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ATAD3 Proteins: Unique Mitochondrial Proteins Essential for Life in Diverse Eukaryotic Lineages

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ATPase family AAA domain–containing 3 (ATAD3) proteins are unique mitochondrial proteins that arose deep in the eukaryotic lineage but that are surprisingly absent in Fungi and Amoebozoa. These ~600-amino acid proteins are anchored in the inner mitochondrial membrane and are essential in metazoans and *Arabidopsis thaliana*. ATAD3s comprise a C-terminal ATPases Associated with a variety of cellular Activities (AAA+) matrix domain and an ATAD3_N domain, which is located primarily in the inner membrane space but potentially extends to the cytosol to interact with the ER. Sequence and structural alignments indicate that ATAD3 proteins are most similar to classic chaperone unfoldases in the AAA+ family, suggesting that they operate in mitochondrial protein quality control. *A. thaliana* has four ATAD3 genes in two distinct clades that appear first in the seed plants, and both clades are essential for viability. The four genes are generally coordinately expressed, and transcripts are highest in growing apices and imbibed seeds. Plants with disrupted ATAD3 have reduced growth, aberrant mitochondrial morphology, diffuse nucleoids and reduced oxidative phosphorylation complex I. These and other pleiotropic phenotypes are also observed in *ATAD3* mutants in metazoans. Here, we discuss the distribution of ATAD3 proteins as they have evolved in the plant kingdom, their unique structure, what we know about their function in plants and the challenges in determining their essential roles in mitochondria.

Keywords: *Arabidopsis thaliana* ● Eukaryotic evolution ● Membrane contact sites ● Nucleoids ● Oxidative phosphorylation ● Protein quality control

Introduction

The large family of ATPases Associated with a variety of cellular Activities (AAA+ proteins) is found in all kingdoms of life and participates in a wide range of functions due to the ability of family members to alter protein or nucleic acid structure using the energy derived from ATP hydrolysis. The signature AAA+ domain comprises N-terminal α/β- and C-terminal α-helical subdomains that are typically attached to additional domains or structures, including membrane anchors and protease domains. This structural elaboration, as well as the association of many AAA+ proteins with adaptor proteins, serves to expand and define AAA+ protein function. The evolution, structural and functional diversity and our current understanding of the molecular mechanism of AAA+ proteins have been reviewed extensively (Iyer et al. 2004, Erzberger and Berger 2006, Snider et al. 2008, Miller and Enemark 2016; Puchades et al. 2020, Jessop et al. 2021, Khan et al. 2022).

Plants express many different AAA+ proteins, which are found in all cellular compartments and membranes. These include the HSP100/ClpB chaperone family (which contains two AAA+ domains) involved in unfolding protein aggregates (Lee et al. 2005, 2007, McLoughlin et al. 2019), the Lon (Tsitsekian et al. 2019), FtsH (Yi et al. 2022) and Clp (Bouchnak and van Wijk 2021) proteases; the chaperone p97/CDC48 (also with two AAA+ domains) (Bègue et al. 2017); the microtubule-severing protein katanin (Komis et al. 2017); subunits of the 19S regulatory particle of the proteasome (Marshall and Vierstra 2019) and others. All of these AAA+ proteins have protein substrates and are engaged in aspects of protein quality control or protein structure modulation. There are also AAA+ family members that act on nucleic acid structures (Iyer et al. 2004, Miller and Enemark 2016). The evolution of many AAA+ proteins is rooted deep in the tree of life (Iyer et al. 2004, Scharfenberg et al. 2015).

The mitochondrial ATPase family AAA domain–containing 3 (ATAD3) proteins are novel members of the AAA+ protein family not previously analyzed in relation to other AAA+ proteins. These approximately 600-amino acid proteins comprise two major domains: a C-terminal AAA+ domain preceded by a transmembrane domain that anchors the protein in the inner mitochondrial membrane and an N-terminal domain of unknown function previously designated as DUF3523 but

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renamed as the ATAD3_N domain (Baudier 2018). ATAD3s are essential in metazoans including Drosophila, *Caenorhabditis elegans*, mammals (Baudier 2018, Peralta et al. 2018, Arguello et al. 2021) and *Arabidopsis thaliana* (Kim et al. 2021). Their essential nature is further underscored by the fact that ATAD3 defects are linked to multiple mitochondrial-based human diseases (Peralta et al. 2019, Zhao et al. 2019, Gunning et al. 2020, Frazier et al. 2021). Why ATAD3s are essential remains enigmatic, as disruption of ATAD3 function results in pleiotropic defects that range from reduced mitochondrial complex I and V and loss of cristae structure to altered lipid metabolism, impaired DNA replication and changes in mitochondrial nucleoid structure (Baudier 2018, Peralta et al. 2018, Arguello et al. 2021, Kim et al. 2021, Ishihara et al. 2022). ATAD3 proteins have also been linked to contact sites between the inner and outer mitochondrial membranes (Zhao et al. 2019) and to mitochondrial–endoplasmic reticulum contact sites, which could allow these proteins to modulate additional functions (Baudier 2018, Csordás et al. 2018, Zhao et al. 2022). Here, we discuss the distribution of ATAD3 proteins as they have evolved in the plant kingdom, their unique structure, what we know about their function in plants and the challenges in determining their essential roles in mitochondria.

Evolutionary History of ATAD3 Proteins

The ATAD3 proteins are not found in the prokaryotic precursors of mitochondria but rather appear to be a eukaryotic innovation and are present in many, though not all, eukaryotic lineages (**Fig. 1**). They are found in the Metazoa and their closest living, single-celled relatives such as the Choanoflagellates (*Monosiga brevicollis*). ATAD3 genes are present in the land plants, the red and green algae (Archaeplastida), and also some protist lineages including the Heterokonts (*Thalassiosira oceanica*) and the Alveolata (*Plasmodium falciparum*). The presence of ATAD3s in these diverse eukaryotic groups suggests that this protein evolved at the base of the eukaryotic tree. However, ATAD3s are notably absent in Fungi and Amoebozoa. It is not unexpected that a mitochondrial-localized protein would be lost in protist lineages that have lost mitochondria, such as *Giardia*. What is perplexing are lineages of eukaryotes with functioning mitochondria that have lost ATAD3. Loss of ATAD3 in the Fungi raises important functional and evolutionary questions. Recent studies indicate that genomes of the earliest diverging fungi all contracted with a continuing loss of ancestral 'protist' genes and gene families (Ocaña-Pallarès et al. 2022, Merényi et al. 2023). We found that ATAD3s are absent in representatives of all major lineages of Fungi, including the Opisthosporidia clade, as well as the better-studied and more recently diverged Basidiomycota. Recent phylogenetic studies indicate that the Opisthosporidia are at the base of the fungal tree and that Nucleariidae are the closest relatives of the Fungi (James et al. 2020, Li et al. 2021, Ocaña-Pallarès et al. 2022, Merényi et al. 2023). Therefore, the absence of ATAD3s in Fungi and the presence of an ATAD3 homolog in the Nucleariidae (*Fonticula alba*) places the loss of ATAD3s after the divergence of the Nucleariidae and other Ophisotokonts but prior to the diversification of extant fungal groups. It is unclear how the Fungi may compensate for the absence of this ancient and conserved protein.

With a few notable exceptions, the number of ATAD3s in each genome is stable across broad expanses of evolutionary time. Most genomes contain only one ATAD3 homolog. However, in humans, there are three ATAD3 proteins; the gene has been duplicated twice in tandem, giving rise to ATAD3A, B and C. ATAD3B differs from the ancestral ATAD3A by having a C-terminal 62-amino acid extension, whereas ATAD3C is truncated, missing the first 70 amino acids. Some but not all non-human primates have three ATAD3 genes, with only a clear homolog of ATAD3A found in all. Mouse or more distantly related metazoans, i.e. frogs or flies, do not have these distinct ATAD3 homologs. The ATAD3A protein in mouse, *Mus musculus*, lies outside of the human ATAD3 clade (**Fig. 1**), indicating that the gene duplications that gave rise to the three human ATAD3s occurred after the divergence of the common ancestor of humans and mice. The ATAD3As from mouse and the other metazoans show a pattern of orthologous relationships—they reflect organismal relationships. This conserved evolutionary pattern suggests that most metazoan ATAD3s share a conserved function. What functional differences exist among the human or other primate ATAD3 proteins remains an open question.

The Archaeplastida lineage of eukaryotes contains the red (Rhodophyta) and green (Chlorophyta and Charophyta) algae as well as the land plants (Embryophytes). The algae have one ATAD3 protein (**Fig. 1**), consistent with most other eukaryotic lineages. The evolutionary history of the ATAD3s in the land plants is intriguing. Instead of loss as seen in the Fungi or selection to maintain one copy as seen in most metazoans, within the plant lineage, there is evidence of ATAD3 gene duplication and divergence. *Physcomitrium patens*, a moss, has two ATAD3s, while *Selaginella*, a lycophyte and Marchantia, a liverwort, both have one, which are not more related to either of the two ATAD3s in *P. patens*. The *P. patens* ATAD3s most likely reflect a recent gene duplication, possibly via a whole genome duplication (WGD), the occurrence of which is well documented in the moss. The evolutionary history of ATAD3s in seed plants is marked by three duplication events (**Fig. 1**). There is clear evidence of a gene duplication at the base of the seed plant lineage. This duplication, which occurred ∼350 million years ago, gave rise to two distinct seed plant lineages, ATAD3A and ATAD3B, found in gymnosperms (*Gnetum* in the Gnetophyta), eudicots (*Citrus* in the Rutaceae and *A. thaliana* in the Brassicaceae) and monocots (*Brachypodium* in the Poaceae). The tree topology in **Fig. 1** indicates that the seed plant ATAD3A and B lineages underwent additional duplication events within the Brassicaceae, generating four ATAD3 proteins: A1, A2, B1 and B2, as seen in *Eutrema* and *A. thaliana*. Based on the estimated divergence times of these taxa, this duplication could be as old as 30 million years ago (mya) or as recent as 20mya (Hohmann et al. 2015, Franzke et al. 2016, Walden 2020).

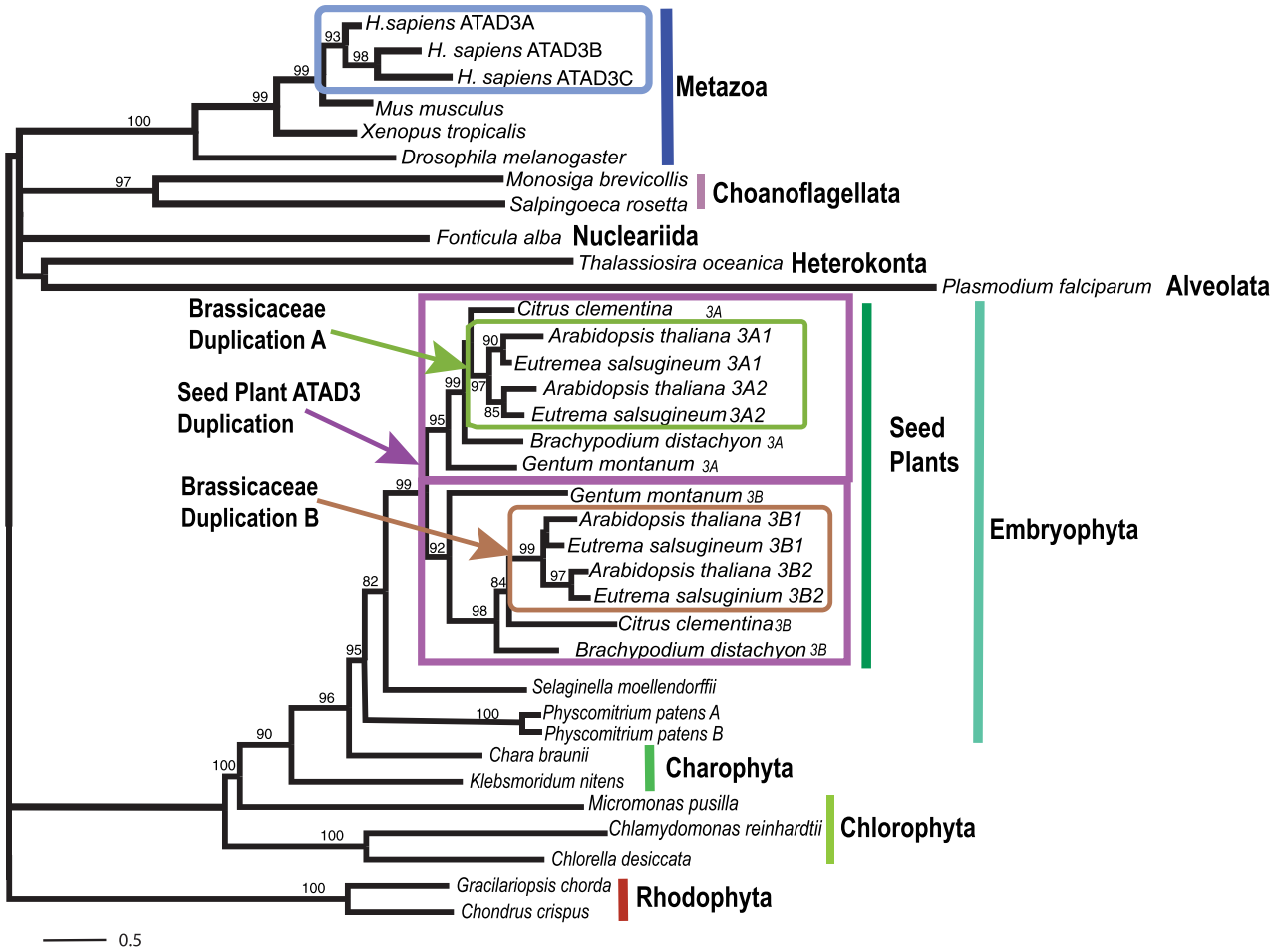

Fig. 1 Evolutionary relationships of eukaryotic ATAD3 proteins. Phylogenetic tree from an amino acid alignment generated with Multiple Alignment using Fast Fourier Transform using the NeighborJoining program with a Jones-Taylor-Thornton matrix (Katoh et al. 2019). The scale bar reflects a distance of 0.50. Branch reliability was estimated with 1,000 bootstrap replicates (shown above or near branches). Only bootstrap values >50% are provided. Higher taxonomic groups are indicated by name and delineated with bars at right. The three human ATAD3s are boxed, highlighting that these duplications are not shared with other metazoans. Arrows indicate branches with gene duplications in the plant lineage. The two seed plant lineages, A and B are boxed. Sequences were obtained using BLASTP searches of the National Center for Biotechnology Information and Ensemble databases using either the Human ATAD3A (NP001164006.1) or *A. thaliana* ATAD3A1 (At3G03060) as queries. Sequences: *Monosiga brevicollis* (EDQ87325); *Fonticula alba* (KCV71360); *Salpingoeca rosetta* (XP_004992339.1); *Plasmodium falciparum* (CAX64041); *Homo sapiens* ATAD3A (NP001164006.1), ATAD3B (ENSP00000500094) and ATAD3C (ENSP00000368062); *Mus musculus* (ENSMUSP00000030903); *Xenopus tropicalis* (ENSXETP00000028394); *Drosophila melanogaster* (FBpp0297140); *Thalassiosira oceanica* (EJK72353); *Gracilariopsis chorda* (PXF48289); *Chondrus crispus* (XP005716765); *A. thaliana* ATAD3A1 (At3G03060), A2 (At5G16930); B1 (At2G18330) and B2 (At4G36580); *Gnetum monantum* A (GMO00032115) and B (GMO00015941); *Brachypodium distachyon* A (XP_003570155.1) and B (XP003570196.1); *Selaginella moellendorffii* (EFJ32552); *Physcomitrium patens* A (XP024376696) and B (XP024376697); *Chara braunii* (OX = 69,332); *Klebsmoridium nitens* (GAQ86794); *Micromonas pusilla* (CCMP1545); *Chlamydomonas reinhardtii* (A0A2K3DV76); *Chlorella desiccate* (KAG7671567); *Citrus clementina* A (ESR38901) and B (ESR5645) and *Eutrema salsugineum* ATAD3A1 (ESQ49800), A2 (ESQ41689), B1 (ESQ50618) and B2 (ESQ53439).

Polyploidy or WGD is common in plants and has shaped plant genomes and plant diversity (Cui 2006, Jiao 2011, Van de Peer et al. 2017, Walden 2020). The timing of the ATAD3 duplications suggests that they could be the result of WGD events that previous studies have placed at the base of the seed plant and Brassicaceae lineages (Soltis et al. 2009, Couvreur et al. 2010, Jiao 2011). Patterns of gene retention or loss after WGD (Blanc and Wolfe 2004, Cheng et al. 2018, Kuzmin et al. 2022) have consistently found that genes whose products localize to organelles are preferentially lost while cytosolically localized proteins are preferentially retained. Retention of the ATAD3 gene duplicates goes against the expected pattern for organelle-localized proteins. In addition, the data indicate that both lineages encode mitochondrion-localized proteins, unlike many other duplicated plant organelle proteins that are dual targeted to chloroplasts and mitochondria or that have evolved specific isoforms targeted to either organelle. The fact that the seed plant ATAD3A and ATAD3B proteins have been retained

for hundreds of millions of years strongly suggests that these two ATAD3 proteins have distinct and conserved functions. Furthermore, the retention of the four ATAD3s in *Arabidopsis* and relatives suggests additional functional diversity among ATAD3s in the Brassicaceae.

The evolutionary history of the ATAD3s in eukaryotes suggests that for most eukaryotes there has been selection to maintain function. In contrast, the duplications within the seed and Brassicaceae lineages suggest diversification of function. Detailed analysis of ATAD3s in plants should illuminate both their conserved functions and the selective forces that have maintained multiple copies within plants.

ATAD3 Protein Structure

A closer look at ATAD3 structure is relevant to considering potential molecular mechanisms of ATAD3 function. **Fig. 2** presents an alignment of the four ATAD3 proteins from *A. thaliana* compared to human ATAD3A. The AAA+ domain is obviously more highly conserved than the ATAD3_N domain. The AAA+ domain contains conserved motifs in the N-terminal α/β subdomain including the Walker A motif (G-x(4)-GK-[TS]) in the 'P-loop' responsible for binding the γ-phosphate of ATP and the Walker B motif ([RK]-x(3)-G-x(3)-LhhhDE), in which the aspartate co-ordinates the magnesium ion essential for ATP hydrolysis. Additional features of the AAA+ domain are a polar residue termed 'Sensor 1', and an arginine residue known as the 'Arg finger', as well as a 'Sensor 2' arginine or lysine in the C-terminal α-helical subdomain (Miller and Enemark 2016). While these features are present in all AAA+ proteins, specific structural insertions divide these proteins into at least seven functional and evolutionary distinct clades (Iyer et al. 2004, Erzberger and Berger 2006, Miller and Enemark 2016). Based on structural analyses described later, we find that the ATAD3 AAA+ domain is most similar to that of AAA+ proteins involved in protein remodeling, termed the 'Classic Clade' by Miller and Enemark (2016), which includes the FtsH and Clp proteases (but ATAD3s lack an associated protease domain or subunit), p97 and CDC48.

As a recently investigated member of the AAA+ protein family, the evolutionary distribution of ATAD3s and their relationship to other family members have not yet been described. Utilizing the structural modeling server Phyre^2 (Kelley et al. 2015) to model the AAA+ domain of ATAD3A1 from *A. thaliana*, the top four proteins to which it structurally aligns are mouse p97/vcp (PDB:3cf1), yeast afg2 (PDB:7x11), yeast cdc48 (PDB:60pc; homolog of mammalian p97) and a proteasome-activating ATPase from *Methanocaldococcus* (PDB:3h4m). ATAD3B1 from *A. thaliana* also models on p97, afg2 and a proteasome subunit. The AlphaFold models for monomers of all four *A. thaliana* ATAD3 proteins are available in the UniProt database (https://www.uniprot.org/). The template structural files used in deriving the AAA+ domain models include human AFGL3 (PDB:6NYY), human TRIP13 (PDB: 6F0X), a human subunit of the 26S proteasome (PDB: 6MSB) and bacterial FtsH proteins (PDBs: 3KDS and 2DHR). These modeling results suggest that the AAA+ domains of the A and B clades are not significantly different. ATAD3A from *Amborella* and humans also both model on proteasome ATPases and afg2, as well as the proteases FtsH and YME1. All the proteins used as templates in these structural predictions act to unfold or remodel protein substrates. This homology, along with the lack of motifs involved in nucleic acid interactions, readily distinguishes ATAD3s from those AAA+ proteins that remodel nucleic acids. Thus, ATAD3 AAA+ structure implicates them as a unique component of the mitochondrial protein quality control network.

The relationship of ATAD3s to other protein-remodeling AAA+ proteins would indicate that the AAA+ domain functions as a hexamer, as described for essentially all members of this family (Jessop et al. 2021). Significantly, the Arg finger residue of the AAA+ domain interacts with another subunit such that completion of the ATPase active site requires an oligomeric structure. Mutational analysis supports the requirement of this interaction for ATP hydrolysis (Miller and Enemark 2016), and the Arg finger also plays a role in intersubunit communication and allosteric regulation (Puchades et al. 2020). The fact that oligomerization is required for activity suggests the possibility that shifts in oligomeric state could act as a regulatory mechanism, and has been suggested (Zhao et al. 2022). No structures of ATAD3 are available although a hexameric form of the AAA+ domain can be modeled on existing AAA+ protein hexamers. The presence of ATAD3 dimers and higher-order oligomers in vivo is supported by several studies in human cells and tissues (Baudier 2018, Peralta et al. 2019, Zhao et al. 2019, 2022, Frazier et al. 2021). Human ATAD3s can also form heterooligomers, as ATAD3A crosslinks to itself and to ATAD3B (Zhao et al. 2019, Zhu et al. 2022). In *A. thaliana*, bimolecular fluorescence complementation experiments demonstrated pairwise interactions of all ATAD3 proteins with themselves and each other (Gordon 2021). Data from the *A. thaliana* mitochondrial complexome map (https://complexomemap.de/at_mito_leaves) identify ATAD3A1, A2 and B1, but not B2, consistent with the reported low copy number of the B2 protein in mitochondria (Fuchs et al. 2020). The bulk of the signal for all three ATAD3 proteins is found at a position indicating the presence of native dimers. However, there is also a weak signal for A2 and B1 at masses consistent with higher-order oligomers, including hexamers, which could be disrupted by the isolation conditions required to remove the proteins from the membranes. Further investigations of ATAD3 in vivo organization are warranted, including the significance of heterooligomers.

There have been extensive structural and biochemical studies of the protein remodeling, hexameric AAA+ proteins (Puchades et al. 2020). Their action involves engaging substrates with loops (pore loops 1 and 2) that protrude into the core of the hexameric structure. These loops bind the substrate as the hexamer adopts a spiral arrangement, and substrate unfolding is accomplished using the energy of ATP hydrolysis. The

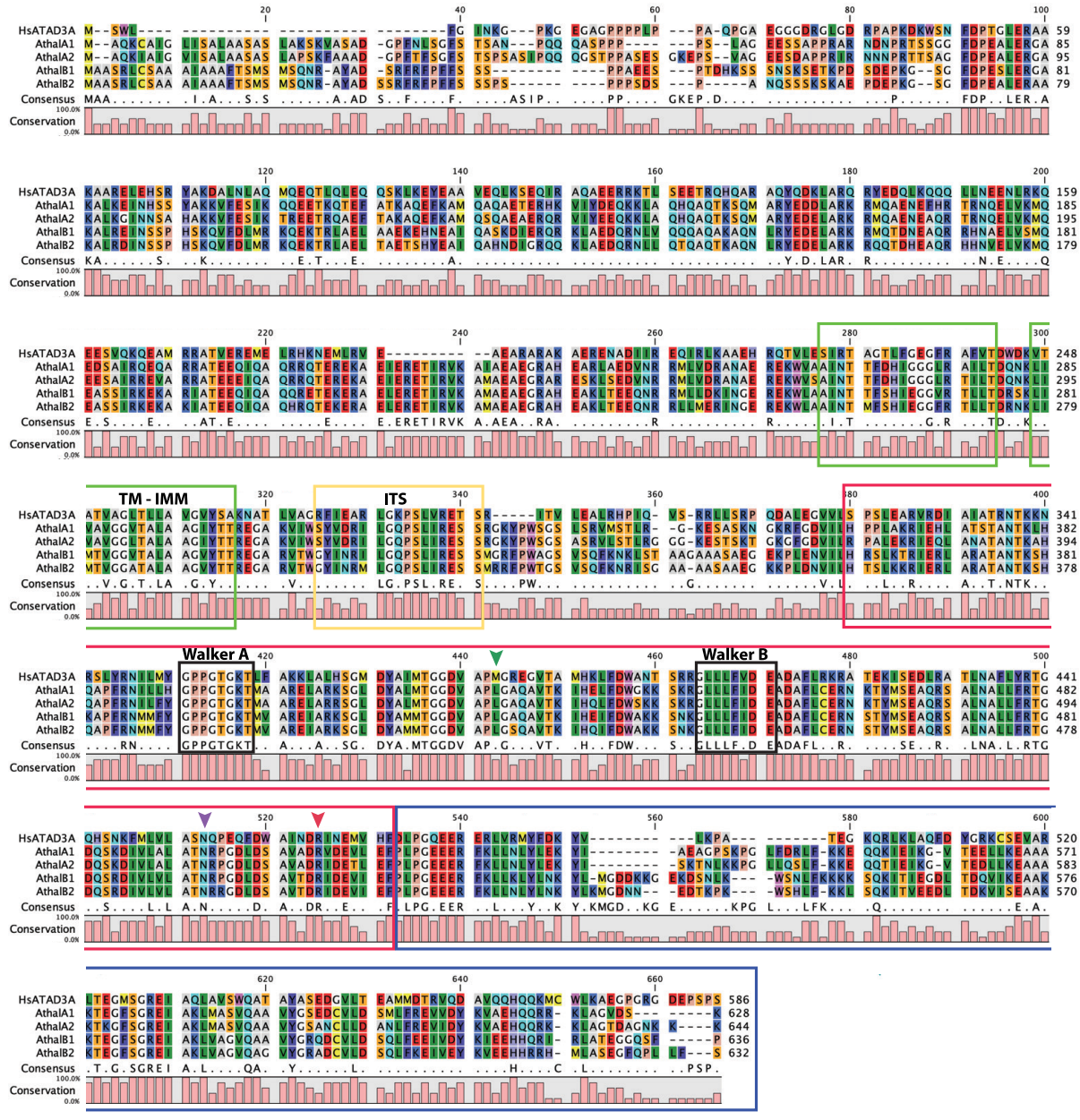

Fig. 2 Amino acid sequence alignment of human ATAD3A and the four ATAD3 proteins from A. thaliana (for accession numbers, see **Fig. 1**). Red box: core AAA+ domain containing Walker A, Walker B motifs (black boxes), Pore Loop 1 (green arrow) Sensor 1 (purple arrow) and Arg finger (red arrow). Blue box: C-terminal AAA+ helical subdomain. Yellow box: Internal mitochondrial targeting sequence. Green boxes: transmembrane helix in the inner mitochondrial membrane (TM-IMM) and a conserved amphipathic helix. Amino acids were colored by property according to the RASMOL scheme (http://openrasmol.org/doc/rasmol.html#chcolours). See the text for additional details.

canonical motif in pore loop 1, which is between the Walker A and B motifs, is X-Ar-ϕ-X, where X is any residue, Ar is aromatic and ϕ is hydrophobic (Miller and Enemark 2016). The aromatic residue is required for activity and has been shown to be involved in substrate binding in many AAA+ protein unfoldases (Puchades et al. 2020). Notably, ATAD3 proteins lack this motif at the corresponding sequence and predicted structural position, having instead the motif Pro-ϕ-Gly, where ϕ is methionine in most metazoans and leucine in the plant lineage (**Fig. 2**). Other variants in pore loop 1 are also found in AAA+ proteins but are less well characterized. One example is RIX7 of *Saccharomyces cerevisiae* (and human homolog NLV2), which is essential for cytosolic ribosome biogenesis; it has a pore loop 1 motif of Gly-M/V-Ser-Gly (Lo et al. 2019). CryoEM data confirm that the hydrophobic residue of this motif contacts the substrate. The pore loop 2 sequence is more variable

both between AAA+ proteins and between ATAD3s in different species. Variations in the X-Ar-ϕ-X motif of pore loop 1 along with differences in pore loop 2 are proposed to dictate aspects of substrate specificity (Erzberger and Berger 2006, Puchades et al. 2020). Determining the substrate specificity of ATAD3s will be a key to understanding their function.

In contrast to the AAA+ domain, the ATAD3_N domain shows no homology to other known proteins and is unique to ATAD3s. Structural predictions in Phyre and AlphaFold indicate with very high confidence that this approximately 250-amino acid domain comprises an intrinsically disordered amino terminus of >50 amino acids followed by α-helical segments. Uniprot also identifies predicted coiled-coil domains at varying positions, consistent with the predicted α-helical structure. This domain shows significant variation between plants and metazoans, and compared to the AAA+ domain, it shows greater variation between the seed plant A and B clades (**Fig. 2**).

Between the ATAD3_N and AAA+ domains, there are two predicted short, ~20-amino acid helices separated by four to six amino acids. The more C-terminal helix is clearly a transmembrane helix, exhibiting a high hydrophobicity, low hydrophobic moment and no net charge. The more N-terminal helix is amphipathic and would be predicted to be 'membrane active' rather than transmembrane (Phoenix and Harris 2002). These helices are discussed further later.

One final feature is the absence of an N-terminal mitochondrion-targeting peptide (Gilquin et al. 2010). Rather, between the transmembrane helix and the start of the AAA+ domain, there is an 18-amino acid sequence that has been identified as an internal targeting sequence (**Fig. 2**). This region shows significant sequence conservation even between *A. thaliana* and human ATAD3s.

Topology of ATAD3 in the Mitochondrion

As noted earlier, all four ATAD3 proteins in *A. thaliana* are found only in mitochondria. C-terminal ATAD3–GFP fusion constructs expressed in transgenic plants localized to mitochondria (Kim et al. 2021). Mitochondrial proteomic experiments in *A. thaliana* recovered all four ATAD3s (Fuchs et al. 2020), and the absence of ATAD3s from databases of chloroplast proteins further supports their exclusive localization to mitochondria. This is in contrast to other organelle quality control AAA+ proteins in plants, such as FtsH, Lon and Hsp100/ClpB proteins, all of which have homologs in both chloroplasts and mitochondria, some of which are dual targeted (Lee et al. 2007, Janska et al. 2013). Unlike these other proteins, which evolved from prokaryotic progenitors, ATAD3 proteins are a eukaryotic innovation as noted earlier. However, despite a long evolutionary history, ATAD3s have not diversified to function in other plant organelles, indicating that their activity likely modulates processes unique to mitochondria.

Gilquin et al. (2010) performed an extensive initial analysis of the topology of ATAD3 in human cells. Their proteolysis experiments with isolated mitochondria placed the AAA+ domain in the mitochondrial matrix, and further experiments in humans support this localization (Zhao et al. 2019, Arguello et al. 2021, Ishihara et al. 2022). The transmembrane helix proximal to the AAA+ domain is anchored in the inner mitochondrial membrane (**Fig. 2**). The adjacent amphipathic helix was suggested by Baudier (2018) to potentially span the outer mitochondrial membrane, which would place the majority of the ATAD3_N domain exposed to the cytosol. However, this scenario can be discounted, as the inner and outer mitochondrial membranes would only be separated by the four to six amino acids between the two helices, an insufficient distance between the two membranes. More likely the more N-terminal, amphipathic helix could orient along the inner mitochondrial membrane or associate with helices of other ATAD3 subunits.

The majority of the ATAD3_N domain is in the intermembrane space between the inner and outer mitochondrial membranes. Interestingly, the length of the predicted helix comprising the majority of the ATAD3_N domain is calculated to be sufficiently long (~20 nm) to span the estimated distance between the two mitochondrial membranes (outside of contact sites). This domain has predicted coiled-coil regions that are necessary for the formation of at least dimeric forms of the protein (Zhao et al. 2019, Ishihara et al. 2022). N-terminal to the predicted helical region there are 50+ amino acids that are 'available' to pass through the outer membrane to the cytosol. Whether or not the N-terminus of ATAD3 penetrates the outer mitochondrial membrane making it capable of interacting with cytosolic components is somewhat contentious due to the possibility of artifacts resulting from the disruption of the outer mitochondrial membrane. Nevertheless, several experiments suggest that the N-terminus is accessible to the cytosol. Zhao et al. (2019) reported recovering ATAD3A by immunoprecipitation with Dynamin-related protein 1 (DRP1, a mitochondrial fission GTPase), which sits on the outer membrane to orchestrate mitochondrial fission; this interaction could be indirect. Gilquin et al. (2010) found that an antibody specific for the first N-terminal 50 amino acids reacts with intact mitochondria, and Zhao et al. (2019) reported similar results. Data from a proximity-dependent biotinylation map of human cells recovered ATAD3 with biotinylated by dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1 isoforms (RPN1 and RPN2) as baits, which are endoplasmic reticulum proteins (Go et al. 2021). The only relevant data in plants come from a study in *P. patens* identifying arginylated peptides—a modification that is known to occur in the cytosol and not in the mitochondrion—which found Gln30 of PpATAD3B to be arginylated (Hoernstein et al. 2016).

The pathway by which this large, complex protein is inserted in this topology, with the C-terminus in the matrix and the N-terminus exposed to the cytosol, requiring the protein to pass through both mitochondrial membranes, is unknown. No other single mitochondrial protein is reported to span both the inner and outer mitochondrial membranes, having domains both within the mitochondrial matrix and exposed to the cytosol, and this topology would place ATAD3 in a prime position to

communicate mitochondrial status to the rest of the cell and vice versa. Indeed, ATAD3 has been associated with proteins of ER–mitochondrial membrane contact sites (Baudier 2018). Confirming ATAD3_N access to the cytosol is critical to defining the potential diverse functions of ATAD3s.

Expression of ATAD3 Proteins in Plants

The abundance and expression of ATAD3s are relevant to considering ATAD3 function. (Fuchs et al. 2020) performed a proteomic analysis of mitochondria isolated from cultured heterotrophic *A. thaliana* cells and estimated the copy number per average mitochondrion for >2,000 proteins over five orders of magnitude. The most abundant three proteins had >37,000 copies per mitochondrion [voltage-dependent ion channel 1 (AT3G01280), ADP/ATP carrier 1 (AT3G08580) and mitochondrial ATP synthase β-subunit (At5g08670/ At5g08680/ At5g08690)], while ∼90 proteins were detected at one copy or less per mitochondrion. ATAD3 copy numbers were estimated as 550, 219 and 740 for A1, A2 and B1, respectively, and only one copy for B2. ATAD3A1, A2 and B1 can be considered moderately abundant mitochondrial proteins as 76% of proteins were less abundant (<219 copies), and 12% of proteins have copy numbers in the same range as the ATAD3s. Some other mitochondrial AAA+ proteins that are involved in protein quality control, including the soluble proteases Lon 1 (AT5G26860) and Clp X (AT5G53350) and the membrane-bound proteases FtsH3 (AT2G29080) and FtsH4 (AT2G26140), and the Hsp100 chaperone family member ClpB4 (AT2G25140), showed a similar copy number. Our proteomic analysis of mitochondria from seedlings grown in culture for 14 d identified 1,998 proteins and ranked the relative abundance of ATAD3s as A2 > B1 > A1 (rank 221, 265, 423, respectively, of 1998), but again, B2 was among the least abundant proteins (ranked 1961 of 1998) (Kim et al. 2021). The significance of the low copy number of B2 is unclear but possibly suggests that B2 is headed to becoming a pseudogene. In total, these data indicate that ATAD3s are present at levels similar to other quality control components in mitochondria.

Databases of transcript expression (https://bar.utoronto.ca/eplant/) provide some additional insights. Under most conditions, the four *A. thaliana* ATAD3 genes have generally the same relative transcript levels as their reported protein abundance, with B1 > A1 > A2 > B2. Their developmental pattern shows the highest transcript levels in the vegetative and floral shoot apex and 24-hour imbibed seeds, likely reflecting mitochondrial biogenesis. Similarly, the *P. patens* ATAD3 genes show the highest expression in imbibed spores. Additionally, heat maps indicate a very consistent pattern of expression in different tissues and different environmental perturbations for the *A. thaliana* and *P. patens* genes. Overall, transcript levels are low, and their response to environmental perturbations is minimal. Maximum transcript levels of the abundant voltage-dependent ion channel 1 and ADP/ATP carrier 1 proteins are an order of magnitude higher than any of the ATAD3s. Coexpression of ATAD3s assessed with ATTED-II (ver.11.1) (Obayashi et al. 2022) finds all four genes are co-expressed with each other and also with Lon1 and mitochondrial Hsp70-1 (AT4G37910) although how this reflects on ATAD3 function is unclear. Overall, ATAD3 proteins are moderately abundant, essential proteins that do not show a strong response to environmental perturbations.

ATAD3 Protein Interactions

In plants, three of the *A. thaliana* ATAD3s (A1, A2 and B1) were specifically recovered by coimmunoprecipitation with mTERF18 (also known as SHOT1; At3g60400) (Kim et al. 2021). The Mitochondrial Transcription tERmination Factor-related (mTERFs) proteins are a family of proteins involved in organelle gene expression that have been found to interact with organellar DNA or RNA and in some cases with proteins (Kleine and Leister 2015). The mTERF18 protein is found in nucleoids, suggesting an indirect link between ATAD3 and mitochondrial DNA. Interestingly, ATAD3 was recovered by immunoprecipitation with human mitochondrial DNA-binding proteins TFAM (mitochondrial transcription factor A) and SSBP1 (He et al. 2012), and earlier studies found ATAD3 enriched in mitochondrial nucleic acid preparations (He et al. 2007, Reyes et al. 2011). Recent studies in human cells also reported that ATAD3A binds TFAM and that ATAD3A deficiency altered nucleoid structure (Ishihara et al. 2022). An additional link to nucleoids was reported by (Sen et al. 2022) who found by proximity labeling that the mitochondrial helicase twinkle (TWNK; Q96RR1) interacts with ATAD3A. Although there is no TFAM homolog in plants, plants do have a homologous twinkle helicase (targeted to chloroplasts and mitochondria) and mitochondrial SSBP1 homologs (Gualberto and Kuehn 2014). Although there is no evidence of direct interaction of ATAD3A with mitochondrial DNA, these observations suggest a conserved link between ATAD3 and the machinery of mitochondrial DNA maintenance and gene expression.

A few studies have reported ATAD3 interactions with other proteins. As mentioned earlier, DRP1, involved in mitochondrial fission, immunoprecipitated ATAD3 from human mitochondria (Zhao et al. 2019). Interaction with human cytochrome c oxidase assembly factor (COA3) has also been documented (Ban-Ishihara et al. 2015). Crosslinking studies and biotinylation experiments (with diverse baits) in human mitochondria have detected interaction of the ATAD3_N domain with proteins in the inner membrane space, consistent with the localization of this helical component of this domain (Go et al. 2021, Zhu et al. 2022). Arguello et al. (2021) reported >200 interactors in human cells from coimmunoprecipitation with ATAD3A including prohibitins, mitochondrial contact site and cristae organizing system (MICOS) complex proteins, membrane-embedded AAA+ proteases (YME1L, AFG3L2 and SPG7), cristae remodeling proteins OPA1 and LETM1 and ATPase synthase subunits, among others. They also performed proximity labeling with BioID fused to the ATAD3A C-terminus and recovered 158 proteins

enriched for subunits and assembly factors of complex I, mitoribosome subunits, ADP/ATP carriers, components of fatty acid metabolism and others. They highlight 12 proteins identified by both methods: subunits or assemble factors of complex I (NDUFAF3, NDUFAF4, NDUFV1, NDUFS7, NDUFS8 and NDUFS1), inner membrane–embedded proteases (YME1L1 and AFG3L2), membrane assembly factors (LETM1 and OXA1L), a component of the protein import motor (TIM16/PAM16) and a ribosomal protein of the large subunit (MRPL47), all of which have homologs in plants except the 20-kDa complex I assembly factor NDUFAF4. The functional significance of these interactions remains to be tested.

Phenotypes Resulting from ATAD3 Disruption

Only a single study has investigated the effects of ATAD3 mutations in plants. Kim et al. (2021) reported that single mutant T-DNA insertion alleles of each of the four *A. thaliana* ATAD3 genes grow like wild type, with no obvious morphological differences, and mutant alleles are transmitted normally. However, homozygous deletion mutants of both ATAD3A1 and A2 or of both ATAD3B1 and B2 could not be recovered, suggesting that one gene from each evolutionary clade (A or B, **Fig. 1**) is required for viability. Mutation of A1 or A2 with B1 was also lethal, while plants lacking either A1 or A2 and B2 were viable. This latter combination might be explained by the fact that B2 is present at very low levels, but then it is surprising that B1 single mutants appear wild type. This might be explained if the total dosage of ATAD3 proteins determines viability. Transmission of the mutant alleles was also impacted by parental genotypes. Further genetic analysis is required to fully explore the complex interactions of these four proteins.

Because *A. thaliana* ATAD3 mutants were either aphenotypic or lethal, insight into the importance of ATAD3A was obtained by studying an *a1b1* homozygote that was partially rescued with an A1:A1-GFP transgene (Kim et al. 2021). The plants exhibited slower growth and reduced complex I, with an accumulation of a complex I assembly intermediate, but had normal levels of complexes III and V. Interestingly, these plants showed enhanced acclimation to high temperature, similar to the mutant of mTERF18, the protein with which they coimmunoprecipitated. Confocal microscopy revealed heterogeneous mitochondrial morphology with many enlarged mitochondria and showed evidence that nucleoids were disrupted, filling the whole organelle, rather than appearing as discrete puncta (**Fig. 3**). These phenotypes are consistent with the link of ATAD3 to nucleoids, as well as the reported interactions with complex I subunits and assembly factors (Arguello et al. 2021).

There has been extensive documentation of the essential nature of ATAD3As in metazoans, as well as a proliferation of studies on how ATAD3 is associated with human disease or how mutations at the ATAD3 locus alter mitochondrial phenotypes (Baudier 2018, Peralta et al. 2019, Teng et al. 2019, Zhao et al. 2019, 2022, Gunning et al. 2020, Frazier et al. 2021, Watanabe et al. 2023). These studies report aberrant mitochondrial cristae morphology, alterations in nucleoid size and trafficking, fragmentation of mitochondria, reduced complex I, a decrease in complex V and disrupted cholesterol and lipid homeostasis. Understanding the primary defects that result in these pleiotropic phenotypes is a goal for future studies.

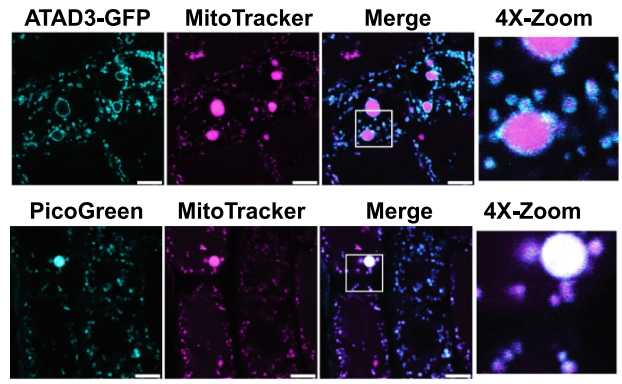

Fig. 3 Confocal microscopy of aberrant mitochondria in roots of *A. thaliana* with disrupted ATAD3 function. Homozygous *a1b1* mutant rescued with a *p*At3A1:At3A1-GFP transgene. Bar = 5 μm. Top: ATAD3A1 is on the mitochondrial periphery as shown by GFP fluorescence, and some mitochondria are greatly enlarged. Mitochondria stained with MitoTracker. Bottom: PicoGreen staining of mitochondrial DNA reveals enlarged nucleoids filling the entire mtiochondrion as labeled with MitoTracker (Kim et al. 2021).

Concluding Remarks

As unique mitochondrial proteins that are essential for life in diverse eukaryotes, ATAD3s clearly contribute to critical biochemical and cellular functions. However, it remains a challenge to uncover their specific molecular mode of action. While the selective forces that drove the initial evolution of ATAD3s in early eukaryotes are unknown, we can hypothesize that they originated with the mitochondrion from an ancestral AAA+ protein. Given that ATAD3 is essential in eukaryotes where it is present, it is puzzling that some eukaryotic lineages have retained, while others have lost ATAD3s. A phylogenetic profiling approach (Pellegrini et al. 1999) may provide information on other proteins with similar distributions and suggest functional relationships. In addition, evolutionary analysis will reveal if the ATAD3s evolved under different selective constraints among the two seed plant clades, as well as between metazoans and plants. These data can illuminate to what extent ATAD3 function is conserved as it relates to ATAD3 structure.

The sequence and structural relationship of ATAD3s to other AAA+ proteins involved in protein quality control indicate that ATAD3s likely function as quality control components in mitochondria. Although multiple different proteins have been found to interact with ATAD3s, whether any of these proteins are substrates of the AAA+ domain is unknown. There is no evidence that ATAD3s process substrates for degradation by mitochondrial proteases as do other AAA+ proteins. Using experiments designed to 'trap' substrates in the AAA+ domain (Rei Liao and van Wijk 2019) could help resolve which

proteins are chaperoned by ATAD3s versus those proteins that may interact in a structural or regulatory mode.

The essential nature of ATAD3s complicates functional studies, and their oligomeric structure further confounds analyses due to potential dominant negative effects. The pleiotropic phenotypes observed as a result of ATAD3 disruption do not point to a molecular mode of action as they can represent secondary effects. The link between ATAD3 and nucleoids in both mammals and plants is interesting, given that their mitochondrial genome structure and transcription are vastly different (Moller et al. 2021); the common substrates of ATAD3s that may impact nucleoids remain to be discovered. Furthermore, it is critical to define the role of the ATAD3_N domain as it may act to control or signal the integration of ATAD3 function within the host cell.

Data Availability

Source data for all figures are provided in the text or figure legends.

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Disclosures

The authors have no conflicts of interest to declare.

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