Alterations in expression levels of deafness dystonia protein 1 affect mitochondrial morphology

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Deafness-Dystonia-Optic Neuropathy (DDON) Syndrome is a rare X-linked progressive neurodegenerative disorder resulting from mutations in the TIMM8A gene encoding for the deafness dystonia protein 1 (DDP1). Despite important progress in identifying and characterizing novel mutations in this gene, little is known about the underlying pathomechanisms. Deficiencies in the biogenesis of hTim23 and consecutive alterations in biogenesis of inner membrane and matrix proteins have been proposed to serve as one possible mechanistic explanation. To shed new light on the role of DDP1 in the biogenesis of mammalian mitochondria, we investigated the effects of reduced or elevated DDP1 levels on mitochondrial dynamics and function. Our results show a reduction in the import of β-barrel proteins into mitochondria from cells overexpressing DDP1. This effect was not observed when the DDON-related mutant form DDP1-C66W was overexpressed. Live cell microscopy of primary fibroblasts derived from DDON patients and of DDP1 downregulated HeLa cells displayed alterations of mitochondrial morphology with notable extensions in the length of mitochondrial tubules, whereas overexpression of DDP1 induced the formation of hollow spherical mitochondria. Of note, knockdown of the TIMM8A gene by RNA interference did not show an influence on the oxygen respiration rate and the mitochondrial membrane potential. Taken together, these results suggest that alterations in the levels of DDP1 can affect the morphology of mitochondria and thus shed new light on the pathogenic mechanisms of DDON.

INTRODUCTION

Deafness-Dystonia-Optic Neuropathy (DDON) Syndrome, previously termed Mohr-Tranebjaerg Syndrome (MTS; MIM 304700), is a rare X-linked progressive neurodegenerative disorder resulting from mutations in the TIMM8A gene encoding for the deafness dystonia protein 1 (DDP1). First reports in 1960 of a large Norwegian family with progressive sensorineural hearing impairment suggested isolated deafness. An extensive clinical restudy of this family in 1995 by Tranebjaerg et al. (1) demonstrated the underlying syndromic character with an X-linked inheritance pattern. Further characterization revealed the causative single base pair deletion in exon 1 in an affected male from the original Norwegian MTS family (2,3). To this date, additional cases of mutations in the DDP1 coding region itself as well as mutations affecting additional genes up- and downstream of the TIMM8A gene locus, resulting in the deletion of the entire TIMM8A gene, have been described. DDON therefore comprises a single gene disorder with various mutations or deletions affecting the TIMM8A gene locus (3–12). An interesting mutation is the replacement of Cys66 by Trp (the so-called C66W mutation) that results in a full-length protein but causes an equally severe clinical phenotype as nonsense mutations (4).

Common clinical manifestations of TIMM8A mutations are progressive postlingual sensorineural hearing impairment in early childhood and a variable onset of slowly progressive dystonia or ataxia. Progressive degeneration of the brain

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stem, corticospinal tract and the basal ganglia was found in those patients that developed the movement disorder (13). In addition, other clinical characteristics such as mental deterioration, paranoia, behavioural abnormalities and cortical blindness were reported, although not in all cases and also not in all individuals of the same affected family (1,3–11). The vast clinical phenotypic variability, both interfamilial and intrafamilial, can therefore not solely be explained by different mutations of the TIMM8A gene but might rather hint at an environmental and genetic modulation of the clinical symptoms (10).

The pathomechanism of DDON is not yet elucidated. Studies in yeast indicate that the yeast homologue of DDP1, Tim8 resides in the intermembrane space (IMS) of mitochondria and forms there a hetero-oligomeric 70 kDa complex with Tim13. This complex was initially suggested to assist the import process of Tim23 precursor protein by stabilizing it as the newly synthesized protein is transferred through the IMS (14–17). Tim23 is an essential subunit of the presequence translocase of the mitochondrial inner membrane (18,19). Truncation or absence of DDP1 as well as the missense mutation C66W in the conserved “twin CX3C” motif (4) causes a defect in assembly of the DDP1/TIMM13 complex (20,21). Hence, DDP1 mutations were suggested to result in impaired hTim23 biogenesis in the affected tissue (17,21). Additionally, reported substrates of DDP1/TIMM13 in mammalian mitochondria are the aspartate/glutamate carriers, citrin and aralar1, participating in the aspartate–malate nicotinamide adenine dinucleotide (NADH) shuttle. Due to shared tissue expression patterns of aralar1, DDPI and TIMM13 and high metabolic demands in large cerebellar neurons, decreased NADH levels in these mitochondria together with a decrease in hTim23 biogenesis and the consecutive alterations in matrix import of precursor proteins might be a possible underlying molecular mechanism of this neurodegenerative disease (22).

However, studies in Saccharomyces cerevisiae and Neurospora crassa point out an involvement of the Tim8–Tim13 complex also in the import pathway of outer membrane β-barrel precursor proteins. Lack of Tim8 and Tim13 partially impairs the assembly pathway Tom40 and Tob55/Sam50 (23–25). Considering the important functions of these β-barrel proteins, their reduced import in DDP1 mutated cells can also contribute to the pathomechanism of DDON.

In the present study, we aimed to improve our understanding of the role of DDP1 in the biogenesis and function of mammalian mitochondria. Specifically, we investigated the effects of reduced or elevated DDP1 levels on mitochondrial morphology, energy production, cellular viability and protein import properties. Our results provide new insights into the molecular function of DDP1 and thus into the pathomechanism of DDON.

RESULTS

Mitochondria with elevated levels of DDP1 are compromised in their capacity to import in vitro β-barrel proteins

The complex of the small chaperones DDP1 and TIMM13 resides in the mitochondrial IMS. This complex plays a multifaceted and not yet completely understood role in the import of multispan inner membrane precursor proteins, such as the aspartate/glutamate carriers and Tim23 (16,17,22). Several reports suggested that the homologous complex in yeast mitochondria also facilitates the transfer of precursors of β-barrel proteins through the mitochondrial IMS (23–25).

To elucidate the potential function of DDP1 in mammalian cells, we monitored the capacity of mitochondria isolated from cells overexpressing DDP1 to import in vitro various precursor proteins. To achieve uniform expression levels of DDP1, we generated a HeLa cell clone expressing DDP1 under a tetracycline-inducible promoter. Importantly, to generate both the wild-type as well as the mutant DDP1 overexpressing HeLa cells (see below), we used the FlpIn system, which ensures that stable integration of the tetracycline-inducible gene occurs at the same genomic locus and only once, thereby ensuring homogenous expression in all cells (26). Radiolabelled precursor proteins of the inner membrane protein hTim23, the inner membrane adenosine diphosphate–adenosine triphosphate (ATP) carrier (AAC), the outer membrane β-barrel proteins hTom40 and voltage-dependent anion channel 1 (VDAC1) and the matrix-destined protein pSu9-DHFR were utilized. The latter one was used to control for the general import capacity of the employed organelles. The import assay was performed with mitochondria derived from either HeLa cells overexpressing DDP1 in an inducible manner or from control cells. Our results suggest that overexpression of DDP1 does not affect the ability of mitochondria to import hTim23 and the control protein pSu9-DHFR. In contrast, import levels of hTom40 and VDAC1 into the organelles from the overexpressing cells were modestly compromised (Fig. 1A and B, and data not shown). Western blot analysis using antibodies against DDP1 confirmed the high expression levels of DDP1 in the overexpression cells (Fig. 1A). We also observed that the overexpression did not cause alterations in the steady-state levels of the matrix proteins MnSOD and Hsp60, as well as the β-barrel proteins Tom40 and VDAC1 (Fig. 1A, lower panels and data not shown). Notably, the import of AAC, which was found in yeast cells to be a substrate of the Tim9–Tim10 complex but not of the Tim8–Tim13 complex (27) was also not affected (Supplementary Material, Fig. S1).

The DDON-related C66W replacement in DDP1 results in a full-length protein that is efficiently imported in vitro into mitochondria but degraded more rapidly than the wild-type counterpart (20). In order to understand whether this variant could possibly interfere with the biogenesis of mitochondrial precursor proteins, we checked the import properties in cells inducibly overexpressing this mutant form. Import efficiencies of all tested precursor proteins into organelles isolated from cells overexpressing the mutant form of DDP1 were similar to those into control mitochondria (Fig. 2A and B). Similarly, the steady-state levels of the monitored mitochondrial proteins were unaffected by the overexpression of the C66W mutant (Fig. 2A). Of note, detected levels of the C66W variant were remarkably lower than those of the native DDP1 (compare Figs 1A and 2A). These observations are in agreement with previous reports regarding the reduced stability of the mutant protein (20). Collectively, we conclude that
overexpression of DDP1, but not of its C66W mutant, specifically reduces the capacity of mitochondria to import in vitro precursor molecules of β-barrel proteins.

The small Tim proteins, Tim8 and Tim13, form a hetero-oligomeric 70 kDa complex in the IMS. Thus, we asked whether the observed reduced β-barrel import upon overexpression of DDP1 is due to an assembly defect of this complex. To address this question, mitochondria overexpressing either the native form of DDP1 or the mutant form were solubilized in buffer containing the detergent digitonin and subjected to blue native-polyacrylamide gel electrophoresis (BN-PAGE) and subsequent western blot analysis (Fig. 3). DDP1 overexpression resulted in a massive formation of a complex with an apparent molecular mass of ~70 kDa that migrates similarly to the complex observed in the wild-type organelle. However, in mitochondria overexpressing DDP1-C66W, we also observed a band with lower apparent Mw that might represent unassembled molecules. In contrast, overexpression of the C66W variant did not lead to accumulation of unassembled molecules and significantly less of the ~70 kDa complex was observed (Fig. 3). Of note, the complex containing the C66W mutation of DDP1 migrated somewhat higher in the native gel system probably due to an altered conformation of the hetero-oligomeric complex. A similar behaviour of the complex was observed when mitochondria were lysed with another detergent, Triton X-100 (Fig. 3). The levels of the translocase of the mitochondrial outer membrane (TOM complex) were not altered in the various samples and served as a loading control (Fig. 3). Taken together, these results suggest that unassembled DDP1 molecules might interfere with the import of β-barrel proteins and further demonstrate that the mutant form is still able to assemble into oligomeric structures.
Next we asked if upregulation of DDP1 could in turn also lead to an increase in the expression levels of its partner protein, TIMM13. Unfortunately, in the absence of a functional TIMM13 antibody, we could not address this question by western blotting and this absence also prevented us from testing whether the increased levels of the 70 kDa complex represent homooligomers of DDP1 or additional hetero-hexamers harbouring both DDP1 and TIMM13. However, we did perform quantitative reverse transcription polymerase chain reaction (qRT–PCR) on these cells and observed similar mRNA levels of TIMM13 in DDP1 overexpressing cells compared with those in control cells (data not shown).

Reduced levels of DDP1 do not affect biogenesis of hTim23 and hTom40

The small Tim proteins residing in the IMS facilitate in yeast the import of β-barrels and multispans inner membrane proteins (15–17,22–25,27). To elucidate whether this holds true also for mammalian mitochondria, we isolated mitochondria from mouse liver and subjected them to osmotic swelling that results in rupturing of the outer membrane and release of IMS elements like DDP1. Next, radiolabelled proteins were added to either intact or swollen mitochondria (mitoplasts) and import capacities of both types of organelles were monitored. Intriguingly, osmotic swelling did not affect hTom40 or hTim23 import as analysed by resistance to alkaline extraction of membrane-embedded proteins (Fig. 4A). Similarly, this treatment also did not compromise the import efficiency of the AAC (Fig. 4B). Effective swelling, the intactness of the mitochondrial inner membrane and the fidelity of the alkaline treatment were confirmed by immunoblot analysis. Membrane-embedded proteins such as hTom70, VDAC1 and hTim23 were detected in the pellet fractions of both intact and swollen mitochondria. As expected, the soluble matrix proteins Hsp60 and MnSOD were found in the supernatant fractions. Their presence in both mitochondria and mitoplasts indicates that the inner membrane was intact in these samples. The osmotic swelling effectively ruptured the outer mitochondrial

**Figure 3.** Complex assembly of DDP1 and its C66W variant. Mitochondria (50 µg) were solubilized for 30 min on ice in buffer containing 1.6% of either Triton X-100 or digitonin. Samples were subjected to BN-PAGE and immunodecoration with antibodies against hTom40 and DDP1. The assembled TOM complex as well as the monomeric and oligomeric forms of DDP1 are indicated. Notably, the TOM complex is not stable in the presence of Triton X-100.

**Figure 4.** Rupturing of the mitochondrial outer membrane does not compromise the import of hTim23, hTom40 and AAC. (A) Isolated mouse liver mitochondria were preincubated in either isotonic or hypotonic buffer resulting in intact organelles or mitochondria with a ruptured outer membrane (mitoplasts), respectively. Mitochondria and mitoplasts were then resolubilized and incubated for 5 or 25 min at 25°C with radiolabelled precursors of hTim23 or hTom40 (A) or with radiolabelled precursor of AAC (B). Samples were subjected to carbonate extraction and the resulting pellet (P) and supernatant (SN) fractions were analysed by SDS–PAGE followed by either autoradiography (upper panels) or immunodecoration with antibodies against mitochondrial proteins. VDAC1 and Tom70, outer membrane proteins; DDP1, soluble IMS protein; Tim23, inner membrane protein; Hsp60 and MnSOD, soluble matrix proteins.
membrane since the DDP1 signal was completely lost in the supernatant fraction of mitoplasts in contrast to intact mitochondria where it behaved like other soluble proteins (Fig. 4A and B). Thus, it seems that DDP1 does not play a crucial role in the import of the investigated precursor proteins. Notably, very minor amounts of the yeast Tim9–Tim10 complex are associated with the mitochondrial inner membrane and thus are not released upon osmotic swelling (28). A similar situation might also occur in mammalian mitochondria, where these residual amounts of Tim9–Tim10 molecules are able to sufficiently promote the import in vitro import assays of small quantities of radiolabelled substrates.

To further analyse the role of DDP1 in the biogenesis of mitochondrial proteins, we tested the steady-state levels of mitochondrial proteins in cells where DDP1 was downregulated or completely absent. Whole cell lysates of HeLa cells specifically downregulated for DDP1 with two different siRNAs were analysed for abundance of the mitochondrial outer membrane proteins hTom40 and VDAC1, as well as for the inner membrane protein hTim23, a suggested putative substrate of the DDP1/TIMM13 complex. Although the levels of DDP1 were markedly decreased in these cells, we did not observe any reduction in the steady-state levels of the investigated proteins (Fig. 5A). Similarly, mitochondria isolated from DDP1-depleted cells were not compromised in their capacity to import in vitro hTom40 and hTim23 (data not shown). A possibility that we cannot exclude is that the observed residual DDP1 molecules are capable of supporting normal biogenesis of hTim23, VDAC1 and hTom40.

To address this point in an unrelated way, we isolated mitochondria from patient fibroblasts, harbouring the c.116delT mutation and of DDP1-downregulated HeLa cells. Cells were incubated with the membrane potential sensitive dye tetramethylrhodamine, ethyl ester (TMRE) and then subjected to fluorescence-activated cell sorting (FACS). No decline in the TMRE signal in DDP1-downregulated cells in comparison to control cells was detected, thus indicating an uncompromised membrane potential in these cells (Fig. 6A). Next, respiration was monitored under normal growth conditions (4.5 g/l glucose medium) and in the growth medium where glucose was substituted for galactose, in order to aggravate a potential respiratory dysfunction by forcing the cells to rely on oxidative phosphorylation for energy production (Fig. 6B). Cells grown in the presence of 10 mM galactose-containing medium showed a higher oxygen consumption rate when compared with rates of cells grown in glucose-containing media, although statistically significant differences between DDP1 downregulated and control cells could not be seen in either case (Fig. 6B). These findings are in agreement with other studies evaluating bioenergetic properties of primary cells derived from DDON patients, showing no major alterations in energy-generating systems (8,21).

Interference with the growth behaviour could also be a consequence of a latent mitochondrial dysfunction. Primary fibroblasts from two DDON patients with the c.116delT mutation in the TIMM8A gene and fibroblasts from a healthy donor were grown in the medium with either low (200 mg/l) or high (4.5 g/l) glucose concentrations as well as in the galactose-containing medium (10 mM). After 12 days, cells were harvested and viable cells were counted. Fibroblasts from patient P1 and fibroblasts from the healthy donor did not show marked differences in cell counts of either growth condition, whereas the cell number of fibroblasts from patient P2 grown in the galactose-containing medium was reduced to half in comparison to the cell count in glucose-containing media (Fig. 6C). Despite this difference, it becomes apparent that the bioenergetic properties of cells with reduced or absent DDP1 protein expression are not drastically altered.

### Changes in the levels of DDP1 influence mitochondrial morphology

As a further step to understand the pathomechanism of DDON, we investigated whether alterations in the levels of DDP1 can cause changes in mitochondrial morphology. To that goal, we performed live cell microscopy of primary fibroblasts with the aforementioned mutation and of DDP1-downregulated HeLa cells. (A) Whole cell lysate (50 μg) from HeLa cells transfected with oligonucleotides directed against either Luciferase Gl2 (as a control, ctrl) or TIMM8A (siRNA1 and 2) were analysed by SDS–PAGE and immunodecoration with antibodies against the indicated mitochondrial proteins. (B) Mitochondria (50 μg) isolated from primary fibroblasts of a healthy person (ctrl) or of two DDON patients diagnosed with the c.116delT mutation (P1 and P2) were analysed by SDS–PAGE and immunodecoration with antibodies against the indicated mitochondrial proteins.

**Figure 5.** Downregulation or absence of DDP1 does not affect the steady-state levels of mitochondrial proteins. (A) Whole cell lysate (50 μg) from HeLa cells transfected with oligonucleotides directed against either Luciferase Gl2 (as a control, ctrl) or TIMM8A (siRNA1 and 2) were analysed by SDS–PAGE and immunodecoration with antibodies against the indicated mitochondrial proteins. (B) Mitochondria (50 μg) isolated from primary fibroblasts of a healthy person (ctrl) or of two DDON patients diagnosed with the c.116delT mutation (P1 and P2) were analysed by SDS–PAGE and immunodecoration with antibodies against the indicated mitochondrial proteins.
cells. To facilitate the visual assessment of the mitochondrial morphology, mitochondria were segmented and depicted in 3D using the Imaris software. Cells harbouring the mutation in the TIMM8A gene displayed an altered mitochondrial morphology in both DDON-patients’ fibroblasts compared with control fibroblasts from a healthy donor. In particular, we noticed that the majority (∼90%) of the primary fibroblasts lacking DDP1 contained long continuous mitochondrial extensions projecting towards the cell periphery (Fig. 7A and B).

A similar picture could be observed in DDP1 downregulated cells, although not to the same extent as in the primary fibroblasts. Transfection with siRNA and subsequent live cell microscopy revealed extended tubular structures stretching towards the cell borders as the predominant pattern in 65–80% of the analysed cells (Fig. 7C and D).

Next, we asked if DDP1 overexpression affects mitochondrial morphology. To that end, we transfected HeLa cells transiently with pCS2-DDP1 and the empty control vector and after ∼36 h incubated these cells with the mitochondria specific dye MitoTracker Red CMXRos. Our images revealed that overexpression of DDP1 resulted in fragmentation of mitochondria and appearance of a fraction of the organelle as hollow, spherical tubes (Fig. 8A). This phenotype was observed in ∼14% (±1% SEM) of more than 500 analysed...
cells, whereas no cells with the same phenotype were found in >600 control cells. The hollow spheres, indicated with arrow heads (Fig. 8A), appeared not to be connected among each other, but were single mitochondrial entities formed around the nucleus. This was further confirmed by segmentation of mitochondria from z-stacks and rendering of a 3D model (Supplementary Material, Movie S1). Notably, similar morphological phenotypes were not observed when TIMM13 was overexpressed alone or in combination with DDP1 (Fig. 8B and C). Hence, it seems that DDP1 does not behave identically to its partner protein, TIMM13. Importantly, overexpression of TIMM13 did not affect the expression levels of DDP1 (Fig. 9). We further observed that when in addition to DDP1 also TIMM13 was overexpressed, the amounts of unassembled DDP1 were reduced concomitantly with the formation of high molecular mass complexes that contain both proteins.

Figure 7. Downregulation or complete lack of DDP1 induces elongated, thread-like mitochondria. (A) Primary fibroblasts as described in the legend to Figure 5B were incubated with MitoTracker Red for 30 min. Subsequently, the medium was replaced by CO2-independent live cell medium and the cells were analyzed by live cell microscopy. Images constitute deconvolved projections of z-stacks (upper panel). Scale bars represent 15 μm. The lower panel shows magnified insets. Mitochondria were segmented and rendered in 3D using the Imaris software. (B) Cells analysed in (A) were categorized into normal or elongated phenotype and bar graphs showing the percentage of cells with elongated mitochondria are presented. Data represent the mean of three independent experiments with SEM. (C) HeLa cells were transfected with control siRNA or siRNA targeting TIMM8A and incubated for 36 h. Then mitochondrial morphology was assessed by live cell microscopy. Representative images for both conditions are shown. Insets display enlarged versions of segmented and 3D rendered mitochondria. Scale bars represent 20 μm. (D) Cells analysed in (C) were categorized into normal or elongated phenotype and bar graphs showing the percentage of cells with elongated mitochondria are presented. Data represent the mean of three independent experiments with SEM.
These results support the notion that the unassembled DDP1 molecules might cause the phenotypes of the DDP1 overexpressing cells. Taken together, enhanced or reduced levels of DDP1 result in distinct and apparent mitochondrial morphology phenotypes.

DISCUSSION

DDON is a rare and poorly understood disease caused by a mutation of the mitochondrial IMS protein DDP1. In the current study, we investigated the function of DDP1 in human cells
by applying in vitro import experiments as well as ex vivo experiments using cells with altered DDP1 levels. Import experiments showed that upregulated amounts of DDP1, but not of the mutant form C66W, reduce the import efficiency of the β-barrel outer membrane proteins hTom40 and VDAC1. Of note, such an effect was not observed in the import of the inner membrane protein hTim23. The precursor form of the latter protein was previously reported to interact with the Tim8/Tim13 complex (15,27) and to rely on this complex for its efficient delivery to the inner membrane (16,17).

We propose that the compromised import of the β-barrel precursors is due to accumulation of unassembled DDP1 molecules in the IMS and the unproductive interactions of this monomeric form with either the precursor molecules or other components in the import pathway. The mutant form was shown in previous studies to be degraded more rapidly than the wild-type form (20). Accordingly, reduced amounts of DDP1-C66W were observed upon its overexpression and no monomeric form was detected. In agreement with this, the amounts of the oligomeric form of DDP1-C66W were lower when the mutant form was upregulated when compared with the situation upon overexpression of native DDP1. Considering the findings by Hofmann et al. (29) that this mutant form is not able to form homo-oligomers in vitro, our data would argue in favour of a hetero-oligomeric complex of DDP1-C66W with TIMM13. This complex formation would thereby be prone to a more rapid degradation and destabilization as also seen previously in fibroblasts derived from patients with the C66W mutation, where the complex could not be detected at all (21). Higher DDP1 levels did not in turn lead to an upregulation of the transcripts of the partner protein TIMM13. This observation might suggest the existence of a free pool of TIMM13 molecules in the IMS that can be recruited for complex formation upon DDP1 upregulation.

Unexpectedly, removal of soluble IMS proteins upon rupturing of the outer membrane did not show any effect on the import of hTom40, AAC or hTim23 into isolated mitochondria. This treatment results in the release of the DDP1-containing complex from the ruptured organelles, but we cannot exclude the possibility that minor amounts of the TIMM9—TIMM10 complex, which are associated with the inner membrane, are still present. Thus, it appears that although the DDP1/TIMM13 complex can interact with hTim23 precursor molecules in the IMS while they are relayed from the TOM complex to the inner membrane, such an interaction does not seem to be absolutely required for their efficient translocation and proper membrane insertion. This is in contrast to the situation in fungi where rupture of the outer membrane or depletion of the Tim8/Tim13 complex results in a severely compromised capacity to import Tim23 or outer membrane β-barrel proteins (23,25,30). This difference might indicate the existence of alternative import pathways in higher eukaryotes when compared with fungi.

Interestingly, neither downregulation nor absence of DDP1 resulted in changes in the steady-state levels of proteins from various intra-mitochondrial compartments. Complete lack of DDP1 in the patient fibroblasts harbouring the c.116delT mutation revealed a similar picture as in the downregulated cells. Thus, either altered protein levels are unlikely to contribute to the basic pathomechanism of the disease or the analysed cells do not reflect the homeostasis of important proteins in the affected neuronal cells. Alternatively, the absence of DDP1 may compromise the steady-state levels of a yet to be identified specific substrate that fulfils an important role in mitochondrial function.

Mitochondrial diseases are often accompanied by changes in the organelles’ bioenergetic properties such as respiration rates and maintenance of membrane potential. Nevertheless, reduced DDP1 levels in HeLa cells neither resulted in alterations of the mitochondrial membrane potential in the examined cells nor compromised oxygen consumption rates even under aggravating metabolic stress conditions. These results argue against an energetic defect as a major cause for the disease and are also in accordance with findings in primary cells from patients with other mutations in the TIMM8A gene (8,21). Our proliferation studies of primary fibroblasts with the c.116delT mutation showed no differences in the growth behaviour of the control fibroblasts and P1 fibroblasts, although a reduced number of cells were counted in the case of P2 fibroblasts in the galactose-supplemented growth medium. Considering the large variability of clinical symptoms among family members with the same genetic mutation, epigenetic factors might be partly responsible for this effect. Comprehensive studies of a large patient cohort would be essential to investigate possible epigenetic factors in detail. In summary, inadequate energy production does not provide a universal explanation for the clinical picture of DDON.

Mitochondrial morphology and the factors regulating the organelles fusion and fission processes are a matter of intense research (31–36). It is appreciated that the cell’s varying requirements result in adaptation in shape by fusion and fission events of the mitochondrial network. Therefore, we examined the morphological implications of reduced or elevated DDP1 levels. Extensive analysis and assessment was performed on >900 cells of each siRNA-treated cell population and indistinguishable morphological changes were found upon DDP1 depletion with both short-interfering oligonucleotides targeting DDP1 mRNA. Elongated, thread-like mitochondria were apparent in the majority of these cells and primary fibroblasts derived from DDON patients with the c.116delT mutation displayed a similar phenotype. Overexpression of DDP1 on the other hand resulted in hollow, spherical mitochondria of varying diameter that were not interconnected. This specific grain-like structure was previously found in Caenorhabditis elegans, where overexpressing DRP-1 cells exhibited severe mitochondrial fragmentation (37).

Currently, the link between alterations in the DDP1 expression levels and the observed morphological changes is not clear. It was reported that in apoptotic cells, DDP1 is released into the cytoplasm where it binds Drp1, a mediator of mitochondrial fission and, promotes its mitochondrial redistribution (38). Despite this report, we favour a scenario where the levels of DDP1 could indirectly affect the import of additional, yet not identified, regulatory substrates, which in turn promote morphological changes in the mitochondrial network.

Although the morphology of the organelle is tightly connected to mitochondrial regulation pathways, these
mitochondrial changes might not result in a direct metabolic deficit in HeLa cells and fibroblasts, but could possibly interfere with mitochondrial transport and/or function in neurons. Since the central nervous system has a high energy demand and is strongly dependent on mitochondrial ATP production (39), alterations would severely affect the cellular metabolism and in the long run lead to neurodegeneration. Fusion events are essential for exchange of contents between dysfunctional and functional mitochondria thereby also ensuring mtDNA integrity and respiration (34). It is very tempting to speculate, considering the morphological changes we found in DDP1-depleted HeLa cells and primary fibroblasts of DDON patients, that this elongated tubules could reflect an intense effort to exchange mitochondrial contents to sustain the basic metabolic functions. Indeed, recent reports suggest that elongated mitochondria are less prone to removal by mitophagy (40,41). Although our study provides initial hints regarding the complex pathomechanism of DDON, extensive further studies employing primarily affected cells and/or a mouse model are required for obtaining a comprehensive understanding of this process and to gain a better knowledge about the correlation of mitochondrial morphology and function in different cell types.

MATERIALS AND METHODS

Cloning procedures

Constructs encoding DDP1 (NCBI acc. no. NG_011734) and TIMM13 (NCBI acc. no. NM_012458.2) were generated by PCR amplification of the corresponding cDNA. The DNA products were sub-cloned into modified versions of pcDNA5/FRT, 6xmyc pCS2 and pCS2 vectors. Site-directed mutagenesis to mutate cystein66 to tryptophan was performed using the QuickChange kit (Stratagene) according to the manufacturer’s instructions. The mutated C66W sequence was sub-cloned into a modified version of the pcDNA5/FRT vector (26). All constructs were verified by DNA sequencing. Constructs pGEM4Z-hTim40 and pGEM4Z-hTim23 are kind gifts of Dr S. Hofmann. The constructs pSu9-DHFR (42) and pGEM4-AAC (43) are described elsewhere.

qRT–PCR

Total RNA from induced and non-induced HeLa cells was extracted using PrepEase RNA Spin Kit (Affymetrix). First-strand cDNA was synthesized from RNA using iScript cDNA Synthesis kit (Biorad) according to the manufacturer’s recommendations. qRT–PCR amplification was performed on first-strand cDNA using TIMM13-specific primers (QT00203266, Qiagen), and glyceraldehyde-3-phosphate dehydrogenase-specific primers (QT1192646, Qiagen) as a control. The Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) kit was used with an AppliedBiosystems StepOne real-time PCR system.

In vitro import

Precursor proteins were synthesized by coupled transcription/translation in reticulocyte lysate (TNT Coupled Reticulocyte Lysate System, Promega) in the presence of [35S]methionine using SP6 RNA polymerase. For in vitro import, isolated mitochondria (25–100 μg) were incubated for the indicated time periods in import buffer (220 mM mannitol, 70 mM sucrose, 1 mM ethylenediaminetetraacetic acid, 20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES)-KOH, pH 7.4 supplemented with 0.5 mM magnesium acetate, 20 mM sodium succinate, 5 mM NADH and 1 mM ATP) with radiolabelled precursor proteins at 25°C and subsequently transferred on ice and treated with proteinase K (100 μg/ml) to remove non-imported material. The protease was inactivated by addition of 2 mM phenylmethylsulphonyl fluoride and then mitochondria were re-isolated and washed with import buffer.

Isolation and manipulation of mitochondria

Mitochondria were isolated from mammalian cells according to the published procedures (44). Cells were grown to confluency, washed with phosphate-buffered saline and harvested. Cells were then pelleted (800g, 5 min, 2°C) and resuspended in import buffer supplemented with 2 mg/ml bovine serum albumin followed by homogenization with a needle and subsequent differential centrifugation steps (44). Mitochondria were isolated from mouse liver by mincing the liver and subsequent homogenization with a potter. The crude suspension was subjected to differential centrifugation essentially as described previously (45), and isolated mitochondria were resuspended in chilled isolation buffer.

To specifically rupture the mitochondrial outer membrane, isolated mitochondria were subjected to osmotic shock treatment with 10 mM HEPES, pH 7.5 for 30 min on ice. Swollen mitochondria (mitoplasts) were resuspended and resuspended in fresh import buffer that was supplemented with 100 μg/ml creatine kinase and 5 mM creatine phosphate before the import reaction.

For alkaline extraction, mitochondria or mitoplasts were isolated and suspended on ice for 30 min in 100 mM sodium carbonate, pH 11.5. The pellet resulting from a centrifugation step (100,000g, 30 min, 2°C) was processed for sodium dodecyl sulfate (SDS)–PAGE. Precipitation of proteins in the supernatant was achieved by addition of 12% trichloroacetic acid followed by centrifugation steps and an acetone wash.

Isolated mitochondria were resuspended in SDS sample buffer and subjected to SDS–PAGE. Endogenous proteins and protein complexes were analysed by western blotting and detection via specific antibodies, whereas radiolabelled proteins were detected by autoradiography. Quantification of electrophoretic bands was done using AIDA Image Analyzer software (Raytest).

Cell culture, transfection and live cell microscopy

HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (PAA) containing 10% (v/v) foetal bovine serum-Gold (PAA) at 37°C under 5% CO2 atmosphere. Transfection was performed using FuGENE6 (Roche) according to the manufacturer’s instructions. For co-transfection experiments, HeLa cells were transfected with a total of 120 ng DNA in a total volume of 250 μl of growth medium for microscopy experiments or a total of 26 μg DNA in a total volume of
40 ml of growth medium for BN-PAGE experiments according to the manufacturer’s instructions. For fluorescence microscopy, 1.5 days after transfection, cells were incubated with the medium containing 40–100 nM MitoTracker Red CMXRos (Molecular Probes) for 30 min which was subsequently replaced by CO₂-independent Live cell medium (Invitrogen). Cells were analyzed on a DeltaVision microscope and deconvolved using softWoRx 4.0 (both Applied Precision) and images 3D segmentation and rendering was performed with Imaris software (Bitplane). For morphological quantification, cells were classified into three categories based on the appearance of the mitochondrial network structure: elongated structures presenting as extended cables, hollow spheres or regular mitochondrial tubules. For live cell imaging, we used either HeLa cells or HeLa cells stably expressing green fluorescent protein-tagged histone H2B.

For RNA interference studies, HeLa cells were transfected using HiPerFect transfection reagent with 5 nm siRNA oligonucleotides 1 and 2 for specific gene sequence. DDP1 (SI00059556, target sequence 5′-CAC GTA GAG GTG CAT GCC TAA-3′; SI03063095, target sequence 5′-CAG AAG CTA ACA GCA GGA ATA CTT CGA-3′) or with control siRNA oligonucleotide directed against Luciferase GL2 (1022070, target sequence 5′-AAC GTA TCA ACA TCA-3′) or with control siRNA oligonucleotide directed against Luciferase GL2 (1022070, target sequence 5′-AAC GTA TCA ACA TCA-3′). Reagents and oligonucleotides were purchased from Qiagen.

Cell lines overexpressing DDP1 variants
HeLa cell lines, stably expressing native DDP1 or the C66W variant in a tetracycline-inducible manner, were based on a HeLa cell line (kind gift of Dr T. U. Mayer) that was generated using the FlpIn system (Invitrogen) following the manufacturer’s protocol. The pFRT/lacZeo vector was used to generate a stable, Zeocin-resistant, clone with only one FRT site. In a second step, this clone was transfected with pcDNA6/TR (for cytomegalovirus promoter-driven expression of the Tet repressor) and stable integrands were isolated by selection with blasticidin and Zeocin. Then the coding sequences of DDP1 or its C66W variant were cloned into a modified pcDNA5/FRT vector. Each construct was cotransfected with pOG44 (for Flp recombinase expression) into the tetr-inducible, FRT site-containing HeLa clone. Positive clones were selected with hygromycin B and analysed for inducible expression of DDP1 and DDP1-C66W by immunodecoration with antibodies against DDP1. Cell lines were grown at 37°C and 5% CO₂ in Dulbecco’s modified Eagle’s medium (PAA) with 10% tetracycline negative fetal bovine serum (FBS) (PAA). For all experiments, DDP1 or C66W expression was induced immediately after cells were seeded with 1 μg/ml tetracycline for 2 days.

Primary fibroblasts and growth analysis
Primary fibroblasts from two DDON patients harbouring the c.116delT mutation (1) and from a healthy donor (matched for gender) were cultivated at 37°C and 5% CO₂ in Dulbecco’s modified Eagle’s medium containing 15% (v/v) FBS-Gold (PAA). For growth analysis, fibroblasts (matched as closely as possible for similar passage numbers and age) were cultivated for 12 days in Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose, 200 mg/l glucose or 10 mM galactose according to previously described procedures (46). Subsequently, the number of viable cells was monitored by Trypan blue staining and cell counting.

Bioenergetic analysis of siRNA-treated cells
Mitochondrial membrane potential (ΔΨₘ) was assessed by FACS of siRNA-treated cells. After transfection (twice within 48 h), HeLa cells were harvested and incubated with 100 nM TMRM at 37°C for 30 min, followed by washing steps and subsequent analysis on a BD LSR II system with FACSDiva software.

For oxymetry studies with a Clark-type electrode, HeLa cells were transfected twice with 25 nM siRNA and cultivated in Dulbecco’s modified Eagle’s medium. The medium, containing either 10 mM galactose or 4.5 g/l glucose, was supplemented with 1 g/100 ml sodium pyruvate and 1 g/100 ml glutamine during measurement recordings.

Miscellaneous
Antibodies specific for DDP1 were raised in rabbits using recombinant MBP-DDP1 full-length protein as an antigen (Pinela). Antibodies against VDAC1 (Abcam), Tom40 (Santa Cruz Biotechnology), Tim23 (BD Transduction Laboratories) and MnSOD (Enzo Life Sciences) were purchased from the indicated manufacturer. Antibodies against Hsp60 and Tom70 were kindly provided by Drs A. Azem and K. Mihara, respectively. Blue native gel electrophoresis (BN-PAGE) was essentially performed as previously described (47).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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