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Short Communication

Mitochondrial damage and cholesterol storage in human hepatocellular carcinoma cells with silencing of *UBIAD1* gene expression

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ABSTRACT

Heterozygous mutations in the *UBIAD1* gene cause Schnyder corneal dystrophy characterized by abnormal cholesterol and phospholipid deposits in the cornea. Ubiad1 protein was recently identified as Golgi prenyltransferase responsible for biosynthesis of vitamin K2 and CoQ10, a key protein in the mitochondrial electron transport chain. Our study shows that silencing *UBIAD1* in cultured human hepatocellular carcinoma cells causes dramatic morphological changes and cholesterol storage in the mitochondria, emphasizing an important role of *UBIAD1* in mitochondrial function.

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Schnyder corneal dystrophy (SCD, MIM [121800]) is an autosomal dominant ophthalmologic disorder characterized by abnormal cholesterol and phospholipid deposits in the cornea that cause progressive opacification and a decrease of visual acuity, frequently requiring surgical transplantation [1]. The disease is linked to heterozygous mutations in the *UBIAD1* gene [2–4], which encodes a transmembrane type 2 protein containing a conserved prenyltransferase domain, similar to those in the *Escherichia coli* UbiA protein [4–6] and in mammalian mitochondrial prenyltransferase COQ2 [7]. The Ubiad1 protein was initially reported to be present in the mitochondria [4], but recent studies have demonstrated that the protein localizes to the Golgi membranes [5,8].

The biological role of Ubiad1 protein has yet to be fully elucidated. Cholesterol accumulation in SCD patients, which has been observed in corneal tissue [9] as well as in fibroblasts [10], suggests the participation of Ubiad1 in cellular cholesterol metabolism. This is indirectly supported by studies demonstrating that the protein plays a tumor suppressor role in cholesterol-dependent bladder and prostate cancers [6,11]. Multiple tumor cell lines with high levels of lipid accumulation also showed a loss of *UBIAD1* expression, while *UBIAD1* overexpression in these lines leads to a decrease in cholesterol, along with an abatement in tumor size and proliferation [6,12]. Furthermore, Ubiad1 protein was identified as an interacting partner of cholesterol-transporting protein, apolipoprotein E [6,13] as well as of HMGCR and SOAT1 enzymes catalyzing, respectively, cholesterol synthesis and storage [1].

Besides its potential role in the cholesterol metabolism Ubiad1 catalyzes the intracellular biosynthesis of vitamin K2 (menaquinone-4, MK-4), involved in the carboxylation of glutamate residues in proteins [14–16]. Most recently Ubiad1 was shown to be a vertebrate Golgi membrane prenyltransferase responsible for production of CoQ10, a key protein in the mitochondrial electron transport chain [8].

Here we studied if *UBIAD1* silencing alters cholesterol levels in cultured human hepatocellular carcinoma cells known to have high capacity for cholesterol synthesis. We demonstrate that *UBIAD1* silencing does not change cholesterol metabolism but causes dramatic morphological changes in the mitochondria, emphasizing an important role of *UBIAD1* in mitochondrial function.

1. Methods and results

1.1. Generation of HepG2 cell lines with the UBIAD1 knockdown

HepG2 cells grown to ~80% confluency in Eagle's Minimum Essential Medium (EMEM) with 20% Fetal Bovine Serum (FBS) were transfected with pRFP-C-RS vectors expressing 3 different 29-mer shRNA for

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human *UBIAD1* (OriGene, TF308518) or a control scrambled 29-mer shRNA of similar base composition (OriGene TF308518) using FastFect reagent (Feldan, 9K-010-0001). All vectors also contained the cDNA encoding Red Fluorescent Protein (RFP) as well as the puromycin resistance gene. After 72 h, 2 μg/mL of puromycin was added to the culture medium for selection of the transfected cells. After the cells demonstrated stable growth the puromycin concentration was reduced to 0.2 μg/mL. Cells expressing plasmids were further purified by cell-sorting. Cells were harvested by trypsinisation, resuspended in a 25 mM Hepes (pH 7.0), 1 mM EDTA, 150 mM NaCl, 1% decomplemented FBS and sorted using RFP-specific gate on FACS Aria™ II instrument. Non-transfected HepG2 cells were used as a negative control. RFP-positive cells were grown in EMEM with 20% FBS and 0.2 μg/mL puromycin. According to the results of western blotting performed on total cellular homogenates using rabbit polyclonal anti-human Ubiad1 antibodies (Abcam ab93413, dilution 1:200) the amount of Ubiad1 protein present in shRNA-expressing cell lines was reduced by ~80% as compared with non-transfected HepG2 cells or cells expressing scrambled shRNA (Supplementary Fig. 1).

1.2. Morphological changes in the cells with the UBIAD1 knockdown

The phenotypes of the wild type cells and those with silenced *UBIAD1* were examined by light and electron microscopy. Cultured cells were detached from the culture dishes with a rubber policeman, washed with Hanks' balanced salt solution, fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 48 h at 4 °C, post-fixed with potassium ferrocyanide-reduced osmium tetroxide, dehydrated in ethanol and embedded in Epon. Semi-thin (1 μm thick) sections were cut, mounted on slides, stained with toluidine blue, and studied with a Leica DMS light microscope, which revealed extensive vacuolization in the cells treated with shRNA expressing vector, but not in the scrambled RNA-treated cells, or untransfected HepG2 cells (Supplementary Fig. 2). For high-resolution analysis ultrathin (120 nm) sections were mounted on 200-mesh copper grids (Polysciences) and stained with uranyl acetate for 5 min, followed by lead citrate for 2 min. The grids were analyzed on a Tecnai FEI electron microscope. Examination of control non-transfected cells showed round or elongated nuclei, with one or two nucleoli. The double nuclear membrane and the sparse profiles of endoplasmic reticulum (ER) had a normal appearance. The elongated mitochondria had a typical double membrane arrangement with infolded inner membrane. The mitochondria were often seen associated with ER profiles (Fig. 1A). Similar morphology was observed in the scrambled RNA-transfected cells (Fig. 1B).

In contrast, examination of *UBIAD1* shRNA-transfected cell lines (Fig. 1C–E) showed round or indented nuclei with enlarged nucleoli. The nucleoli were made of large interlacing strands of granular material containing a less dense matrix. All three cell lines also exhibited a remarkable increase in the number of mitochondria, which presented with round profiles, swollen mitochondrial matrix and disorganized or reduced inner membranes (Fig. 1C–D insets).

1.3. Cholesterol storage and oxidative stress in UBIAD1-silent cells

Total cholesterol content in the transfected and non-transfected cell lines was measured by Amplex Red Assay (Life Technologies). Briefly, 1×10^6 cells in 3 ml of DMEM containing 20% FBS and 0.2 μg/ml of puromycin were seeded in 60 mm dishes. The following day the media was replaced with 2 ml of DMEM containing 10% CLPDS. After 16 h the cells were washed twice with PBS and harvested with 50% methanol in water. Cholesterol was extracted from cell pellet by Folch method. The organic phase was washed twice with a mixture of methanol/0.58% NaCl/$CHCl_3$ (45:47:3) and dried under the flow of nitrogen. Amplex red assay was performed according to the manufacturer's protocol by measuring the fluorescence of the sample using an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

We did not detect a significant difference between the control, scrambled RNA-transfected and shRNA-transfected cell lines in the content of total cholesterol or cholesterol esters (Supplementary Fig. 3). As this analysis could not rule out the possibility of changes in distribution of these lipids in the cell, we tested cells for the presence of cholesterol deposits by Filipin staining. Briefly, cells cultured on glass coverslips were stained or not in culture with mitochondrial or lysosomal markers, MitoTracker Green, MitoTracker Deep Red and LysoTracker Green (Invitrogen), rinsed with PBS, fixed with 4% PFA for 30 min at room temperature, washed again with PBS and treated for 10 min with glycine in PBS (1.5 mg/mL) to quench the excess of PFA. Cells were further stained in the dark for 2 h with PBS containing 10% FBS and 50 μg/mL of Filipin (Sigma F9765) and mounted on glass slides with Prolong-Gold AntiFade (Life Technologies). Slides were studied using an Ultraview spinning disc confocal unit mounted on a Leica DM6000B microscope equipped with a 63 × 1.4 NA objective and an Orca-R2 camera (Hamamatsu). Imaging was performed using the 405/488/561/640 dichroic in emission discrimination mode. Filipin fluorescence was detected using dual pass band 445(w60) 615(w70) emission filter following excitation with 405 nm laser. MitoTracker Green was detected using single pass band 527(w55) emission filter following excitation with a 488 nm laser. MitoTracker Deep Red was detected using 705(w90), 485 (w60) emission filter following excitation with a 640 nm laser. Images were captured within the linear range of the Hamamatsu Orca R2 camera (maximal pixel intensities were in the 2000s, while saturation is at 4095) and processed with Volocity and Photoshop software.

Our results (Fig. 2A and Supplementary Fig. 4A) demonstrate that cells transfected with *UBIAD1* shRNA constructs show increased Filipin staining as compared with non-transfected cells or those transfected with scrambled RNA construct. The staining shows punctate cytoplasmic pattern resembling that in cultured skin fibroblasts from a Niemann–Pick type C patient, however Filipin staining does not co-localize with LysoTracker Green-stained lysosomal/endosomal compartment (Supplementary Fig. 5) but shows partial co-localization with mitochondrial marker, MitoTracker Deep Red (Fig. 2A). MitoTracker staining also revealed signs of fragmented mitochondrial network and collapsed mitochondria in shRNA-transfected cells containing cholesterol deposits (Fig. 2A, Supplementary Fig. 4B). These signs not present in control or scrambled RNA-transfected cells were consistent with mitochondrial damage detected by EM (Fig. 1).

Finally to test whether mitochondrial damage observed in the *UBIAD*-silent HepG2 cells is associated with oxidative stress we tested the cells for the presence of reactive oxygen species (ROS) using CellROX Green Reagent (Life Technologies). The reagent was added to the culture medium in the final concentration of 5 μg/mL. After 30 min of incubation at 37 °C cells were fixed with 4% PFA, mounted on glass slides and analyzed by confocal microscopy as described above. Our data (Fig. 2B) showed that *UBIAD1* shRNA-transfected cell lines, but not control non-transfected HepG2 cells or those transfected with scrambled RNA, had intense CellROX Green staining suggesting increased levels of ROS and oxidative stress.

2. Discussion

This study presents evidence that the suppression of *UBIAD1* gene expression in cultured hepatocellular carcinoma cells causes damage to the mitochondrial network, which becomes fragmented and disorganized as revealed by histochemical analysis. At the EM level, cells show increased number of pleomorphic mitochondria in the cytoplasm, most of them being swollen or with reduced and disorganized cristae. Our data also demonstrate an increase in ROS generation in the cells transfected with *UBIAD1* shRNA constructs. All these events are consistent with ROS-mediated mitochondrial damage in the *UBIAD1*-silent cells.

The recent study [8] showed that silencing *UBIAD1* in cultured human primary endothelial cells resulted in eNOS dysfunction and ROS increase, suggesting that production of the Golgi CoQ10 pool by Ubiad1 is

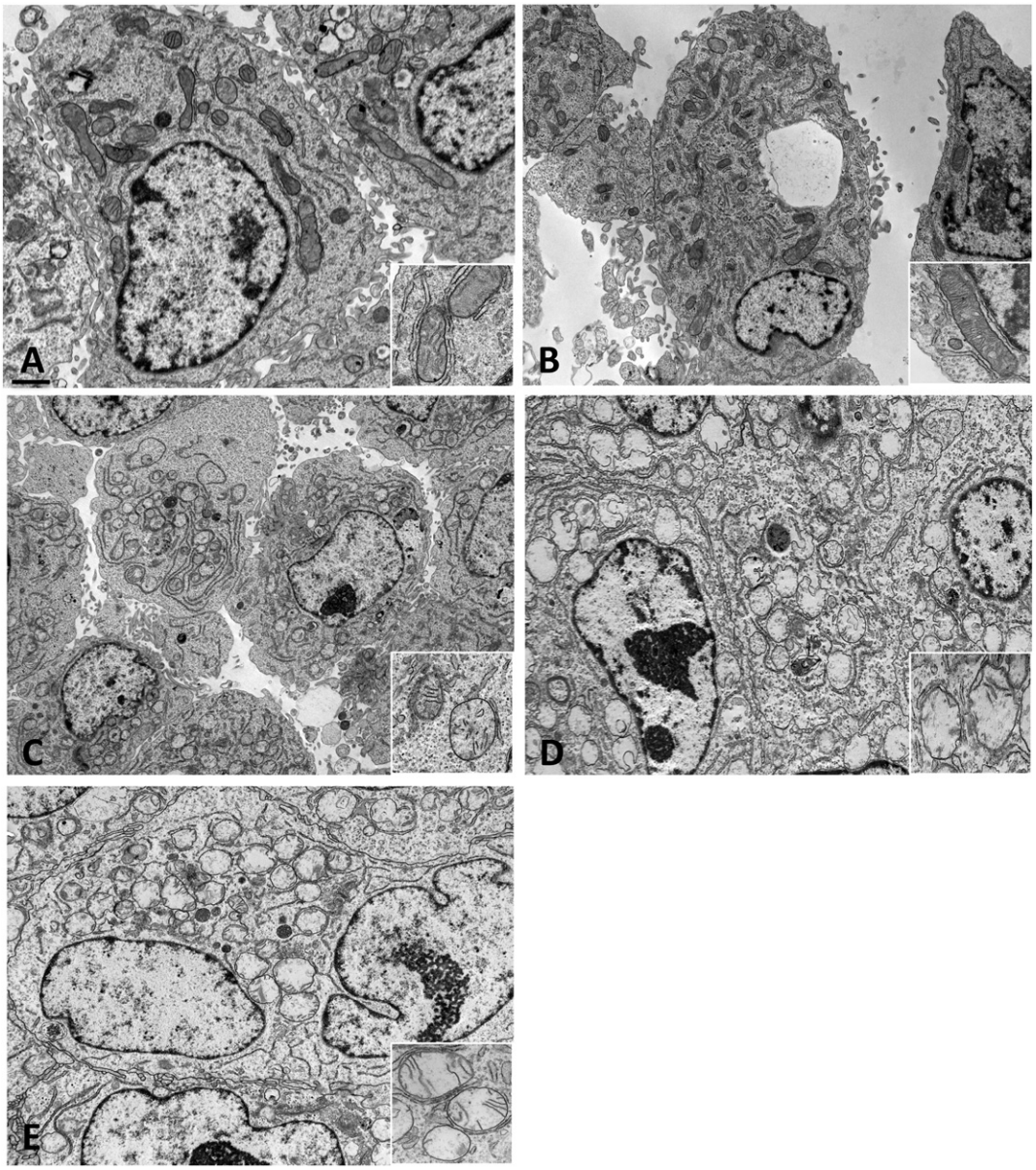

Fig. 1. Mitochondrial damage in *UBIAD1*-silent HepG2 cells. (A) Non-transfected control cells showing normal morphology; the mitochondria are relatively abundant and elongated (inset). (B) Control cells transfected with scrambled RNA construct show normal morphology similar to that of non-transfected control cells; the inset shows a mitochondrion at higher magnification. (C), (D) and (E) *UBIAD1* shRNA-transfected cell lines (sh65, sh66 and sh67) show an increase in number and a change in shape of the mitochondria, which have signs of swelling (inset). Bar is equal to 2 μm and applies to all figures. Panels show representative images of at least 30 studied for each cell line.

important for membrane redox signaling and protection of the cells from lipid peroxidation. At the same time, the authors did not detect mitochondrial abnormalities in *UBIAD1*-silent cells, which could be due to the use of a different cell type (hepatocellular carcinoma versus primary endothelial cells). Also, the morphological alterations in the mitochondria revealed by EM in the current work could avoid detection by confocal microscopy used in the work of Mugoni and coauthors [8]. Interestingly, mutations in the *Drosophila UBIAD1* ortholog, *Heix*, result in abnormal mitochondrial morphology and malfunction of the electron transport chain phenotypes, both rescued by the addition of menaquinone-4 [17].

In contrast with the previously proposed role of the Ubiad1 protein in cholesterol modulation, we did not detect increased levels of cholesterol or cholesterol esters in the *UBIAD1*-silent cells. We however, detected Filipin/MitoTracker-positive cytoplasmic punctate in *UBIAD1*-silent cells consistent with cholesterol storage in the mitochondria. Such unusual location for cholesterol deposits can be a result of mitochondrial damage causing accumulation of the cholesterol pool normally metabolized into pregnenolone in the inner mitochondrial membranes [18]. Further studies are required to understand if the cellular phenotype characterized by oxidative stress and mitochondrial damage observed in the *UBIAD1*-silent HepG2 cells is related to that in Schnyder corneal dystrophy, where crystalline cholesterol deposits are restricted to the cornea and observed in ~50% of patients [1], and lipid metabolism is not systemically affected [9,19]. It also remains to be clarified whether cellular phenotypes depend on the role of Ubiad1 in biosynthesis of CoQ10 or vitamin K or both. Regardless, our data implicate Ubiad1 protein as a cellular component crucial for mitochondrial function.

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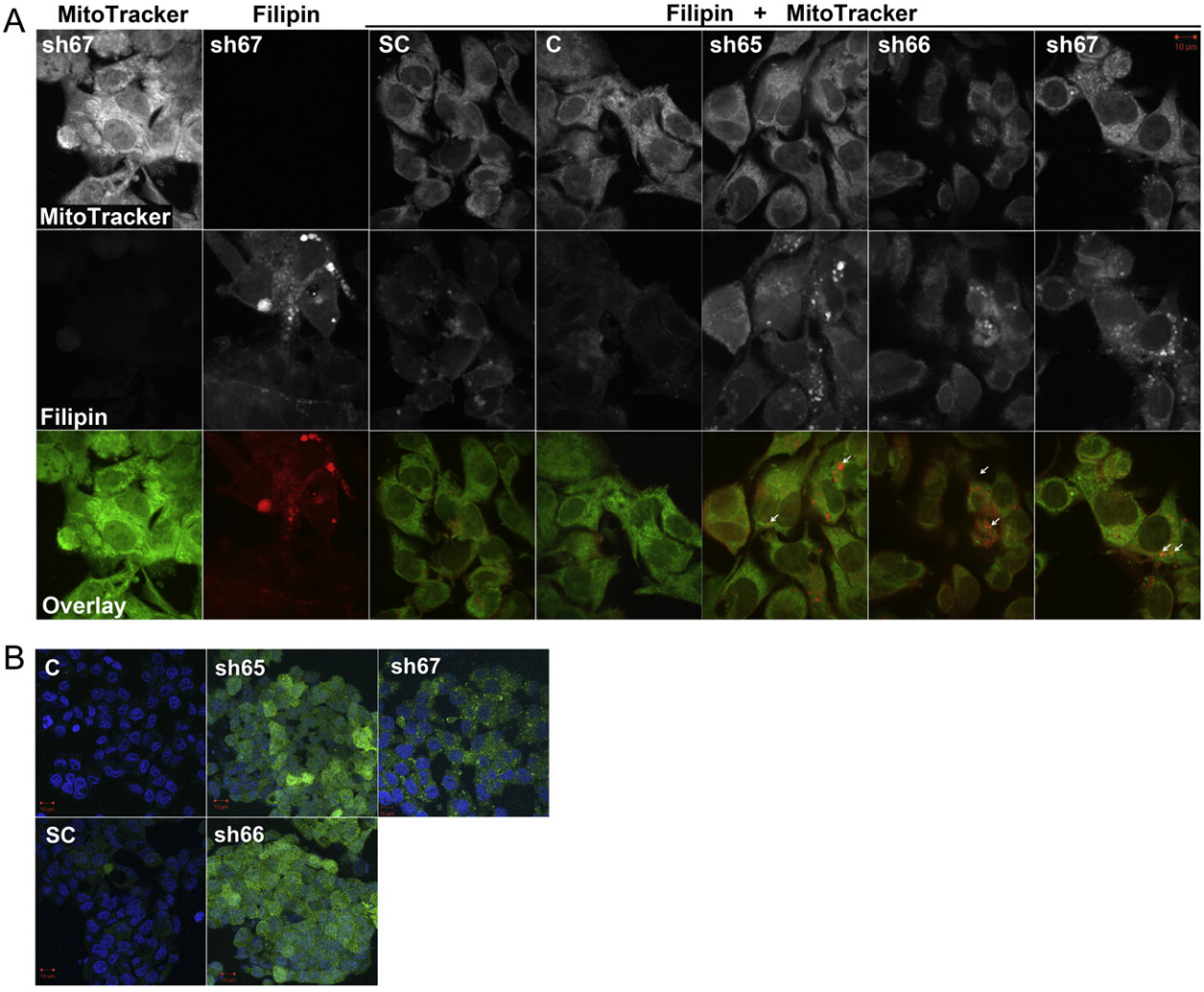

Fig. 2. Accumulation of cholesterol and oxidation stress in *UBIAD1*-silent HepG2 cells. Non-transfected HepG2 cells, cells transfected with scrambled RNA construct and *UBIAD1* shRNA-transfected cell lines were stained with cholesterol-specific stain, Filipin and/or a mitochondrial marker, MitoTracker Deep Red or with a ROS-specific CellROX Green Reagent and DAPI. (A) *UBIAD1* shRNA-transfected cell lines (sh65, sh66 and sh67) show increased cytoplasmic Filipin staining partially co-localized with MitoTracker Deep Red (arrowheads) as compared with control (*C*) or scrambled RNA-transfected cells (SC). (B) *UBIAD1* shRNA-transfected cell lines (sh65, sh66 and sh67), but not control non-transfected HepG2 cells (*C*) or those transfected with scrambled RNA (SC) show CellROX Green staining suggesting increased level of ROS and oxidative stress. Slides were studied on an Ultraview spinning disc confocal microscope (A) or Zeiss LSM510 confocal microscope (B). Panels show representative images of at least 10 studied for each cell line. Bar is equal to 10 μm and applies to all figures.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ymgmr.2014.09.001.

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