Drp1 phosphorylation by MAPK1 causes mitochondrial dysfunction in cell culture model of Huntington's disease

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Abstract

Mitochondrial dysfunction is a major cytopathology in Huntington's disease (HD), a fatal and inherited neurodegenerative disease. However, the molecular mechanisms by which the disease-causing gene, mutant Huntingtin (mtHtt), affects mitochondrial function remains elusive. This study aims to determine the role that Mitogen-activated protein kinase 1 (MAPK1) plays in the over-activation of Dynamin-related protein 1 (Drp1), the mitochondrial fission protein, which leads to mitochondrial dysfunction and neurodegeneration seen in HD. We show that MAPK1 binds to and phosphorylates Drp1 *in vitro*. Drp1 phosphorylation at serine 616 is increased in HD knock-in mouse derived striatal cells, which is abolished by treatment with U0126, a potent inhibitor of MEK1/2. A phosphorylation-deficient mutant of Drp1, Drp1S616A, corrects mitochondrial fragmentation associated with HD. Treatment with U0126 also reduces mitochondrial fragmentation, but has no additional effect in correcting aberrant mitochondrial morphology in cells expressing Drp1S616A. Finally, treatment with U0126 reduces mitochondrial depolarization and mitochondrial superoxide production in HD mutant striatal cells when compared to wildtype cells. This study suggests that in HD, MAPK1 activation leads to the aberrant mitochondrial fission and mitochondrial function by phosphorylating Drp1. Therefore, inhibition of Drp1-mediated excessive mitochondrial fission might be a strategy for development of therapy for treating HD.

Keywords

Mitochondrial fission; Dynamin-related protein 1; Mitogen-activated protein kinase 1; Huntington's disease; Phosphorylation

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1. Introduction

Mitochondria are vital for energy production, and neurons, that have high electrical activity and cannot use glycolysis as a source of ATP, are extremely dependent on mitochondria for the metabolic demand of regulating calcium, maintaining resting membrane potential, allowing for axonal and dendritic transport as well as the release and re-uptake of neurotransmitters [1,2]. Mitochondrial dysfunction is featured in many neurodegenerative diseases and has therefore become a main hypothesis to explain the cellular mechanism of these diseases [3,4].

Dynamin-related protein 1 (Drp1) is a cytosolic GTPase that regulates the mitochondrial fission, which is important for mitochondrial renewal, proliferation, and redistribution [5,6]. Upon activation Drp1 translocates to the mitochondria, oligomerizes, and binds to its molecular adaptors at the constriction sites, leading to division of the mitochondria [5,6]. Excessive mitochondrial fission causes mitochondrial fragmentation, which leads to permeabilization of the outer mitochondrial membrane, ATP depletion, increase of ROS, and release of apoptotic factors [5,7]. Drp1 can undergo post-translational modification, including phosphorylation, ubiquitination, and sumoylation that can modify its GTPase activity, cellular location, and oligomerization [8,9]. Phosphorylation of Drp1 by different serine/threonine kinases in normal physiology and pathophysiology has different outcomes on the activity of the protein. Phosphorylation of Drp1 at serine 616 promotes Drp1-mediated mitochondrial fission, whereas phosphorylation at serine 637 inhibits both Drp1 enzyme activity and translocation to mitochondria [8,10].

Mitogen-activated protein kinase 1 (MAPK1/ERK2) is a serine/ threonine kinase and is a component of the MAP kinase signal transduction pathway. This pathway when activated has many cellular effects including regulation of cell growth, survival, and differentiation. MAPK1 has been implicated in the induction of mitochondrial fragmentation that leads to apoptosis [11] and cancer [12,13]. In this study, we aim to determine the role of MAPK1 plays in regulation of Drp1 activation, especially in HD. We have found that MAPK1 bound to and phosphorylated Drp1 at serine 616 residue. Moreover, suppression of MAPK1 by an inhibitor U0126, inhibited mitochondrial fragmentation and mitochondrial dysfunction in HD cell culture model. These data provide a new line of evidence showing the mechanism by which Drp1 hyperactivation mediates mitochondrial dysfunction in HD.

2. Materials and methods

2.1. Antibodies

Antibodies for phospho-(Ser/Thr), phospho-Drp1S616 and phospho-Drp1S637 were purchased from Cell Signaling; Anti-DLP1 was from BD Transduction, anti-ERK1/2 (MAPK1) was from Proteintech. Antibodies for Tom20, actin and Myc were from Santa Cruz technology.

2.2. In vitro phosphorylation of Drp1 by MAPK1

Five hundred nanograms of recombind human Drp1 (GST-tagged protein, Abnova, Tainwan) was incubated with 250 ng of recombind human MARK1 (Prospec) in a 30 μl

reaction mixture (40 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 10 mM $MgCl_2$, and 100 μM ATP). After incubation at 30 °C for 30 min, the reaction was stopped by boiling in the sample-loading buffer for SDS-PAGE. The phosphorylation of Drp1 was detected by anti-serine/threonine phosphorylation antibody.

2.3. Immunoprecipitation

Soluble protein was incubated with the indicated antibody overnight at 4 °C and protein A/G beads for 1 h. Immunoprecipitates were washed three times with cell lysate buffer and analyzed by SDS-PAGE and immunoblotting with antibodies.

2.4. Mass spectrometry analysis

Recombinant human MAPK1 (500 ng, Prospec) and recombinant human Drp1 (1 μg, Abnova) were incubated in 30 μL of reaction mixture (40 mM Tris-HCl pH 7.5, 2 mM DTT, 10 mM $MgCl_2$, 100 μM ATP) at 30 °C for 30 min. Samples were then subjected to 10% SDS-PAGE followed by Coomassie blue staining. The bands of Drp1 were excised from the gel, washed/destained in 50% ethanol with 5% acetic acid, dehydrated in acetonitrile, reduced with DTT, alkylated with iodoacetamide, and digested with either trypsin or chymotrypsin. After digestion, the peptides were extracted from the polyacrylamide using 50% acetonitrile with 5% formic acid, and analyzed by capillary column liquid chromatography-tandem mass spectrometry (LC-MS).

2.5. HD cell culture

HdhQ7 and Q111 mouse striatal cells were derived from a knock-in transgenic mouse model with either 7-polyglutamine repeats (Q7, wild-type) or 111-polyglutamine repeats (Q111, HD) in the *Huntingtin* gene. These cells were maintained in HyClone™ Dulbecco's modified Eagle's medium (DMEM, GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (FBS, Corning), 1% penicillin/streptomycin, and 0.4 mg/mL G418. Cultures were maintained at 33 °C in a humidified atmosphere containing 5% CO_2. The cells were maintained below 12 passages.

2.6. Preparation of total cell lysates

HD mouse striatal cells were washed with 1X PBS and then incubated in total lysis buffer (10 mM HEPES-NaOH pH 7.8, containing 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, protease inhibitor, and phosphatase inhibitor) for 20 min at 4 °C. Cells were scraped and then homogenized 5X using a 25-gauge syringe. Samples were centrifuged at $12,000 \times g$ for 20 min at 4 °C. Supernatants were saved as total lysates (stored at −20 °C).

2.7. Western blot analysis

Protein concentrations were determined by Bradford assay. Thirty micrograms of proteins was resuspended in Laemmli buffer, loaded on SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed with the indicated antibody, followed by visualization by ECL.

2.8. Immunocytochemistry

Cells cultured on coverslips were washed with cold PBS, fixed in 4% formaldehyde and permeabilized with 0.1% Triton X-100. After incubation with 2% normal goat serum (to block non-specific staining), fixed cells were incubated overnight at 4 °C with antibodies against Tom20 (1:500, Santa Cruz Biotechnology, USA). Cells were washed with PBS and incubated for 60 min with FITC-labeled goat anti-rabbit antibody and rhodamine-labeled goat anti-mouse antibody (1:500, Invitrogen, USA), followed by incubation with Hoechst dye (1:10000, Invitrogen, USA) for 10 min. Coverslips were mounted and slides were imaged by confocal microscopy (Olympus, Fluoview FV100). To determine mitochondrial superoxide production in cultures, cells were incubated with 5 μM MitoSOX™ red mitochondrial superoxide indicator (Invitrogen) for 10 min at 37 °C. To measure the membrane potential of mitochondria in cultures, cells were incubated with 0.25 μM Tetramethylrhodamine (TMRM) (Invitrogen) for 20 min at 37 °C. The staining was imaged by microscope, and quantification was carried out using NIH Image J software.

2.9. Statistical analysis

Results are expressed as mean ± SEM. Unpaired Student's *t*-test was used for differences between two groups to assess significance. One-way ANOVA with *post-hoc* Holm-Sidak test was used for comparison of multiple groups. Statistical significance was considered achieved when the value of p was <0.05.

3. Results

3.1. MAPK1 binds to and phosphorylates Drp1 in vitro

In order to determine whether or not Drp1 is a substrate for MAPK1 phosphorylation, recombinant human Drp1 (GST-Drp1) was subjected to an *in vitro* phosphorylation assay with recombinant human MAPK1 in the presence of ATP. Immunoprecipitation analysis showed Drp1 interacting with MAPK1 (Fig. 1A). Moreover, we found that Drp1 was phosphorylated in the presence of MAPK1 when antibodies recognize phosphor-serine/threonine were used (Fig. 1B). Next, mass spectrometry analysis revealed two conserved sites, Ser637 and Ser616, as a target of MAPK1-dependent phosphorylation (data not shown), the sites which have been found to be phosphorylated by multiple kinases [14–17].

3.2. Drp1 Ser616 phosphorylation by MAPK1 is increased in HdhQ111 striatal cells

Hdh mouse striatal cells were derived from a knock-in transgenic mouse model with either 7-polyglutamine repeats (Q7, wild-type) or 111-polyglutamine repeats (Q111, HD) in the mouse *Huntingtin* gene [18], and is considered as a genetically accurate cell culture model to study HD. In order to determine the phosphorylation of Drp1 at specific serine sites, total lysates were harvested from HdhQ7 and HdhQ111 cells, and western blot analysis was performed with anti-p-Drp1 S616, anti-p-Drp1 S637, or anti-Drp1 antibodies. We found that Drp1 phosphorylation at Ser616 was increased in mutant HdhQ111 striatal cells compared to that in wild-type HdhQ7 striatal cells (Fig. 2A and B), whereas no change in Ser637 phosphorylation between mutant HdhQ111 striatal cells and wild-type HdhQ7 striatal cells was observed (Fig. 2A).

U0126 is a highly selective and potent MEK1 and MEK2 inhibitor [19]. To determine if MAPK1 has a role in the phosphorylation of Drp1 in HD, HdhQ7 and HdhQ111 striatal cells were treated with DMSO (control) or 10 μM U0126 for 4 h. We found that treatment with U0126 abolished enhanced Drp1 S616 phosphorylation in HdhQ111 striatal cells (Fig. 2). However, MAPK1 inactivation had no effects on Ser637 phosphorylation of Drp1 in either mutant HdhQ111 striatal cells or wildtype HdhQ7 cells (Fig. 2A). These results suggest that MAPK1 mediates Drp1 phosphorylation at Ser616 residue under HD-related cell culture conditions.

3.3. MAPK1 inactivation decreases mitochondrial fragmentation in HdhQ111 striatal cells

To better understand the functional significance of Drp1 phosphorylation by MAPK1, mitochondrial morphology in culture was determined by immunostaining with anti-Tom20 antibody (Tom20, an outer membrane protein of mitochondria). Consistent with our previous studies [20], HdhQ111 mutant striatal cells exhibited excessive mitochondrial fragmentation featured with dot- or spheres-like Tom20-positive staining. Treatment with U0126 improved mitochondrial network; we observed elongated mitochondria with interconnected mitochondrial tubular network (Fig. 3A). This result suggests that MAPK1 plays a role in regulation of mitochondrial morphology in this cell culture model of HD.

To determine if phosphorylation of Drp1 at Ser616 by MAPK1 mediates mitochondrial fragmentation, we transfected Myc-tagged Drp1 S616A mutant [16], in which serine was replaced by alanine, in HdhQ111 striatal cells and determined mitochondrial morphology in the presence of U0126. Imaging analysis found that expression of Myc-Drp1 Ser616A reduced the extent of mitochondrial fragmentation in HdhQ111 striatal cells. Treatment with U0126 under the same transfected conditions had no additional benefit in reducing mitochondrial fragmentation (Fig. 3B). These findings suggest that MAPK1, at least in part, mediates Drp1-phosphorylation-induced mitochondrial fragmentation in HD mutant cells.

MAPK1 inactivation restores the mitochondrial membrane potential, and decreases the mitochondrial superoxide production in HdhQ111 striatal cells.

Mitochondrial fragmentation leads to the permeabilization of the mitochondrial outer membrane, decreasing the mitochondrial membrane potential, and causing the release of Reactive Oxygen Species (ROS) [16,21]. Next, we determined the effect of inactivation of MAPK1 on mitochondrial function in HdhQ111 striatal cells. Imaging analysis showed mutant HdhQ111 striatal cells with a decreased mitochondrial membrane potential and an increase in mitochondrial superoxide production, when compared to those in wild-type HdhQ7 striatal cells (Fig. 4). In contrast, the addition of U0126 restored the mitochondrial membrane potential and suppressed superoxide production in mutant HdhQ111 striatal cells (Fig. 4). Therefore, suppression of MAPK1 activation by U0126 reduced mitochondrial functional abnormalities in HD mutant cells.

4. Discussion

In this study, we show that MAPK1 binds to and phosphorylates Drp1 in HD cell culture. Our experiments have also demonstrated an increased phosphorylation of Drp1 at Ser616

that is responsible for the increased fission of mitochondria seen in HD. Using U0126, a potent inhibitor of the MAP kinase signaling pathway, we show this phosphorylation, at Ser616, being abolished, as well as the recovery of more elongated mitochondria. With the inhibitor U0126, we also see mitochondrial function return to a normal physiological state with the increase in mitochondrial membrane potential and the decrease in mitochondrial ROS production. Moreover, the phosphorylation-deficient mutant Drp1S616A shows less fragmented mitochondria in the mutant cells, confirming this serine residue as the site of activation. After testing together the phosphorylation-deficient mutant of Drp1 and the MEK1/2 inhibitor, we confirm that Drp1 fission and MAPK1 phosphorylation act through the same pathway leading to mitochondrial fragmentation and dysfunction seen in HD.

Huntington's disease is due to a genetic mutation in the gene *huntingtin* that causes a toxic gain-of-function [22] on the ubiquitously expressed protein that it transcribes. The fact that this ubiquitously expressed protein, when mutated, only manifests into a neurological disease can be explained by the mitochondrial fragmentation that is observed with HD, as well as other neurological diseases such as Parkinson's disease and Alzheimer's disease [3]. This selective mitochondrial damage in neurons is due to the unique dependence of neurons on mitochondria, compared to most other cell types in the body. Mitochondria are considered the powerhouse of the cell and neurons are highly electrically active cells that cannot use glycolysis as an alternative source of ATP [1,2]. There have been several proposed theories, with evidence to back up each, of how mutant huntingtin protein causes mitochondrial dysfunction, but one in particular has been well documented to cause mitochondrial dysfunction, and not just with neurological diseases. Changes in mitochondrial morphology have been associated with ATP production [23], ROS generation [21], movement [24], and apoptosis [25]. Mitochondrial morphology is maintained through a balance of fusion and fission events that are regulated by GTPases to maintain shape, number, and functionality. Optic atrophy 1 (Opa1) on the inner mitochondrial membrane and mitofusions 1 and 2 (Mfn1 and Mfn2) on the outer mitochondrial membrane regulate mitochondrial fusion; whereas, dynamin-related protein 1 (Drp1) in the cytosol regulates mitochondrial fission through its molecular adaptors, namely Fis1, Mff, and MiD49/51, on the outer mitochondrial membrane [1,2]. It is possible that mtHtt protein either directly or indirectly interacts with these GTPases to disrupt the balance of fusion and fission events that normally occur with mitochondria.

Drp1, the cytosolic GTPase that regulates mitochondrial fission events, can undergo post-translational modifications, such as phosphorylation, that can alter the activity and location of the protein [6,8]. Ser637 of Drp1 is phosphorylated by protein kinase A (PKA), which decreases its GTPase activity, protecting mitochondria [14,15]. Thr595 of Drp1 is phosphorylated by a mutated LRKK2 kinase in Parkinson's disease, leading to increased GTPase activity, and excessive mitochondrial fission [26]. Cyclin-dependent kinase 1 (Cdk1) phosphorylates Drp1 at Ser616 during mitosis, where it is necessary to proliferate mitochondria for division and allow for the proper distribution of mitochondria between the two daughter cells [17]. Protein kinase C δ (PKCδ) phosphorylates Ser616 of Drp1 during oxidative stress, leading to mitochondrial fragmentation and dysfunction [16]. MAPK1 phosphorylates Drp1 at Ser616 leading to tumor growth and cancer [11,13]. In all cases of phosphorylation of Drp1 at Ser616 by a kinase, enhanced Ser616 phosphorylation has

correlated with increased mitochondrial fission. Excessive mitochondrial fission thus causes mitochondrial fragmentation, which leads to permeabilization of the outer mitochondrial membrane, ATP depletion, increase of ROS, and release of apoptotic factors.

Besides Drp1's phosphorylation, formation of Drp1 tetramer at constriction sites on the outer mitochondrial membrane has been shown to be required for mitochondrial fission, by accelerating the GTP hydrolysis necessary to sever the mitochondrial membrane [2]. We investigated the oligomerization of Drp1 in HD mouse striatal cells and observed the increased formation of Drp1 tetramer in mutant HdhQ111 cells compared to wild-type HdhQ7 cells. However, we also noted no change in the formation of the Drp1 tetramer in HdhQ111 cells when inhibiting the MAP kinase signaling pathway with U0126 (data not shown).

Activation of MAPK1 is involved in diverse cellular effects. Inhibition of MAPK1 is not a feasible option for treatment of HD. Therefore, it is important to look upstream of MAPK1 phosphorylation of Drp1 and link MAPK1 to mtHtt. The activation of MAPK1 by mtHtt can be either direct or indirect. It has been shown that mtHtt directly binds to Drp1 increasing its GTPase activity [27,28]. This interaction between mtHtt and Drp1 can make the fission protein more susceptible to phosphorylation by MAPK1. As discussed earlier, mutant huntingtin *N*-terminal fragments can enter the nucleus and alter gene transcription [29]. This altered gene transcription can lead to over-activation of the MAP kinase signaling pathway.

Acknowledgments

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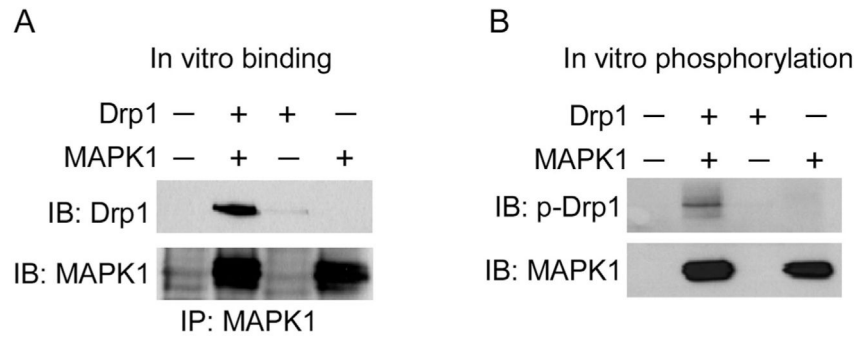
Fig. 1. MARK1 binds to and phosphorylates Drp1 in vitro
GST-Drp1 and MAPK1 recombinant proteins were incubated as described in Method. (A)
Immunoprecipitation (IP) with anti-MAPK1 antibodies followed by western blot analysis
with the indicated antibodies was performed. (B) Immunoblot for anti-serine/threonine
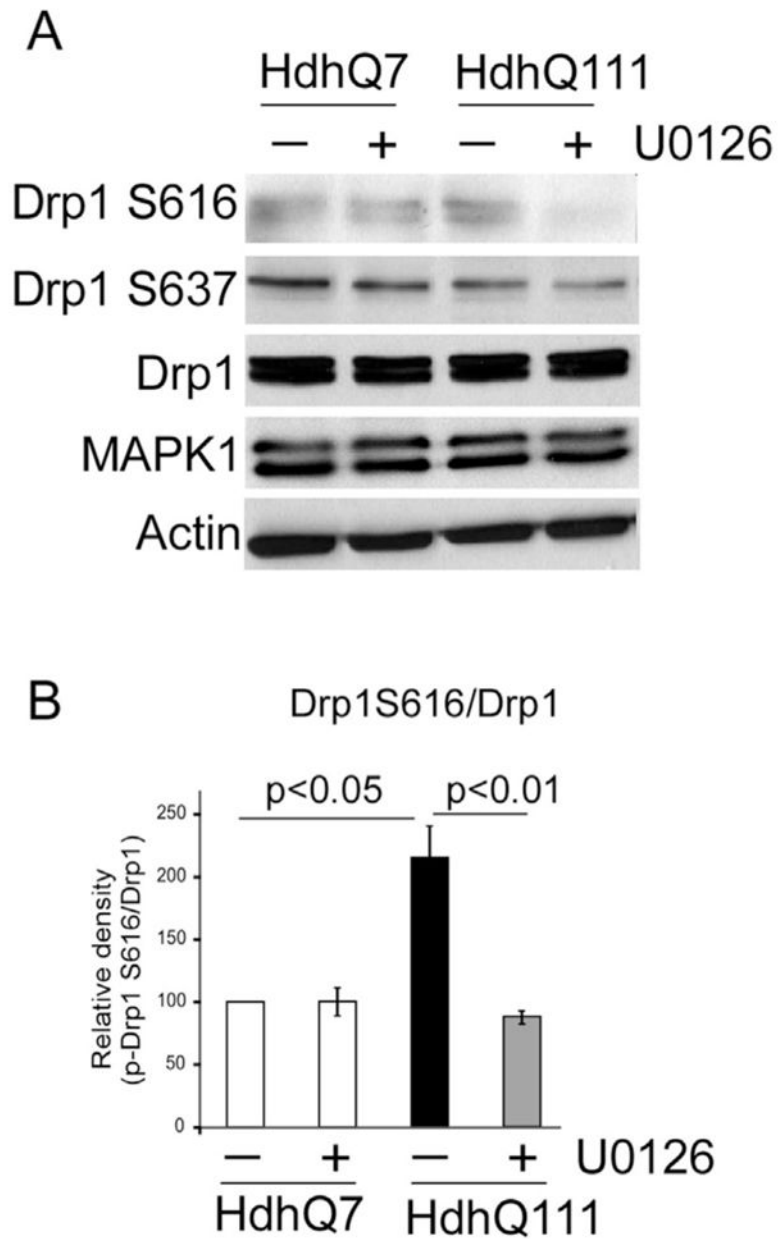antibodies shows Drp1 being phosphorylated by MAPK1.

A

HdhQ7   HdhQ111

−   +   −   +   U0126

Drp1 S616

Drp1 S637

Drp1

MAPK1

Actin

B   Drp1S616/Drp1

p<0.05   p<0.01

−   +   −   +   U0126

HdhQ7   HdhQ111

Fig. 2. Inhibition of MAPK1 by U0126 suppresses Drp1 phosphorylation at serine 616 in HD mutant cells

(A) HdhQ7 and HdhQ111 cells were treated with U0126 (10 μM) for 4 h. Total cell lysates were harvested and western blot analysis with the indicated antibodies was performed. Actin was used as a loading control. (B) Histogram shows the relative density of Drp1 S616 versus Drp1 total level. Data represents mean ± SEM of three independent experiments.
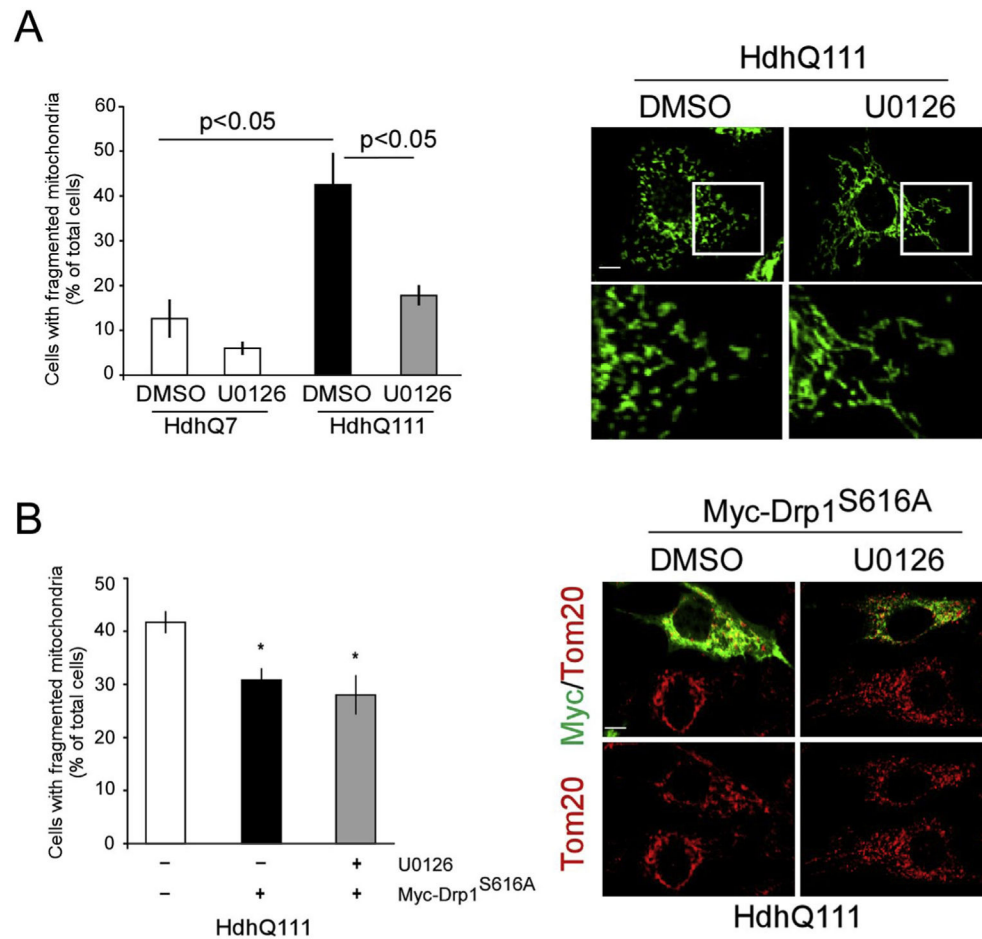
Fig. 3. U0126 treatment and Drp1 S616A mutant both decrease mitochondrial fragmentation in HdhQ111 cells

(A) Striatal cells were treated with 10 μM U0126 for 16 h and then stained for the mitochondrial protein, Tom20. (B) HdhQ111 striatal cells were transfected with the phosphorylation deficient mutant Myc-Drp1S616A, followed by treatment with 10 μM U0126 for 16 h. The cells were then stained with both anti-Tom20 and anti-Myc antibodies. Histogram: The percentage of cells with fragmented mitochondria versus total number of cells was calculated. Data represents mean ± SEM of three independent experiments.
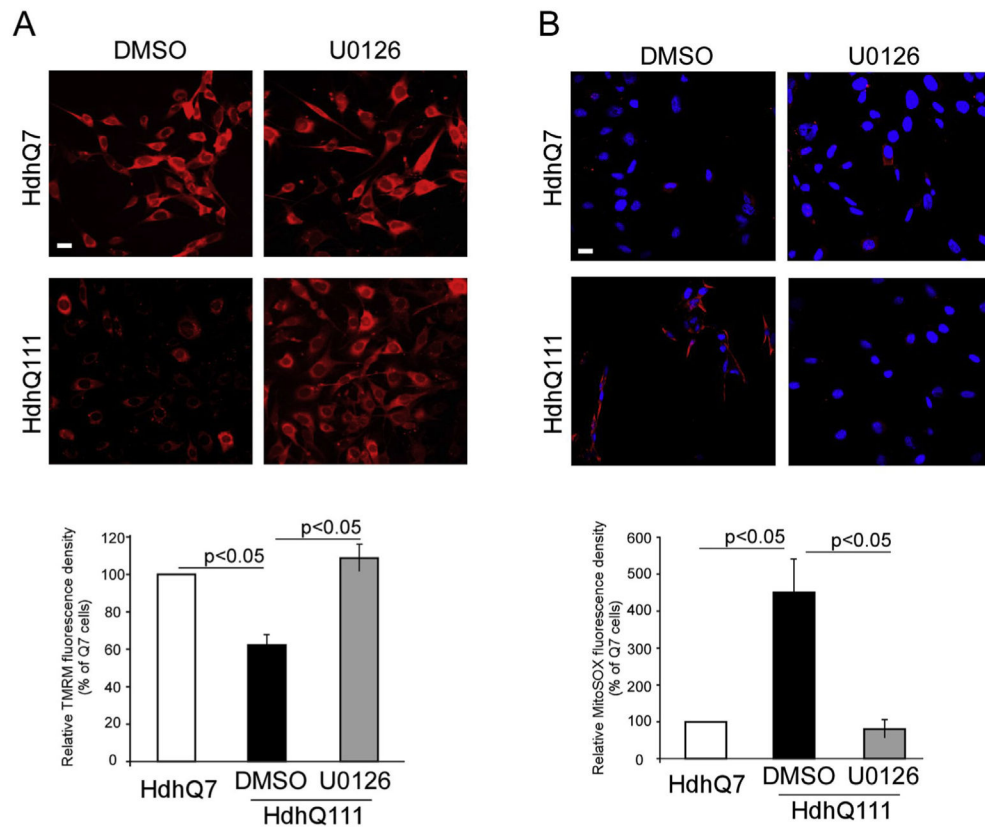
Fig. 4. U0126 treatment restores mitochondrial membrane potential and decreases mitochondrial ROS production in HdhQ111 cells

(A) Striatal cells were treated with 10 μM U0126 for 16 h and then stained with TMRM to determine mitochondrial membrane potential. The histogram shows the quantitation of fluorescence density of TMRM in cells. (B) Striatal cells were treated with 10 μM U0126 for 16 h and then stained with MitoSox to determine mitochondrial ROS production. The histogram shows the quantitation of fluorescence density of mitoSOX in cells. Data represents mean ± SEM of three independent experiments.