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SET overexpression in HEK293 cells regulates mitochondrial uncoupling proteins levels within a mitochondrial fission/reduced autophagic flux scenario

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ABSTRACT

We hypothesized that SET, a protein accumulated in some cancer types and Alzheimer disease, is involved in cell death through mitochondrial mechanisms. We addressed the mRNA and protein levels of the mitochondrial uncoupling proteins UCP1, UCP2 and UCP3 (S and L isoforms) by quantitative real-time PCR and immunofluorescence as well as other mitochondrial involvements, in HEK293 cells overexpressing the SET protein (HEK293/SET), either in the presence or absence of oxidative stress induced by the pro-oxidant *t*-butyl hydroperoxide (*t*-BHP). SET overexpression in HEK293 cells decreased UCP1 and increased UCP2 and UCP3 (S/L) mRNA and protein levels, whilst also preventing lipid peroxidation and decreasing the content of cellular ATP. SET overexpression also (*i*) decreased the area of mitochondria and increased the number of organelles and lysosomes, (*ii*) increased mitochondrial fission, as demonstrated by increased *FIS1* mRNA and FIS-1 protein levels, an apparent accumulation of DRP-1 protein, and an increase in the VDAC protein level, and (*iii*) reduced autophagic flux, as demonstrated by a decrease in LC3B lipidation (LC3B-II) in the presence of chloroquine. Therefore, SET overexpression in HEK293 cells promotes mitochondrial fission and reduces autophagic flux in apparent association with up-regulation of UCP2 and UCP3; this implies a potential involvement in cellular processes that are deregulated such as in Alzheimer's disease and cancer.

1. Introduction

Mitochondria perform oxidative phosphorylation, which involves the oxidation of adenine nucleotides through the respiratory chain in the inner mitochondrial membrane, allowing the generation of an electrochemical gradient whose dissipation is coupled to ATP synthesis; alterations in oxidative phosphorylation lead to adjustments that are necessary to maintain cellular metabolic homeostasis [1]. Reactive oxygen species (ROS) are generated concomitantly with oxidative phosphorylation. In normal cells, low ROS levels are necessary for signal transduction pathways. Meanwhile, cancer cells exhibit accelerated metabolism to maintain their increased proliferation rate, and then generate high ROS levels [2]. ROS are, in turn, potent mutagens that increase the genomic instability contributing to cancer initiation and progression [3], and are potentially associated with the pathogenesis of several diseases such as Alzheimer's and Parkinson's disease [4].

Mitochondrial uncoupling is a process which impairs dissipation of the electrochemical gradient from ATP synthesis; in this regard, mild mitochondrial uncoupling is believed to reduce mitochondrial ROS generation [5]. Uncoupling proteins (UCPs) regulate mitochondrial uncoupling; changes in their levels have been observed in many diseases [6–8]. UCPs are inner mitochondrial membrane proteins, the function of which includes the reentry of protons into the mitochondrial matrix, dissipating the electrochemical gradient and therefore, the mitochondrial membrane potential [9]. UCP1 has been found in brown adipose tissue where it dissipates the redox energy, providing heat [10]. UCP2 is

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ubiquitously expressed, and UCP3 is expressed in brown adipose tissue, skeletal muscle and heart [5,11].

It has been proposed that UCP2 and UCP3 are acutely activated by reactive oxygen species (ROS) and regulated by glutathionylation; their actions reduce the generation of ROS by dissipating the mitochondrial membrane potential [5]. UCP2 has been found to be overexpressed in many cancer types [12] and associated with decreased cell death in response to treatments with UV radiation and chemotherapy [8,12]. UCP3 is expressed in cancer cells and up-regulated by insulin-like growth factor 1 [13]; a role for UCP3 in lipid metabolism has also been proposed [14].

The SET protein is accumulated in Alzheimer's disease [15] and different types of cancer [16,17], such as head and neck squamous cell carcinoma (HNSCC), in which it contributes to cellular survival and response to oxidative stress, up-regulating the cell antioxidant defense genes [18]. SET is an inhibitor of protein phosphatase 2A (PP2A) [19], which has been associated with multiple cellular processes and the regulation of several key proteins involved in cellular proliferation and metabolism, such as myc, Akt and p53 [20–23].

In cancer cells exposed to oxidative stress conditions, autophagy represents a mechanism of stress tolerance [24]. When autophagy is implicated in mitochondrial degradation, it corresponds to mitophaghy, which in turn involves mitochondrial dynamic processes such as fission and fusion [25]. Considering that (*i*) mitochondrial metabolism generates ROS and metabolism is accelerated in cancer cells, (*ii*) SET contributes to cell survival and response to oxidative stress, and (*iii*) SET has been found to be accumulated in HNSCC, we hypothesized that SET is involved in cell death through mitochondrial mechanisms. In this context, we addressed the mRNA and protein levels of the mitochondrial uncoupling proteins UCP1, UCP2 and UCP3, as well as other mitochondrial involvements, in HEK293 cells overexpressing the SET protein, either in the presence or absence of oxidative stress.

2. Materials and methods

2.1. Cell culture and treatments

The HEK293 (human embryonic kidney; ATCC) cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma–Aldrich) supplemented with 10% fetal bovine serum (Life Technologies). The cells were exposed to 50 μM *tert*-butyl hydroperoxide solution (*t*-BHP), 20 μM chloroquine (CQ) or 10 μM valinomycin (Val) (Sigma–Aldrich).

2.2. Plasmids and reagents

The DNA constructs for full length SET were used for transfection into the HEK293 cell line, as previously described [26].

2.3. RNA extraction and cDNA

RNA was isolated from cultured cells with Trizol reagent (Invitrogen) according to the manufacturer's protocol. RQ1 RNase-Free DNase (Promega) was utilized to treat and precipitate the RNA. cDNA synthesis was performed using the GoScript™ Reverse Transcription System (Promega).

2.4. Protein extraction

Protein subcellular fractionation was achieved by differential extraction using the ProteoExtract Subcellular Proteome Extraction kit (Calbiochem, EDM Bioscience). Total proteins were extracted using CelLytic MT (Sigma–Aldrich). Quantification of proteins was performed with the Bradford protein assay (BioRad, #500-0006).

2.5. Immunofluorescence assay

Cells were placed on glass coverslips, fixed with absolute methanol and blocked with 0.5% Triton X-100 in phosphate buffered saline and bovine serum albumin. UCP1 (sc-6528), UCP2 (sc-6526) and UCP3 (sc-31385) (Santa Cruz Biotechnology), and FIS-1 (Cell Signaling) primary antibodies, as well as Alexa Fluor 488 and 546-conjugated secondary antibodies (Life Technologies), were used; nuclei were stained with DAPI (Sigma–Aldrich). Cells were visualized using a Zeiss Axiovert 40 CFL Microscope and Zeiss AxioVision 4.8.2 software.

2.6. Quantitative real-time PCR

Quantitative real-time PCR for *UCP1*, *UCP2*, *UCP3L* (long isoform), and *UCP3S* (short isoform) was performed with Fast EvaGreen Master Mix (Uniscience), as previously described [26]. Quantitative real-time PCR for *FIS1* was performed with the TaqMan assay (Hs00211420; Life Technologies). *GAPDH* and *β-actin* were used as housekeeping genes.

2.7. Lipid peroxidation assay

The formation of thiobarbituric acid-reactive substances (TBARS) was used to estimate the lipid peroxidation level. Cell lysate was obtained in SDS buffer and incubated with thiobarbituric acid (TBA) at 95 °C for 60 min, butanol was extracted, and the samples were measured spectrophotometrically at 532 nm. TBARS concentration in the samples was calculated through a malondialdehyde (MDA) calibration curve.

2.8. ATP content

An ATP bioluminescence assay (flaA, Sigma–Aldrich) was used to measure cellular ATP. After 48 h, the cells, either treated or not with 50 μM *t*-BPH or 1 μM valinomycin for 2 h, were lysed and the protein lysates were incubated at 100 °C to inhibit ATPase activity. The ATP reaction was performed according to the manufacturer's protocol. An ATP standard curve was also prepared. The reading was taken immediately using a plate luminometer (AutoLumat LB 953, Berthold Technologies).

2.9. Transmission electron microscopy

Cells were collected after trypsin digestion, fixed by immersion in glutaraldehyde, post-fixed in osmium tetroxide, stained in a block with uranium acetate, dehydrated and embedded in resin (Embed 812, EM Sciences). Ultrathin sections (60 nm) were collected on Pioloform (Ted Pella, Redding, CA) and carbon-coated single sloth grids, and contrasted with uranyl acetate and lead citrate. Transmission electron micrographs were obtained using a Jeol JEM 100 CXII electron microscope equipped with a digital Hamamatsu ORCA-HR camera. The amount of mitochondria and lysosomes was determined using five images at 14 Kx. The mitochondria area was determined using fifteen randomly selected mitochondria per image. Data represent average ± SEM of two independent experiments.

2.10. Confocal microscopy

Cells were plated on *Chamber Slide™ System* 4 wells (177437-Lab Tek), and incubated with 40 nM LysoTracker® Deep Red (L12492-Molecular Probes) or 30 nM MitoTracker® Red CMXRos (M-7512-Molecular probes) at 37 °C for 15 min. After cells were fixed with absolute methanol at 25 °C for 10 min and stained

with DAPI (Sigma–Aldrich). Cells were imaged with a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystem) using the 63× objective with 3× digital zoom or without the zoom. The quantification of fluorescence was performed using the ImageJ software. A common threshold for all images was established and the value considered to compare the intensity of the fluorescence was the average of Raw Integrated Density (sum of pixel values).

2.11. Mitochondrial DNA quantification

The mitochondrial DNA was quantified by real-time PCR with the TaqMan assay (Life Technologies), as previously described [27]. Briefly, DNA was extracted using phenol–chloroform. Quantitative PCR was performed using the *LPL* gene to detect nuclear DNA and the *ND1* gene to detect mitochondrial DNA. The number of mitochondrial genomes per cell was assessed by the ratio *ND1*/*LPL*.

2.12. Immunoblotting assay

The protein concentration was determined through the DC protein assay (Bio-Rad). Total proteins (30–50 μg) were resolved in SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated at 4 °C with primary antibodies for LC3B (#3868; Cell Signaling), DRP1 (#8570; Cell Signaling), p62 (SC-28359; Santa Cruz Biotechnologies), FIS-1 (SC-37646; Santa Cruz), VDAC (#4661; Cell Signaling), SET (SC-5655; Santa Cruz) and GAPDH (#2118; Cell Signaling), the secondary antibodies conjugated with horseradish peroxidase were added for 1 h at room temperature. Immunocomplexes were detected using the ECL Western blotting system (GE Health Care).

2.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 5.0, USA). Student's *t*-test or ANOVA was used to

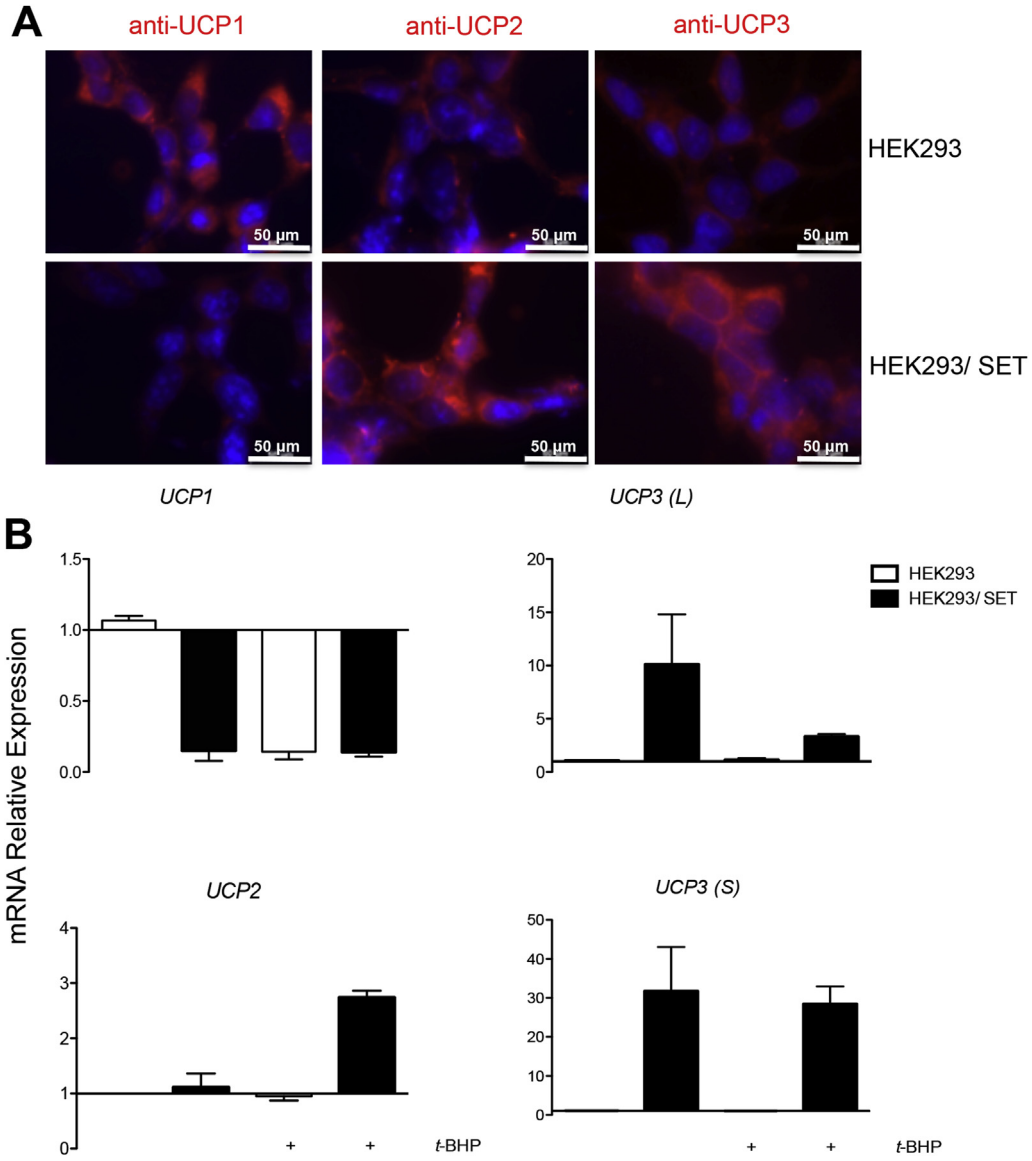

Fig. 1. SET overexpression in HEK293 cells regulates the levels of UCPs protein and mRNA. (A) Immunofluorescence microscopy assay for UCPs. Cells were incubated with antibodies for UCP1, UCP2 and UCP3 and nuclei were stained with DAPI; cells were visualized at 400× magnification. (B) Quantitative real-time PCR for *UCP* mRNA, in the presence or absence of 50 μM *t*-BHP (+*t*-BHP, two hours); mRNA relative expression was calculated by the $2^{-\Delta\Delta CT}$ method and data are reported as means ± SD of three independent experiments in triplicate.

examine the association between media and treatments. *P* values <0.05 were considered significant.

3. Results

3.1. SET overexpression in HEK293 cells regulated the levels of UCP proteins and UCP mRNAs, prevented lipid peroxidation and decreased ATP content

The levels of UCP proteins were assessed by immunofluorescence microscopy in HEK293 control (HEK293) and SET-overexpressing HEK293 (HEK293/SET) cells (Fig. 1A). In HEK293/SET cells, the staining for UCP1 was lower, for UCP2 was slightly higher and for UCP3 was markedly higher in relation to HEK293 cells. Quantitative real-time PCR was performed to assess whether SET overexpression in HEK293 cells also influences the levels of *UCPs* mRNA (Fig. 1B). Accordingly, HEK293/SET cells presented a lower level of *UCP1* mRNA, a slightly higher level of *UCP2* and markedly higher levels of *UCP3S* and *UCP3L* in relation to HEK293 cells. Concomitantly, we assessed the levels of *UCPs* mRNA in cells exposed to oxidative stress induced by the pro-oxidant *tert*-butyl hydroperoxide (*t*-BHP, 50 μM) (Fig. 1B). This condition did not significantly influence the observed decrease in the level of *UCP1*, or the increase in the level of *UCP3S*, but promoted a significant additional increase in the level of *UCP2* and decrease in the level of *UCP3L*. Therefore, the levels of UCP1 protein and *UCP1* mRNA tend to decrease whereas the levels of UCP2/UCP3 protein and *UCP2*/*UCP3* mRNA tend to increase, even if an oxidative stress condition is required in the case of *UCP2*. In this context, we addressed whether SET overexpression in HEK293 cells could also influence aspects of UCPs activity, by assessing both lipid peroxidation status (Fig. 2A) and cell ATP content (Fig. 2B). Accordingly, SET overexpression in HEK293 cells prevented lipid peroxidation and decreased the cellular ATP content, both in the presence and absence of oxidative stress induced by 50 μM *t*-BHP. These results suggest that SET overexpression in HEK293 cells regulates the levels of UCPs, in an apparent association with antioxidant protection (lipid peroxidation prevention) and energetic impairment (ATP content decrease).

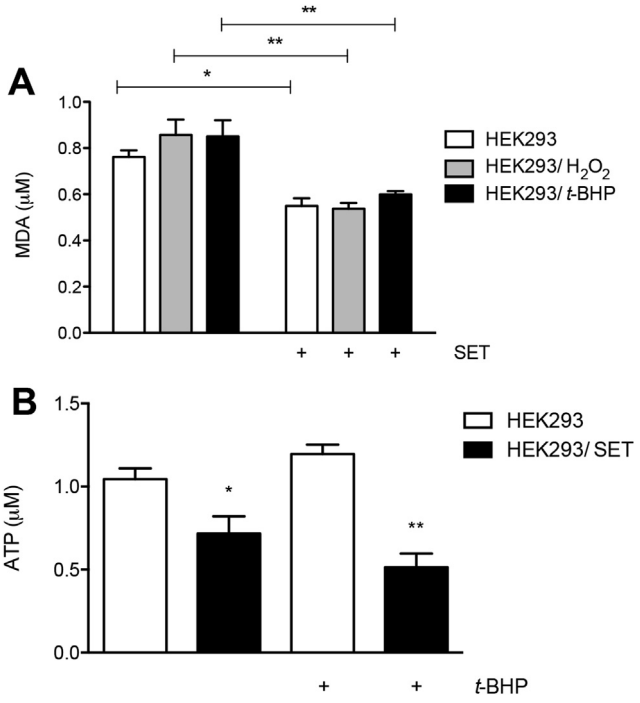

Fig. 2. SET overexpression in HEK293 cells inhibits lipid peroxidation and decreases the content of cellular ATP. (A) Lipid peroxidation, assessed as malondialdehyde (MDA), in the presence or absence of 5 μM H_2O_2 (positive control) and 50 μM *t*-BHP. (B) Cellular ATP content in the presence or absence of 50 μM *t*-BHP. Data are reported as means ± SD of three independent experiments in triplicate; statistical difference: $^{*}p < 0.05$; $^{**}p < 0.01$.

3.2. SET overexpression in HEK293 cells decreased the area of mitochondria and increased the number of the organelles and lysosomes

HEK293 and HEK293/SET cells, exposed or not to oxidative stress induced by 50 μM *t*-BHP, were analyzed by transmission electron microscopy. SET overexpression in HEK293 cells decreased the area of mitochondria (Fig. 3A and B-1) and increased the number of organelles (Fig. 3A and B-2) and lysosomes (Fig. 3A and B-3). Alterations of mitochondria cristae shape were observed in HEK293/SET cells, which could indicate alterations in the mitochondrial activity for cell survival [28]; moreover, SET overexpression in HEK293 cells did not promote alterations in mtDNA amount (data not shown). Accordingly, confocal microscopy for mitochondria and lysosomes (Fig. 3C) showed that their levels were higher in HEK293/SET cells. Specifically, the fluorescence intensity detected with MitoTracker® was ~80% higher: 10.4 ± 1.2 arbitrary units for HEK293 *vs.* 18.1 ± 4.6 for HEK293/SET.

3.3. HEK293/SET cells presented evidence of increased mitochondrial fission and reduced autophagic flux

In the above context, we searched for evidence of mitochondrial fission in HEK293/SET cells: the level of *FIS1* mRNA by quantitative real time PCR (Fig. 4A), the levels of DRP-1 and FIS-1 proteins as well as of VDAC protein (voltage-dependent anion channel) as a mitochondrial marker by immunoblotting (Fig. 4B), and the level of FIS1 protein by immunofluorescence (Fig. 4C). A slight increase in *FIS1* mRNA level was observed in HEK293/SET cells. Accordingly, the FIS-1 protein level was increased in HEK293/SET cells and the DRP-1 protein was accumulated with FIS-1. In addition, VDAC, present in the outer mitochondrial membrane, was increased in HEK293/SET cells. The autophagic flux, assessed through the verification of LC3B lipidation (LC3B-II) in the presence of chloroquine (Fig. 4D), was reduced in HEK293/SET cells.

4. Discussion

Mitochondrial uncoupling, which can reduce ROS generation (mild condition), has been found to be altered in cancer [5,7]; either the up- or down-regulation of UCPs appears to be involved in this process [6]. In the present study, we found that SET overexpression in HEK293 cells down-regulates UCP1 and up-regulates UCP2/UCP3. UCPs have attracted attention in cancer due to their role in reprogramming cancer cell metabolism [6,7]. UCP1 has been associated with heat in brown adipose tissue. UCP2 was described to modulate energy metabolism in response to high ROS levels [12]; UCP2 was found to be increased in human colon cancer [29], leukemia [7] and lung tumors [8]. Notably, the level of UCP3 was most positively influenced by the overexpression of SET in HEK293 cells. In humans, the UCP3 gene is present in two isoforms: UCP3 long (L) and UCP3 short (S). In human muscle, a greater expression of the UCP3S isoform was observed [14]. In addition, increased UCP3 gene level has been associated with lung carcinoma [14] and a direct link between UCP3 expression and autophagy has been observed in chondrocytes [30].

The ROS action on lipids generates and accumulates lipid oxidation products, which is an established oxidative stress marker. Tissues and organs can accumulate large amounts of these

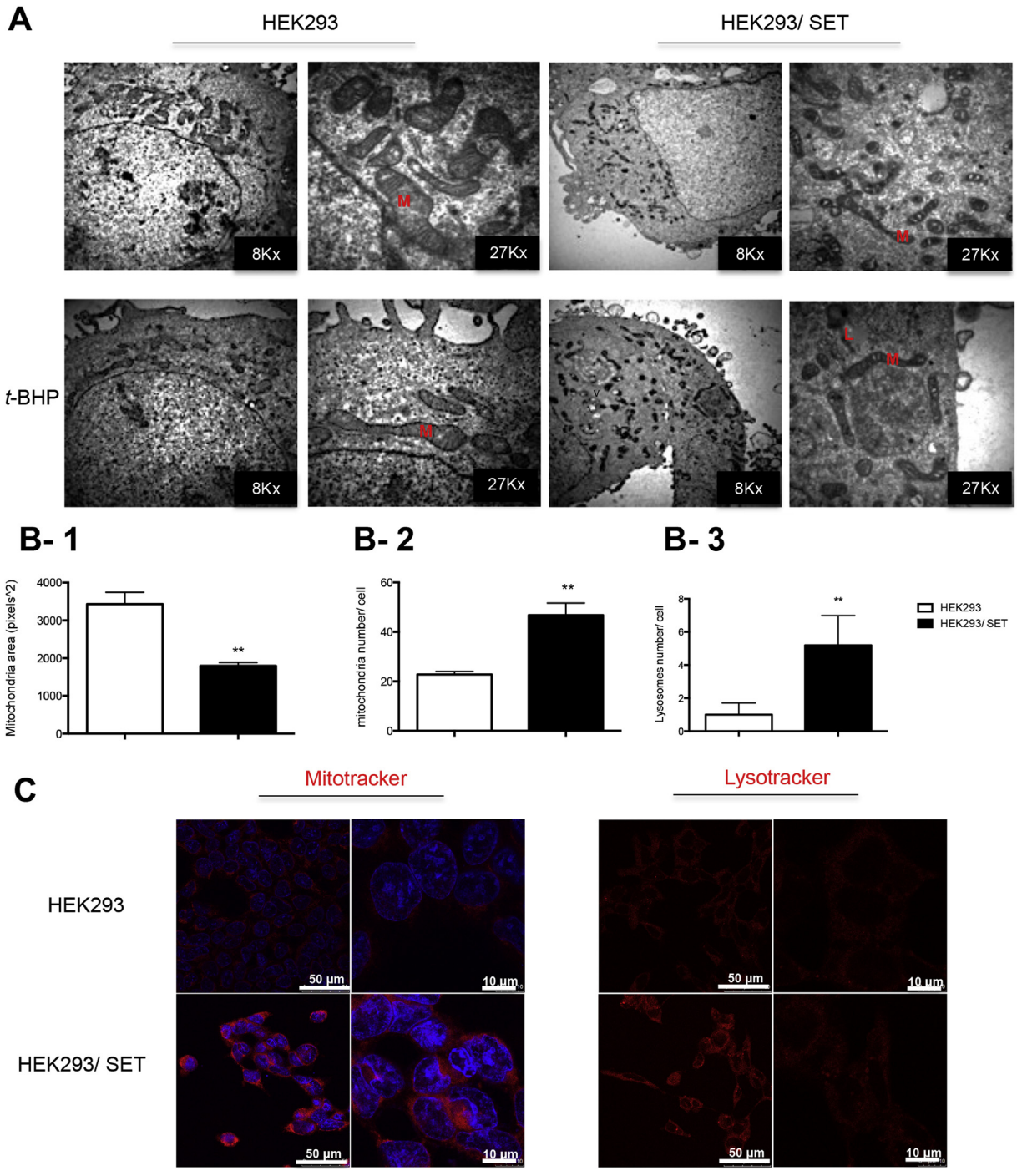

Fig. 3. SET overexpression in HEK293 cells decreases the area of mitochondria and increases the number of the organelles and lysosomes. (A) Electron transmission microscopy in the presence or absence of 50 µM *t*-BHP: (*M*) mitochondria, (*L*) lysosomes. (B) Estimation of mitochondria area (B-1) and number (B-2) and lysosomes number (B-3) in the presence of *t*-BHP, using micrographs and analysis by ImageJ software. Data are presented as means ± SD of two independent experiments in triplicate; statistical difference: $^{**}p < 0.01$. (C) Confocal microscopy for mitochondria (Mitotracker®) and lysosomes (Lysotracker®). The images are representative of 4 different fields for each condition.

products, leading to oxidative stress-related processes such as aging, cardiovascular diseases, Alzheimer's disease [31] and cancer [32]. Attack of ROS in the lipids of inner mitochondrial membrane promotes the accumulation of lipid peroxides, which may damage components of the mitochondrial matrix. In this regard, UCP3 has been implicated in the transport of lipid peroxides out of the mitochondria [33]. HEK293/SET cells presented decreased lipid peroxidation status in relation to HEK293 cells, which could be due to a protective action of UCP3 against lipid peroxides in the organelles. Compatible with this protective action of SET on mitochondria, the protein overexpression was associated with condensation of the organelles and an increase in their number, apparently within a mitochondrial dynamics context.

Mitochondrial dynamics have been implicated in the maintenance of mitochondrial morphology, mtDNA stability, respiratory capacity, apoptosis, and response to cellular stress [25]. Maintenance of the shape, size, and number of mitochondria is driven by mitochondrial fusion and fission, which may be associated with

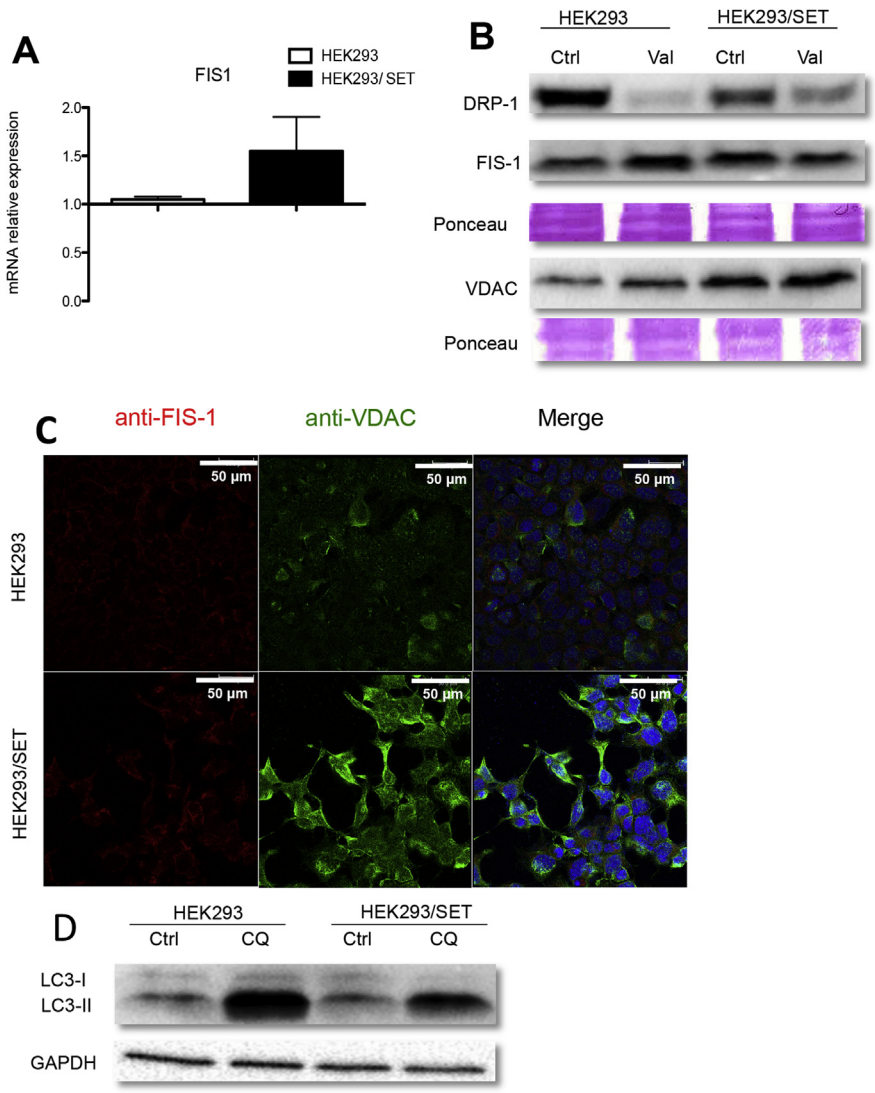

Fig. 4. HEK293 cells with SET overexpressed present evidence of increased mitochondrial fission and reduced autophagic flux. (A) Quantitative real-time PCR for *FIS1* mRNA, calculated by the $2^{-\Delta\Delta CT}$ method. (B) Immunoblotting for DRP-1, FIS-1 and VDAC, in the presence or absence of 10 μM valinomycin (Val, 2 h incubation), a positive fission control, using enriched mitochondrial subcellular fraction (Ctrl, control in relation to Val); ponceau staining was used as a protein loading control. (C) Immunofluorescence microscopy assay for FIS-1; nuclei were stained with DAPI. (D) Western blotting for LC3B (I and II) in the presence or absence of chloroquine (CQ); GADPH vas used as a protein loading control.

starvation [34]. In cases where mitochondrial fusion is reduced, mitochondria become fragmented and small because of unbalanced fission, whereas when mitochondrial fission is reduced, mitochondria become elongated and excessively interconnected because of unbalanced fusion [34]. Our results suggest that SET overexpression promotes mitochondrial fission because of the accumulation of small mitochondria associated with increased levels DRP-1 and FIS-1, as well as of VDAC. Included in the functions of fusion/fission are the protection of mitochondrial DNA, alterations of cellular energetics, and the regulation of cell division [35]. DRP-1 and FIS-1 are the most important proteins involved in the fission process in mammalian species [36]. It has been reported that high FIS-1 levels promote mitochondrial fragmentation and stimulate autophagy [37], and that elongated mitochondria precedes macroautophagy [28]. In this context, we analyzed the LC3B protein in cells exposed to chloroquine and observed a relatively low autophagic flux in HEK293/SET cells.

These results suggest that SET overexpression protects HEK293 cells from death by promoting the up-regulation of UCP2 and UCP3 under oxidative stress conditions, thereby reducing ROS and preventing autophagy (mitophagy). In addition, our findings showing increased VDAC levels in HEK293/SET cells, indicate a potential role of SET in the control of hexokinase 2 and metabolism, once VDAC binds to this enzyme, and both are involved in the "Warburg effect" in cancer [38]. We therefore speculate that this either reflects an increase in the number of mitochondria or may be a tentative way of compensating for a reduction in oxidative phosphorylation due to uncoupling. The present work is the first to show an association between SET overexpression, UCP2/UCP3, and mitochondrial fission, which could be involved in the imbalance between cell survival/death and metabolism alterations, particularly in Alzheimer's disease and cancer.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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