REVIEW

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A role for the *GDAP1* gene in the molecular pathogenesis of Charcot-Marie-Tooth disease

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In 2002 a series of mutations in the *GDAP1* gene were reported in patients suffering from Charcot-Marie-Tooth disease manifesting as early–onset, progressive distal-muscle wasting and weakness. The molecular etiology of Charcot-Marie-Tooth -*GDAP1* disease has been elucidated but its pathogenesis remains unclear, especially given the seemingly contradictory function of the GDAP1 protein. Expression of *GDAP1* is observed almost exclusively in neuronal cells, however, the GDAP1 protein is present in mitochondria, where it plays a role in fission, a ubiquitous process occurring in all cells. While GDAP1 contains two glutathione S-transferase (GST) domains, its GST activity is in fact very limited. Additionally, despite GDAP1 affecting mitochondrial functionality, and hence being of great importance to cellular function, the *GDAP1*-associated Charcot–Marie–Tooth disease is mainly characterized by axonal degeneration. Finally, mutations in the *GDAP1* gene may be inherited in a recessive or dominant manner. Given the way such varied observations are hard to reconcile with one another, the investigation of GDAP1 is at one and the same time a difficult but also challenging endeavour. The purpose of this review is to summarize the current knowledge on the GDAP1 protein and its function in the cell. A further part is the characterization of *GDAP1*-associated Charcot–Marie–Tooth disease, its symptoms and course, as well as an outlining of the possible mechanisms underpinning the disorder.

Key words: GDAP1, Charcot–Marie–Tooth disease, neuropathy, mitochondrial dynamics

INTRODUCTION

Charcot-Marie-Tooth disease (CMT) is characterized by progressive weakness and muscle atrophy (mainly encompassing the distal muscles of the upper and lower limbs) and sensory loss. In general terms, CMT is divided into two main groups i.e. CMT1, which is a demyelinating form associated with reduced nerve-conduction velocities and segmental demyelination and remyelination, and CMT2, axonal neuropathy, characterized by axonal loss without demyelinating lesions (Dyck and Lambert 1968). The basic criterion for CMT1/CMT2 division is the value of 38 m/s of the nerve conduction velocity in the motor fibers of the median nerve.

While CMT is caused by mutations in many genes involved in various cellular pathways, the different mutations in the *GDAP1* (ganglioside induced differentiation associated protein 1) gene are associated with an axonal or intermediate form of CMT with recessive or dominant modes of inheritance and a wide range of severities (Cassereau et al. 2011). The *locus* for CMT associated with *GDAP1* gene mutations was first identified in cases of recessive CMT in Tunisian families, on chromosome 8q13-q21 (Ben Othmane et al. 1993). The corresponding gene was found in 2002 by two independent groups (Baxter et al. 2002, Cuesta et al. 2002). In 2005 the first dominantly-inherited mutation in the *GDAP1* gene was reported (Claramunt et al. 2005).

GDAP1 protein is involved in many aspects of mitochondrial morphology and functioning, thus its associations with CMT disease is not very surprising, as several neurodegenerative diseases result from alterations in mitochondrial dynamic processes (Bertholet et al. 2016, Gao et al. 2017). For example, mutations in the mitofusin 2 (*MFN2*) gene, encoding protein required

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for mitochondrial fusion (Chen et al. 2003, Santel and Fuller 2001), lead to autosomal-dominant CMT (Züchner et al. 2004); while mutations in the gene encoding optic atrophy protein 1 (*OPA1*), the main mediator of inner membrane fusion in mammals, promote autosomal-dominant optic atrophy (ADOA), an inherited form of optic-nerve degeneration (Alexander et al. 2000, Delettre et al. 2000).

GDAP1 protein seems to be a very interesting object for study in terms of structure and function. Certain of its aspects are still unexplored and wait for discovery and others are confusing, as some of published reports appear contradictory. This review has therefore sought to bring together pieces of knowledge concerning the function of the protein and its involvement in the appearance of CMT. Every effort is also made to shed light on the possible molecular mechanism underpinning *GDAP1*-neuropathy, with some indications given as to processes capable of contributing to progression of the disease.

The *GDAP1* gene and its protein

The *GDAP1* gene was originally identified as one of 10 cDNAs whose expression increased upon differentiation of a neuroblastoma cell line (Liu et al. 1999). It spans almost 14 kilobases of genomic DNA, with coding sequences consisting of six exons (Cuesta et al. 2002). Two transcript variants of *GDAP1* produced by alternative splicing have been identified. The longer variant encodes a 358-amino acid protein, while transcript variant 2 encodes a 290-amino acid protein shortened at the N-terminal. *GDAP1* is mainly expressed in neurons and, albeit at much lower levels, in Schwann cells (Niemann et al. 2005, Pedrola et al. 2005). However, rather high level of *GDAP1* transcript was also found in cancer cell lines of different tissue origin (Ratajewski and Pulaski 2009). The GDAP1 protein contains two glutathione S-transferase (GST) domains (GST-N and GST-C) separated by an alpha helical loop (α-loop), a C-proximal hydrophobic domain (HD1) essential to GDAP1-induced mitochondrial and peroxisomal fission, and a C-terminal transmembrane domain (TMD) or tail-anchor, responsible for the correct locating of the GDAP1 protein (Cuesta et al. 2002, Huber et al. 2013, 2016, Marco et al. 2004, Niemann et al. 2009, Wagner et al. 2009) (Fig. 1).

The multiple roles of GDAP1 protein

Once the link between GDAP1 and CMT disease had been identified, bioinformatics analysis showed that GDAP1 belongs to the glutathione S-transferase (GST) family (Cuesta et al. 2002, Marco et al. 2004). These enzymes catalyze the conjugation of the reduced form of glutathione with xenobiotic substrates, the purpose being detoxification. However, until very recently, considerable efforts to demonstrate GST activity in purified GDAP1 had been unsuccessful (Pedrola et al. 2005, Shield et al. 2006). Recently, theta-class-like GST activity was clearly demonstrated in the case of the recombinant GDAP1 protein (Huber et al. 2016). It seems, that the glutathione-conjugating activity of GDAP1 is regulated by its C-terminal HD1 domain in an autoinhibitory manner, given that a full-length protein and

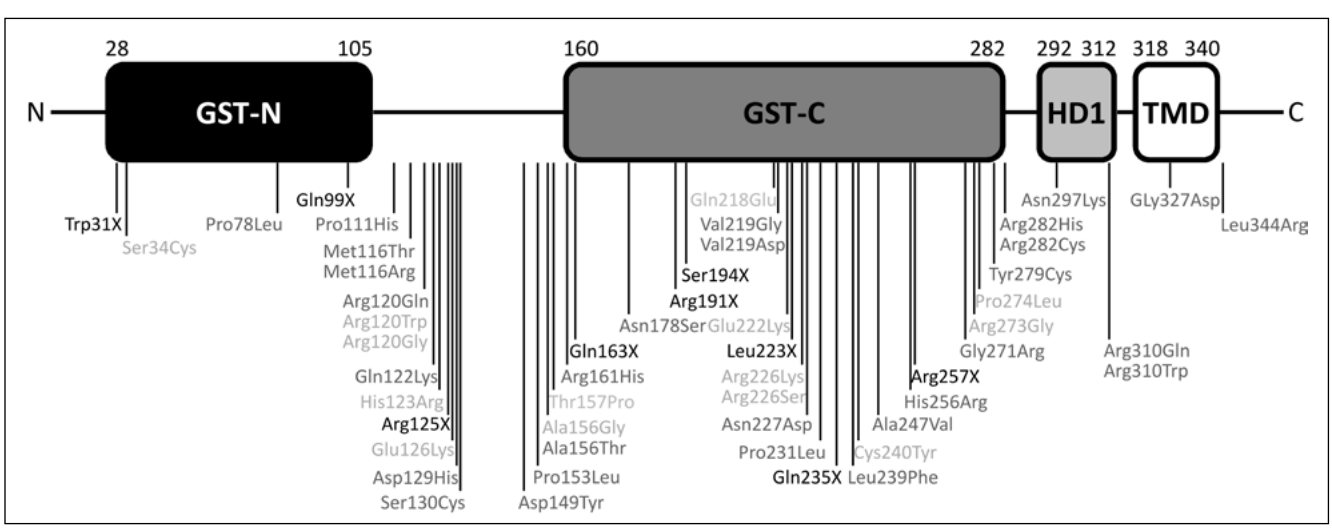

Fig. 1. Graphic representation of the GDAP1 protein domain structure. The borders of individual domains were determined by reference to the Pfam database (http: //pfam.xfam.org). Amino-acid substitutions found in patients with CMT are indicated. Recessively-inherited changes are represented in black (nonsense) and dark gray (missense), while dominant ones are in light gray. Domains: (GST) glutathione S-transferase domain; (HD1) hydrophobic domain; (TMD) transmembrane domain.

its fragments deprived of both TMD and HD1 or only TMD domain were found to be catalytically inactive *in vitro* (Huber et al. 2016). GDAP1 also regulates cellular glutathione content *in vivo* and protects cells against oxidative stress. It was found that Gdap1 is upregulated in oxidative-stress-resistant mouse neuronal cells. Overexpression of *Gdap1* increases the total cellular glutathione level and protects against endogenous oxidative stress caused by glutathione depletion. In contrast, Gdap1 downregulation increases the susceptibility of mouse neuronal cells against glutathione reduction (Noack et al. 2012). Together, it seems that GDAP1 is regulated by the redox state of the cells. It was suggested that GDAP1's GST domains serve as a redox sensor, rather than a *bona fide* GST enzyme, modulating its function by changing its conformational state upon stimulation (Huber et al. 2016). In line with this, glutathione depletion resulted in a marked upregulation of Gdap1, whereas increasing the cellular glutathione content had the opposite effect in mouse neuronal cells (Noack et al. 2012). This offers new intriguing insight into the function and the way GDAP1 activity is regulated.

The finding that GDAP1 is an integral membrane protein of the outer mitochondrial membrane (Niemann et al. 2005, Pedrola et al. 2005) pointed to an involvement of the protein in mitochondrial processes. Neurons are extremely polarized cells demanding a high level of energy that mitochondria are able to provide through adenosine triphosphate (ATP) production induced by the process of oxidative phosphorylation (OXPHOS). Mitochondria are essential to neuronal function and involved in many neuronal processes, such as calcium (Ca^{2+}) homeostasis, maintaining plasma membrane potential, axonal and dendritic transport, and the release and reuptake of neurotransmitters at synapses (Sheng and Cai 2012, Vos et al. 2010). The production of an extra protein regulating mitochondrial functionality thus seems explicable and understandable enough. This led to a presentation of GDAP1 as a participant in the mitochondrial fission process (Niemann et al. 2005, 2009). Overexpression of *GDAP1* induces fragmentation of mitochondria while not inducing apoptosis, the effect being to impair mitochondrial transmembrane potential, or to interfere with mitochondrial fusion (Niemann et al. 2005). In *Drosophila melanogaster*, *Gdap1* RNAi leads to progressive aggregation and fusion of mitochondria, and eventually to the presence of large, elongated mitochondria in the fly's thorax muscle. Mitochondria in the retina are also of larger size. In turn, smaller mitochondria in the retina and, more evidently, in the muscle were observed after overexpression of *Gdap1* (López Del Amo et al. 2015). However, GDAP1 would not seem to be a canonical fission protein, as its homologues have not been found in either *Saccharomyces cerevisiae* or *Caenorhabditis elegans* (López Del Amo et al. 2015), albeit with the expression of human *GDAP1* being in a position to correct for deficiency in yeast *FIS1* (Estela et al. 2011). What is more, GDAP1-fission is seen to depend on the other well-known fission factors Fis1 and Drp1 (Niemann et al. 2009). These findings can be put together with reports that overexpression of CMT-patient missense mutant alleles results in fragmented mitochondrial distribution very similar to that found in cells overexpressing the wild-type allele (Pedrola et al. 2008, Pla-Martín et al. 2013). That gives rise to a suggestion that GDAP1 rather have another function than mitochondrial fission *per se*.

Some clue as to the actual role of GDAP1 in cells is provided by a recent report revealing that GDAP1 is present not only in mitochondria, but also in mitochondria-associated membranes (MAMs), a place of interface of mitochondria and endoplasmic reticulum (ER) (Pla-Martín et al. 2013). Formation of the contact sites between ER and mitochondria seems to be required not only for the regulation of mitochondrial morphology, dynamic and function, but also for the transport of calcium from the ER to the mitochondria, the import of lipids into mitochondria, the formation of autophagosomes, and cell survival (Herrera-Cruz and Simmen 2017, Vance 2014). The ER has been shown to wrap around mitochondria and to facilitate mitochondrial division, and this step precedes Drp1 recruitment (Friedman et al. 2011). GDAP1 may be involved in the formation and/or modulation of the ER-mitochondria contacts and this way regulate the fragmentation of mitochondria, especially that GDAP1 fission is Drp1-dependent (Niemann et al. 2009). Depletion of GDAP1 leads to changes in the mitochondrial network, reducing co-localization between mitochondria and ER and limiting Ca^{2+} entry in mitochondria following store-operated calcium entry (SOCE) in human neuroblastoma cells (Pla-Martín et al. 2013). Consistently, overexpression of *GDAP1* in HeLa cells is seen to induce a redistribution of mitochondria that show increased contacts with ER (Pla-Martín et al. 2013). Defects in the maintenance of intracellular Ca^{2+} homeostasis were also observed in *gdap1*$^{-/-}$ mice neurons (Barneo-Muñoz et al. 2015).

As GDAP1 interacts with trafficking-associated proteins (caytaxin and RAB6B) and β-tubulin (Estela et al. 2011, Pla-Martín et al. 2013), it may form contacts between mitochondria and microtubules and directly participate in mitochondrial transport. Abnormal post-translational modifications of microtubules were observed in primary sensory and motor neuron cultures of *gdap1*$^{-/-}$ mice (Barneo-Muñoz et al. 2015). Also,

Gdap1 RNAi at the retina of *Drosophila melanogaster* results in a tendency for peripheral localization of mitochondria to be lost (López Del Amo et al. 2015). In more complex polarized cells such as neurons, mitochondria are the subject of active transport to synaptic regions, which have high demands for energy and calcium buffering. This means that the expression of additional protein like GDAP1 facilitating this process is highly probable.

Beyond its involvement in mitochondrial processes, GDAP1 also targets peroxisomes to regulate peroxisomal morphology. Loss of GDAP1 leads to elongated peroxisomes, whereas overexpression promotes peroxisomal fragmentation. Like mitochondrial fission, GDAP1-induced fission of peroxisomes is Drp1-dependent (Huber et al. 2013).

To sum up, however the precise molecular function of GDAP1 is unclear, the available data indicate an important role of GDAP1 in several key cellular processes, like maintenance of mitochondrial morphology, motility, distribution and functioning, as well as in the cellular homeostasis of Ca^{2+} and glutathione. It is likely that GDAP1 interacts with different protein partners and is responsible for formation of the contacts between mitochondria or peroxisomes and other cellular structures, like ER and microtubules. These interactions are extremely important for correct mitochondrial/peroxisomal functioning and dynamics and also prevent the damage to mitochondria and stress resulting from it. Thus it is not surprising that the neurons relying heavily upon energy generated by mitochondria are cells which are most affected by mutations of *GDAP1*.

CMT disease is caused by mutations in the *GDAP1* gene

As shown above, GDAP1 is involved in the regulation of the key cellular processes that neuronal cell viability and survival entail. It is not surprising, therefore, that mutations in the gene encoding this protein may lead to pathological defects. Indeed, changes in the *GDAP1* gene are associated with CMT disease, one of the hereditary motor and sensory neuropathies, characterized by progressive sensory deficiency and distal progressive muscle debility. To date, above 80 mutations associated with the *GDAP1* gene have been described (http: //www.hgmd.cf.ac.uk/ac/index.php). Most are missense/nonsense mutations (Fig. 1), though some small deletions, insertions and mutations interfering with splicing have also been reported (Auer-Grumbach et al. 2008, Cuesta et al. 2002, De Sandre-Giovannoli et al. 2003, Kabzińska et al. 2005, Nelis et al. 2002).

CMT disease phenotypes associated with *GDAP1* mutations

GDAP1 mutations are responsible for primary axonal damage, however in sural biopsies specimens obtained from some patients harboring *GDAP1* gene mutations additional dysmyelination has also been detected (Kabzinska et al. 2006, Fu et al. 2017). Phenotype-genotype correlations for mutations are hard to determine for several reasons. Firstly, patient symptomology is very diverse. In some cases, especially considering dominantly-inherited *GDAP1* mutations, there is marked variability for age of onset and the level of functional disability in patients carrying the same mutations, as well as significant intra-familial variability (Azzedine et al. 2003, Manganelli et al. 2012). Secondly, the majority of mutations have been described in only a few unrelated patients (Cassereau et al. 2011). Finally, it is possible that other genes mutations may act as modulators of the disease phenotype. A recent finding of this kind concerned the possible contribution of the *JPH1* Arg213Pro allele to CMT, with the *GDAP1* mutation resulting R120W responsible for more severe clinical manifestations (Pla-Martín et al. 2015). However, it is clear that the autosomal recessive (AR) and autosomal dominant (AD) forms of CMT (*GDAP1*) show numerous distinct clinical and electrophysiological features. In the AR form penetrance of the *GDAP1* gene mutations is complete and there is rather small inter- and intra-familial variability. This form is usually severe with early onset (symptoms are usually observed before the end of adolescence) and is characterized by muscle weakness and wasting resulting in disabilities in the first or second decade of life (Cassereau et al. 2011). Some patients with the AR trait of inheritance additionally manifest dysphonia and respiratory dysfunction (Sevilla et al. 2008, Sivera et al. 2017). Among the recessively-inherited changes, nonsense and frameshift mutations leading to the truncation of the protein produced are very often associated with a most severe phenotype of CMT showing a more-rapid course of the disease (Cassereau et al. 2011). In turn, it is common for dominantly-inherited mutations to be associated with late-onset and rather mild CMT. Of note, in the group of patients harbouring dominant *GDAP1* gene mutations (AD-CMT) the penetrance at least at the clinical level seems to be limited, but minimal electrophysiological abnormalities may be observed almost in all clinically asymptomatic individuals. Similarly, in some of the clinically asymptomatic individuals harbouring *GDAP1* gene mutations, some abnormalities may be detected in the feet and distal leg muscles (Cassereau et al. 2011, Manganelli et al. 2012, Sivera et al. 2017, Zimon et al. 2011) (Fig. 2).

A model organism to investigate the function of GDAP1 and *GDAP1*-related CMT diseases

Some challenges have been experienced identifying a suitable model to study GDAP1 protein function and the pathomechanisms leading to CMT. As mentioned above, *GDAP1* genes are not found in simple model organisms like the yeast *Saccharomyses cerevisiae* and the nematode *Caenorhabditis elegans*. On the other hand, work does show that human *GDAP1* complements almost all phenotypes involving deletion of the *FIS1* gene in yeast (Estela et al. 2011). This opens up a new possibility for *S.cerevsiae* to be used in *in vivo* testing of the potential pathogenicity of the mutations found in human beings. However, neurons are very complex cells with high demands where energy and transport are concerned. This means that results obtained in yeast may not offer a full reflection of the conditions the neuronal cells experience.

Recently, a true *GDAP1* ortholog was confirmed in *Drosophila melanogaster*, with alterations in the level of

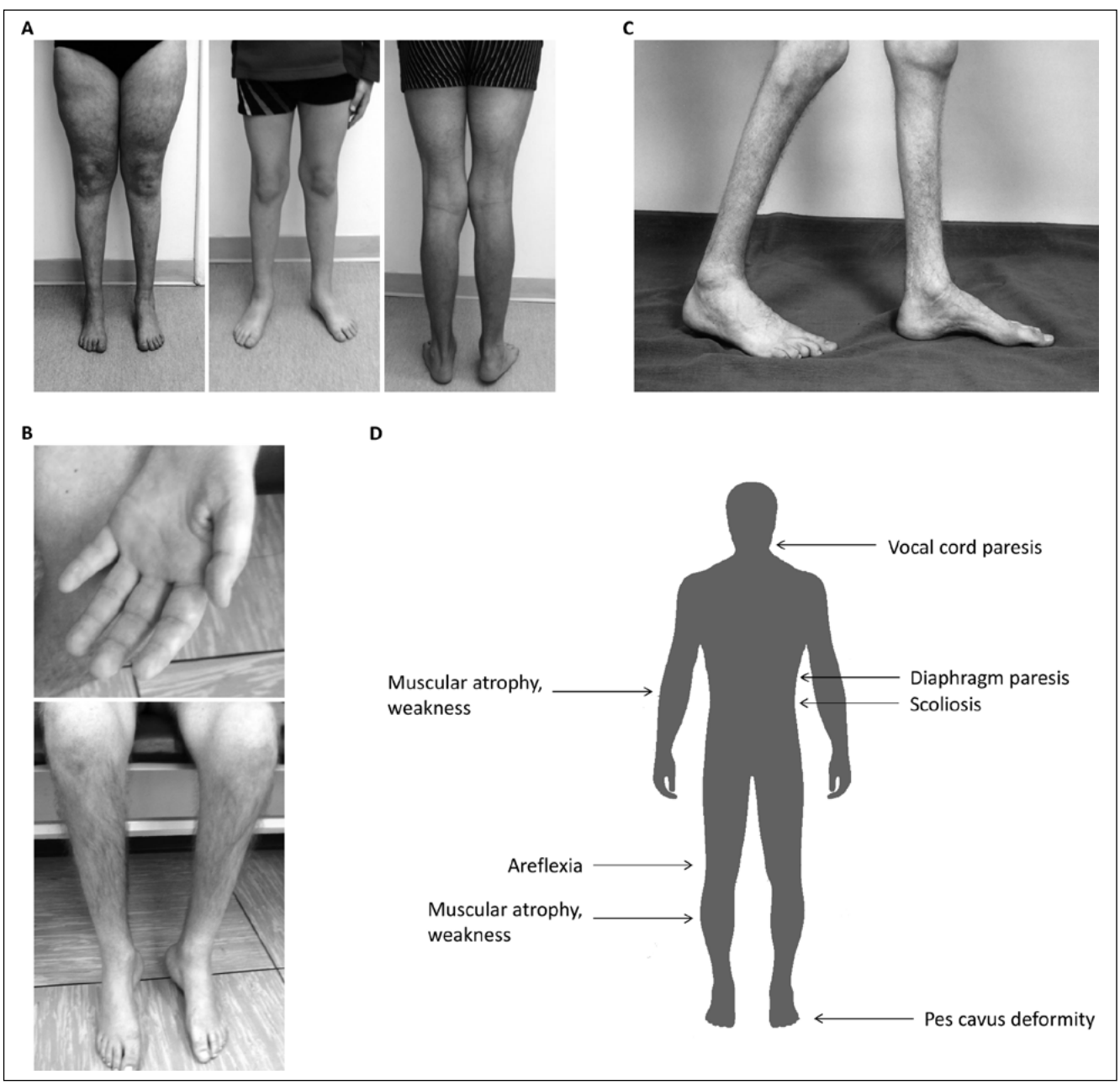

Fig. 2. Clinical presentation of CMT disease caused by *GDAP1* mutations varies from a mild form (A) via a moderate form (B) to a severe form (C). In patient (A), CMT disease is inherited as an autosomal-dominant trait, whereas the (B) and (C) patients suffer from a recessive type of CMT. Note the varying degrees of distal-muscle wasting of the upper and lower limbs. (D) Scheme of clinical characteristics of CMT caused by mutations in *GDAP1* gene.

Gdap1 in specific tissues found to lead to neuronal and muscular degeneration. Importantly, co-expression of human *GDAP1* with *Gdap1* RNAi can correct the neurodegenerative phenotype in the retina (López Del Amo et al. 2015). However, it is noteworthy that muscular degeneration in *Drosophila* is tissue-autonomous and not dependent on innervation, which is not observed in patients (López Del Amo et al. 2015). This may be a result of an expression pattern different from that found in human beings, as expression of *Gdap1* was also observed in fly muscle (López Del Amo et al. 2015). In line with this, general alteration of the level of Gdap1 has severe consequences, such as a reduction in maximum lifespan, not only resulting from impaired neuromuscular competence (López del Amo et al. 2017).

Thus far, two mouse models lacking *Gdap1* (*gdap1*$^{-/-}$) have been reported. They present different phenotypes, though in both cases Western blot analysis confirmed the absence of GDAP1 protein. In the first model, 19-month-old *Gdap1* knockout mice lacking exon 5 develop a peripheral neuropathy with reduced nerve-conduction velocity and mild hypomyelination, but no detectable axonal loss (Niemann et al. 2014). In contrast, in the second mouse model, involving the deletion of exon 1 from the *Gdap1* gene, no evident signs of demyelination were observed despite the occurrence of motor behavioural deficits from 3 months of age. This model also lacks evidence of morphological axonopathy, though proteomic studies of energetic metabolism in peripheral nerves reveal some physiological damage. Some changes have also been observed in motor neuron somas and at neuromuscular junctions (Barneo-Muñoz et al. 2015). Of note, mutations in the *GDAP1* gene of patients are associated predominantly with the axonal form of CMT (Cassereau et al. 2011), what is not observed in both mouse models. Also, it is unclear why the results presented in these two studies are so different, since they investigate the same phenomenon, at least in theory.

Summarizing, the use of model organisms to study the function of GDAP1, and the pathogenesis of GDAP1-related CMT, has obvious limitations. In less-complex organisms, such as yeast and nematode worms, orthologs of the GDAP1 gene are not observed. While this fact does not preclude their use, it does necessitate painstaking investigations of appropriate phenotypes, which may prove unsuccessful. On the other hand, in the more complex organism, some features of CMT disease do indeed present, and are without doubt informative and helpful. However, they cannot be said to offer an exact reflection of phenotypes observed in patients. Some important data regarding the mechanism behind *GDAP1*-associated CMT may thus be lacking, while others may not relate to human cells.

The role of *GDAP1* gene mutations in CMT pathogenesis

The GDAP1 protein regulates many key aspect of neuronal function and is responsible for maintenance of bioenergetic homeostasis. Many processes requiring GDAP1 are interconnected and affect one other. Thus, the pathogenic mechanism of *GDAP1* mutations found in patients with CMT may involve many multiple pathways and minor defects, in the end, leading to CMT disease. Mutations in the *GDAP1* gene may be inherited dominantly or recessively and these two traits are characterized by distinct phenotypes. It seems reasonable to suggest that the mechanisms underpinning the dominantly- and recessively-inherited mutations are different. In the following sections we provide an overview on the potential pathological mechanisms associated with different *GDAP1* mutations, according to inheritance trait.

GDAP1 gene mutations transmitted as an autosomal-recessive trait

It is usual for recessively-inherited mutations to be associated with a loss-of-function phenotype, where two genomic copies of the given gene are inactive and the protein cannot perform its function. This is observed in the case of complete deletion of the genes, mutations leading to the appearance of the premature STOP codon (nonsense, insertions, deletions) or missense mutations which significantly impair protein function. Similarly, we can expect mutations transmitted in a recessive mode in the *GDAP1* gene to caused dysfunction of the protein resulting in CMT phenotypes. Indeed, those mutations, which lead to a premature stop of translation or which are present within the C-terminal tail of GDAP1, the mitochondrial and peroxisomal targeting domain, affect anchoring to the mitochondrial outer membrane, or destabilize the protein, resulting in its rapid degradation and causing the most severe forms with disease onset in the first decade of life (Cassereau et al. 2011, Kabzińska et al. 2011, Niemann et al. 2005). The situation is less obvious for the missense mutations, which constitute the majority of those described so far. We cannot exclude the possibility that some also provoke rapid degradation of the mutant protein. It was noted that, unlike wild-type recombinant GDAP1 protein, all tested mutants isolated from insect cells were non-soluble, suggesting non-functional folding (Huber et al. 2016). Nevertheless, we cannot compare the heterologous production of the recombinant protein with the situation in the native host cell, with this observation supporting the

idea of inactivation and very rapid degradation of the mutant GDAP1 protein through multiple protein quality-control systems.

GDAP1 regulates several aspects of mitochondrial dynamics. Impaired mitochondrial fission may be a candidate mechanism underlying *GDAP1*-associated axonopathy. Mitochondrial division facilitates the transport, distribution, and quality control-mediated degradation of the organelle. The balance between mitochondrial division and fusion is required to maintain the form and function of mitochondria and thus neuronal viability and survival (Lackner 2014, Safiulina and Kaasik 2013). Nevertheless, data on fission activity of particular GDAP1 mutant proteins is contradictory. Some reports indicate that recessively-inherited mutations in the *GDAP1* gene result in reduced fission activities (Niemann et al. 2005, 2009, Noack et al. 2012), while others have shown overexpression of missense mutant alleles resulting in a fragmented mitochondrial distribution very similar to that found in cells overexpressing the wild-type allele (Pedrola et al. 2008, Pla-Martín et al. 2013). However, not all recessively-inherited mutations would seem to impair the GDAP1 fission-activity to the same degree (Niemann et al. 2005, Noack et al. 2012), suggesting something other than loss-of-function mechanisms or impairment of other aspects of mitochondrial functioning.

Disrupted transport of mitochondria offers a very potent mechanism which may account for axonal loss in CMT patients with *GDAP1* mutations. As was mentioned above (under, The multiple roles of GDAP1 protein), GDAP1 may play a direct role in the active transport of mitochondria inside neuronal cells. Thus, mutations may alter the interaction between GDAP1 and transport protein, leading to alterations in mitochondrial transport and movement. Missense mutations are clustered predominantly in two regions: the α-loop between two GST domains and the GST-C domain (Fig. 1). The α-loop region is responsible for the interaction between GDAP1 and β-tubulin (Estela et al. 2011), perhaps suggesting that changes here at least may impair the interaction between the two. Indeed, mutant proteins have been shown to interact more strongly with β-tubulin than does the wild-type protein, with the interaction being more intense for those mutations located within or near the α-loop domain (Estela et al. 2011). Interactions between GDAP1 and transport proteins are also most likely required as mitochondria are being located close to SOCE sites. Their perturbation by mutations in *GDAP1* is probably responsible for the inhibition of SOCE activity, as recessively-inherited *GDAP1* mutations located inside the α-loop are unable to compensate for a lack of *GDAP1* in SOCE activity in human neuroblastoma cells (González-Sánchez et al. 2017). Additionally, research using yeast *S. cerevisiae* makes it clear that *GDAP1* alleles containing missense mutations rescue all phenotypes of the null *FIS1* (*fis1Δ*) mutant, except for cell-cycle delay; and this effect is independent of the inheritance pattern of particular mutations. The authors thus propose that the defect in the cell cycle in the *fis1Δ* strain may be a result of an anomalous interaction between mitochondria and microtubules of the mitotic spindle (Estela et al. 2011). What is interesting, similar effect may be observed in mammalian cells, as significant down-regulation of cell cycle pathways and G2/M growth arrest in *Gdap1*-null mouse cells undergoing reprogramming were described (Prieto et al. 2016). In neurons, which are highly complex cells, mitochondria must be transported actively to (and maintained in) regions requiring a high energy level, like synapses. The movement of mitochondria is modulated in response to physiological signals and directed at dendrites and axons, i.e. sites that are far from the cell bodies (Schwarz 2013). Transport of mitochondria is so crucial in determining neuronal survival that a disruption of the synaptic translocation of mitochondria affects neuronal function adversely (Li et al. 2004, Verstreken et al. 2005). Axonal transport defects are further shown to play an important role in the pathology of neurodegenerative disorders (Millecamps and Julien 2013) and neuropathies. Mutations in *MFN2* gene, encoding mitochondrial fusion protein, MFN2 resemble those in GDAP1 in also being implicated in the appearance of CMT (Züchner et al. 2004). An abnormal mitochondrial distribution was observed in the Purkinje cells of Mfn2-deficient mice (Chen et al. 2007). Also, the accumulation of mitochondria in the distal part of sural nerve axons has been observed in CMT patients with *MFN2* mutations (Vallat et al. 2008), while MFN2 is found to interact with the Miro/Milton complex, which in turn links mitochondria to kinesin motors (Misko et al. 2010). All these findings suggest that impairment of mitochondrial transport may be the key mechanism involved in the pathophysiology of the MFN2-dependent CMT. Additionally, *MFN2* mutants can cause degeneration specific to long motor and sensory axons only, as these highly metabolic regions are most distant from the cell body, and thus more sensitive to impaired mitochondrial recruitment (Misko et al. 2010). It is probable that similar mechanism is responsible for the axonopathy in *GDAP1*-related CMT and this is the one of the main reasons of axons injury and death.

As GDAP1 is involved in many aspects of mitochondrial functioning, we cannot preclude other factors contributing to or modulating the symptoms of CMT disease. The alteration of the mitochondrial network and calcium homeostasis caused by decreased level of GDAP1 may also disturb the autophagy flux (Haidar and

Timmerman 2017). Autophagy is a degradation process required for the removal of proteins aggregates, superfluous or damaged organelles and other dysfunctional cells components. Neuronal cells are especially sensitive to disruption of the autophagic pathways and defects in this process are observed in many neurodegenerative diseases, also in hereditary neuropathies (Haidar and Timmerman 2017). Thus, deregulation of autophagy in GDAP1-deficient cells may also contribute to the pathology of CMT.

It is well known that mitochondria are producers of reactive oxygen species (Murphy 2009), with impairment of function leading to increased generation of oxygen radicals, highly reactive molecules interacting with and damaging nucleic acids, proteins, carbohydrates and lipids. Oxidative stress accompanies many neurodegenerative diseases (Chen et al. 2012) and mitochondrial diseases (Hayashi and Cortopassi 2015). Indeed, some reports suggest an involvement of oxidative stress in the development of CMT, since fibroblasts from autosomal-recessive CMT patients with reduced *GDAP1* levels also have reduced glutathione concentration and reduced mitochondrial membrane potential (Noack et al. 2012). Additionally, mutations cluster within the coding region of the GST-C domain, suggesting that the function of GDAP1's GST domain is impaired in these mutants. Mild but persistent oxidative-stress conditions in *Gdap1*$^{-/-}$ mice have also been documented (Niemann et al. 2014). On the other hand, metabolic analysis in *Drosophila melanogaster* suggests that alterations in oxidative stress are not a primary cause of the neuromuscular degeneration, but a long-term consequence of the underlying mitochondrial dysfunction (López Del Amo et al. 2015). Consistently, short-term knockdown of *GDAP1* is not shown to affect intracellular glutathione levels, or cause oxidative stress in mouse neuroblastoma cells (Huber et al. 2013). As the involvement of the reactive oxygen species in the CMT disease is still being debated, it is possible that they may exacerbate the symptoms, and contribute to a more rapid course of the disorder.

The recent finding that GDAP1 is also associated with MAM (see, The multiple roles of GDAP1 protein) gives the possibility that the defects described above may result from alteration in formation or functioning of the ER-mitochondria contacts. Several key cellular processes are regulated by MAM including lipid metabolism, calcium homeostasis, mitochondrial fission-fusion events, transport of mitochondria and autophagy (Krols et al. 2016). Thus, it is not surprising that several proteins associated with these junctions are involved in neurodegeneration (Krols et al. 2016). However, this aspect of GDAP1 functioning is unclear and need further extensive studies.

GDAP1 gene mutations transmitted as an autosomal-dominant trait

Dominantly-inherited mutations represent a minority of all the changes found in the *GDAP1* gene. This, combined with relatively weak and heterogeneous phenotypes, has meant these mutations are investigated less extensively than recessively-inherited counterparts. The present data do not allow for clear identification concerning the dominant mechanism reflects haploinsufficiency, dominant-negative loss of activity or a toxic function gained. The first option does not seem reliable, as the nonsense mutations leading to the shortening and inactivation of the protein are recessively-inherited. Two other options are more plausible, and all the more so given that GDAP1 is able to form heterodimers, with the mutations not disturbing protein dimerization, irrespective of the mode of inheritance (Huber et al. 2016). There is thus a possibility that mutant proteins may interact with wild-type ones, giving impairment of the overall GDAP1 function.

Like the mutations transmitted in a recessive trait, dominant mutations may affect mitochondrial transport and distribution, and similarly, this may be one of the most important mechanisms involved in axonal loss. However, it seems that dominantly-inherited mutations impair mitochondrial dynamics in other way than those inherited recessively. Overexpression of the dominant mutant *GDAP1* alleles increases mitochondrial motility, but – unlike wild-type or recessive variants - was not found to induce significant changes in mitochondria–ER interactions (Pla-Martín et al. 2013). Additionally, these variants promote significantly increased SOCE activity, as compared with wild-type GDAP1 – a fact that might reflect altered mitochondrial distribution vis-à-vis the plasma membrane, under base conditions (González-Sánchez et al. 2017). Taken together, these results suggest that the motility and proper positioning of mitochondria in respect of plasma membrane are affected. This may lead to the impairment of mitochondrial functioning and energy production. It was presented that the c.719G > A (Cys240Tyr) dominantly-inherited *GDAP1* mutation is shown to be associated with impaired activity of mitochondrial complex I (Cassereau et al. 2009). However, we cannot preclude that observed defect is independent from mitochondrial transport and additionally contributes to the course of the CMT disease. Complex I is the largest of the electron transport chain complexes, and the major site of superoxide production (Kudin et al. 2004). Its activity and expression are reduced in many neuronal diseases, such as Parkinson's disease (Parker et al. 2008, Schapira et al. 1990), Leber hereditary optic neuropathy (Brown et al. 2000, Kor-

sten et al. 2010, Wallace et al. 1988) and primary open angle glaucoma (Lee et al. 2012, Van Bergen et al. 2015). Complex I deficiency leads to reduced NADH oxidation and electron transfer, causing a sharp reduction in ATP synthesis. Decreased energy production may result in defects in all highly energy-consuming processes, such as mitochondrial fusion and transport. Indeed, dominantly-inherited mutations in *GDAP1* have been found to contrast with recessively-inherited ones, in that they impair normal mitochondrial fusion, with mild damage to mitochondria sustained in the process (Niemann et al. 2009). Dysfunction of Complex I is also associated with increased generation of reactive oxygen species, with chronic oxidative stress ensuing. This factor may also contribute to the pathogenesis of dominantly-inherited mutations.

The overall disruption of mitochondrial physiology as observed in the dominantly-transmitted mutation may lead to increased vulnerability of the cells to apoptosis and necrosis, with axonal loss ensuing. Indeed, overexpression of the dominant mutant allele of GDAP1 was seen to increase sensitivity of cells to apoptosis-inducing factors (Niemann et al. 2009).

CMT (GDAP1) pathogenesis – a general view

To sum up, the dominantly- and recessively-inherited mutations appear to be characterized by diverse modes of action, as the mutant alleles behave in different ways, depending on the mode of inheritance. However, both types of mutation may alter the

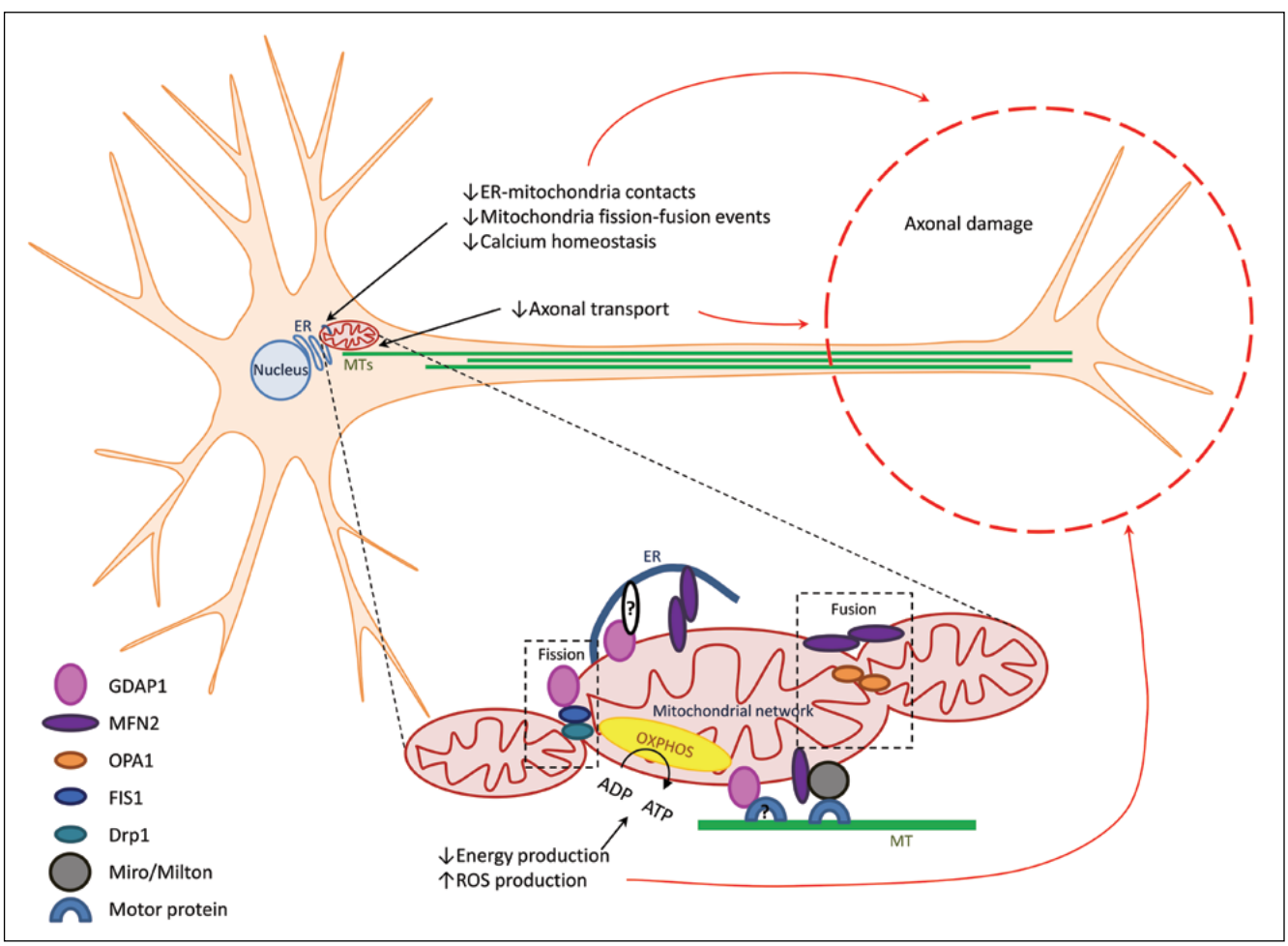

Fig. 3. The possible mechanism of axonal damage in CMT patients with *GDAP1* mutations.

GDAP1 may be involved in many processes associated with mitochondria, like fission events, the formation of ER-mitochondria contacts and the transport of mitochondria along microtubules (MTs). CMT-associated *GDAP1* mutations may impair each of the events leading to the appearance of general defects in mitochondrial functioning as the mitochondrial network is disrupted, interaction between mitochondria and the ER altered, calcium homeostasis impaired, ATP synthesis reduced, oxidative stress due to the excessive presence of reactive oxygen species (ROS) increased and axonal transport impaired. All of these defects exert a negative influence on the axon, and contribute to axonal damage resulting in CMT disease. Question marks indicate unknown elements and/or interacting partners.

same processes, for example axonal transport, which seems to be one of the most important reasons for axonal loss. However, in both cases the mechanisms underpinning CMT disease are complex and may include many pathological events, like disruption of the mitochondrial network, altered interactions between mitochondria, the ER and microtubules, reduced ATP production and increased oxidative stress and impairment of calcium homeostasis (Fig. 3). Because all these processes are inextricably linked and interdependent, it is hard to say which one indeed represents the primary cause of *GDAP1*-associated CMT. It is possible that all the defects observed in cells carrying mutant alleles contribute to the symptoms of the disease and may modulate their severity and course. Certain mutations may affect different pathway in their own way and observed axonopathy is a result of very complex effects. In general, dysfunction of the GDAP1 protein may lead to overall mitochondrial impairment. This observation is supported by a recent study using a model organism. In the peripheral nerves of *Gdap1*$^{-/-}$ mice (as compared with wild-type mice), expression analysis relating to selected mitochondrial proteins reveals changes in expression of all markers of glycolysis, oxidative phosphorylation and mitochondrial dynamics, as well as catalase of oxidative stress (Barneo-Muñoz et al. 2015). In turn, global proteomic analysis in *D. melanogaster* has shown that both up- and down-regulation of Gdap1 results in deregulation of the insulin-signaling pathway, an accumulation of carbohydrates and an increase in the β-oxidation of lipids, indicating changes in energy metabolism that favor the use of lipids as an energy source (López del Amo et al. 2017). Similarly, studies in mouse Mfn2 have revealed deffects in mitochondrial functioning, altering gluconeogenesis and impairing insulin signaling in the liver and muscle (Sebastián et al. 2012).

CONCLUSIONS

Since the *GDAP1* gene is mainly expressed in neurons, neuronal dysfunction seems a logical consequence of its depletion or mutation. Indeed, changes in the *GDAP1* gene result in the CMT disease. Our basic knowledge about GDAP1 is growing and many cellular processes requiring this protein have been discovered. Currently, it is known that GDAP1 is not only required for fission of mitochondria, but also regulates mitochondrial interaction with ER and plasma membrane, transport, calcium entry, production of energy and ROS, and also morphology of peroxisomes. However, many aspects of GDAP1 function require further investigation, for example what specific role(s) GDAP1 plays in neuronal cells that make it an essential requirement for normal functioning and alteration of which pathway contributes most to phenotypes observed in GDAP1-depleted cells. Also, the mechanism resulting in the dominant or recessive trait of *GDAP1* gene mutations remains unclear, especially given the lack of regions in which only the one of these two types of mutations is clustered. Both types are located in the central part of the protein (the α-loop and GST-C domain), which may suggest that particular mutations affect GDAP1 protein activity in various ways, or impair different functions of the protein. Taking these facts into account, it is possible that there are many factors contributing and modulating the *GDAP1*-CMT and each of the mutations leads to the disease in a different way, affecting some processes more and some less, thereby explaining such heterogeneous symptoms, course and severity of CMT disease. This may complicate the thinking of any future therapy for this disorder. Finally, we cannot definitively exclude that the majority of the reported so far biochemical and cellular alterations accompanying *GDAP1* mutations reflect not yet identified primary disturbances. Despite all the complexity of the picture that emerges from the currently available literature, there is still a possibility that, at least, some *GDAP1* gene mutations share a common pathogenic pathway, which may represent a potential target for CMT therapy.

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