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DHTKD1 is essential for mitochondrial biogenesis and function maintenance

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ABSTRACT

Maintaining the functional integrity of mitochondria is crucial for cell function, signal transduction and overall cell activities. Mitochondrial dysfunctions may alter energy metabolism and in many cases are associated with neurological diseases. Recent studies have reported that mutations in dehydrogenase E1 and transketolase domain-containing 1 (*DHTKD1*), a mitochondrial protein encoding gene, could cause neurological abnormalities. However, the function of DHTKD1 in mitochondria remains unknown. Here, we report a strong correlation of *DHTKD1* expression level with ATP production, revealing the fact that DHTKD1 plays a critical role in energy production in mitochondria. Moreover, suppression of *DHTKD1* leads to impaired mitochondrial biogenesis and increased reactive oxygen species (ROS), thus leading to retarded cell growth and increased cell apoptosis. These findings demonstrate that DHTKD1 contributes to mitochondrial biogenesis and function maintenance.

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1. Introduction

Mitochondria are constituted of about 1500 proteins. The majority are encoded by nuclear genome, while only about 1% are encoded by mitochondrial DNA (mtDNA) independently [1]. Thus, maintain the integrity and interaction between mitochondrial and nuclear genomes is necessity for well-functioning mitochondria [2]. Mitochondria were long viewed as vital but static energy factories, furthermore, mitochondria have been found to maintain overall mitochondrial morphology and function in accordance with the cell cycle, cell differentiation, metabolic rates and energy requirement [3]. The precise control of the mitochondrial amount through regulation of biogenesis and degradation is one of the key elements involved in appropriate mitochondrial and cell function [2]. Mitochondrial dysfunction caused by various abnormalities can lead to apoptosis, mtDNA mutations, and a severe reduction in energy production. Mitochondrial abundance, integrity, activity, and normal morphology is crucial for a cell, thus damage of mitochondria can cause many cell dysfunctions, even impairing cell survival leading to death [1]. Indeed, dysfunction of mitochondria is linked to the development of various human diseases, including neurodegenerative conditions, metabolic syndromes and cancers [4–6].

Dehydrogenase E1 and transketolase domain containing protein 1 (*DHTKD1*) gene is localized in chromosomal region 10p14 [7]. *DHTKD1* was first cloned by Nagase's group in 2000 [8], and it is consisted of 17 exons with an open reading frame (ORF) of 5202 base pairs length, encoding a proposed mitochondrial protein with 919 amino acids [9,10]. The product of this gene was similar to oxoglutarate dehydrogenase (OGDH) and OGDH like protein (OGDHL) [7], and was presumed to function as an E1 component of dehydrogenase complex in tricarboxylic acid (TCA) cycle or elsewhere, and might play an critical role in energy production in mitochondria. Recently, we reported a heterozygous nonsense mutation in *DHTKD1* was the cause of Charcot–Marie–Tooth disease type 2Q (CMT2Q), one of the most common inherited neurodegenerative diseases. We demonstrated that *DHTKD1* mRNA transcripts containing premature stop codons were degraded through the nonsense-mediated mRNA decay (NMD) and thus led to a significantly lower protein level [10]. At the same time, Prokisch's group reported their findings about *DHTKD1* mutations in human 2-aminoadipic and 2-oxoadipic aciduria, which was an L-lysine metabolism disorder, featuring with mild to severe neurological symptoms, caused by impaired turnover of decarboxylation 2-oxoadipate to glutaryl-CoA [11]. These studies implied a critical

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role for DHTKD1 in mitochondrial metabolism and neurological development.

In humans, mitochondrial dysfunction is associated with various neurological disorders [12]. Here, mutations in the mitochondrial protein DHTKD1, leading to a number of neuropathies, deserve special attention. However, it remains unknown how DHTKD1 mediates TCA cycle. There also remain concerns about why mutations in *DHTKD1* would have a negative impact on neurological function. To explore the function of DHTKD1 on mitochondria, we silenced *DHTKD1* with siRNAs. It was found that suppression of *DHTKD1* could decrease ATP production. Moreover, reduction of DHTKD1 led to impaired mitochondrial biogenesis, as well as increased cellular ROS level. These findings demonstrate that DHTKD1 may participate in energy metabolic regulation, mitochondrial biogenesis and early apoptosis induction. The mechanism of DHTKD1 related to human metabolic dysfunction, neurological diseases, and even cancer are suggested.

2. Materials and methods

2.1. Cell culture

Hepatic carcinoma cell HepG2, glioma cell line U87MG and gastric carcinoma cell NCI-N87 were grown in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640-based complete medium respectively, supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% humidified CO_2 atmosphere.

2.2. Immunoblotting

Total cell lysates were prepared in lysis buffer. Proteins were separated by SDS–PAGE, transferred to nitrocellulose membranes and probed with anti-DHTKD1 (Abnova).

2.3. RNA isolation and quantitative real-time PCR

Total RNA was isolated with TRIzol reagent (Takara) according to the manufacturer's instructions, and then was reverse transcribed with AMV reverse transcriptase (Takara). Quantitative PCR was carried out with SYBR Green PCR kit (Takara) according to the manufacturer's instructions. Amplifications were performed in Mastercycler ep realplex (Eppendorf). Relative transcript quantities were calculated using the ΔΔCt method with *β-ACTIN* as the endogenous reference gene. Each sample was analyzed in triplicate. Primer sequences were listed in Table 1.

2.4. Gene silencing by siRNA transfection

siRNA oligonucleotides targeting human *DHTKD1* and control oligonucleotides were from RIBOBIO. Transfections were carried out using siRNA transfection reagent (Roche) according to the manufacturer's instructions. Cells were seeded in six-well plates and transfected after 24 h with 100 nM *DHTKD1* or negative control siRNA. After 48–72 h, cells were harvested for subsequent assays.

2.5. OGDHC assays

OGDHC preparation and activity analysis procedures has been described previously [13]. Transfected HepG2 cells were first resuspended with buffer 1, and then lysed with one third volumes of buffer 2 at least 20 min on ice before the assay. 3–5 μl cell lysates were added to the total 1 ml of the OGDHC assay medium. Reaction rates of OGDHC were determined by the accumulation of the reaction product NADH at 340 nm. The product accumulation curves were followed for 30–120 s. The values were normalized to total protein amount.

Table 1
Primers used in this work.

Primer	Sequence
SOD1	5′-GGTGGGCCAAAGGATGAAGAG-3′ 5′-CCACAAGCCAAACGACTTCC-3′
CAT	5′-TTGCCTATCCTGACACTCACCG-3′ 5′-TGGAGCACCACCCTGATTGT-3′
GPX1	5′-CAGTCGGTGTATGCCTTCTCG-3′ 5′-GAGGGACGCCACATTCTCG-3′
PGC1B	5′-TCAGACAGAACGCCAAGCATC-3′ 5′-CGCACTCCTCAATCTCACCAAA-3′
TFAM	5′-ATGGCGTTTCTCCGAAGCAT-3′ 5′-TCCGCCCTATAAGCATCTTGA-3′
NRF1	5′-CCCTGATGGCACTGTCTCACTT-3′ 5′-ATGCTTGCGTCGTCTGGATG-3′
ATP5G3	5′-CCAGAGTTGCATACAGACCAAT-3′ 5′-CCCATTAAATACCGTAGAGCCCT-3′
CYCS	5′-CTTTGGGCGGAAGACAGGTC-3′ 5′-TTATTGGCGGCTGTGTAAGAG-3′
COX5a	5′-ATCCAGTCAGTTCGCTGCTAT-3′ 5′-CCAGGCATCTATATCTGGCTTG-3′
COX6B1	5′-CTACAAGACCGCCCCTTTTGA-3′ 5′-GCAGAGGGACTGGTACACAC-3′
mt-COX1	5′-CCTACGCCAAAATCCATTTCA-3′ 5′-ATGTGGTGTATGCATCGGGG-3′
mt-CYTB	5′-GCCCACTAAGCCAATCACTTT-3′ 5′-CGGATGCTACTTGTCCAATGA-3′
H19	5′-GTACGAGTGTGCGTGAGTGTG-3′ 5′-ATGGAATGCTTGAAGGCTGC-3′

2.6. ATP quantitation

ATP contents were determined using the luciferin/luciferase-based ATP Bioluminescence Assay Kit (Beyotime) according to the manufacturer's instructions. Transfected cells were lysed in lysis buffer and 10 μl supernatant was added to 100 μl reaction buffer for the immediately detection. All samples were measured in triplicates and the values were normalized as nmol/mg protein.

2.7. ROS assays

ROS level was measured by a fluorescent method with flow cytometry. DCFH-DA (Beyotime) was used as indicator of cellular ROS. Transfected cells were incubated with DCFH-DA at 37 °C for 20 min. The intensity of fluorescence was analyzed to quantify the ROS level.

2.8. Real-time PCR of mtDNA

Mitochondrial DNA (mtDNA) copy number was quantified by qPCR from isolated total DNA using previously described method [14]. All samples were measured in triplicates and qPCR results obtained were confirmed by three independent experiments. We first normalized the amount of mtDNA to the amount of the nDNA and then calculated the percentage of mtDNA present in *DHTKD1*-siRNA-transfected cells as a ratio relative to mtDNA of ctrl-siRNA-transfected cells. The primers used were listed in Table 1.

2.9. MTT assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure cell proliferation. Logarithmically negative control and *DHTKD1*-siRNA-transfected

HepG2 cells were seeded at 2.0×10^3 cells/well in 96-well plates and incubated in a humidified atmosphere (37 °C and 5% CO_2). After culturing cells for an appropriate time, 10 μl of 5 mg/ml MTT (Sigma) was added into each well and continued to culture for 4 h. Then, the cell culture medium was replaced by 100 μl of dimethyl sulfoxide (Sigma). Thirty minutes after dimethyl sulfoxide addition, optical density was read at 490 nm wavelength and cell growth curves were determined according to the optical density values.

2.10. Cell apoptosis and cell cycle analysis

Cells transfected with siRNA were harvested and stained with Annexin V-FITC apoptosis detection kit (KeyGEN) and then analyzed by flow cytometry. In brief, transfected cells were collected, washed, and resuspended in 500 μl binding buffer and then incubated with 5 μl FITC-conjugated annexin-V and PI (propidium iodide) in the dark for 15 min at room temperature. For cell cycle analysis, harvested cell were stained with PI after fixed with ethanol. All samples were analyzed by flow cytometry, and data were processed with the FlowJo software.

2.11. Statistical analysis

All quantitative data in this study were presented as mean ± S.D. A two-tailed Student's *t*-test was used to analyze comparisons between two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. DHTKD1 is involved in mitochondrial functions

DHTKD1 was presumed to be similar to OGDH [7]. Previously, DHTKD1 was proved to localize to mitochondria [10], raising the possibility that DHTKD1 plays a role in mitochondria through the TCA cycle. Mitochondrial energy production critically depends on the proper functioning of multiple enzyme complexes, such as multienzyme OGDHC, which catalyzes a highly regulated step of the mitochondrial TCA cycle, being essential for the organism's development and survival [15]. To disrupt this balance by modulating the expression level of *DHTKD1* is expected to affect the mitochondrial function. In the present work, we sought experimental evidence to support this hypothesis. We silenced *DHTKD1* using siRNA oligomers [10] in hepatic carcinoma cell HepG2, glioma cell U87MG and gastric carcinoma cell NCI-N87. The result showed that *DHTKD1* was effectively silenced (Fig. 1A and Supplementary Fig. 1A). Along with *DHTKD1* suppression, initial OGDHC activity was reduced by 14% as compared to controls ($P = 0.0314$), whereas it was not affected by *DHTKD1* overexpression (Fig. 1B). The observed response to the *DHTKD1* inhibition, ATP level displayed a modest decrease as compared to the control cells ($P = 0.0318$) (Fig. 1C), which was associated with the repression of oxidative phosphorylation (OXPHOS) genes (ATP synthase, cytochrome C and cytochrome C oxidase) (Fig. 1D and Supplementary Fig. 1C). It is important to note here that up-regulation of DHTKD1 increased ATP level markedly (Fig. 1C), suggesting an

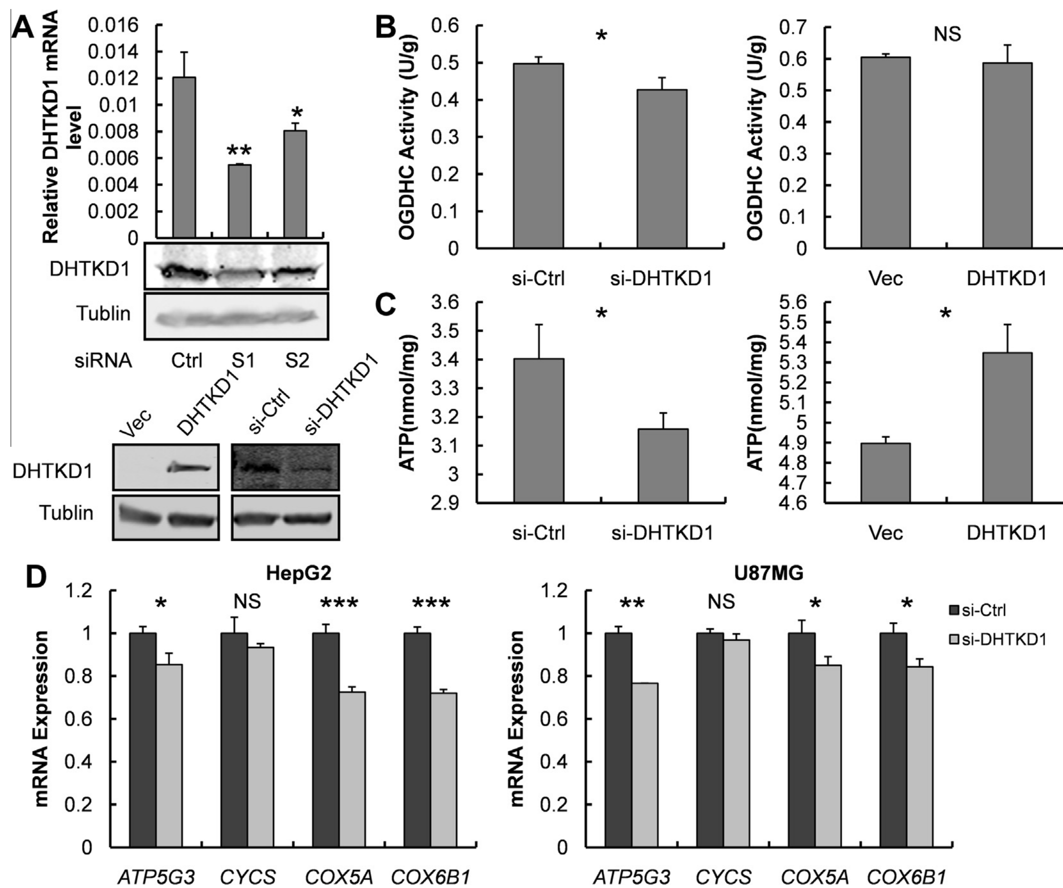

Fig. 1. DHTKD1 is involved in normal mitochondrial functions. (A) Knock-down and overexpression of *DHTKD1* in HepG2 cells via transfected with *DHTKD1* siRNA and *His-DHTKD1* vector, respectively. DHTKD1 protein was detected with His or DHTKD1 antibody. (B) OGDHC activity was measured in *DHTKD1* knock-down (left) and overexpressed (right) HepG2 cells. (C) ATP content was measured in the same cells as shown in (B). (D) OXPHOS genes expression was detected by RT-qPCR in HepG2 and U87MG cells. All quantitative data is presented in means ± S.D. of three independent experiments. NS, non-significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

important role of DHTKD1 in maintaining mitochondrial catalytic function and mitochondrial energy metabolism. Taken together, these results further reveal the fact that suppression of *DHTKD1* displays impairment of mitochondrial function.

3.2. DHTKD1 inhibition impairs mitochondrial biogenesis

Given the functional evidence for mitochondrial dysfunction, we first evaluated mitochondrial amount in cultured cells. We stained cells with mitochondrial specific probe, MitoTracker Red. We found that fluorescence produced by DHTKD1 siRNA-transfected cells was weaker than that of controls (Fig. 2A). Then, we further assessed mtDNA copy number via real-time qPCR described by Hayashi [14]. The amounts of *mt-COX1* (cytochrome C oxidase subunit 1) and *mt-CYTB* (cytochrome b) DNA relative to the levels of *H19*, a nuclear DNA (nDNA)-encoded gene, were markedly reduced in *DHTKD1* silenced cells compared to controls (Fig. 2B and Supplementary Fig. 1B). These results demonstrated DHTKD1 deficiency led to reduced mitochondrion. Furthermore, we detected the expression of several genes involved in mitochondrial biogenesis. Consistently, *PGC-1β*, *NRF1* and *TFAM* expression was lower in *DHTKD1* silenced cells (Fig. 2C), implying disabled mitochondrial biogenesis when DHTKD1 insufficient. These findings suggest that DHTKD1 controls mtDNA copy number through its effect on mitochondrial biogenesis.

3.3. DHTKD1 suppression causes increased ROS level

It is now well established that OGDHC is a source of ROS in mitochondria, in addition to that produced in the mitochondrial electron transport chain [16]. It was recently discovered that mitochondrial ROS directly stimulates pathological conditions as diverse as malignancies, autoimmune diseases, and neurodegenerative diseases which all share the common phenotype of increased mtROS production [17]. Studies of ROS in *DHTKD1*-insuffcent HepG2 showed increased resting ROS compared to controls (Fig. 3A), indicating reduced ability to handle ROS in *DHTKD1*-insuffcent cancer cells. Overexpression of *DHTKD1* also produced more ROS (Fig. 3A), implying that change of DHTKD1 dosage could alter cellular oxidative stress. Among the enzymes of TCA cycle, OGDHC is especially vulnerable to ROS [18], suggesting more mitochondrial ROS signals playing a role in OGDHC inactivity confirmed as above. Owing to the high reactivity and toxicity of ROS, mammalian cells have evolved a number of antioxidant enzyme systems to scavenge mtROS as soon as they are generated [17]. Decreased expression of ROS detoxifying enzymes including superoxide dismutase (SOD1), catalase (CAT) and glutathione peroxidase 1 (GPX1) was observed in *DHTKD1*-silenced cells (Fig. 3B), which aligned with increased ROS levels by FACS as mentioned in the previous section. Together, these data point to a scenario where the mitochondrial dysfunction induced by the partial silencing of *DHTKD1* caused the status of mitochondrial dysfunction with sustained ROS imbalance is sufficient to exacerbate mtDNA reduction and aberrant mitochondrial gene expression.

3.4. Silencing of DHTKD1 induces cell apoptosis

To investigate whether insufficient ATP and excessive ROS level would influence cell growth, we detected cell viability using MTT assay. The results showed that suppression of *DHTKD1* in HepG2

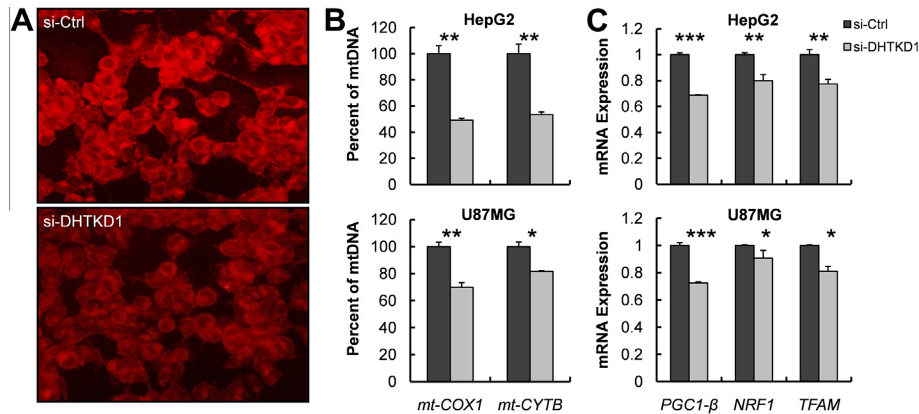

Fig. 2. Impaired mitochondrial biogenesis in *DHTKD1* silenced cells. (A) MitoTracker Red staining showed decreased mitochondrial mass in *DHTKD1* silenced HepG2. (B) Decreased mtDNA copy numbers in *DHTKD1* silenced HepG2 and U87MG cells. mtDNA (*COX1* and *CYTB*)/nDNA (*H19*) ratio was determined by real-time qPCR on DNA template isolated from the indicated cells. (C) Mitochondrial biogenesis associated genes were decreased in *DHTKD1* silenced cells. mRNA expression level of these genes were detected by RT-qPCR. All quantitative data is presented in means ± S.D. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

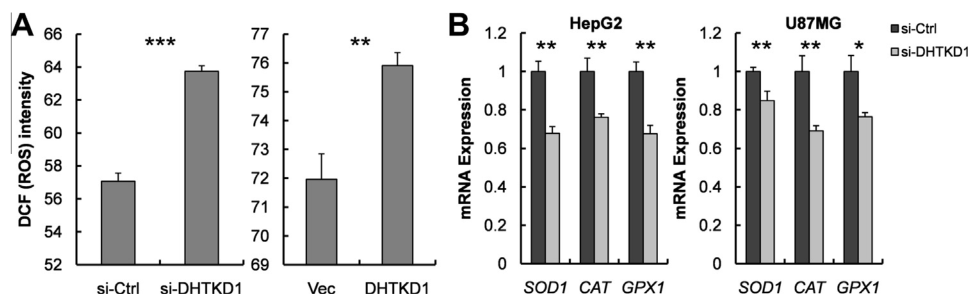

Fig. 3. Increased ROS level in *DHTKD1* deficient cells. (A) Knock-down (left) and overexpression (right) of *DHTKD1* increased cellular ROS level determined with flow cytometry. DCF was used as a fluorescent indicator of cellular ROS. (B) Decreased ROS defense genes expression in *DHTKD1* deficient cells determined through RT-qPCR. All quantitative data is presented in means ± S.D. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

significantly inhibited cell growth. *DHTKD1* silenced cells grew much more slowly than controls (Fig. 4A). To examine whether cells undergo apoptosis, *DHTKD1* silenced cells were stained with annexin V and propidium iodide (PI). As shown, lack of *DHTKD1* resulted in higher early apoptotic population compared to controls (17.44 ± 0.1861% and 18.61 ± 0.2768% vs. 10.53 ± 1.574%) (Fig. 4B and C). Next we examined the cell cycle distribution; we found that inhibition of *DHTKD1* would not impact cell cycles (Fig. 4D). Thus, these results indicate that energy shortage in *DHTKD1*-insufficient human cancer cells inhibits cell proliferation and induces early apoptosis, suggesting an essential role for DHTKD1 in normal cell growth and development.

4. Discussion

Mitochondrial dysfunction is a fundamental problem associated with many neurological diseases, such as Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis and many peripheral entrapment neuropathies [19]. Central nervous system functions greatly depend on the efficient mitochondrial function because of a high requirement for energy in brain tissue. mtDNA mutation, mitochondrial dynamic defect, ROS production and other environmental factors could change energy metabolism [20].

For a long time, we have always thought that the main function of mitochondria is to produce energy via OXPHOS [19]. Now, people are more inclined to believe that the dynamic network of mitochondria not only connects distal cellular compartments physically, but also makes great contribution to cellular life and death [21,22]. In addition to ATP generation, mitochondria also participate in various crucial pathways, such as calcium ions balance [23], iron–sulfur clusters synthesis [24] and apoptosis regulation [25]. To illuminate mechanisms of DHTKD1 in the impact of energy production in mitochondria, we suppressed *DHTKD1* by using specific oligomers against different *DHTKD1* mRNA regions. Response to the suppression, ATP level in *DHTKD1*-insuffiecient HepG2 cells was significantly less than that in controls. Corresponding to this change, decreased expression levels of OXPHOS genes have been found. To further understand the mechanism linking mitochondrial function to ATP generation, we measured the activity of OGDHC in the HepG2 cells. Consistent with the decreased ATP level, OGDHC, the rate-limiting multienzyme complex showed modest downward trends. As a multienzyme complex in TCA, the direct correlation of OGDHC activity and TCA dysfunction is demonstrated. However, it remains unknown whether this impairment of OGDHC activity is secondary to ATP failure or a contributor to the progression of ATP decrease. We demonstrate that decreased ATP production is presumed to be substantial evidence pointing to a critical role of DHTKD1 in mitochondrial functions.

Mitochondria use OXPHOS to produce ATP, as well as ROS, which can cause respiratory chain defect, DNA damage and cell cycle checkpoint activation [26]. In this study, we find that inhibition

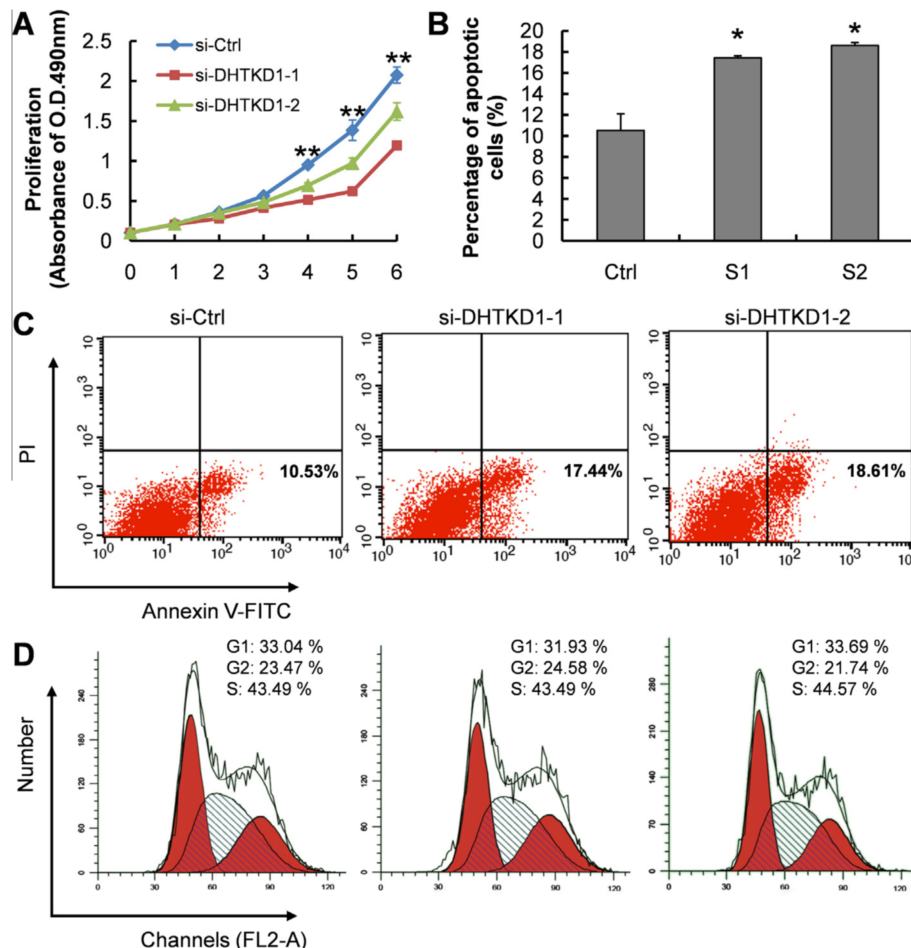

Fig. 4. *DHTKD1* deficiency leads to cell apoptosis. (A) Retarded cell proliferation in *DHTKD1* silenced cells. Proliferation curve of cells transfected with the indicated siRNAs based on MTT method. (B and C) Apoptosis assays of the indicated cells. siRNA transfected cells were stained with FITC-annexin V and PI for flow cytometric analysis. The four-quadrant diagrams (C) and quantitative data (B) of percentage of apoptotic cells were shown. (D) Cell cycle analysis of the indicated siRNAs transfected cells. All quantitative data is presented in means ± S.D. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

of *DHTKD1* was associated with increased ROS generation and marked depressed expression of a number of antioxidant genes, which would further exacerbate the ROS damage. The reductions in *PGC-1β* and its target genes *NRF1* and *TFAM*, which control many aspects of mitochondrial biogenesis, further confirm that increased ROS would substantially induce the mitochondrial defect and associate with neurodegenerative diseases.

Mitochondrial dysfunction results in a decline mitochondrial biogenesis which can cause mtDNA reduction and thus would drive further decline in mitochondrial function [20]. Besides oxidative damage, mtDNA depletion leading to mitochondrial dysfunction emerges as an important element of neurological disorders. In the present study, a significant different content of mtDNA in *DHTKD1*-insufficent HepG2 cells and controls was proved. We demonstrate that the decrease in mtDNA amount may be a result of the defect of mitochondrial biogenesis and function. Production of substantial amounts of ROS reduces mtDNA. In turn, mtDNA deficiency in *DHTKD1*-insufficent cells in our study may have contributed to this ROS accumulation.

Cellular abnormalities are associated with different types of mitochondrial dysfunctions. Without normal function maintenance of mitochondria, cell function, signaling pathways and overall cell activities were influenced, leading to cell apoptosis and other dynamic movements defect. There is much evidence indicating that increased oxidative stress and abnormal apoptosis are associated with the pathogenesis of several ageing-related neurodegenerative disorders [27]. Further work define the abnormal mitochondrial function involved in the silencing of *DHTKD1* cause retarded cell proliferation and increased trend forward cell apoptosis. Our study confirms the role of mitochondria in the pathogenesis of cell growth.

Given the central importance of DHTKD1in mitochondria, we reveal the fact that any genetic mutation in *DHTKD1* resulting in significantly decreased mitochondrial biogenesis or function could cause mitochondrial dysfunction and oxidative stress, involved in the onset and progression of neurological decline like CMT2Q. From this perspective, it is worth noting that there are many important roles of DHTKD1 in mitochondrial biogenesis and function. In summary, identification of the molecular mechanisms of this highly conserved gene underlying neurological diseases is of great importance in evaluating its role in mitochondrial function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.08.047.

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