



Mitofusin-2 regulates inflammation-mediated mouse neuroblastoma N2a cells dysfunction and endoplasmic reticulum stress via the Yap-Hippo pathway

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Abstract

Endoplasmic reticulum (ER) stress is involved in inflammation-induced neurotoxicity. Mitofusin 2 (Mfn2), a member of the GTPase family of proteins, resides in the ER membrane and is known to regulate ER stress. However, the potential role and underlying mechanism of Mfn2 in inflammation-induced neuronal dysfunction is unknown. In our study, we explored the potential of Mfn2 to attenuate inflammation-mediated neuronal dysfunction by inhibiting ER stress. Our data show that Mfn2 overexpression significantly ameliorated tumor necrosis factor alpha (TNF α)-induced ER stress, as indicated by the downregulation of the ER stress proteins PERK, GRP78 and CHOP. Mfn2 overexpression also prevented the TNF α -mediated activation of caspase-3, caspase-12 and cleaved poly (ADP-ribose) polymerase (PARP). Cellular antioxidant dysfunction and reactive oxygen species overproduction were also improved by Mfn2 in the setting of TNF α in mouse neuroblastoma N2a cells in vitro. Similarly, disordered calcium homeostasis, indicated by disturbed levels of calcium-related proteins and calcium overloading, was corrected by Mfn2, as evidenced by the increased expression of store-operated calcium entry (SERCA), decreased levels of inositol trisphosphate receptor (IP3R), and normalized calcium content in TNF α -treated N2a cells. Mfn2 overexpression was found to elevate Yes-associated protein (Yap) expression; knockdown of Yap abolished the regulatory effects of Mfn2 on ER stress, oxidative stress, calcium balance, neural death and inflammatory injury. These results lead us to conclude that re-activation of the Mfn2–Yap signaling pathway alleviates TNF α -induced ER stress and dysfunction of mouse neuroblastoma N2a cells. Our findings provide a better understanding of the regulatory role of Mfn2–Yap–ER stress in neuroinflammation and indicate that the Mfn2–Yap axis may be a focus of research in terms of having therapeutic value for the treatment of neurodegenerative diseases.

Keywords Yap · Mfn2 · ER stress · Neuroinflammation · Dysfunction of mouse neuroblastoma N2a cells

Introduction

Huntington's disease (HD), a neurodegenerative disorder primarily caused by instable and abnormally long expansion of the polyglutamine (polyQ) tract, is characterized by a chronic loss of neurons in the basal ganglia [1]. Motor ability and cognitive function are impaired during the progression of HD. Emerging evidence suggests that neuroinflammation due to neuro-immune interactions plays a pivotal

role in the pathogenesis of intractable neurogenic disorders, especially in the development of HD. Uncontrolled inflammation evokes reactive oxygen species (ROS) overloading which in turn mediates oxidative stress in neurons, an effect that is accompanied with neuronal dysfunction and/or death. Inflammatory cytokines are also released from inflammatory cells and enhance the inflammation-mediated damage in the brain. Given the functional significance of inflammatory injury in the pathogenesis of HD, investigation of the pathophysiological mediator(s) of neuroinflammation is a primary focus of researchers.

Mitofusin 2 (Mfn2), which resides in the outer membrane of the endoplasmic reticulum (ER), plays a pivotal role in ER function, such as calcium balance and protein synthesis. Mfn2 is also involved in mitochondrial regulation, such as mitochondrial fusion, mitophagy and

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mitochondrial fission, and it has been shown to have anti-inflammatory actions in several disease models [2, 9]. For example, Mfn2 activation attenuates tumor necrosis factor alpha (TNF α)-mediated hair follicle stem cell apoptosis [2, 3]; Mfn2 overexpression sustains mitochondrial function and elevates the cellular survival rate in lipopolysaccharide-treated human kidney-2 cells [4, 5]; in hyperglycemic stress, increased Mfn2 interrupts inflammatory signals in pancreatic β -cells [6]. In addition, the anti-inflammatory property of Mfn2 has also been noted in pulmonary arterial hypertension [7], airway smooth muscle [8] and alveolar epithelial cells [9]. Although a number of studies have indicated that Mfn2 is associated with inflammation-mediated cell stress, the potential role and underlying mechanism of Mfn2 in neuronal dysfunction resulting from inflammation are not yet fully characterized.

The ER, an intracellular organelle, executes several essential functions in cellular pathophysiological processes [10]. The primary action of the ER is to modulate calcium balance via the recycling and releasing of calcium into the cytoplasm. ER also exerts a critical effect on protein generation, synthesis, modification and transport [11–13]. The primary result of ER dysfunction is ER stress, an effect that is accompanied with an accumulation of misfolded proteins and calcium overloading [14, 15]. Subsequently, disruption of calcium homeostasis has been found to be associated with oxidative stress that contributes to the activation of apoptosis in a mechanism through ER stress [3, 16]. The molecular features of ER stress-mediated apoptosis is associated with the upregulation of CHOP, an ER stress protein, and activation of caspase-12, a cysteine protease [17]. Based on previous studies, ER stress has been identified as a potential molecular mechanism that is involved the initiation and progression of several neurodegenerative disorders, such as HD [18, 19]. Given the beneficial effects of Mfn2 on ER function and the inflammation response, we investigated whether Mfn2 could attenuate inflammation-mediated neuronal dysfunction by inhibiting ER stress.

The Yes-associated protein (Yap)–Hippo signaling pathway is a novel pathway that is responsible for cell survival. Specifically, Yap regulates cerebral hypoxia–reoxygenation injury by modulating the ROCK1/F-actin pathways [20, 21]. Yap activation modulates the activity of glioblastoma A172 cells in a manner dependent on the MAPK–ERK axis [22, 23] and is also associated with microglial BV-2 cell survival through attenuation of Bnip3-related mitophagy in the setting of an inflammatory microenvironment [24, 25]. Similar results have also been found in gastric cancer [26] and rectal cancer [27]. In the study reported here, we investigated whether Yap is augmented by Mfn2 and promotes the survival of neurons in the context of inflammatory injury.

Materials and methods

Cell culture and treatments

Mouse neuroblastoma N2a cells (ATCC® CCL-131™; American Type Culture Collection, Manassas, VA, USA) were cultured in high-glucose Dulbecco's Modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco). After the cells reached 80% confluence, different doses of TNF α were added into the medium for 12 h based on a previous report [28].

Cell viability and TUNEL staining

The MTT colorimetric assay was used to assess metabolic activity and cellular viability. Cells were seeded onto a 96-well plate, and the MTT tetrazolium compound was then added to the medium (2 mg/ml; Sigma-Aldrich, St. Louis, MO, USA); the cells were then cultured in the dark for 4 h and then DMSO was added to the medium. The optical density of each well was observed at 490 nm using a spectrophotometer (Epoch 2; BioTek Instruments, Inc., Winooski, VT, USA) [29]. The TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay was used to detect apoptotic cells. Cells were fixed in 4% paraformaldehyde at room temperature for 30 min and the tissue sliced into sections. A TUNEL kit (Roche Apoptosis Detection kit; Roche, Mannheim, Germany) was used to stain the slices according to the instructions of the manufacturer. Finally, the sections were amplified to 400 \times , and the apoptotic cells in at least ten fields were randomly chosen for detailed examination. The apoptotic index was taken to be the proportion of apoptotic cells to total cells, in accordance to a previous study [30].

Enzyme-linked immunosorbent assay

Mouse neuroblastoma N2a cells (1.5×10^3 /well) were seeded into 24-well plates. After incubation with TNF α , the protein was isolated, and the contents of cellular antioxidants, including superoxide dismutase (SOD), glutathione (GSH), and glutathione peroxidase (GPX), were analyzed using enzyme-linked immunosorbent assay (ELISA) kits. In addition, lactose dehydrogenase content and caspase-3 activity were determined using ELISA kits purchased from Beyotime Biotechnology (Nanjing, China: LDH Cytotoxicity Assay Kit, Cat. No.: C0016; Caspase-3 Activity Assay Kit, Cat. No.: C1115), according to a previous study [31]. Caspase-12 activity was determined using the ELISA kit purchased from Abcam (Cambridge, UK: Cat. No.: #ab65664).

Transfection

Small, interfering RNA (siRNA) against the macrophage-stimulating 1 (Mst1) gene and the pDC315-Mst1 vector were obtained from GenePharm (Shanghai, China). Transfection was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's instructions [32]. After 6 h, the cells were transferred to complete growth medium, and 48 h later, the cells were harvested and used in subsequent experiments. The siRNA knockdown efficiency and overexpression efficiency were confirmed by western blotting [33]. Western blots were used to verify the overexpression and knockdown efficiency of recombinant human adenovirus ad-Mfn2 infection and Yap siRNA transfection, respectively.

Western blotting

Total protein was extracted by RIPA (R0010; Solarbio Science and Technology, Beijing, China), and the protein concentration of each sample was detected using a bicinchoninic acid (BCA) kit (20201ES76; Yeasen Biotech Co., Ltd, Shanghai, China). Deionized water was added to generate 30- μ g protein samples for each lane. A 10% sodium dodecyl sulphate (SDS) separation gel and a concentration gel were prepared [34]. The following diluted primary antibodies were added to the membrane and incubated overnight at 4 °C: Bcl2 (1:1000; Cell Signaling Technology Inc., Danvers, MA, USA; Cat. No.: 3498), Bax (1:1000; Cell Signaling Technology; Cat. No.: 2772), pro-caspase3 (1:1000; Abcam; Cat. No.: 13847), cleaved caspase3 (1:1000; Abcam; Cat. No.: ab49822), cyt-c (1:1000; Abcam; Cat. No.: ab90529), Tom20 (1:1000; Abcam; Cat. No.: ab186735), JNK (1:1000; Cell Signaling Technology; Cat. No.: 4672), p-JNK (1:1000; Cell Signaling Technology; Cat. No.: 9251), Mst1 (1:1000; Cell Signaling Technology; Cat. No.: 3682). The membranes were washed three times with phosphate-buffered saline (PBS) (5 min each time), supplemented with horseradish peroxidase-marked second antibody (1:200; Bioss, Beijing, China), oscillated and then incubated at 37 °C for 1 h. After incubation, each membrane was washed three times with PBS (5 min each time) and reacted with enhanced chemiluminescence solution (ECL808-25; Biomiga Inc., San Diego, CA, USA) at room temperature for 1 min; then, the extra liquor was removed, and the membranes were covered with preservative film [35]. Each membrane was observed with an X-ray machine (36209ES01; Qian Chen Biological Technology Co. Ltd., Shanghai, China) to visualize protein expression. Glyceraldehyde 3-phosphate dehydrogenase was used as the internal reference. The relative protein expression was the ratio of the gray value of the target band to the inner reference band.

Immunofluorescence

Cells were plated on glass slides in a 6-well plate at a density of 1×10^6 cells per well, following which the cells were fixed in ice-cold 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 and blocked with 2% gelatin in PBS at room temperature [36]. The cells were then incubated with the primary antibodies overnight at 4 °C: cyt-c (1:1000; Abcam; Cat. No.: ab90529), p-JNK (1:1000; Cell Signaling Technology; Cat. No.: 9251), Mst1 (1:1000; Cell Signaling Technology; Cat. No.: 3682). After being washed with PBS, the cells were incubated with secondary antibody and 4',6-diamidino-2-phenylindole (DAPI; 1:1000 dilution in PBS) for 1 h at room temperature. Images were obtained using a fluorescence microscope [37].

Flow cytometry analysis for ROS

Cell suspensions were collected. The liquor (50 g, digested two times) was collected, centrifuged for 2 min with the supernatant removed, supplemented with the superoxide indicator dihydroethidium, incubated at room temperature for 10 min, centrifuged, and washed with PBS [38]. The cells were resuspended by adding binding buffer (1 \times) in the dark; then, the cells were incubated at room temperature for 30 min and filtered through a nylon mesh (40- μ m well). ROS production was measured by fluorescence-activated cell sorting (FACS) [39].

Statistical analysis

The software program SPSS version 17 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The results are presented as the mean \pm standard error of the mean. Data for analysis consisted of three replications, and statistical analyses were conducted using one-way analysis of variance followed by Tukey's test to compare variable groups. $p < 0.05$ was considered to be statistically significant.

Results

Mfn2 and Yap are repressed by TNF α in mouse neuroblastoma N2a cells

In the present study, we used TNF α (10 ng/ml for 24 h) to establish the inflammatory environment and then determined cell viability using the MTT assay. As shown in Fig. 1a, compared to the control group TNF α treatment reduced the cellular viability of mouse neuroblastoma N2a cells, indicating that inflammation injury had developed in the TNF α -treated mouse neuroblastoma N2a cells. This model was used in all subsequent functional assays. Western blotting was used to

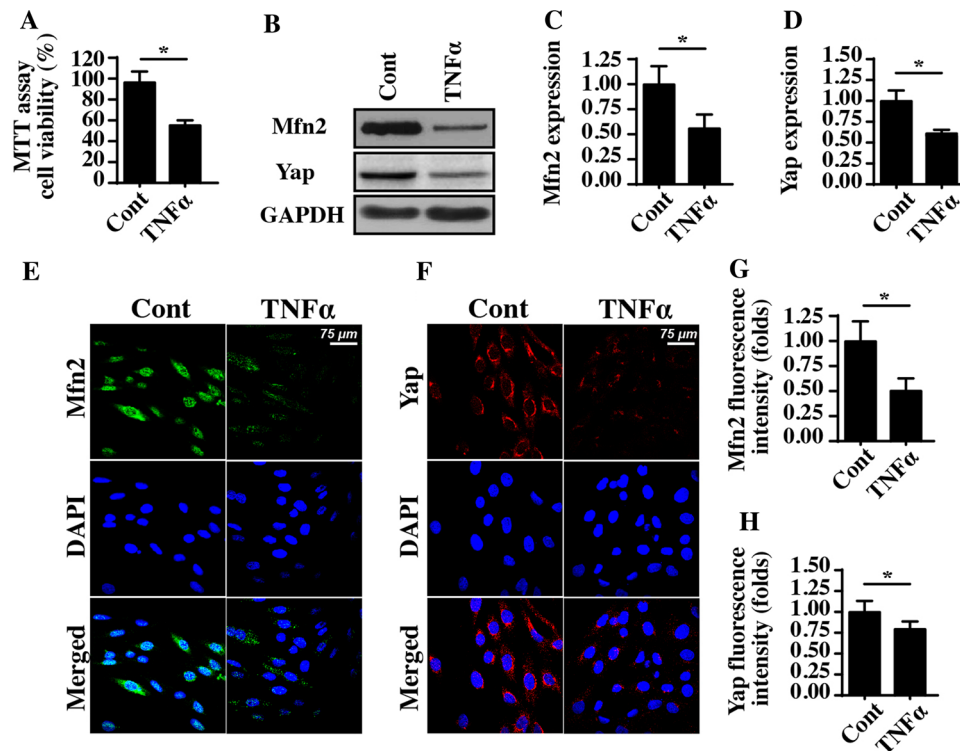


Fig. 1 Tumor necrosis factor alpha (*TNF α*) reduces the levels of mitofusin 2 (*Mfn2*) and Yes-associated protein (*Yap*) in mouse neuroblastoma N2a cells. **a** N2a cells were treated with *TNF α* and then the viability of the treated N2a cells was determined using the MTT assay. *TNF α* treatment reduced the viability of the N2a cells. **b–d** Proteins were isolated from N2a cells and then western blotting was used to confirm alterations in the expression of *Yap* and *Mfn2*. **e–h** Immunofluorescence assay was used to analyze the expression of

Mfn2 and *Yap* in N2a cells. *TNF α* treatment reduced the levels of *Mfn2* and *Yap* in N2a cells. Data are presented as the mean of three replications with the standard error of the mean (SEM). Asterisk above columns linked by horizontal line indicates a significant difference between treatments at $p < 0.05$. *Cont* Control, *DAPI* 4',6-diamidino-2-phenylindole, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase (internal control)

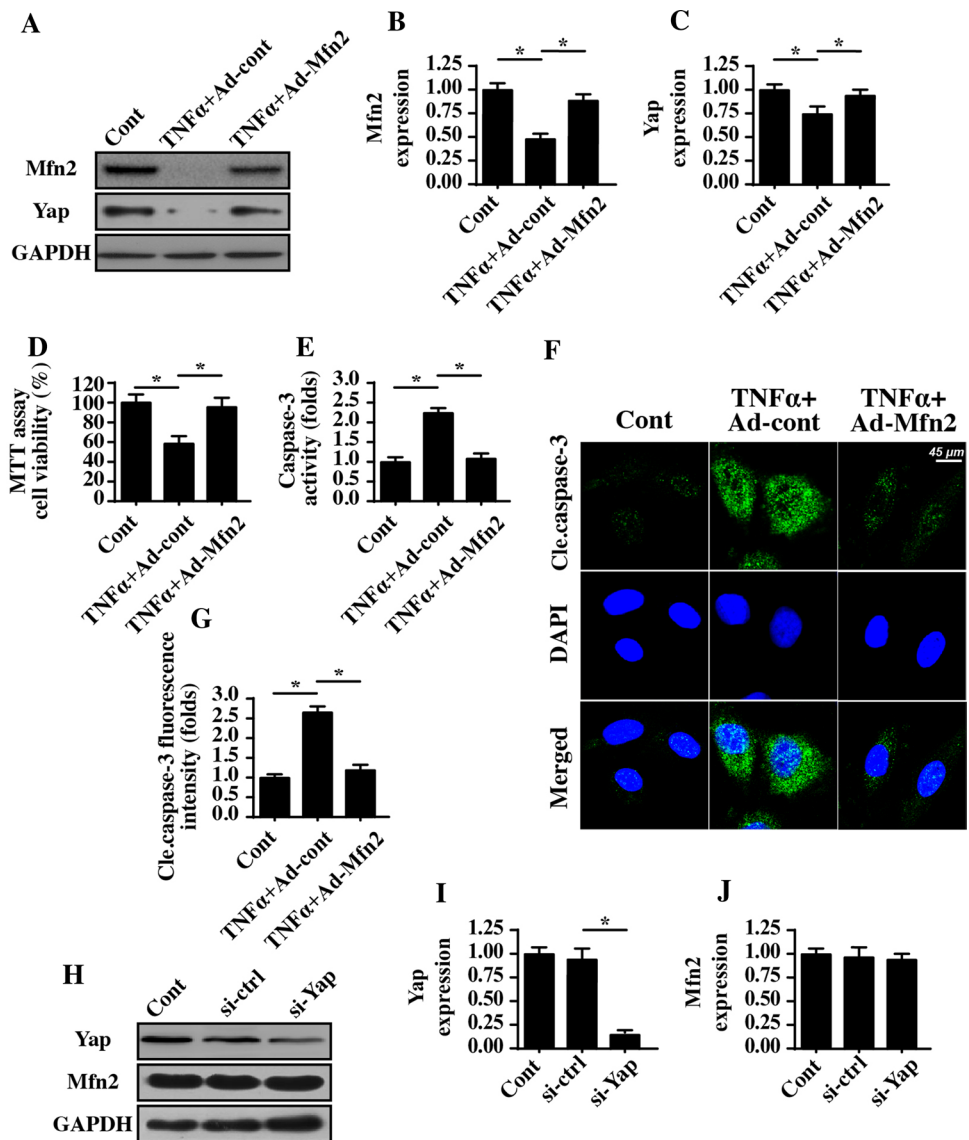
observe the changes in *Mfn2* and *Yap* in the setting of the *TNF α* treatment. As shown in Fig. 1b–d, compared to the control group, *Mfn2* expression was downregulated in response to *TNF α* treatment and *Yap* expression was significantly repressed. These results indicate that *Mfn2* and *Yap* were both inhibited by the *TNF α* -mediated inflammatory microenvironment. This finding was further supported by the results of the immunofluorescence assay. As shown in Fig. 1e–h, compared to the control group, the fluorescence intensity of *Mfn2* was obviously suppressed in *TNF α* -treated cells, an effect that was accompanied with a drop in the levels of *Yap* (Fig. 1e–h). Taken together, the above data provide evidence that *TNF α* -induced inflammation stress promoted the decrease in both *Mfn2* and *Yap* expression in mouse neuroblastoma N2a cells.

Overexpression of *Mfn2* reverses *Yap* expression and the survival of mouse neuroblastoma N2a cells in the setting of *TNF α* -induced inflammation stress

The following set of experiments was conducted to verify the roles of *Mfn2* downregulation and *Yap* inactivation

in *TNF α* -mediated damage to mouse neuroblastoma N2a cells. First, adenovirus-loaded *Mfn2* was transfected into mouse neuroblastoma N2a cells to reverse the expression of *Mfn2*; the overexpression efficiency was confirmed by western blotting. As shown in Fig. 2a–c, compared to the control group *TNF α* reduced the levels of *Mfn2* in the N2a cells transfected with the adenovirus vector, and this effect was occurred concomitantly with a decline in *Yap* expression. Interestingly, overexpression of *Mfn2* upregulated *Yap* expression in *TNF α* -treated mouse neuroblastoma N2a cells, indicating that *Mfn2* would appear to be the upstream mediator of *Yap* in mouse neuroblastoma N2a cells (Fig. 2a–c). The MTT assay was then performed to observe cell viability in response to *Mfn2* overexpression. As shown in Fig. 2d, compared to the control group *TNF α* treatment reduced the cell viability of mouse neuroblastoma N2a cells, and this effect could be reversed by *Mfn2* overexpression, indicative of the anti-apoptotic effect of *Mfn2* on inflammation-attacked mouse neuroblastoma N2a cells. Caspase-3 activity was also measured as a reflection of the activation of the caspase family during cell apoptosis. As shown in Fig. 2e,

Fig. 2 Overexpression of Mfn2 abolishes TNF α -mediated apoptosis in mouse neuroblastoma N2a cells. **a–c** Proteins were isolated from N2a cells and the expressions of Mfn2 and Yap were determined by western blotting. Adenovirus-loaded Mfn2 (*Ad-Mfn2*) was transfected into N2a cells to overexpress Mfn2. **d** Cell viability was determined by the MTT assay. Overexpression of Mfn2 reversed cell viability in TNF α -treated N2a cells. **e** An enzyme-linked immunosorbent assay (ELISA) was used to observe the changes in caspase-3 in response to TNF α treatment and/or Mfn2 overexpression. Mfn2 overexpression reduced TNF α -mediated caspase-3 upregulation. **f, g** The immunofluorescence assay was used to observe the changes in caspase-3 in N2a cells. **h–j** The knockdown efficiency of small interfering RNA (siRNA) against Yap (*si-Yap*) was determined using western blotting. Mfn2 expression was also measured in response to Yap siRNA transfection. Data are presented as the mean of three replications with the SEM. Asterisk above columns linked by horizontal line indicates a significant difference between treatments at $p < 0.05$. *Cont* Control



compared to the control group TNF α elevated the activity of caspase-3, illustrating that TNF α induced the death of mouse neuroblastoma N2a cells in an apoptosis-dependent manner. Interestingly, Mfn2 overexpression attenuated TNF α -mediated caspase-3 expression upregulation (Fig. 2e). These results were further supported by the results of the immunofluorescence assay. As shown in Fig. 2f, g, compared to the control group the fluorescence intensity of cleaved caspase-3 was augmented in response to TNF α treatment. However, Mfn2 overexpression attenuated TNF α -mediated caspase-3 activation (Fig. 2f, g). Finally, to determine which protein was the upstream mediator, siRNA against Yap was tested, and the knockdown efficiency is shown in Fig. 2h, i. Compared to the control group, Yap knockdown had no effect on the expression of Mfn2. Based on this result, combined with the data shown in Fig. 2a, we conclude that Mfn2 can be considered to be a primary upstream mediator of the

Yap–Hippo pathway. Taken together, the above data verify that Mfn2 was the upstream mediator of Yap and increased Mfn2 sustained mouse neuroblastoma N2a cell viability in the setting of TNF α -mediated inflammation stress.

Knockdown of Yap abolishes the protective effect of Mfn2 overexpression in TNF α -treated mouse neuroblastoma N2a cells

Having found that Mfn2 overexpression was beneficial to N2a cells in the setting of the TNF α -mediated inflammation microenvironment [40, 41], we next looked at whether Yap, the downstream effector of Mfn2, was also involved in the survival of mouse neuroblastoma N2a cells. To this end, siRNA against Yap was transfected into Mfn2-overexpressed cells, and then cell viability was determined by the MTT assay. As shown in Fig. 3a, compared to the control

group TNF α reduced cell viability, and this effect could be reversed by Mfn2 overexpression. Interestingly, the loss of Yap was able to abolish the pro-survival action of Mfn2 overexpression on mouse neuroblastoma N2a cells in the setting of TNF α stress (Fig. 3a). This finding was further supported by the results of TUNEL staining. As shown in Fig. 3c, compared to the control group the number of TUNEL-positive cells increased in response to TNF α treatment. However, Mfn2 overexpression suppressed the ratio of TUNEL-positive cells (Fig. 3b, c), an effect that was negated by Yap siRNA transfection, as evidenced by the increased number of TUNEL-positive cells in Yap-deleted cells. These results indicate that Mfn2 overexpression alleviated

TNF α -mediated death in mouse neuroblastoma N2a cells via the upregulation of Yap.

Furthermore, to confirm whether apoptosis was modulated by Mfn2 in a Yap-dependent manner, western blotting was used to observe the alterations in caspase-3 and its substrate PARP. As shown in Fig. 3d–f, compared to the control group TNF α enhanced the expression of cleaved caspase-3 and its substrate cleaved PARP. Interestingly, Mfn2 overexpression repressed the levels of cleaved caspase-3 and its substrate cleaved PARP (Fig. 3d–f), and these alterations could be reversed by Yap deletion. These data indicate that Yap, as a downstream effector of Mfn2, was significantly required for

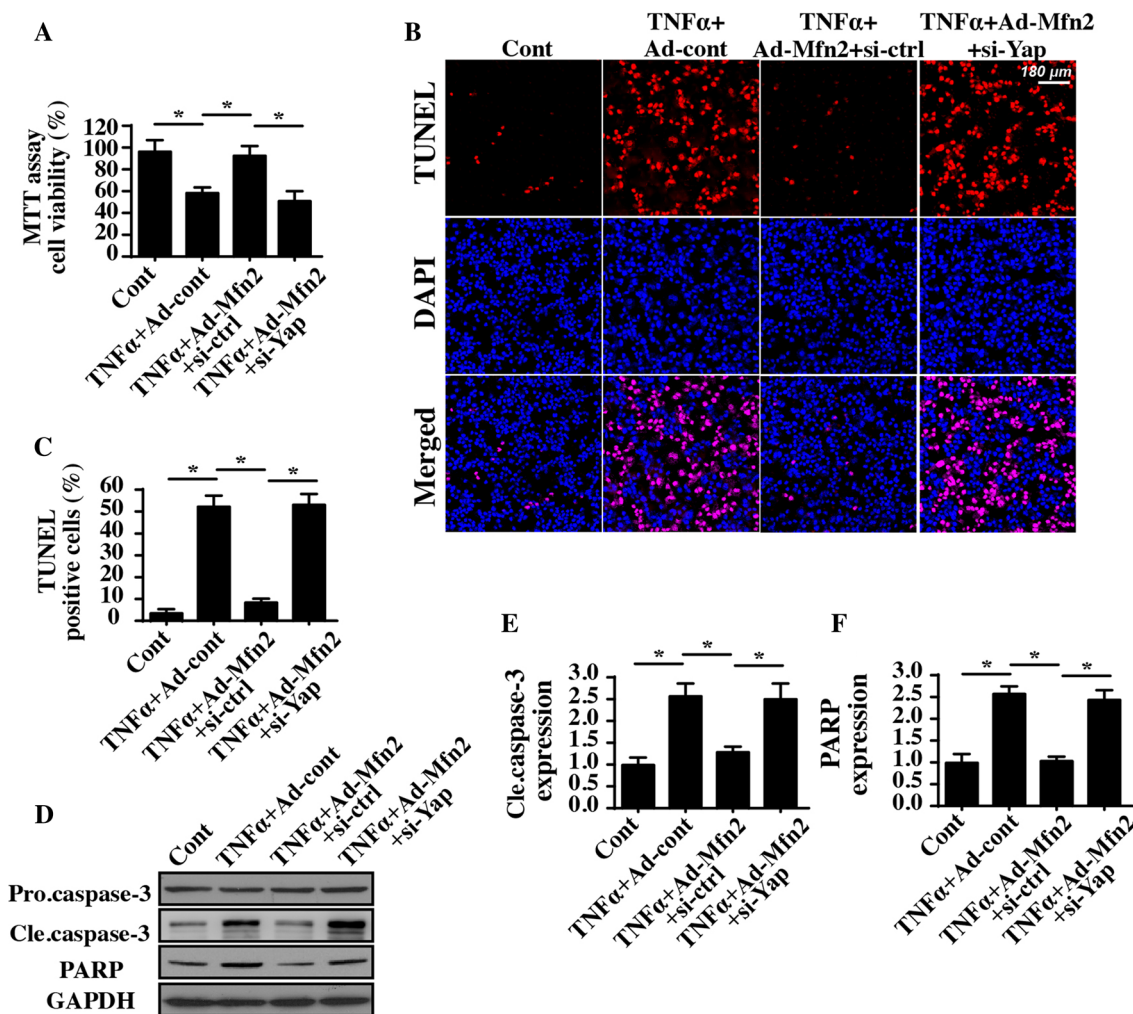


Fig. 3 Yap is activated by Mfn2 and contributes to the survival of mouse neuroblastoma N2a cells under TNF α treatment. **a** Cell viability was measured by the MTT assay. siRNA against Yap (*si-Yap*) was transfected into N2a cells to repress Yap expression. **b, c** Cellular death was measured by the terminal deoxynucleotidyl transferase dUTP nick end labeling (*TUNEL*) assay. The number of TUNEL-positive cells was evaluated to reflect the role of Yap in TNF α -mediated N2a cell death. **d–f** Proteins were isolated from N2a cells and then

western blotting was used to observe the changes in caspase-3 and cleaved poly (ADP-ribose) polymerase (*PARP*). siRNA against Yap was transfected into N2a cells to repress Yap expression. Data are presented as the mean of three replications with the SEM. Asterisk above columns linked by horizontal line indicates a significant difference between treatments at $p < 0.05$. *Pro* pro-form of caspase-3, *Cle* cleaved, *Cont* control

Mfn2-mediated mouse neuroblastoma N2a cell survival in the setting of TNF α -mediated inflammatory injury.

Mfn2 attenuates ER stress in mouse neuroblastoma N2a cells

Endoplasmic reticulum stress has been shown to be an early hallmark of neuronal dysfunction in mouse neuroblastoma N2a cells [42, 43]. One of the aims of our study was to determine whether apoptosis in TNF α -mediated mouse neuroblastoma N2a cells was activated by ER stress and whether the Mfn2–Yap signaling pathway protected mouse neuroblastoma N2a cells against TNF α stress through an inhibition of ER stress. Western blotting revealed that the expression of ER stress markers such as PERK, CHOP and GRP78 were elevated in response to TNF α treatment (Fig. 4a–d). Interestingly, ER stress parameters were significantly repressed by Mfn2 overexpression (Fig. 4a–d), indicative of the protective effects of Mfn2 overexpression on ER function in the setting of the inflammatory microenvironment. However, loss of Yap re-activated ER stress in Mfn2-overexpressed cells, suggesting that Yap was required for Mfn2-mediated ER protection (Fig. 4a–d). These data support the notion that ER stress, activated by TNF α , was markedly inhibited by Mfn2 in a Yap-dependent manner in mouse neuroblastoma N2a cells.

Excessive ER stress is known to activate the caspase-12-related apoptotic pathway [44, 45]. To confirm this, we used the immunofluorescence assay to observe caspase-12 expression in response to TNF α treatment and/or Mfn2 overexpression. As shown in Fig. 4e, f, compared to the control group the intensity of caspase-12 fluorescence was increased by TNF α , i.e. there was an upregulation of caspase-12 expression, whereas Mfn2 overexpression suppressed the TNF α -mediated caspase-12 augmented expression. Interestingly, loss of Yap abolished the regulator effects of Mfn2 overexpression on caspase-12 modification (Fig. 4e, f). The ELISA assay was then used to observe the activity of caspase-12 in response to Mfn2 overexpression and Yap deletion. As shown in Fig. 4g, compared to the control group TNF α enhanced the activity of caspase-12, and this effect could be attenuated by Mfn2 overexpression. Loss of Yap was able to prevent Mfn2-mediated caspase-12 down-regulation (Fig. 4g), as evidenced by increased caspase-12 activity in Yap-deleted cells. Taken together, this information indicates that TNF α -activated ER stress was negatively modulated by the Mfn2–Yap signaling pathway.

Cellular oxidative stress and calcium balance are modulated by Mfn2 in mouse neuroblastoma N2a cells

At the molecular level, ER stress is associated with maintenance of calcium homeostasis and modification of the redox

balance [46, 47]. Previous studies have reported that ER stress would induce the disorder of calcium-related proteins, contributing to the oxidative stress through the upregulation of calcium-related ROS overproduction [48, 49]. In the present study, western blotting was used to observe the changes in calcium-related proteins. As shown in Fig. 5a–d, compared to the control group TNF α increased the expression of the inositol trisphosphate receptor (IP3R), which modulates calcium release from the ER, whereas it reduced the levels of store-operated calcium entry (SERCA); this result is indicative of the imbalance in calcium-related proteins. Interestingly, Mfn2 overexpression was able to correct the disorder between calcium-related proteins, and this effect was dependent on Yap, as evidenced by the loss of Yap abolishing the regulatory effects of Mfn2 on calcium-related proteins regulation (Fig. 5a–d). Cytoplasmic calcium content was then measured by immunofluorescence. As shown in Fig. 5e, f, compared to the control group TNF α increased the content of calcium in mouse neuroblastoma N2a cells, indicating that TNF α interrupted calcium recycling in mouse neuroblastoma N2a cells. Interestingly, Mfn2 overexpression reversed the calcium balance, and this effect was negated in Yap-deleted cells (Fig. 5e, f), indicating that the Mfn2–Yap axis was involved in calcium regulation in the setting of TNF α -induced inflammatory injury.

Oxidative stress was also measured by immunofluorescence. As shown in Fig. 5g, h, compared to the control group TNF α increased the production of ROS in mouse neuroblastoma N2a cells, whereas this effect was significantly repressed by Mfn2 overexpression, as evidenced by the decreased ROS production. Interestingly, loss of Yap abolished the anti-oxidative impact of Mfn2 overexpression on TNF α -treated mouse neuroblastoma N2a cells (Fig. 5g, h). This result was further validated by analyzing the alterations in the levels of cellular antioxidants. As shown in Fig. 5i–k, compared to the control group, TNF α reduced the levels of SOD, GSH and GPX, which is indicative of the inactivation of the anti-oxidative system in the inflammation microenvironment. In contrast, Mfn2 overexpression reversed the content of SOD, GSH and GPX, and this effect could be abolished by Yap deletion, suggesting that Mfn2 reversed the cell anti-oxidative capacity in a mechanism involving the upregulation of Yap (Fig. 5i–k). Taken together, the above data support the functional importance of the Mfn2–Yap cascade in the regulation of TNF α -mediated calcium imbalance and oxidative stress in mouse neuroblastoma N2a cells.

Discussion

Neuroinflammation has been identified as the primary pathogenesis of HD, a neurodegenerative disorder, and anti-inflammatory drugs have been found to be effective

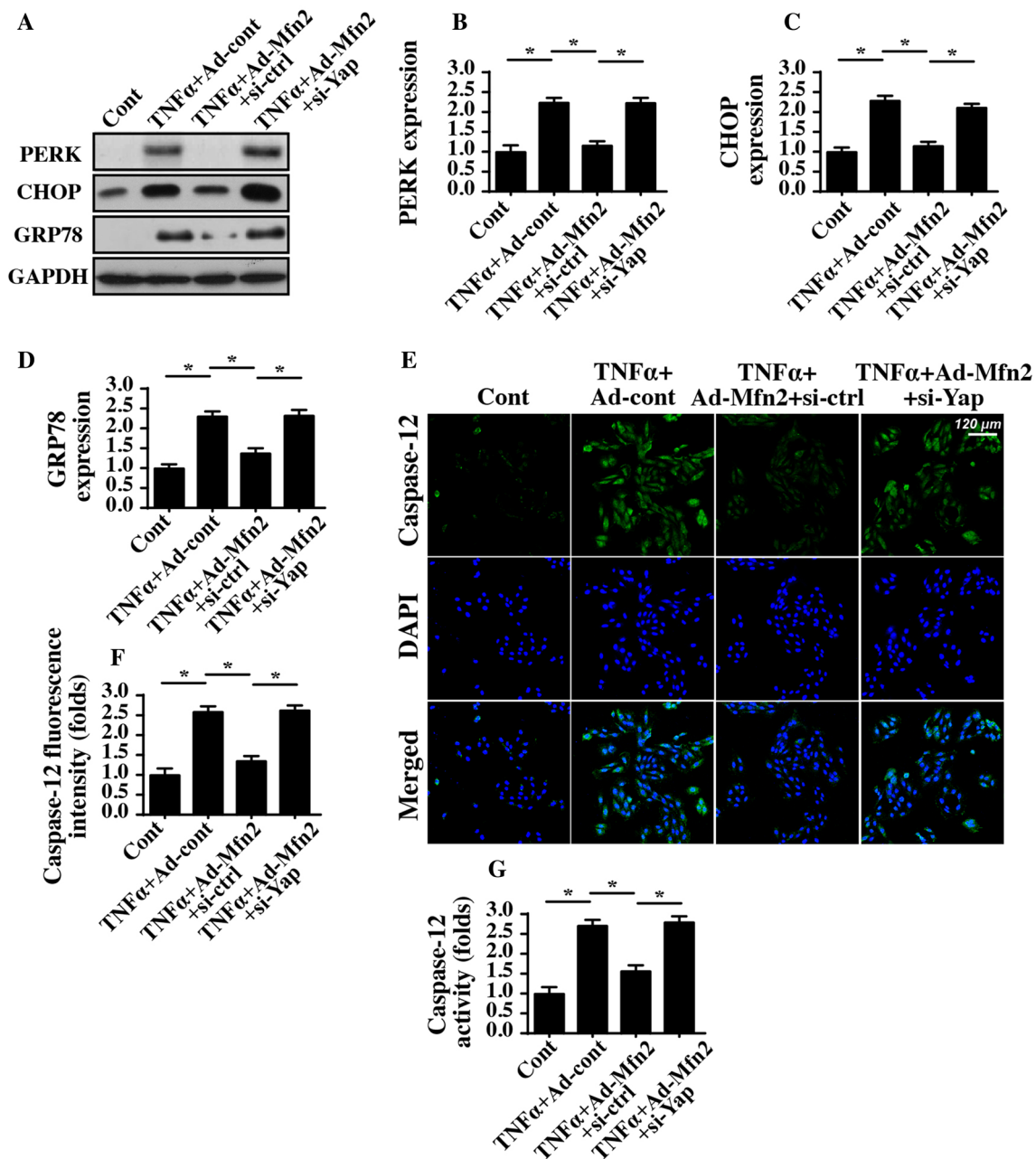


Fig. 4 The Mfn2–Yap signaling pathway modulates endoplasmic reticulum stress in N2a cells subjected to TNF α treatment. **a–d** Proteins were isolated from N2a cells and the expressions of the ER stress proteins PERK, CHOP and GRP78 were measured by western blotting. siRNA against Yap (*si-Yap*) and adenovirus-loaded Mfn2 (*Ad-Mfn2*) were transfected into N2a cells. **e, f** Immunofluorescence assay was used to observe the changes in caspase-12 in N2a cells.

Mfn2 overexpression attenuated caspase-12 activity and this effect could be negated by Yap deletion. **g** ELISA assay was used to verify the activity of caspase-12 in response to Yap deletion and/or Mfn2 overexpression. Data are presented as the mean of three replications with the SEM. Asterisk above columns linked by horizontal line indicates a significant difference between treatments at $p < 0.05$. *Cont* Control

in the treatment of patients with HD. Consequently, in attempts to reduce the progression of HD, the authors of several carefully thought-out studies have tried to determine the molecular mechanism by which inflammation exacerbates neuronal dysfunction [50]. In the present study, we used TNF α to mimic neuroinflammation

in vitro and observed molecular alterations in response to TNF α treatment. We found that Mfn2 expression was downregulated, an effect that was followed by a decrease in Yap expression. Interestingly, Mfn2 overexpression upregulated Yap expression, and the activated Mfn2–Yap signaling pathway promoted neuron survival in

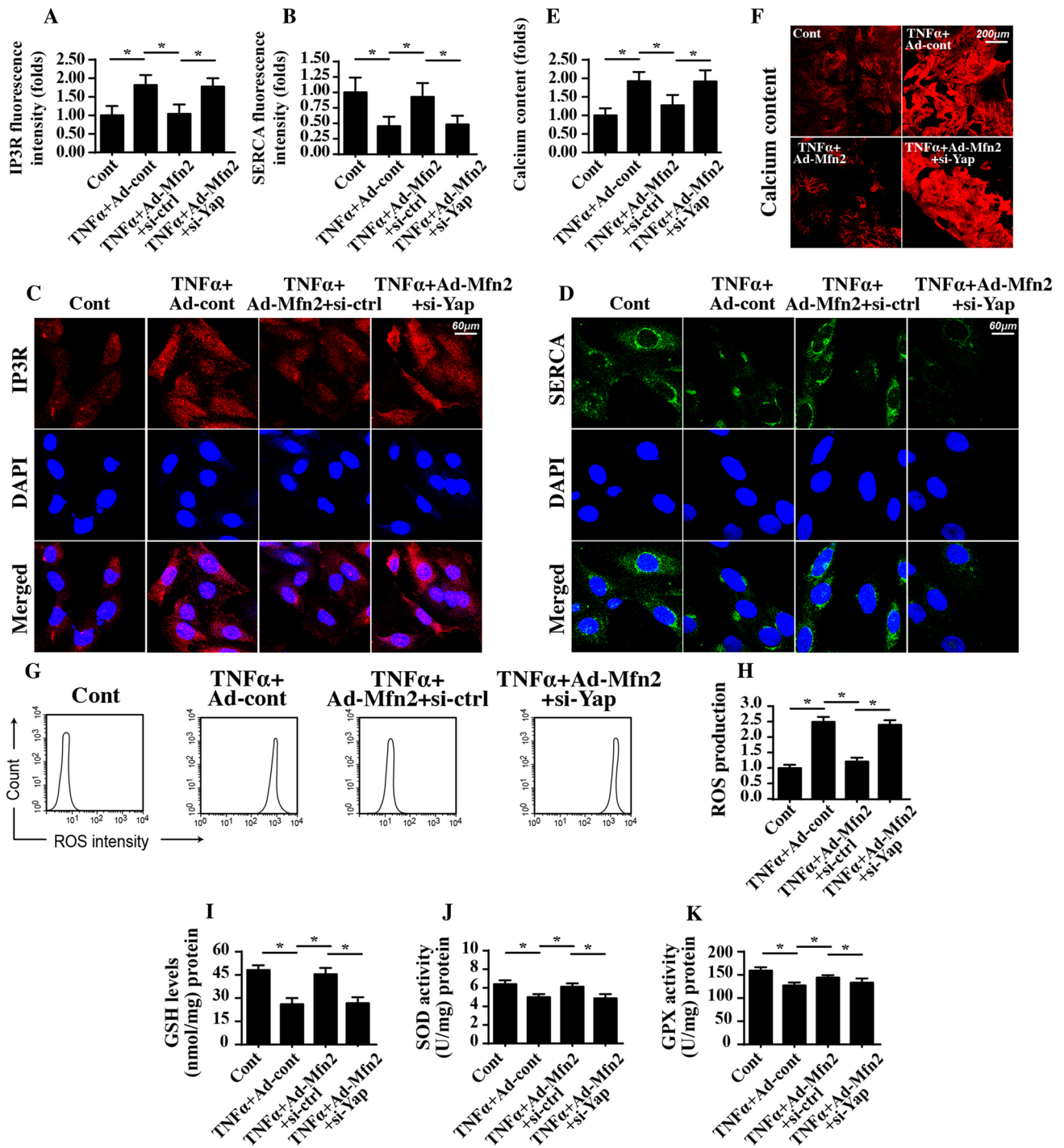


Fig. 5 The Mfn2–Yap signaling pathway affects calcium overloading and oxidative stress in N2a cells. **a–d** Immunofluorescence assay was used to observe the alterations in store-operated calcium entry (*SERCA*) and the inositol trisphosphate receptor (*IP3R*). siRNA against Yap (*si-Yap*) and adenovirus-loaded Mfn2 (*Ad-Mfn2*) were transfected into N2a cells. **e, f** Cellular calcium was labeled with Fura-2AM and then the fluorescence intensity of calcium was measured as a reflection of calcium overloading. **g, h** Reactive oxygen species (*ROS*) production was measured by the immunofluores-

cence assay. siRNA against Yap and adenovirus-loaded Mfn2 were transfected into N2a cells. **i–k** ELISA assay was used to observe the changes in cellular antioxidants. Mfn2 overexpression attenuated TNF α -mediated ROS overproduction and this effect could be negated by Yap deletion. Data are presented as the mean of three replications with the SEM. Asterisk above columns linked by horizontal line indicates a significant difference between treatments at $p < 0.05$. *Cont* Control

the TNF α -induced microenvironment. To our knowledge, this is the first investigation to identify the Mfn2–Yap axis as a novel signaling pathway involved in the progression of HD.

We found that ER stress was activated by inflammation injury, as evidenced by an increase in the level of markers related to ER stress, elevated ROS production and activated caspase-12-related apoptotic pathway. These results indicate that ER stress could be regarded as a primary downstream effector of inflammation injury, and they are in accordance with previously reported results [51, 52]. For example, in a colonic epithelial cell line, inhibition of ER stress attenuated inflammation injury [53]. ER stress has also been observed to modulate the viability of pancreatic β -cells [54], human monocytic leukemia cell [55], alveolar epithelium [56] and bronchial epithelial cells [57] in the context of an inflammation-induced microenvironment. These findings, together with our results, illustrate the functional importance of ER stress in initiating and transmitting the inflammation signals. Our data also indicate that the protective mechanism of anti-inflammation therapy may be associated with the inhibition of ER stress.

We observed that ER stress resulted in the downregulation of the Mfn2 level after exposure to TNF α treatment. However, Mfn2 overexpression attenuated the expression of ER stress-related proteins, and this effect ultimately repressed the activation of caspase-12, sending pro-survival signals to mouse neuroblastoma N2a cells in the inflammatory microenvironment. This finding is consistent with those from previous studies [51, 58]. For example, in HepG2 cells, increased Mfn2 expression attenuates ER stress, sustaining cellular viability and mitochondrial function [59]. In addition, in Parkinson's disease, Mfn2-related ER stress also modulates neurodegeneration in a manner dependent on the PINK1/Parkin pathway [60, 61]. Moreover, in patients with remethylation defects, ER stress is also modified via Mfn2 through autophagy [62]. In brain endothelial cells, Mfn2 affects ER stress and mitochondrial homeostasis through cytoskeletal alterations [34]. To that end, Mfn2 in proopiomelanocortin (POMC) neurons connects ER stress with leptin resistance and energy imbalance. Therefore, we consider that these findings verify that ER stress is a targetable pathogenetic component of the phenotypes caused by Mfn2 downregulation in the setting of neuroinflammation. Not only ER stress but mitochondrial fission also seems to be affected by Mfn2 downregulation in the setting of neuroinflammation. For example, an imbalance in Sirt3 deficiency-mediated brain mitochondrial dynamics seems to be associated with Mfn2 downregulation [63]. Mfn2 dysfunction also contributes to the development of neurodegenerative disorders by inducing mitochondrial damage [64]. Accordingly, more

studies are necessary to puzzle out whether the Mfn2–Yap signaling pathway is also associated with a disordering of mitochondrial dynamics in response to TNF α stress.

Yap is a pro-survival signal for cells subjected to various stress environments. For example, Yap modulates the viability of thoracic cancer by affecting the activity of programmed death-ligand 1 (PD-L1) [65]. The inhibition of Yap expression can activate breast cancer cell apoptosis; and in cardiac reperfusion injury, the Yap–Hippo pathway is inhibited and contributes to the progression of cardiac dysfunction by augmenting cardiomyocytes [66]. Moreover, in prostate cancer, Yap activation modulates docosahexaenoic acid-induced apoptosis in a manner dependent on the FFAR4 pathway [67]. Notably, the beneficial effects of Yap in brain tissues have also been widely explored. The Yap–Hippo pathway is inactivated in cerebral ischemia reperfusion injury, thereby contributing to mitochondrial dysfunction and neuron death. The integrity of the blood–brain barrier is also sustained by Yap and its substrate PIK3CB in subarachnoid hemorrhage. Moreover, neuron differentiation of human pluripotent stem cells is closely affected by Yap. Notably, several studies have indicated that the inflammation response modulates the transcription of Yap and Mfn2, leading to a drop in the expression of Yap and Mfn2. This mechanism may contribute to the downregulation of Mfn2 and Yap in the setting of TNF α stress.

Overall, our findings define a mechanism by which the Mfn2–Yap pathway mediates protection against inflammation-induced apoptosis of mouse neuroblastoma N2a cells *in vitro*. However, there are some limitations to our results. First, only *in vitro* cell experiments were performed, and thus more animal studies and/or animal primary neuron assays are necessary to further support our observations. Second, we did not address the regulatory mechanisms by which Mfn2 modulates the expression of Yap. More studies are required to explore whether Mfn2 affects the transcription of Yap. In addition, although we observed an association between Yap deficiency and increased N2a cell death, it remains unknown whether Yap overexpression is sufficient to confer neuroprotective effects against TNF α similar to those achieved by Mfn2 overexpression.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Data availability All data generated or analyzed during this study are included in this published article.

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