THE CHARCOT-MARIE-TOOTH RELATED GENE *GDAP1* COMPLEMENTS CELL CYCLE DELAY AT G2/M IN *S. cerevisiae fis1* DEFECTIVE CELLS

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Running title: GDAP1 complements *fis1Δ* phenotype in *S. cerevisiae.*

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Mutations in the *GDAP1* gene are responsible of the Charcot-Marie-Tooth CMT4A, ARCMT2K, and CMT2K variants. GDAP1 is a mitochondrial outer membrane protein that has been related to the fission pathway of the mitochondrial network dynamics. As mitochondrial dynamics is a conserved process, we reasoned that expressing *GDAP1* in *Saccharomyces cerevisiae* strains defective for genes involved in mitochondrial fission or fusion could show some knowledge on GDAP1 function. We have discovered a consistent relation between Fis1p and the cell cycle since *fis1Δ* cells showed G2/M delay during the cell cycle progression. *fis1Δ* phenotype, which includes cell cycle delay, is fully rescued by *GDAP1*. By contrast, clinical missense mutations rescued *fis1Δ* phenotype except for the cell cycle delay. In addition, both Fis1p and the human GDAP1 interact with β-tubulins Tub2p and TUBB, respectively. Defect in the *fis1* gene may induce abnormal location of mitochondria during budding mitosis causing the cell cycle delay at G2/M due to its anomalous interaction with microtubules from the mitotic spindle. In the case of neurons harboring defects in GDAP1, mitochondria and microtubule cytoskeleton interaction would be altered, which might affect mitochondrial axonal transport and movement within the cell, and may explain the pathophysiology of the *GDAP1*-related Charcot-Marie-Tooth disease.

Mitochondria are highly dynamic organelles, continuously undergoing fission and fusion, and play a crucial role in many cellular functions such as respiration, substrate oxidation, ATP production, Ca^{2+} economy and apoptosis (1). The inheritance of mitochondria, the maintenance of their characteristic shape, and also their positioning inside the cell are mediated by active transport along cytoskeletal elements and depend on continuous fusion and fission of the organelles (2). The function of mitochondrial fission and fusion processes is not firmly established but these processes are mediated by specific molecular complexes which are best characterized in the budding yeast *Saccharomyces cerevisiae*. One of them, Fis1p, is an 18-kDa type-II integral membrane-anchored fission protein, which is evenly distributed in the mitochondrial outer membrane (3). Deletion of this gene from yeast (*fis1*) or the reduction in protein levels by RNA interference (RNAi) in mammalian cells (*FIS1*) results in a network of interconnected tubes (4), but its function still remains enigmatic (5). Deletion of other genes encoding proteins involved in mitochondrial fission such as the GTPase Dnm1p, which is responsible for the driving force at specific points of constriction, shows a similar mitochondrial net-like pattern (4), (6), (7). On the other hand, mitochondrial fusion process is directed by Fzo1p and Mgm1p, both GTPase proteins located or associated to the mitochondrial outer and inner membrane, respectively. Deletion of Fzo1p leads to mitochondrial fragmentation, a petite phenotype, and the loss of mitochondrial DNA, indicating an important function of Fzo1p in mitochondrial biogenesis (8).

Ganglioside-induced differentiation associated protein-1 (GDAP1) has been related to the mitochondrial fission process. It is located in the mitochondrial outer membrane and is mainly expressed in neurons (9), (10), (11). Although GDAP1 sequence has high level of similarity with the glutathione S-transferases (GST) (12), (13), no GST activity has been reported before (10), (14).

Mutations in *GDAP1* are the cause of the Charcot-Marie-Tooth (CMT) disease, either autosomal demyeliniting recessive CMT4A (15), or axonal recessive AR-CMT2K, or dominant CMT2K. The human counterparts of Fzo1p and Mgm1p, MFN1/MFN2 and OPA1, respectively, are also related to human disease. Mutations in *MFN2* cause the most frequent form of autosomal dominant axonal CMT disease, CMT2A (16), (17). Mutations in *OPA1* cause autosomal dominant optic atrophy (ADOA) (18), (19). No pathogenic mutations in the human *FIS1* gene has been described; by contrast, *DRP1* (the human homologue of yeast *dnm1*) has been associated with human (20) and mouse diseases (21).

It could be argued that GDAP1 should have a specific function in the mitochondrial fission pathway of mammalian cells. Furthermore, there is no ortholog of human *GDAP1* in *S. cerevisiae*. However, we reasoned that complementation experiments expressing *GDAP1* in yeast strains defective for genes involved in mitochondrial fission or fusion could add some knowledge on the GDAP1 possible role in relation to the mitochondrial network. Here we demonstrate that cells lacking Fis1p show abnormalities in cell cycle and mitotic spindle structures. Cell cycle delay at G2/M and other phenotypes in *fis1*Δ cells are fully recovered by *GDAP1*, which suggests a possible new function shared by Fis1p (also the human FIS1) and GDAP1. We hypothesize that *fis1Δ* cell cycle delay at G2/M is the consequence of the aberrant spindle formation during cell division and nuclei separation. However, the expression of several pathogenic *GDAP1* forms could not improved the cell cycle delay and the aberrant spindle formation in *S. cerevisiae fis1Δ* cells, although it could improve other processes, indicating that the correct GDAP1 sequence and structure is important for their complete functionality inside the cell.

EXPERIMENTAL PROCEDURES

Yeast strains and growth assay conditions- All strains used in this study were isogenic to FY833 (22): RJ1289 (*dnm1::kanMX4*), RJ1366 (*fis1::URA3*), RJ1368 (*mdv1::URA3*) (23), and YHS74 (*fzo::kanMX4*). Cell growth was assayed in YPEG medium with a source of glucose (0.05%), and the addition of the appropriate amino-acids, by spotting serial dilutions onto plates at 30ºC for 4 days.

Genetic procedures and yeast transformation- *GDAP1* constructs harboring pathological missense mutations R120Q, R120W, T157P, R161H, and R282C, and *GDAP1* lacking its transmembrane domains (*GDAP1*$^{\Delta 320\text{-}358}$ or *GDAP1-TMD)* were generated as described previously (10). Complete *GDAP1*, *fis1* and *FIS1* cDNA were cloned in *pRS425* vector (empty vectors were used for controls). Yeast transformation was performed by the lithium acetate method as described elsewhere (24). For mitochondrial structure visualization, *pRS314-ADH1-Su9-GFP* (*pSu9-GFP*) was expressed in the cells (25).

Yeast two-hybrid assay- A yeast two-hybrid screening (26) for proteins that interact with GDAP1 was carried out in the *S. cerevisiae* TAT7 strain (*MAT*a *ade2 his3 leu2 trp1 gal4 gal80 LYS2*::*lexAop-HIS3*, *URA3*::lexAop-lacZ) that was kindly provided by Dr. P. Sanz. Briefly, the GDAP1 cytosolic domain fused to LexA was cloned in the pBTM116 vector. A commercial human brain cDNA library cloned in the pACT2 vector (Clontech Laboratories, Inc., San Jose, CA) was transformed in the TAT7 strain. Transformants were selected in SC + 2% glucose plates lacking tryptophan, leucine, and histidine, and were then subsequently screened for β-galactosidase activity using a filter lift assay (27). 250.000 independent clones have been tested and the positive plasmids obtained were sequenced. GDAP1 protein structure and partial GDAP1: GST-Nt (aminoacids 24-105), loop (aminoacids 106-152), GST-Ct (aminoacids 153-309), and TMD (aminoacids 310-358) were cloned in *pACT2* vector and tested for β-galactosidase filter assay as described before (28) as following.

Mitochondrial purification- Cells were grown in SC medium until late log phase at 30ºC, harvested by centrifugation, and suspended with freshly prepared buffer A (100 mM Tris-SO_4, pH 9.4, and 10N DTT) (3-4 ml/g pellet weight). Cell suspension was incubated at 30ºC, and treated with zymoliase 20T in buffer B (1.2 M sorbitol, 20mM KPi, pH 7.4) in an orbital shaking chamber. Samples were then centrifuged, washed with cold buffer B, and suspended with cold buffer C (0.8 M sorbitol, 20mM K-MES pH 6.0) plus 100 µl PMSF

100mM. Cell lysis was carried out with a douncer glass-glass homogenizer. The supernatant was centrifuged at 12000g at 4ºC, suspended with cold buffer C and centrifuged again at 2000g at 4ºC. The mitochondria enriched fraction was obtained after centrifugation of supernatant from previous step at 12000xg for 15 min at 4ºC.

Antibodies- The following antibodies were used: anti-GDAP1 (Abnova, Taipei, Taiwan), anti-α-tubulin (Sigma, St Louis, MO, USA). The ECL™ anti-mouse-IgG, horseradish peroxidase linked antibody was from GE Healthcare (Little Chalfont Buckinghamshire, UK). A mouse-IgG antibody coupled was purchased from Molecular Probes (Gibco Invitrogen, Grand Island, NY, USA). The antibodies anti-c-myc and anti-LexA, and the anti-HA for coimmunoprecipitation experiments were purchased from Sigma.

Indirect immunofluoresce and imaging- Immunofluorescence experiments were performed as described previously (29), but with minor modifications. Cells were fixed, blocked, and then incubated with the α-tubulin antibody in blocking solution (PBS/ 3% BSA) o/n at 4ºC. Nuclei were counterstained with DAPI (Sigma). Mitochondria was visualized after *pS314-Su9-GFP* expression in all the strains (25). Wide-field fluorescence and differential interference contrast (DIC) images were captured using a Leica DM RXA2 light microscope (Nussloch, Germany), and photographed with a Hamamatsu digital camera (Tokyo, Japan).

Synchronization experiments and cell cycle analysis- S. cerevisiae cells were grown until early log-phase in YPEG medium. Cells synchronization was performed as following: at G1 phase with α-factor, in early S-phase with hydroxyurea, and in metaphase with nocodazole (all purchased from Sigma) as described elsewhere (29). Then, 1 ml aliquots were taken every 30 min, and cells were sonicated, fixed with 80% cold ethanol, and then subjected to RNase (Sigma) and pepsin treatment. Nuclei were stained with phosphate iodide (PI, Sigma). The BD FACSCanto™ flow cytometer apparatus used was from BD Biosciences. The budding index (BI) indicated the proportion of budding cells in the cell culture.

Immunoprecipitation- Hela cells were lysed in cold lysis buffer [50 mM Tris–HCl (pH 7.5), 10 mM NaCl, 2 mM EDTA, 1% NP-40, 15 % glycerol and protease inhibitors (complete, Mini, EDTA-free, (Roche Applied Science))]. Cell suspensions were immunoprecipitated using Dynabeads protein G (Invitrogen) according to manufacturer's instructions, and immunoblotting was performed as described elsewhere (30). *S. cerevisiae* FY250 cells were grown (until OD_{600}=0.6), pelleted and washed twice with distilled water. Cell Lysis was performed with IPS buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1 % Triton X-100, 10 % glycerol] plus 1M DTT, 100 mM PMSF, and protease inhibitors. Glass beads (0.5 mm diameter, Sigma) were added to the sample and incubated with zymoliase 20T (Sigma) for 30 min for cell wall degradation. Protein extracts were obtained after centrifugation and immunoprecipitation was proceeded as described (30).

RESULTS

Fis1Δ cells show increased cell size and abnormal distribution of mitochondria- We wanted to determine the effect GDAP1 produces on mitochondrial morphology in *S. cerevisiae* strains. First, we confirmed that *GDAP1* expression in the budding yeast that human GDAP1 is not toxic for their growth and is targeted to mitochondria (supplemental material and supplemental Fig. S1, *A* and *B*). To investigate whether GDAP1 could restore the altered mitochondrial patterns found in mutant cells defective in mitochondrial dynamics, we expressed *GDAP1* in the fission-defective *dnm1Δ*, *fis1Δ*, and *mdv1Δ* strains, and in the fusion-defective *fzo1Δ* strain. We visualized the mitochondrial morphology by expressing *pSu9-GFP* in all the strains tested, and five different patterns of the mitochondrial network were defined: fusioned 'aggregated', predominantly tubular 'tubular', tubular and vesicular 'mixed', predominantly vesicular 'vesicular' and completely fragmented 'fragmented'. Wild-type (WT) cells showed predominantly tubular reticulum-shaped mitochondria, whereas fission-defective strains (*dnm1Δ, fis1Δ*, and *mdv1Δ)* presented a fusion-like (aggregated) pattern (Fig. 1, *A* and *B*). Expression of *GDAP1* did not produce any effect on the morphological pattern in the WT or in the mutant strains tested (Fig. 1*B*). However, we observed a 1.2-fold significant cell body size enlargement in

fis1Δ compared to the WT. This phenotype was rescued by expression of *fis1* and, more interestingly, by expression of *GDAP1* (Fig. 1*C*). No cell size changes were observed in the other mutant strains.

To check mitochondrial distribution in *S. cerevisiae* cells, we defined three different patterns based on mitochondrial position inside the cell body as follows: lateral, when mitochondrial mass is distributed towards the cell periphery; central, when mitochondria is predominantly located in the middle of the cell; and mixed, which is an intermediate structure. In *fis1Δ* cells, mitochondria showed a lateral topology whereas the WT cells had increased cells with mitochondrial central pattern (Fig. 1*D*). Surprisingly, *GDAP1* expression in *fis1Δ* recovered the central pattern found in WT cells.

In order to determine whether GDAP1 could recover other functions that were altered in the *fis1Δ* strain and also in the other strains tested, we investigated cell growth and cell viability as described previously (31). We did not observe any significant growth defects in the dynamics-defective strains analyzed when cells were spotted onto YPD and SC media (data not shown); in contrast, cell growth was affected in *fis1Δ*, *dnm1Δ* and *fzo1Δ* strains under respiratory conditions in YPEG medium. This phenotype was rescued by *GDAP1* expression in both *fis1Δ* and *dnm1Δ* (Supplemental Fig. S2*A*). When inducing apoptosis by acetic acid (AA) or hydrogen peroxide (H_2O_2), cell viability was reduced only in *fis1Δ*. Such phenotype was reverted after *GDAP1* expression (supplemental material and supplemental Fig. S3, *A* and *B*). O_2 consumption measured with a Clark electrode was diminished in *fis1Δ* cells, and could be also recovered after *GDAP1* expression (supplemental material and supplemental Fig. S2*B*). Taken together all our results, GDAP1 seems to mimic several Fis1p functions in the budding yeast.

Fis1Δ cells show defects at G2/M phase of the cell cycle and spindle aberrant formation, which were recovered by GDAP1- The observation that *fis1Δ* cells displayed an enlarged cell phenotype prompted us to investigate the relation between mitochondrial dynamics and the cell cycle, and test how GDAP1 affect this phenotype. First, we estimated the cell cycle status in all the strains, either by flow cytometry or by calculating the Budding Index (BI). *Fis1Δ* showed decreased percentage of cells at G1 and increased number of cells at G2/M when compared to the WT strain (Fig. 2*A*, and supplemental table S1). Accordingly, BI was 1.6-fold increased in *fis1Δ* (supplemental table S2), confirming a cell cycle alteration in these cells. As previously observed with other *fis1*Δ phenotypes, *GDAP1* expression reverted cell cycle delay (Fig. 2*A*). This finding suggests that Fis1p and GDAP1 may participate or interfere somehow in the regulation of the cell cycle progression. To further investigate such a phenomenon, we checked the cell cycle progression in *fis1Δ* by cell synchronization at different stages using alpha-factor, hydroxyurea or nocodazole (producing G1, S, and G2/M arrests, respectively). *fis1Δ* cells failed to correctly achieve and then exit from the arrest after any of the treatments tested, indicating that Fis1p absence seems to delay normal cell cycle progression. Again *GDAP1* expression could completely recover normal cell cycle physiology (Fig. 2*B*).

To further characterize the cell cycle defects found in the *fis1Δ* strain, three classes of morphology were assigned based on the pattern observed after nuclear staining in dividing cells: an undivided nucleus in one cell body (class I, pre-M), an undivided nucleus in the bud neck (class II, early-M), and divided nuclei in two cell bodies (class III, late-M) as described before (32). In the WT strain, the class III type morphology was predominant (Fig. 3, *A* and *B*) indicating that cell division is progressing correctly. In contrast, *fis1Δ* showed increased number of cells belonging to the class II, and this abnormality was reverted after expression of *GDAP1*, yeast *fis1* (Fig. 3) or its human counterpart *FIS1* (data not shown). Moreover, we studied in detail the position of the mitotic spindle by immunofluorescence assays, and observed an increased number of aberrant shorter mitotic spindle formations in the *fis1Δ* cells that could be conditioning proper nuclei separation during cell division; such a phenotype was reverted by *GDAP1* expression (Fig. 3, *A* and *C*).

GDAP1 mutations could not recuperate cell cycle defects, spindle formation and increased cell size in fis1Δ- Since mutations in *GDAP1* have been associated to a more or less severe phenotype depending on, for example, their mode of inheritance in CMT patients (33), (34), we checked the effect of some pathological missense mutations

(R120W and T157P, both dominants, and R120Q, R161H, and R282C, which are recessively inherited) on *fis1Δ* cells. We included a truncated GDAP1 lacking its C-terminal hydrophobic domains, GDAP1-TMD (Fig. 4, *A*) and tested cell growth onto YPEG, cell viability after apoptotic stimuli, cell cycle, spindle formation, and cell body size. We confirmed that every mutant constructs was expressed in yeast (Fig. S1, *C*). The results obtained showed that the GDAP1 missense mutations recovered normal cell growth and improved cell viability after exposure to apoptotic stimuli in *fis1Δ* as the original GDAP1 (GDAP1[WT]) (Fig. 4*B* and supplemental Fig. S3*C*, respectively). GDAP1-TMD could not restore such functions indicating that the transmembrane domains are necessary for GDAP1 correct position and function (Fig. 4*B*, and supplemental Fig. S3*C*). Moreover, none of the missense mutations tested and neither GDAP1-TMD could recover normal cell cycle, correct spindle formation and the increased type II nuclear topology according to previously described criteria(32) as the original GDAP1 (Fig. 4, *C, D,* and *E*), although there was a variation in response depending on the GDAP1 mutation tested.

Interaction between Fis1p, GDAP1 and β-tubulin may explain mitotic spindle defects recovery in fis1Δ cells- In an attempt to define the pathophysiology of *GDAP1* neuropathies we have investigated possible GDAP1 protein interactors by a two-hybrid experiment. We found interaction between GDAP1 and the human β-tubulin (TUBB), which was confirmed by a coimmunoprecipation assay (Fig. 5*A*). Furthermore, mammalian FIS1 and GDAP1 interact (Fig. 5*B*), so it is reasonable to think that both proteins may participate in the interaction between mitochondria and microtubules. Thus, we hypothesized that the correction during G2/M progression produced by *GDAP1* expression in *fis1Δ* cells is accomplished by complementation of the interaction of Fis1p with yeast β-tubulin, Tub2p. To validate such a hypothesis we performed a coimmunoprecipitation assay between Fis1p and Tub2p, observing that both proteins interact in yeast (Fig. 5*C*).

We also hypothesized that no recovering effect on cell cycle delay by *GDAP1* missense mutations could be related to abnormal interaction between Tub2p and GDAP1 mutants. To address this point we performed both a coimmunoprecipitation assay and β-galactosidase liquid assay in permeabilized yeast cells (35). We observed that GDAP1 mutant proteins still interact with TUBB (Fig. 6, *A*). Unexpectedly, the interactions were increased for all mutant proteins when compared with WT GDAP1. Interestingly, the interaction was more intense for those mutations located within or near the α-loop domain (Fig. 6, *B*). Furthermore, we then determined that the GDAP1-TUBB interaction is achieved through the α-loop domain but not with the two GST domains and the transmembrane domain (Fig. 6, *C*). Such an anomalous interaction might affect the proper complementation of *fis1Δ* cells by GDAP1.

DISCUSSION

The morphology and the number of mitochondria in a eukaryotic cell are two dynamic processes that are essential for mitochondrial physiology, including oxidative phosphorylation, metabolic reactions and calcium homeostasis. Maintenance of the mitochondrial network is precisely regulated by mitochondrial fusion and fission, which involves specific proteins participating either in the fusion pathway such mitofusins 1 and 2 (MFN1 and MFN2), and OPA1, or in the fission pathway such as DRP1, FIS1 and Mff (5). Mutations in the *MFN2*, *OPA1* and *DRP1* genes cause Mendelian disorders (17), (18), (19), (20), (21). Mutations in *GDAP1* cause a peripheral neuropathy similar to that produced by *MFN2*, and have also been related to mitochondrial dynamics. Furthermore, overexpression of *GDAP1* in mammalian cells induces mitochondrial fragmentation, thus its participation in the fission pathway has been postulated (10), (9). *GDAP1* does not have any homologue gene in yeast. However, to investigate the putative role GDAP1 has on mitochondrial dynamics, we performed complementation experiments by expressing *GDAP1* in *S. cerevisiae* strains defective for genes involved either in mitochondrial fission (*fis1*, *Dnm1* and *Mdv1)* or fusion (*Fzo1).*

In *S. cerevisiae, GDAP1* heterologous expression did not fragment mitochondria, and did not produce any effect on the mitochondrial morphology pattern in any of the mutant strains tested. Interestingly, we observed an abnormal

increased cell size in the *fis1Δ* strain. This finding agrees with previous studies performed in mammalian cells lacking *FIS1* where sustained mitochondrial elongation and cell enlargement and flattening were found (36). Further characterization of *S. cerevisiae fis1Δ* cells showed increased sensitivity to cell death when cells were exposed to oxidative stress agents. We then analyzed in depth the effect that *GDAP1* produces on the *fis1Δ* strain and found that it could fully recover *fis1Δ* phenotype. First, *GDAP1* expression reverted the increased cell death when exposed to oxidative stress agents, the diminish cell growth under forced mitochondrial respiration conditions, and the reduced oxygen consumption found in *fis1Δ* cells. Moreover, the aberrant lateral mitochondrial network topology found in *fis1Δ* cells was also rescued after *GDAP1* expression. Interestingly, we observed a G2/M delay in the cell cycle phenotype in *fis1Δ* cells that might be linked to the cell enlargement. Again, cell size enlargement and cell cycle delay found in the *fis1Δ* cells were complemented after *GDAP1* expression. These findings suggest that human GDAP1 might share some relevant biological functions with Fis1p, and probably with the human FIS1, such as the role on mitochondrial fission.

Cell cycle defects found in the *fis1Δ* strain were unexpected. Furthermore, the results obtained after cell synchronization experiments with different agents indicated that lack of Fis1p delays normal cell cycle progression and could be related to the aberrant spindle structure formations found in *fis1Δ* cells. A possible explanation for this phenomenon is that the DNA replication normally occurs in *fis1Δ* cells and these cells then start the mitosis, but division gets stacked or delayed and affect correct nuclei separation and migration towards the cell pole into the daughter cell later on. Since *fis1Δ* cells show aberrant spindle formation and the interaction between Fis1p and Tub2p was confirmed by coimmunoprecipitation assays in this work, we reasoned that one possible explanation to the aberrant spindle formation in *fis1Δ* cells could be an abnormal interaction between mitochondria and the microtubules through Fis1p and Tub2p, respectively. Thus, lack of Fis1p might affect mitochondrial correct localization inside the cell and proper spindle microtubules formation.

It has been postulated that the abnormal phenotype observed in the *fis1Δ* cells could be the result of the selection for compensatory mutations since this situation might provide an advantage for the organism (37). To exclude this possibility, we demonstrated by genomic sequencing that the previously described selected mutation in the stress response gene *WHI2* found in *fis1Δ* strains with other genetic backgrounds (37) was not present in our *fis1Δ* strain (data not shown). Furthermore, *fis1* and also *FIS1* expression could recover *fis1Δ* defects.

In humans, mutations in *GDAP1* cause the Charcot-Marie-Tooth neuropathy. Recently, it has been proposed that the different pathomechanisms found for mitochondrial dynamics and apoptosis in the GDAP1 mutations could rely on the disease mode of inheritance (34). We reasoned that one possible approach to test the biological effect of some clinical *GDAP1* missense mutations found in CMT patients, either recessive or dominant, could be investigated using complementation assays on the *fis1Δ* strain. Some of the observed altered functions in *fis1Δ* phenotype (growth under forced mitochondrial respiratory conditions and cell death after exposition to some agents) were improved by expressing the pathological *GDAP1* missense mutations tested as when expressing the original *GDAP1*. However, the other phenotypes linked to *fis1Δ* phenotype (increased cell size, spindle aberrant formation during mitosis and cell cycle delay) could not be complemented by either the expression of the *GDAP1* missense mutations or the *GDAP1-TMD* construct. This finding suggests that the molecular mechanism associated with the cycle delay could be related with the physiological function of both GDAP1 in humans and Fis1p in yeast. Furthermore, since there is an interaction between Fis1p and Tub2p in yeast and between GDAP1 and TUBB in mammalian cells, it is reasonable to predict that the mutant forms of GDAP1 unable to rescue cell cycle would be impaired in tubulin binding. The biological consequence would be an inappropriate link between mitochondria and the cytoskeleton, which may affect cytoskeleton-mitochondria precise connection and correct spindle formation later on. This aberrant situation would determine the whole process onwards and unleash irregularities in the cell cycle structure and its progression. Biochemical assays showed that every tested

GDAP1 mutant protein interacted with TUBB interaction was not affected by amino acid changes in GDAP1; thus, the spindle aberrant formation during mitosis and its consequences could not be explained by a loss of function mechanism caused by amino acid changes in GDAP1. On the contrary, we observed that the GDAP1 mutant forms and TUBB binding was increased, and this interaction varies depending on the mutation tested, being the interaction even stronger for mutations located near or within the α-loop domain. For that reason, we think that complementation failure of *GDAP1* mutants could be the consequence of an abnormal gain of function mechanism that might affect spindle formation and other associated phenotypes. This mechanism would be mediated by the α-loop domain for which no structural and biological information has been defined yet (14).

Mitochondrial dynamics is a conserved pathway along evolution since most of the molecules involved in mitochondrial fission and fusion are highly conserved among species. However, GDAP1 has appeared recently in evolution in multicellular organisms, especially in vertebrates (14), and it is mainly expressed in the nervous system (11). Furthermore, GDAP1 defects cause a peripheral neuropathy (12), (13), (33), (11), and the affected neurons are postmitotic non-dividing cells. Consequently, we wonder how we could reconcile our complementation results in the budding yeast with the physiological role GDAP1 has in neurons. We speculate that what the budding yeast and neurons have in common could be the mitochondria-cytoskeleton interaction by means of the molecular interaction between the mitochondrial fission molecules Fis1p/FIS1 and GDAP1, and tubulins, Tub2p or TUBB, as these molecules are highly conserved along evolution. In yeast, during early mitotic cell division in the cell cycle, such interaction might help to actively transport mitochondria and other organelles into the developing bud, where mitochondria continue accumulating until cytokinesis is completed (2). However, mitochondrial transport in the budding yeast displays cell cycle coordinated motility mostly via the actin cytoskeleton, whereas nuclei is distributed towards the developing bud along the intranuclear mitotic spindle (38). In our work, interaction between mitochondrial fission proteins and tubulin has been observed so we speculate that microtubules could be also important for mitochondrial minor interaction with the cytoskeleton, possibly providing the required energy for cytokinesis accomplishment. In contrast, in non-dividing neurons, mitochondrial fission proteins might help mitochondria being effectively distributed and transported along the axonal cytoskeleton from the soma to the synapse for their correct position and function with other organelles. Integration of mitochondrial dynamics with axonal transport in neurons may help to explain the pathophysiological mechanisms underlying GDAP1 peripheral neuropathies.

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FOOTNOTES

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The abbreviations used are: GDAP1, Ganglioside-induced differentiation associated protein-1; *GDAP1*$^{\Delta 320\text{-}358}$ or *GDAP1-TMD*, *GDAP1* lacking its transmembrane domains; CMT, Charcot-Marie-Tooth disease; AA, acetic acid; Tub2p, *S. cerevisiae* β-tubulin; TUBB, human β-tubulin.

FIGURE LEGENDS

Fig. 1. Effect of *GDAP1* expression on mitochondria and cell body size in *S. cerevisiae* strains. *A.* Representative images of mitochondrial structure in the indicated strains before and after *GDAP1* expression by fluorescence microscopy. Differential interference contrast (DIC) images were also captured. Scale bar: 5 µm. *B.* Quantification of mitochondrial morphology. We observed five different mitochondrial architectures: fusioned 'aggregated', predominantly tubular 'tubular', tubular and vesicular 'mixed', predominantly vesicular 'vesicular' and completely fragmented 'fragmented'. *Dnm1Δ, fis1Δ*, and *mdv1Δ* showed predominantly an aggregated pattern, whereas *fzo1Δ* mitochondria are mostly fragmented. *GDAP1* expression did not produce any effect on mitochondrial morphology. *C.* Quantification of cell diameter in large-budded cells. *fis1Δ* showed enlarged cell body size that was reverted by *GDAP1* or *fis1* expression. *D.* Quantification of mitochondrial position inside the cell. We observed three different mitochondrial localization patterns: predominantly lateral 'lateral', lateral and central 'mixed', and predominantly central 'central'. *fis1Δ* showed increased lateral position, and this situation was reverted after *GDAP1* expression. At least 300 cells were counted. Errors bars indicate SEM (n = 3/4). P values are generated from a two-tailed unpaired Student's t test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 2. Effect of *GDAP1* expression on cell cycle in *S. cerevisiae*. *A.* Proportion of cells at each stage of the cell cycle determined by flow cytometry in asynchronous cultures. Results showed that *fis1Δ* has increased number of cells at the G2/M phase, and this situation was reverted by *GDAP1* or *fis1* expression. *B.* Cell cycle progression after cell arrest with α-factor, hydroxyurea and nocodazole. Exit from each treatment was measured at 30, 60, 90, 120 and 150 min. Results shows *fis1Δ* could not exit from arrest and *GDAP1* expression recovered the normal situation. Data are represented by the mean ($n \geq 4$).

Fig. 3. *GDAP1* expression reverted abnormal budding and mitotic spindle formation in *fis1Δ*. *A.* Representative images from asynchronous cell cultures immunostained with α-tubulin to detect the spindle formation. Cells were counterstained with DAPI. DIC images were also included. Scale bars: 10 µm. *B.* Quantification of the G2/M defects upon nuclear DNA staining in large-budded cells as described before (32). Class I: an undivided nucleus in one cell body; Class II: undivided nuclei in the bud neck; and class III: two divided nuclei separated in two cell bodies. Minimum 300 cells were counted in each experiment. *C.* Detailed images from cell cultures showing the mitochondria (mito), spindle formation (tubulin), and nuclei position (DAPI). *GDAP1* expression recovered *fis1Δ* defects. At least four independent experiments were carried out. Scale bars: 10 µm.

Fig. 4. Effect of *GDAP1* missense mutations expression on *fis1Δ* phenotype. *A.* GDAP1 predicted domains (Glutation-S-transferase, GST; and hydrophobic domains, named 1 and 2) and the position of the

missense mutations (R120Q, R120W, T157P, R161H, and R282C) tested. GDAP1 lacking its transmembrane domains (GDAP1-TMD) is included. *B.* Representative images of cells growth onto YPGE. GDAP1 (original and missense mutations) and Fis1p improve *fis1Δ* aberrant growth. *C.* Quantification of the effect the *GDAP1* missense mutations have on *fis1Δ* cell cycle. *D.* Representative images of cell cultures immunostained with α-tubulin to follow spindle formation. Cells were counterstained with DAPI. DIC images were also included. Scale bars: 10 μm. *E.* Quantification of the G2/M defects upon nuclear DNA staining in large-budded cells as described (32). Class I: an undivided nucleus in one cell body; Class II: undivided nuclei in bud neck; and class III: two divided nuclei separated in two cell bodies. Minimum 300 cells were counted in each experiment. None of the GDAP1 mutations neither GDAP1-TMD could revert G2/M alteration and the aberrant spindle formation in *fis1Δ* cells. Data represent the mean of at least four independent experiments.

Fig. 5. Analysis of the interactions between FIS1/Fis1p, GDAP1 and β-tubulin by coimmunoprecipitation. *A. TUBB,* and *B. FIS1* fused to *HA* tag was expressed with either an empty vector or *GDAP1* fused to *c-myc* tag in Hela cells. An immunoprecipitate with the anti-c-myc antibody from 400 μg of the cell lysate was probed with an antibody against the HA or c-myc tag. Cell lysates (40 μg) without immunoprecipitation were included (input). *C. S. cerevisiae fis1* fused to *HA* tag was expressed either with an empty vector or *Tub2* fused to *LexA* tag in FY250 cells. An immunoprecipitate with the anti-LexA antibody from 400 μg of the cell lysate was probed with an antibody against the HA or LexA tag. Cell lysates (40 μg) without immunoprecipitation were included (input).

Fig. 6. Study in depth of the interactions between GDAP1 and β-tubulin. *A. TUBB,* fused to *HA* tag was expressed with either *GDAP1* (WT, and missense mutations R120Q, R120W, T157P, R161H and R282C) or an empty vector fused to *c-myc* tag in Hela cells. An immunoprecipitate with the anti-c-myc antibody from 400 μg of the cell lysate was probed with an antibody against the HA or c-myc tag. Cell lysates (40 μg) without immunoprecipitation were included (input). *B.* β-galactosidose liquid assay to test the intensity of the interaction between GDAP1 and the GDAP1 mutant forms R120Q, R120W, T157P, R161H and R282C. Data are represented by the mean ($n \geq 3$). P values are generated from a two-tailed unpaired Student's t test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Results show that the missense mutations can bind TUBB and that this interaction is increased in all of them. *C.* A β-galactosidose lift assay to test the interaction between GDAP1 (first lane), and GDAP1 domains: GST-Nt (aminoacids 24-105), α-loop aminoacids (106-152), GST-Ct (aminoacids 153-309), TMD (aminoacids 310-358) (14). TUBB specifically interacts to the α-loop domain.