochondrial (mt-AAA) metalloproteases are essential for at least some of their biological roles. For example, mutations in the HEXXH motif of Yme1p abolish metal-binding, cause loss of proteolytic activity and result in loss of ability to complement a null mutant [13]. Mutations that abolish protease activity of either Afg3p [14] or Rca1p [15] do not lead to a respiratory defect, but the double mutant that combines loss of both activities is respiration-deficient [16]. The two mt-AAA metalloprotease complexes appear to be involved in the turnover of different mitochondrial translation products. Notably, Cox2p turnover requires the i-AAA complex [17], whereas that of the remaining translation products, especially Cox1p and Cobp, depends on the m-AAA complex [10,14].

In humans, four genes that appear to encode members of the mt-AAA metalloprotease family have been partially characterized. These comprise a proposed orthologue of yeast *YME1*, designated *YME1L* [18,19], located on chromosome 10, plus three genes that are more related to yeast *RCA1* and *AFG3* than to *YME1*. These are *AFG3L1* [20] and *SPG7* [21], both on chromosome 16, and *AFG3L2* on chromosome 18 [22]. Thus far only a partial sequence is available for *AFG3L1*, although the complete coding sequences of the other three members of the family are known. Reporter analysis shows that YME1L is mitochondrially targeted in human cells [18,19], and its expression in yeast can also complement a *yme1* null mutant, indicating functional conservation [18]. The physiological roles of AFG3L2 and paraplegin, the *SPG7* gene product, are unknown. Paraplegin was originally identified on the basis that null mutations in the *SPG7* gene cause one form of hereditary spastic paraplegia [21]. The protein is mitochondrially targeted, and patients have some evidence of mitochondrial dysfunction resembling mtDNA disease, although they do not have an overt respiratory chain deficiency. Paraplegin expression in yeast does not complement *afg3* or *rca1* null mutants (G.A.J. Hakkaart and L. de Jong, personal communication). AFG3L2 is also mitochondrially localized in human cells [22].

The precise functions of these various genes and gene prod-

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ucts remain to be elucidated. An essential first step towards understanding their biological significance is to characterize fully the gene family, and establish the phylogenetic relationships between the family members. Since complete and properly annotated genomic sequence information is not yet available for any mammal, it is instructive to consider the published genomic DNA sequences of two model metazoans, the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*.

## 2. Invertebrate genes for mt-AAA metalloprotease family members

Since the *C. elegans* and *D. melanogaster* genome sequences are continuously being updated and modified, errors undoubtedly remain, both in the actual sequences as well in their interpretation, notably the assignment of coding sequences. Remaining sequence gaps and regions of sequence withdrawn for re-finishing also potentially complicate the analysis. Therefore, in order to maximize the chance of extracting correct and complete information relating to mt-AAA metalloprotease family members (or any gene of interest), it is necessary to consider not only the annotated genome data, but also all available expressed sequence tags (ESTs), extensive flanking genomic regions, and any accessible unfinished or unannotated sequences.

TBLASTN analysis of the *Drosophila* genome, using as query sequences each of the yeast mt-AAA metalloproteases, reveals just three clear members of the family, namely computer-predicted genes *CG6512* on chromosome 3L, *CG3499* on chromosome 2R and *CG2658* (*EG:100G10.7*) on chromosome X. All other hits are of coding sequences no more closely related to yeast mt-AAA metalloproteases than to other members of the AAA superfamily, such as proteasomal subunits, and can therefore be rejected from the analysis. Percent identities in pairwise comparisons with yeast and human mt-AAA metalloproteases (Table 1) indicate clear affinities: the CG6512 protein with human AFG3L2 and yeast Afg3p and Rca1p, CG3499 with human YME1L and yeast Yme1p, and CG2658 with human paraplegin, and to a lesser extent

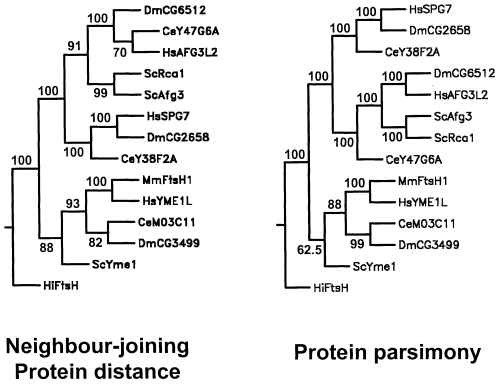


Fig. 1. Phylogenetic relationships amongst mt-AAA family members. Output trees created by PHYLIP programs as indicated, showing bootstrap values for each node. Database sources for the input sequences were as listed in the legend to Table 1, plus *H. influenzae* FtsH (GenBank AAC22979) as outgroup, here abbreviated as HiFtsH, and a proposed homologue of yeast Yme1p from mouse (GenBank AAC35558), here abbreviated as MmFtsH1. Other sequences are abbreviated as follows: DmCG6512, DmCG2658 and DmCG3499 – *Drosophila* computer-predicted polypeptides CG6512, CG2658 and CG3499; CeY47G6A, CeM03C11 and CeY38F2A – *C. elegans* computer-predicted polypeptides Y47G6A.10, M03C11.5 and Y38F2AR.para; ScYme1, ScAfg3 and ScRca1 – yeast mt-AAA genes of these names; and HsAFG3L2, HsYME1L and HsSPG7 – human AFG3L2, YME1L and paraplegin.

with yeast Rca1p and Afg3p. In each case, the best match is to a human rather than to a yeast protein sequence.

In the case of *C. elegans*, a similar exercise reveals two predicted genes listed in the wormpep database that are clear members of the family, namely Y47G6A.10, encoded on chromosome I, and M03C11.5, encoded on chromosome III. A putative third gene can be found in an unfinished region of chromosome IV (unannotated clone Y38F2AR, EMBL: AC025715), from which a plausible coding region can be assembled by a combination of EST analysis (consensus of GenBank sequences D36561 and C62714) and TBLASTN searching of the available genomic DNA sequence, respecting the usual splice boundary rules. The assembled open reading frame (ORF) of 758 amino acids, encoded by 11 exons, and

Table 1
Pairwise identity comparisons[a] of putative *Drosophila* and *C. elegans* mt-AAA metalloproteases with those of yeast and mammals[b]

| | Yeast Yme1p | Yeast Afg3p | Yeast Rca1p | Human AFG3L2 | Human paraplegin | Human YME1L | Yeast Yta2p |
|---|---|---|---|---|---|---|---|
| *Drosophila* CG6512 | 34 | 50 | 48 | **65** | 41 | 35 | 35 |
| *Drosophila* CG3499 | 41 | 34 | 34 | 35 | 34 | **48** | 31 |
| *Drosophila* CG2658 | 32 | 39 | 38 | 43 | **51** | 34 | 33 |
| *C. elegans* Y47G6A.10 | 33 | 51 | 47 | **60** | 41 | 34 | 35 |
| *C. elegans* M03C11.5 | 42 | 37 | 36 | 37 | 39 | **45** | 26 |
| *C. elegans* Y38F2AR.para | 35 | 43 | 40 | 43 | **49** | 33 | 29 |
| Yeast Yme1p | | 36 | 34 | 35 | 33 | 42 | 30 |
| Yeast Afg3p | | | 55 | 51 | 42 | 33 | 34 |
| Yeast Rca1p | | | | 48 | 40 | 34 | 33 |
| Human AFG3L2 | | | | | 45 | 36 | 33 |
| Human paraplegin | | | | | | 33 | 33 |
| Human YME1L | | | | | | | 28 |

[a]Pairwise identities computed using GCG program GAP. Best matches to each invertebrate polypeptide are shown in bold.
[b]Sequences analyzed were as follows: *Drosophila* CG6512 (SPTREMBL: Q9VVE6), CG3499 (SPTREMBL: Q9W1Y0) and CG2658 (SPTREMBL: O76867), *C. elegans* Y47GA6.10 (GenBank AAF60660), M0C311.5 (GenBank CAA88955.1) and Y38F2AR.para (assembled manually from the sequence of *C. elegans* genomic clone Y38F2AR (EMBL: AC025715; see http://www.uta.fi/imt/group/metazoan.html)), yeast Yme1p (Yta11p, GenBank CAA89278.1), Rca1p (Yta12p, GenBank CAA89236.1), Afg3p (Yta10p, GenBank AAB64550.1) and proteasomal subunit Yta2p (Rpt3p, GenBank AAB64836.1), human AFG3L2 (GenBank NP_006787), YME1L (GenBank CAB51858) and paraplegin (GenBank NP_003110).

provisionally designated Y38F2AR.para can be accessed at http://www.uta.fi/imt/group/metazoan.html.

A sequence related to portions of clone Y38F2AR is found at a second location in the genome, near the telomere of chromosome X (cosmid Y47C4, EMBL: AC024789). This includes three non-contiguous segments potentially encoding a polypeptide similar to exons 6, 8 and 9 of Y38F2AR.para, but lacking other coding sequences from the gene (see http://www.uta.fi/imt/group/metazoan.html for details). It seems reasonable to regard these as fragments of a Y38F2AR.para pseudogene, although it cannot be ruled out that these exons are incorporated into the coding region of a different polypeptide.

All other AAA proteins found in the *C. elegans* genome are just as similar to proteasomal subunits as to the mt-AAA metalloprotease family and need not to be considered further. Percent identities in pairwise comparisons with yeast and human mt-AAA metalloproteases (Table 1) again indicate clear affinities: Y47G6A.10 with human AFG3L2 and yeast Afg3p and Rca1p, M03C11.5 with human YME1L and yeast Yme1p, Y38F2AR.para with human paraplegin. The N-terminal regions, including putative mitochondrial targeting information, show the least conservation at the primary sequence level, and hence attract the greatest uncertainty. However, the above affinities (for both *Drosophila* and *C. elegans*) are even more clear-cut if the N-terminal 150 amino acids are ignored in each case.

## 3. Phylogenetic analysis of mt-AAA metalloproteases

The above findings strongly suggest that the mt-AAA metalloprotease gene family comprises three main branches, with each invertebrate species possessing a single gene from each branch. Phylogenetic analysis using programs from the PHYLIP package confirms this inference. Both protein parsimony and neighbor-joining using protein distances produce trees with the same three main branches, as shown in Fig. 1, in which a eubacterial homologue (FtsH of *Haemophilus influenzae*) is used as outgroup. The first and deepest branch groups all of the Yme1p-related sequences, with the yeast sequence as the deepest sub-branch. The second group contains human paraplegin (SPG7), along with apparent orthologues from other metazoans. The third group, sister to the second, contains human AFG3L2, one homologue each in *Drosophila* and *C. elegans*, plus the two remaining yeast members of the family Rca1p and Afg3p grouped together down one sub-branch.

The two methods produce slightly different tree topologies for the metazoan representatives of each of the three main branches, and some bootstrap values for these nodes are relatively low. However, given the limitations of the method, the data are broadly consistent with monophyly both for a *YME1*-like gene that has been retained in single-copy in all taxa, and for a paraplegin-like gene which has been lost (or never possessed) by yeast. The history of the third branch is harder to reconstruct with confidence. Once again, the hypothesis of a single ancestral *AFG3*-like gene is attractive, the two yeast proteins Afg3p and Rca1p clearly having resulted from a gene duplication subsequent to the divergence of animals and fungi. Although complete sequence information on human AFG3L1 is not yet available, it seems to be most closely related to human AFG3L2 than to any other of the full-length sequences currently available, again consistent with a lineage-specific gene duplication. EST contig building is consistent with a single *YME1*-like gene and a single paraplegin gene in both human and mouse. The exact number of *AFG3*-like genes in these taxa remains unclear, but it may be no higher than two.

## 4. Subcellular localization of putative mt-AAA proteins

In the absence of direct biochemical or genetic data, information on the subcellular localization and biological functions of the putative mt-AAA metalloprotease proteins of metazoans can only be tentatively deduced in silico. Mitochondrial localization of AFG3L2, paraplegin and YME1L has been inferred from reporter assays. A mitochondrial localization is predicted by at least one of the programs PSORT II and MITOPROT for each of the invertebrate mt-AAA metalloproteases except for *Drosophila* CG6512, although by both programs for just two of the *C. elegans* proteins and for none from *Drosophila* (see http://www.uta.fi/imt/group/metazoan.html). The predictive value of these programs is low, however, since the results for human YME1L and AFG3L2 are no more convincing. In the case of YME1L, neither program can identify the protein as mitochondrially targeted. However, given that no other subcellular localization is strongly supported for any of the proteins, mitochondrial targeting remains a reasonable working hypothesis.

It should be noted, however, that the poor conservation of the N-terminal regions, even of the mature mt-AAA proteins, combined with the unreliability of exon prediction programs, means that some of the N-termini of these proteins may have been misassigned or misidentified. Final assignment of subcellular localization, cleavage sites and exact amino acid sequences must await the availability of full-length cDNA sequences, combined with proteomic or other biochemical analyses.

## 5. Functional analysis of mt-AAA proteins

Mutant alleles have been obtained for a significant fraction of *Drosophila* genes. It is therefore useful to consider whether any mutants have already been mapped to regions of the fly genome in which the mt-AAA genes lie, and for which they might be a candidate. The *AFG3*-like gene *CG6512* lies in a 500 kb interval between genes for two transcription factors, *sina* (*seven in absentia*) and *tap*, in which 49 other predicted coding sequences reside (Fig. 2a). As far as we have been able to ascertain, no unassigned mutants have been mapped to intervals covering cytogenetic bands 74A2-A3, where *CG6512* seems to be located. One male sterile, *ms(3)73D*, as well as a number of lethals, including some P-element insertions, map to adjacent bands, but unless cytogenetic positions are grossly in error these are not plausible candidates.

*CG3499* (*YME1*-like) lies within a 40 kb interval (Fig. 2b) between *inaD*, encoding an ion channel involved in phototransduction, and the site of a lethal P-element insertion, *l(2)10444*, in the gene for nucleoporin (*CG3820*). *CG3499* seems a plausible candidate for only one mutant, *l(2)59ABb* [23], but this lethal has been mapped only to a very wide region, cytogenetic bands 59A1-B2, containing over 20 known and predicted genes. Therefore *CG3499* is not a likely candidate. Other mutants, such as *pa* (*patulous*) or *tft* (*tufts*), map to even larger intervals and are probably not worth considering. One other mutant, *mei-P14*, with a phenotype of impaired

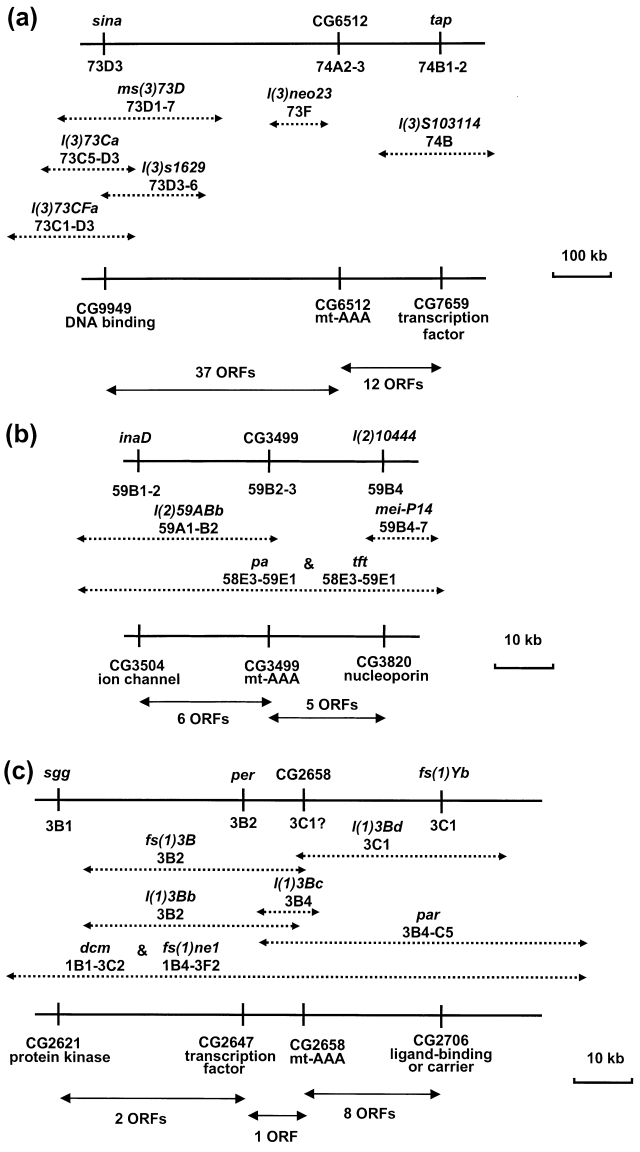


Fig. 2. Reconciled genetic and physical maps of the genomic regions around putative *Drosophila* mt-AAA metalloprotease genes (a) *CG6512*, (b) *CG3499* and (c) *CG2658*. In each case, the top line shows the nearest genetically mapped loci currently identified in the genomic DNA sequence, together with their currently assigned cytogenetic locations. The bottom line shows the physical map, the computer-predicted gene names (CG...) and proposed functional assignments. The number of ORFs that lie in the intervals shown includes both identified and unidentified gene products. In the central region of each diagram are shown the previously isolated mutants from the region that remain to be mapped precisely, together with their estimated cytogenetic positions, and approximate physical intervals shown by dashed arrows. A short region of sequence at the P-element insertion site in *l(3)s1629* corresponds with that found at a far upstream site in band 71E1: therefore this insertion may have been mapped erroneously.

meiotic recombination, maps in the same general area as *l(2)1044*, but once again, unless cytogenetic mapping is highly erroneous, it cannot be relevant to *CG3499*.

*CG2658* (paraplegin-like) lies, together with nine other predicted coding sequences, between *per (period)* and the female sterile *fs(1)Yb* in cytogenetic interval 3B2-C1, in which three lethals, *l(1)*Bb, *l(1)3Bc* and *l(1)3Bd*, and one other female sterile, *fs(1)3B*, also potentially map (Fig. 2c). *CG2658* has been studied previously in the context of investigations of the nearby *per* locus, which encodes a transcription factor controlling biological rhythms, including circadian behavior [24]. *CG2658* does not appear to be involved directly in *per* function, but the expression of the adjacent gene *CG2650* (*anon*-3B1.2) is under circadian regulation [24]. In addition, the mutant *par* (*paralog*) also maps to this region, but within a very wide cytogenetic interval (3B4-C5). Its phenotype of temperature-sensitive female sterility, involving the production of few if any eggs [25], could conceivably be related to a defect in mitochondrial protein quality control although many other biological functions impinge on oogenesis. *Par* is probably not allelic to *fs(1)Yb*, based on details of the phenotype and its genetic properties [25,26]. A number of other mutants map in very wide intervals covering the region of *CG2658*. Based on a similar analysis, no *C. elegans* mutants have been obtained, for which mt-AAA genes are candidates.

Overall, given that no strong candidates emerge from such an analysis, the most fruitful approach to obtain fly (or worm) mutants with altered mt-AAA metalloprotease function would probably be site-selected mutagenesis. An alternative possibility might be to attempt to overexpress dominant-negative transgenic variants, predicted to lack protease activity.

## 6. Conclusions and speculations

The absence of a paraplegin-like gene from yeast, and the duplication of the *AFG3*-like gene in different lineages, raise many questions. The paraplegin-like protein may, for example, be a component of the m-AAA complex in metazoans, whose function is at least in part fulfilled by a duplicated *AFG3*-like gene in yeast (i.e. *RCA1*). However, given that the different components of the m-AAA complex may have distinct proteolytic substrates, it is tempting to speculate that the paraplegin-like protein found in metazoa is involved in assembly or turnover functions relating to proteins that are present in animals but absent from *Saccharomyces cerevisiae*, i.e. those of complex I. This does not, however, account for other *AFG3*-like gene duplications.

The results of phylogenetic analysis permit a conceptual simplification of the mt-AAA gene family into a single *YME1*-like gene encoding the i-AAA, plus at least two genes encoding most probably two distinct m-AAA metalloprotease complexes. The fact that expression of human *YME1L* can complement a yeast *yme1* mutant [18], but that human paraplegin cannot complement either *rca1* or *afg3*, supports the idea that paraplegin may have a distinct function in mitochondrial protein metabolism from that of the *AFG3* subfamily. Since in all cases examined (including mammals) the paraplegin-like gene is single-copy, we propose that, like Yme1p, it functions as a homo-oligomeric complex. The third gene or genes would encode a second m-AAA complex, still retained by yeast, which may be either homo- or hetero-oligomeric, depending on the extent of gene duplication and diversification that has taken place.

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