Charcot-Marie-Tooth-related Gene GDAP1 Complements Cell Cycle Delay at G2/M Phase in Saccharomyces cerevisiae fis1 Gene-defective Cells*

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Mutations in the GDAP1 gene are responsible for 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 increase our knowledge of GDAP1 function. We discovered a consistent relation between Fis1p and the cell cycle because fis1Δ cells showed G2/M delay during cell cycle progression. The fis1Δ phenotype, which includes cell cycle delay, was fully rescued by GDAP1. By contrast, clinical missense mutations rescued the fis1Δ phenotype except for the cell cycle delay. In addition, both Fis1p and human GDAP1 interacted with β-tubulins Tub2p and TUBB, respectively. A 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, the interaction between mitochondria and the microtubule cytoskeleton 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, Ca2+ 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 that are best characterized in the budding yeast Saccharomyces cerevisiae. One of them, Fis1p, is an 18-kDa type II integral membrane-anchored fission protein that is evenly distributed in the mitochondrial outer membrane (3). Deletion of this gene from yeast (fis1) or a reduction in protein levels by RNA interference 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 on or associated with the mitochondrial outer and inner membranes, respectively. Deletion of Fzo1p leads to mitochondrial fragmentation, a petite phenotype, and loss of mitochondrial DNA, indicating an important function of Fzo1p in mitochondrial biogenesis (8).

GDAP1 (ganglioside-induced differentiation-associated protein-1) has been related to the mitochondrial fission process. It is located in the mitochondrial outer membrane and is expressed mainly in neurons (9–11). Although GDAP1 sequence has a high level of similarity to glutathione S-transferase (GST) (12, 13), no GST activity has been reported previously (10, 14). Mutations in GDAP1 are the cause of the Charcot-Marie-Tooth (CMT)2 disease: autosomal recessive demyelinating CMT4A (15) autosomal recessive axonal ARCMT2K or dominant axonal CMT2K. The human counterparts of Fzo1p and Mgm1p, MFN1/MFN2 (mitofusin-1/mitofusin-2) 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 have been described; by contrast, DRPI (the human homolog of yeast dnm1) has been associated with human (20) and mouse (21) diseases.

It could be argued that GDAP1 should have a specific function in the mitochondrial fission pathway of mammalian cells.
**GDAP1 Complements the fis1Δ phenotype in S. cerevisiae**

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 would result in some knowledge about the possible role of GDAP1 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 fis1Δ cells are fully recovered by GDAP1, which suggests a possible new function shared by Fis1p (also 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 separation of nuclei. However, the expression of several pathogenic GDAP1 forms could not improve 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 are 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 YEPG medium (0.67% yeast nitrogen base without amino acids, 3% glycerol, 2% ethanol) with a source of glucose (0.05%) and the addition of the appropriate amino acids by spotting serial dilutions onto plates at 30 °C for 4 days.

**Genetic Methods and Yeast Transformation**—GDAP1 constructs harboring pathological missense mutations R120Q, R120W, T157P, R161H, and R282C and GDAP1 lacking its transmembrane domains (GDAP1Δ310–358 or GDAP1-TMD) were generated as described previously (10). Complete GDAP1, fis1, and FIS1 cDNAs were cloned in the pRS425 vector; empty vectors were used for controls. Yeast transformation was performed by the lithium acetate method as described (24). For mitochondrial structure visualization, pRS314-ADH1-Su9-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 TAT7 strain (MATa ade2 his3 leu2 trp1 gal4 gal80 lys2::lexAop-HIS3, URA3::lexAop-lacZ), which was kindly provided by Dr. P. Sanz. Briefly, the GDAP1 cytosolic domain fused to LexA was cloned into the pBTM116 vector. A commercial human brain cDNA library cloned in the pACT2 vector (Clontech) was transformed in the TAT7 strain. Transformants were selected in synthetic complete medium (SC, 0.67% yeast nitrogen base, 2% glucose, 30 mg/l amino acids) plates lacking tryptophan, leucine, and histidine plates, and were then subsequently screened for β-galactosidase activity using a filter lift assay (27). 250,000 independent clones were tested, and the positive plasmids obtained were sequenced. GDAP1 protein structure and partial GDAP1 (GST-N terminus (amino acids 24–105), loop (amino acids 106–152), GST-C terminus (amino acids 153–309), and TMD (amino acids 310–358)) were cloned in the pACT2 vector and tested using the β-galactosidase filter assay as described (28).

**Mitochondrial Purification**—Cells were grown in SC until late log phase at 30 °C, harvested by centrifugation, and suspended in freshly prepared buffer A (100 mM Tris-SO4, pH 9.4, and 10 mM DTT; 3–4 ml/g of pellet weight). The cell suspension was incubated at 30 °C and treated with Zymolyase 20T (Sigma) in buffer B (1.2 ml sorbitol and 20 mM potassium P, pH 7.4) in an orbital shaking chamber. Samples were then centrifuged, washed with cold buffer B, and suspended in cold buffer C (0.8 M sorbitol and 20 mM K-MES, pH 6.0) plus 100 μl of PMSF (100 mM). Cell lysis was carried out with a Dounce glass-glass homogenizer. The supernatant was centrifuged at 12,000 × g at 4 °C, suspended with cold buffer C, and centrifuged again at 2000 × g at 4 °C. The mitochondrial enriched fraction was obtained after centrifugation of the supernatant from the previous step at 12,000 × g for 15 min at 4 °C.

**Antibodies**—Anti-GDAP1 (Abnova, Taipei, Taiwan) and anti-α-tubulin (Sigma) antibodies were used. The horseradish peroxidase-linked ECL® anti-mouse IgG antibody was from GE Healthcare. An anti-mouse IgG antibody was purchased from Invitrogen. Anti-c-Myc and anti-LexA antibodies and the anti-HA antibody used for co-immunoprecipitation experiments were purchased from Sigma.

**Indirect Immunofluorescence and Imaging**—Immunofluorescence experiments were performed as described previously (29) but with minor modifications. Cells were fixed, blocked, and then incubated with the anti-α-tubulin antibody in blocking solution (PBS and 3% BSA) overnight at 4 °C. Nuclei were counterstained with DAPI (Sigma). Mitochondria was visualized after p5314-Su9-GFP expression in all of the strains (25). Wide-field fluorescence and differential interference contrast images were captured using a Leica DM RXA2 light microscope and photographed with a Hamamatsu digital camera.

**Synchronization Experiments and Cell Cycle Analysis**—*S. cerevisiae* cells were grown until early log phase in YPEG medium. Cell synchronization was performed as follows: in G1 phase with α-factor, in early S phase with hydroxyurea, and in metaphase with nocodazole (all purchased from Sigma) as described previously (29). 1 ml aliquots were taken every 30 min, and cells were sonicated, fixed with 80% cold ethanol, and subjected to RNase (Sigma) and pepsin treatment. Nuclei were stained with propidium iodide (Sigma). The FACScanto® flow cytometer apparatus used was from BD Biosciences. The budding index 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% Nonidet P-40, 15% glycerol, and Complete protease inhibitors (mini, EDTA-free; Roche Applied Science)). Cell suspensions were immunoprecipitated using Dynabeads-protein G (Invitrogen) according to the manufacturer’s instructions, and immunoblotting was performed as described previously (30).

*S. cerevisiae* FY250 cells were grown to A600 = 0.6, pelleted, and washed twice with distilled water. Cell lysis was performed with 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol plus 1 mM DTT, 100 mM PMSF, and protease inhibitors. Glass beads (0.5-mm diameter; Sigma) were added to the sample and incubated with Zymolyase 20T for 30 min for cell wall degradation. Protein extracts were obtained after centrif-
ugation, and immunoprecipitation was performed as described (30).

RESULTS

fis1Δ Cells Show Increased Cell Size and Abnormal Distribution of Mitochondria—We wanted to determine the effect that GDAP1 produces on mitochondrial morphology in S. cerevisiae strains. First, we confirmed that GDAP1 expression in the budding yeast cells is not toxic for their growth and is targeted to mitochondria (supplemental “Experimental Procedures” and Fig. S1, A and B). To investigate whether GDAP1 can 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 pRS314-ADH1-Su9-GFP in all of the strains tested, and five different patterns of the mitochondrial network were defined: fused (“aggregated”), predominantly tubular (“tubular”), tubular and vesicular (“mixed”), predominantly vesicular (“vesicular”), and completely fragmented (“fragmented”). 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 mutant strains tested (Fig. 1B). However, we observed a 1.2-fold significant cell body size enlargement in fis1Δ cells compared with WT cells. This phenotype was rescued by expression of fis1 and, more interestingly, by expression of GDAP1 (Fig. 1C). 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 toward the cell periphery; central, when mitochondria are located predominantly in the middle of the cell; and mixed, which is an intermediate structure. In fis1Δ cells, mitochondria showed a lateral topology, whereas WT cells had increased cells with a mitochondrial central pattern (Fig. 1D). Surprisingly, GDAP1 expression in fis1Δ recovered the central pattern found in WT cells.

To determine whether GDAP1 can 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 (yeast extract-peptone-glucose medium) and SC (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. S2A). When inducing apoptosis by acetic acid or hydrogen peroxide (H2O2), cell viability was reduced only in fis1Δ. Such a phenotype was reverted after GDAP1 expression (supplemental “Experimental Procedures” and Fig. S2B). Taken together, our results suggest that GDAP1 mimics several Fis1p functions in the budding yeast.

fis1Δ Cells Show Defects at the G2/M Phase of the Cell Cycle and Aberrant Spindle Formation, Which Are 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 to test how GDAP1 affects this phenotype. First, we estimated the cell cycle status in all of the strains either by flow cytometry or by calculating the budding index. fis1Δ showed a decreased percentage of cells at G1 and an increased number of cells at G2/M compared with the WT strain (Fig. 2A and supplemental Table S1). Accordingly, the budding index was increased by 1.6-fold in fis1Δ (supplemental Table S2), confirming a cell cycle alteration in these cells. As observed previously with other fis1Δ phenotypes, GDAP1 expression reverted cell cycle delay (Fig. 2A). This finding suggests that Fis1p and GDAP1 may participate or interfere somehow in the regulation of cell cycle progression.

To further investigate such a phenomenon, we checked the cell cycle progression in fis1Δ by cell synchronization at different stages using α-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 an absence of Fis1p seems to delay normal cell cycle progression. Again, GDAP1 expression could completely recover normal cell cycle physiology (Fig. 2B).

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 phase), an undivided nucleus in the bud neck (class II, early M), and divided nuclei in two cell bodies (class III, late M), as described previously (32). In the WT strain, the class III morphology was predominant (Fig. 3, A and B), indicating that cell division was progressing correctly. In contrast, the fis1Δ strain showed an increased number of cells belonging to 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 affect proper separation of nuclei during cell division; such a phenotype was reverted by GDAP1 expression (Fig. 3, A and C).

GDAP1 Mutations Cannot Recover Cell Cycle Defects, Spindle Formation, and Increased Cell Size in fis1Δ—Because mutations in GDAP1 have been associated with 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 pathologic missense mutations (R120W and T157P (dominant) and R120Q, R161H, and R282C (recessive)) on fis1Δ cells. We included a truncated GDAP1 lacking its C-terminal hydrophobic domains, GDAP1-TMD (Fig. 4A), and tested cell growth on YPEG medium, cell viability after apoptotic stimulus, cell cycle, spindle formation, and cell body size. We confirmed that every mutant construct was expressed in yeast (supplemental Fig. S1C). The results obtained showed that the GDAP1 missense mutations recovered normal cell growth and
GDAP1 Complements the fis1Δ phenotype in S. cerevisiae

A

|       | empty vector | GDAP1 |
|-------|--------------|-------|
|       | DIC          | mito  |
| WT    | DIC          | mito  |
| fis1Δ | DIC          | mito  |
| dnm1Δ | DIC          | mito  |
| mdv1Δ | DIC          | mito  |
| fzo1Δ | DIC          | mito  |

B

![Graph showing cell morphology percentages](image)

C

![Graph showing cell diameter](image)

D

![Graph showing cell morphology](image)
improved cell viability after exposure to apoptotic stimuli in fis1Δ as the original GDAP1 (WT GDAP1) (Fig. 4B and supplemental Fig. S3C, respectively). GDAP1-TMD could not restore such functions, indicating that the TMDs are necessary for the correct position and function of GDAP1 (Fig. 4B and supplemental Fig. S3C). Moreover, neither the missense mutations nor GDAP1-TMD could recover the 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–E), although there was a variation in response depending on the GDAP1 mutation tested.

FIGURE 1. Effect of GDAP1 expression on mitochondria and cell body size in S. cerevisiae strains. A, representative images of mitochondrial structure (mito) in the indicated strains before and after GDAP1 expression obtained by fluorescence microscopy. Differential interference contrast (DIC) images were also captured. Scale bars = 5 μm. B, quantification of mitochondrial morphology. We observed five different mitochondrial architectures: fused (aggregated), predominantly tubular (tubular), tubular and vesicular (mixed), predominantly vesicular (vesicular), and completely fragmented (fragmented). dnm1Δ, fis1Δ, and mdv1Δ mitochondria showed predominantly an aggregated pattern, whereas fzo1Δ mitochondria were 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 S.E. (n = 3/4). P values were generated from Student’s two-tailed unpaired t test: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

FIGURE 2. Effect of GDAP1 expression on the cell cycle in S. cerevisiae. A, proportion of cells at each stage of the cell cycle determined by flow cytometry in asynchronous cultures. The results show that fis1Δ had an 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 after each treatment was measured at 30, 60, 90, 120, and 150 min. The results show that fis1Δ could not exit from arrest and that GDAP1 expression recovered the normal situation. Data are represented as the mean (n = 4).
GDAP1 Complements the fis1Δ phenotype in S. cerevisiae

Interaction between Fis1p, GDAP1, and β-Tubulin May Explain Mitotic Spindle Defect Recovery in fis1Δ Cells—In an attempt to define the pathophysiology of GDAP1 neuropathies, we investigated possible GDAP1 protein interactors by a two-hybrid experiment. We found an interaction between GDAP1 and human β-tubulin (TUBB), which was confirmed by a co-immunoprecipitation assay (Fig. 5A). Furthermore, mammalian FIS1 and GDAP1 interacted (Fig. 5B), so it is reasonable to believe 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 co-immunoprecipitation assay with Fis1p and Tub2p and observed that both proteins interacted in yeast (Fig. 5C).

We also hypothesized that the lack of a 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 co-immunoprecipitation assay and a β-galactosidase liquid assay in permeabilized yeast cells (35). We observed that GDAP1 mutant proteins still interacted with TUBB (Fig. 6A). Unexpectedly, the interactions were increased for all mutant proteins compared with WT GDAP1. Interestingly, the interaction was more intense for those mutations located within or near the α-loop domain (Fig. 6B). We then determined that the GDAP1-TUBB interaction was achieved through the α-loop domain but not the two GST domains and the TMD (Fig. 6C). Such an anomalous interaction might affect the proper complementation of fis1Δ cells by GDAP1.

DISCUSSION

The morphology and 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 involve specific proteins participating either in the fusion pathway, such as MFN1, 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 (17–21). 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 fusion–fission pathway has been postulated (9, 10). GDAP1 does not have any gene homolog in yeast. However, to investigate the putative role that GDAP1 has on mitochondrial dynamics, we performed complementation experiments by expressing GDAP1 in S. cerevisiae strains defective for genes involved in either mitochondrial fission (fis1, dnm1, and mdv1) or fusion (fzo1).

In S. cerevisiae, heterologous GDAP1 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 has on the fis1Δ strain and found that it could fully recover the fis1Δ phenotype. First, GDAP1 expres-
sion reverted the increased cell death when exposed to oxidative stress agents, the diminished cell growth under forced mitochondrial respiration conditions, and the reduced oxygen consumption found in \textit{fis1}\textsuperscript{\textminus} cells. Moreover, the aberrant lateral mitochondrial network topology found in \textit{fis1}/H9004 cells was also rescued after GDAP1 expression. Interestingly, we observed a G2/M delay in the cell cycle phenotype in \textit{fis1}/H9004 cells that might be linked to the cell enlargement. Again, the cell size enlargement and cell cycle delay found in the \textit{fis1}/H9004 cells were complemented after GDAP1 expression. These findings suggest that human GDAP1 might share some relevant biological functions with Fis1p and probably with human FIS1, such as the role in mitochondrial fission.

Cell cycle defects found in the \textit{fis1}\textsuperscript{\textminus} 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 \textit{fis1}\textsuperscript{\textminus} cells. A possible explanation for this phenomenon is that DNA replication normally occurs in \textit{fis1}\textsuperscript{\textminus} cells, and these cells then start mitosis, but division gets stacked or delayed and affects the correct separation of nuclei and migration toward the cell pole into the daughter cell later on. Because \textit{fis1}\textsuperscript{\textminus} cells show aberrant spindle formation and because the interaction between Fis1p and Tub2p was confirmed by co-immunoprecipitation assays in this work, we reasoned that one possible explanation for the aberrant spindle formation in \textit{fis1}\textsuperscript{\textminus} cells could be an abnormal interaction between mitochondria and the microtubules through Fis1p and Tub2p, respectively. Thus, lack of Fis1p might affect the correct mitochondrial localization inside the cell and proper spindle microtubule formation.

It has been postulated that the abnormal phenotype observed in \textit{fis1}\textsuperscript{\textminus} cells could be the result of the selection for compensatory mutations because 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 \textit{WHI2} found in \textit{fis1}\textsuperscript{\textminus} strains with other genetic backgrounds (37) was not present in our \textit{fis1}\textsuperscript{\textminus} strain (data not shown). Furthermore, \textit{fis1} and also \textit{FIS1} expression could recover \textit{fis1}/H9004 defects. In humans, mutations in GDAP1 cause the CMT neuropathy. Recently, it has been proposed that the different patho-

**FIGURE 4. Effect of GDAP1 missense mutation expression on the \textit{fis1}\textsuperscript{\textminus} phenotype.** A, predicted GDAP1 domains (GST and hydrophobic domains 1 and 2) and positions of the missense mutations (R120Q, R120W, T157P, R161H, and R282C) tested. GDAP1 lacking its transmembrane domains (GDAP1-TMD) is included. \textit{Nt}, N terminus; \textit{Ct}, C terminus. \textit{B}, representative images of cells grown on YEPG medium. GDAP1 (original and missense mutations) and Fis1p improved aberrant \textit{fis1}\textsuperscript{\textminus} growth. \textit{C}, quantification of the effect of the GDAP1 missense mutations on the \textit{fis1}\textsuperscript{\textminus} cell cycle. \textit{D}, representative images of cell cultures immunostained with \textit{\textgamma}\textsuperscript{2}tubulin to follow spindle formation. Cells were counterstained with DAPI. Differential interference contrast (DIC) images were also obtained. Scale bars = 10 \textmu m. \textit{E}, quantification of the G2/M defects upon nuclear DNA staining in large budded cells as described (32). Class I represents an undivided nucleus in one cell body; class II represents undivided nuclei in bud neck; and class III represents two divided nuclei separated in two cell bodies. A minimum of 300 cells were counted in each experiment. Neither the GDAP1 mutations nor GDAP1-TMD could revert the G2/M alteration and aberrant spindle formation in \textit{fis1}\textsuperscript{\textminus} cells. Data represent the mean of at least four independent experiments.
mechanisms 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 fis1/H9004 strain. Some of the observed altered functions in the fis1/H9004 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 the fis1/H9004 phenotype (increased cell size, aberrant spindle formation during mitosis, and cell cycle delay) could not be complemented by expression of either the GDAP1 missense mutations or the GDAP1-TMD construct. This finding suggests that the molecular mechanism associated with the cycle delay could be related to the physiological function of both GDAP1 in humans and Fis1p in yeast. Furthermore, because there is an interaction between Fis1p and Tub2p in yeast cells and between GDAP1 and TUBB in mammalian cells, it is reasonable to predict that the mutant forms of GDAP1 unable to rescue the cell cycle would be impaired in tubulin binding. The biological consequence would be an inappropriate link between mitochondria and the cytoskeleton, which may affect the precise connection between the cytoskeleton and mitochondria and correct spindle formation later on. This aberrant situation would determine the whole process onwards and unleash irregularities in the

**FIGURE 5. Analysis of the interactions between Fis1p/FIS1, GDAP1, and β-tubulin by co-immunoprecipitation.** TUBB (A) and FIST (B) fused to an HA tag were expressed with either an empty vector or GDAP1 fused to a c-Myc tag in HeLa cells. An immunoprecipitate with the anti-c-Myc antibody from 400 μg of 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 an HA tag was expressed either with an empty vector or tub2 fused to a LexA tag in FY250 cells. An immunoprecipitate with the anti-LexA antibody from 400 μg of cell lysate was probed with an antibody against the HA or LexA tag. Cell lysates (40 μg) without immunoprecipitation were included (input). WB, Western blot; CoIP, co-immunoprecipitation.

**FIGURE 6. In-depth study of the interactions between GDAP1 and β-tubulin.** A, TUBB fused to an HA tag was expressed with either GDAP1 (WT and missense mutations R120Q, R120W, T157P, R161H, and R282C) or an empty vector fused to a c-Myc tag in HeLa cells. An immunoprecipitate with the anti-c-Myc antibody from 400 μg of cell lysate was probed with an antibody against the HA or c-Myc tag. Cell lysates (40 μg) without immunoprecipitation were included (input). WB, Western blot; CoIP, co-immunoprecipitation. B, a β-galactosidase liquid assay was performed to test the intensity of the interaction between GDAP1 and the GDAP1 mutants R120Q, R120W, T157P, R161H, and R282C). Data are represented as the mean (n = 3). p values were generated from Student’s two-tailed unpaired t test: *, p < 0.05; ***, p < 0.001. Results show that the missense mutations could bind TUBB and that this interaction was increased in all of them. C, a β-galactosidase lift assay was performed to test the interaction between GDAP1 (first lane) and the following GDAP1 domains: GST-N terminus (Nt; amino acids 24–105), GST-C terminus (Ct; amino acids 153–358), and the TMD (amino acids 310–358) (14). TUBB specifically interacted with the α-loop domain.
Mitochondrial dynamics is a conserved pathway throughout evolution because 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 expressed mainly in the nervous system (11). Furthermore, GDAP1 defects cause a peripheral neuropathy (11–13, 33), and the affected neurons are post-mitotic non-dividing cells. Consequently, we wondered how we could reconcile our complementation results in the budding yeast with the physiological role that GDAP1 has in neurons. We speculated that what the budding yeast and neurons in human could be the interaction between mitochondria and the cytoskeleton by means of the molecular interaction between the mitochondrial fission molecules Fis1p/FIS1 and GDAP1 and tubulins Tub2p and TUBB, as these molecules are highly conserved throughout evolution. In yeast, during early mitotic cell division in the cell cycle, such an 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 are distributed toward the developing bud along the intranuclear mitotic spindle (38).

In this work, we observed an interaction between mitochondrial fission proteins and tubulin, so we speculate that microtubules could also be important for minor mitochondrial interaction with the cytoskeleton, possibly providing the required energy for cytokinesis. In contrast, in non-dividing neurons, mitochondrial fission proteins might help the mitochondria to be 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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