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SOD1^{G93A} Induces a Unique PSAP-Dependent Mitochondrial Apoptosis Pathway via Bax–Bak Interaction

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Abstract: Amyotrophic lateral syndrome (ALS) is a progressive degenerative disorder characterized by motor neuron death and axon degeneration. Mitochondrial dysfunction plays a key role in the pathogenesis of ALS, the mechanism of which remains poorly understood. The B-cell lymphoma-2 (Bcl-2) family of proteins that control and mediate mitochondrial function and apoptosis, including the pro-apoptotic members Bcl2-Associated X (Bax), are involved in ALS development. The death receptor 6 (DR6) regulates motor neuron death in ALS, and DR6 antibodies can prevent axon degeneration and motor neuron damage by blocking DR6. Previous studies demonstrated that PSAP localized to mitochondria and was required for DR6-induced apoptosis. In this study, SOD1^{G93A} was transfected into the motor neuron cell line NSC-34 to serve as an ALS cell model *in vitro*. The data assessed the role of PSAP in SOD1^{G93A}-induced apoptosis and demonstrated that the overexpression of SOD1^{G93A}, but not wtSOD1, induced PARP cleavage, caspase-3 activation, cytochrome c release, and Bax translocation. PSAP, Bax, and Bak were necessary for SOD1^{G93A}-induced apoptosis, as silencing PSAP inhibited SOD1^{G93A}-mediated cell death that was dependent on Bax–Bak interaction.

Introduction

Amyotrophic lateral syndrome (ALS) is a progressive neuronal degenerative disease that causes damage to motor neurons.

ALS can be divided into familial (fALS) and sporadic ALS (sALS) (Wang *et al.*, 2018). fALS accounts for 5–10% of the total number of ALS cases. Mutations in superoxide dismutase 1 (SOD1) account for ~20% of fALS and ~5% of sALS (Alonso *et al.*, 2009). The motor neurons in the brain, brainstem, and spinal cord of patients with ALS are invaded; therefore, the muscles undergo atrophy, weakness, and even paralysis. The onset and early progression of ALS are usually insidious, and neurologists often fail to identify ALS during early disease stages. Following disease progression, up to ~80% of motor neurons are lost when clinical signs of weakness appear (Pasinelli and Brown, 2006). Unfortunately, the etiology of ALS remains unknown. Generally, wild-type SOD1 (wtSOD1) is located in the cytosol, and mutant forms are found within the mitochondria of ALS neurons. SOD1 mutation is the most studied factor in ALS. It is found in about a quarter of fALS cases, and 7% of common sALS cases and human mutSOD1^{G93A}-transgenic mice showed the typical phenotypes of patients with ALS in the clinic.

Mitochondrial dysfunction is critical in the pathogenesis of neurodegenerative disorders, such as ALS and Alzheimer's disease (Chaturvedi and Beal, 2013). Mitochondrial homeostasis regulates neuronal apoptosis via the intrinsic pathway. Indeed, pro-apoptotic members of the Bcl-2 family, such as Bak and Bax, induce mitochondrial cytochrome c (Cyt C) release from the mitochondria into the cytosol, promoting apoptosome formation, including cytoplasmic apoptotic protease activating factor-1 (Apaf-1), Cyt C, and caspase-9, which activates downstream caspases that induce apoptosis (Xu *et al.*, 2017). The decreased expression of anti-apoptotic members of the Bcl-2 family, including Bcl-xl, coupled to the increased expression of Bcl-2-associated death domain (Bad) and Bax, is frequently observed in transgenic

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$SOD1^{G93A}$ mice and patients with ALS. The interaction of mutant SOD1 and Bcl-2 can trigger mitochondrial-dependent neuronal death in ALS. In addition, Bcl-2 overexpression can rescue motor neurons in fALS (Chen *et al.*, 1997). The number of damaged motor neurons, the rescue of axonal degeneration, and muscle and movement activity significantly improved in $Bax^{-/-}$ or P53 up-regulated modulator of apoptosis ($Puma^{-/-}$) mice (Gould *et al.*, 2006; Kieran *et al.*, 2007). However, how mitochondrial dysfunction regulates motor neuron death in ALS remains unknown.

Elevated DR6 protein levels have been detected in $SOD1^{G93A}$ mice and patients with ALS. DR6 antibodies can inhibit motor neuron death via blocking DR6, highlighting the role of DR6 in ALS (Huang *et al.*, 2013). Xu *et al.* (2017) identified presenilin 1 (PS1)-associated protein (PSAP) through its interaction with PS1 via the double yeast hybrid system (Mao *et al.*, 2008; Cozzolino *et al.*, 2013). PSAP was specifically located in the mitochondria and was required for DR6-induced apoptosis (Zhang *et al.*, 2020). This study investigated the role of PSAP in $SOD1^{G93A}$-induced apoptosis in the mouse motor neuron cell line NSC-34.

The present study demonstrated that $SOD1^{G93A}$ overexpression in NSC-34 cells led to the induction of apoptosis. The knockdown of PSAP, Bax, or Bak abolished PARP cleavage and caspase 3 activation, induced by $SOD1^{G93A}$, indicating that PSAP, Bax, and Bak mediated cell death following $SOD1^{G93A}$ overexpression. Furthermore, the immunoprecipitation (IP) analysis showed that PSAP interacted with Bax and Bak on SOD1 overexpression. In addition, PSAP was required for $SOD1^{G93A}$-induced apoptosis, which was dependent on Bax–Bak complex formation.

Materials and Methods

Reagents

Lipofectamine 2000 was purchased from Thermo Fisher (CA, USA). A mitochondria isolation kit was procured from Transgen Biotech (Beijing, China). Complete protease inhibitor cocktail tablets, Cell Counting Kit-8 (CCK-) 8, and anti-Cyt C antibody were purchased from Bimake (TX, USA). Anti-myc, Bak, β-actin, and Cox I antibodies, were purchased from Santa Cruz Biotechnology (TX, USA). An anti-PARP antibody was purchased from Cell Signaling Technology (MA, USA). An anti-Bax antibody was purchased from R&D Systems (MN, USA). Both anti-mouse IgG and anti-rabbit IgG secondary antibodies were purchased from Bioss (Beijing, China). pcDNA3.1/LacZ-Myc-Flag, pcDNA3.1/SOD1-Myc-Flag, and pcDNA3.1/$SOD1^{G93A}$-Myc-Flag plasmids were amplified as described previously (Zeng *et al.*, 2012).

Cell culture and transfection

NSC-34 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone) supplemented with fetal bovine serum (10%), streptomycin (100 μg/mL), and penicillin (100 units/mL) at 37°C and in the presence of 5% CO_2. The cells were transfected with pcDNA3-LacZ, wtSOD1, or $SOD1^{G93A}$ constructs using Lipofectamine 2000 following the manufacturer's protocols.

Cell viability assay

The cell survival activity was measured by the CCK-8 method. Briefly, NSC34 cells were seeded into 24-well plates at a density of 105 cells/mL. The cells were transfected with LacZ, wtSOD1, or $SOD1^{G93A}$ plasmids for 24 h. After 24 h-transfection, the CCK-8 reagent was added and cultured for 0.5–5 h at 37°C. The absorbance was finally measured at 450 nm following the manufacturer's protocols.

Caspase-3 activity

A caspase-3 activity kit was purchased from Bioworld (Nanjing, China). NSC34 cells were seeded into six-well plates at a density of 5×10^5 cells/mL. The cells were transfected with LacZ, wtSOD1, or $SOD1^{G93A}$ plasmids for 24 h. Activated caspase-3 cleaved Ac-DEVD-pNA to generate pNA. The absorbance was measured at 405 nm to indicate caspase-3 activity.

siRNA silencing

siRNA duplexes to PSAP, Bax, and Bak, and scrambled siRNA and RNAi-mate reagent were purchased from Gene Pharma (Shanghai, China). The cells were treated with each siRNA for 48 h and transfected with LacZ, wtSOD1, or $SOD1^{G93A}$ plasmids using Lipofectamine 2000 reagent. The cells were harvested and lysed for further analysis 24 h after transfection.

Subcellular fractionation and mitochondrial preparation

The cytosolic and mitochondrial fractions were extracted using the mitochondria isolation kit following the manufacturer's protocols to explore Cyt C release and Bax translocation.

Flow cytometry

Cell apoptosis was determined using an Annexin V/propidium iodide (PI) kit and analyzed by flow cytometry. The cells were harvested after 24 h transfection, washed twice with an ice-cold phosphate-buffered solution (PBS), and stained with Annexin V/PI solution.

Western blot analysis, and IP

IP and immunoblotting were performed as previously described (Zeng *et al.*, 2012).

For Western blot analysis, cells were harvested and lysed by sonication for 25 s on ice in lysis buffer. Denatured protein (20 μg) ofeach sample was separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked with 5% skim milk powder in Tris-buffered saline tween (TBST) for 45 min. The protein blot was then incubated with appropriate primary antibodies and HRP-conjugated secondary antibody separately. For immunoprecipitation (IP), cells were harvested and lysed by sonication for 20 s on ice in IP lysis buffer, and samples were centrifuged at 14,000 × *g* for 5 min at 4°C. Then the supernatants were incubated with anti-myc, anti-PSAP, or anti-Bax together overnight at 4°C. The samples were added with protein A-sepharose (Bimake, TX, USA) for 2 h. After washing five times with cold PBS, the immunoprecipitates were separated by SDA-PAGE and incubated with the primary antibodies.

Statistical analysis

The data were analyzed by using SPSS 22.0 statistical analysis software, and groups of data are presented as mean ± standard

deviation (SD).Statistical significance was performed on the data by analysis of variance for significance test. $^{**}P < 0.01$ was used as the criterion for determining statistical difference.

Main Text

SOD1^{G93A} overexpression induced apoptotic cell death in NSC-34 cells

NSC-34 cells were transfected with flag-tagged LacZ or wtSOD1, SOD1^{G93A} to investigate whether SOD1^{G93A} promoted apoptosis. Fig. 1A shows the percentage of viable cells after the transfection of LacZ, wtSOD1, and SOD1^{G93A}. The results indicated that the overexpression of SOD1^{G93A} decreased cell survival by ~45% compared with lacZ- or wtSOD1-transfected cells ($P < 0.01$).

As shown in Figs. 1B and 1C, SOD1^{G93A} triggered PARP cleavage after 24 h-transfection. In contrast, no apoptotic events were observed on the transfection of LacZ or wtSOD1 constructs. Annexin V/PI double staining assay was performed to detect cells in the necrotic or apoptotic stage to further confirm the apoptotic activity of SOD1^{G93A}. As shown in Fig. 1D, the number of apoptotic and necrotic cells was ~40% (B2 and B4) in SOD1^{G93A}-transfected cells, while 5% of cells were B4 (Annexin V$^+$/PI$^-$) or B2 (Annexin V$^+$/PI$^+$) in LacZ- or wtSOD1-transfected cells, suggesting low levels of cell death. Taken together, these results strongly suggested that the overexpression of SOD1^{G93A} induced apoptosis in NSC-34 cells.

SOD1 ^{G93A} -induced apoptosis was mediated in Cyt C release and Bax translocation

Since mitochondrial damage promotes motor neuron cell death, the study next examined whether SOD1^{G93A} induced the release of mitochondria-related molecules into the cytoplasm. As shown in Fig. 2, increased levels of Cyt C were detected in cytosolic extracts, while a concomitant decrease in the levels of these molecules was observed in the mitochondrial fractions on SOD1^{G93A} overexpression ($P < 0.01$). In contrast, Bax expression reduced in the cytosolic fractions and was higher in the mitochondrial fraction on exogenous SOD1^{G93A} expression ($P < 0.01$).

Silencing of Bax and Bak attenuated SOD1^{G93A}-induced apoptosis

SOD1^{G93A} triggered Cyt C release and Bax translocation regulated by the Bcl-2 family of proteins, which are known regulators of the mitochondrial apoptotic pathway. The study therefore determined the effects of Bax and Bak on SOD1^{G93A}-induced apoptosis. Prior to SOD1^{G93A} overexpression, the cells were silenced with Bax, Bak-specific siRNAs, or scrambled controls. The cell viability was then assessed following the transfection of LacZ, wtSOD1, and SOD1^{G93A}. Fig. 3A shows that both Bax and Bak were involved in SOD1^{G93A}-induced apoptosis as their silencing enhanced NSC-34 cell viability compared with nonsilencing cells ($P < 0.01$). As shown in Fig. 3B, SOD1^{G93A} increased the activity of caspase-3 by ~40% compared with LacZ-transfected cells. However, SOD1^{G93A}-triggered caspase-3 activation was inhibited by Bax or Bak silencing. Furthermore, as shown in Figs. 3C, 3E, and 3F, SOD1^{G93A}-induced PARP cleavage was completely ablated following Bax or Bak silencing. The effect of Bax- or Bak-silenced cells on SOD1^{G93A} overexpression-induced cell death was confirmed by Annexin V/PI staining (Fig. 3G), indicating that the percentage of apoptotic cells was ~60% (B2 and B4) following SOD1^{G93A} overexpression, which decreased to

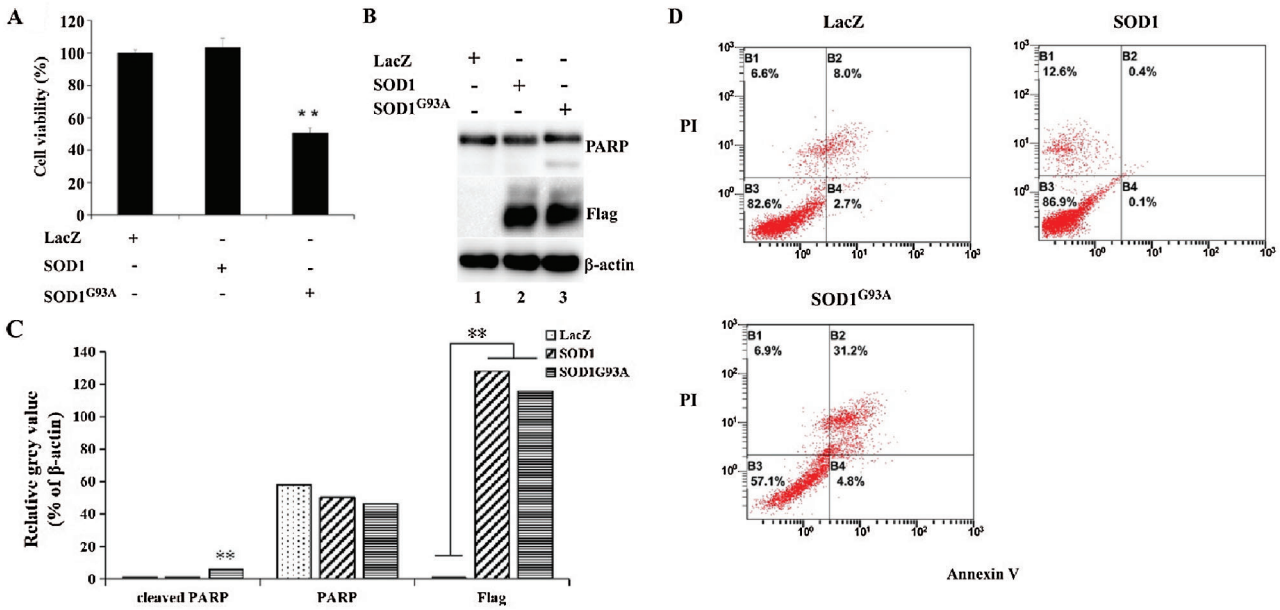

FIGURE 1. SOD1^{G93A} overexpression induced apoptotic cell death in NSC-34 cells. (A) NSC-34 cells were transfected with LacZ, wtSOD1, and SOD1^{G93A} for 24 h, and the cell viability was assessed using CCK-8 assay. Experiments were performed in triplicate. Data represent the mean ± standard deviation. $^{**}P < 0.01$, significant difference between SOD1^{G93A}-transfected and LacZ-transfected groups. (B) Top panel: PARP; Panel 2: wtSOD1 or SOD1^{G93A}-flag. β-actin was used as a loading control. (C) Experiments were performed in triplicate. Data represent the mean ± standard deviation. $^{**}P < 0.01$, significant difference between SOD1^{G93A}-transfected and LacZ-transfected groups. (D) Percentage of apoptotic cells was determined in LacZ-, wtSOD1-, and SOD1^{G93A}-transfected cells using Annexin V/PI staining and flow cytometry analysis.

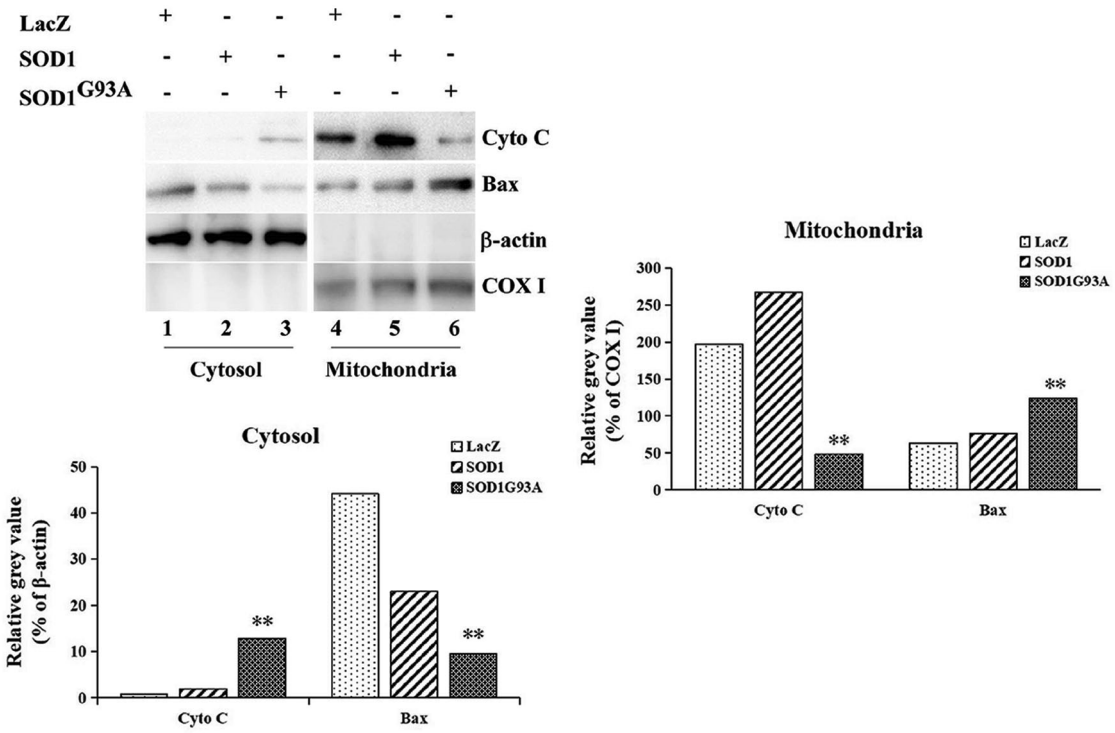

FIGURE 2. SOD1^{G93A}-induced apoptosis involved cytochrome c release and Bax translocation. LacZ, wtSOD1, or SOD1^{G93A} were transiently transfected for 24 h. The cells were then subjected to cytosolic (left panels) and mitochondrial extractions (right panels) as described under "Experimental Procedures." Top panel: Cyt c release. Panel 2: Bax translocation. Panel 3: Bak release. Panel 4: β-actin. Bottom panel: COX I was only detected in the mitochondrial fraction, confirming the accuracy of the experimental procedures.Data presented as mean ± SEM. ANOVA, $^{**}P < 0.01$ compared with control.

~10% in Bax- or Bak-silenced cells. These data indicated that both Bax and Bak were required for SOD1^{G93A}-induced apoptosis.

PSAP silencing abolished SOD1^{G93A}-induced apoptosis

PSAP specifically localized to the outer membrane of the mitochondria and was associated with Bax or Bak. In addition, PSAP was required for DR6-induced apoptosis (Zhang *et al.*, 2020). DR6 was highly expressed in samples from mice and patients with ALS and played a crucial role in ALS development. The effects of DR6 on PSAP-specific siRNAs were assessed to further determine the role of PSAP in SOD1^{G93A}-induced apoptosis. Similarly, the silencing of PSAP attenuated SOD1^{G93A}-triggered cell death (Fig. 3A), PARP cleavage (Figs. 3C and 3F), and caspase-3 activation (Fig. 3B). Annexin V/PI staining confirmed these effects. Taken together, these data revealed that PSAP was required for SOD1^{G93A}-induced apoptosis (Fig. 3G).

PSAP regulated SOD1^{G93A}-induced apoptosis through Bax–Bak binding

The possible interaction of Bax, Bak, and PSAP upon SOD1^{G93A} overexpression was explored to further investigate the mechanism by which Bax, Bak, and PSAP mediated SOD1^{G93A}-induced apoptosis. As shown in Fig. 4A, Bax co-immunoprecipitated with SOD1-myc using anti-myc antibodies (lanes 3 and 4, top panel) but not IgG (lane 5) on transfection with wtSOD1 or SOD1^{G93A}. The levels of this complex modestly decreased in SOD1^{G93A}-transfected cells (lane 4) compared with wtSOD1-transfected cells (lane 3). Similarly, Bak co-immunoprecipitated with anti-myc (SOD1), but the interaction did not increase in cells overexpressing SOD1^{G93A}. Furthermore, only a negligible change in the levels of PSAP–Bax or PSAP–Bak complex formation was observed on SOD1^{G93A} transfection compared with LacZ-transfected cells. On transfection with wtSOD1, Bax co-immunoprecipitated with PSAP, while PSAP–Bak complex formation decreased. In addition, Bak–Bax complexes were detected in SOD1^{G93A}-expressing cells (lanes 4 and 5). The effects on PSAP-specific siRNAs were assessed to further confirm the role of PSAP in SOD1^{G93A}-induced Bak–Bax interaction. Similarly, the silencing of PSAP (Figs. 4C and 4D) attenuated SOD1^{G93A}-triggered Bak–Bax complex formation (lane 7 compared with lane 4, Fig. 4B). These data indicated that PSAP regulated SOD1^{G93A}-induced apoptosis via Bax–Bak interaction.

Discussion

This study demonstrated that a specific mitochondrial protein PSAP played an important role in SOD1^{G93A}-induced apoptosis through Bax-Bak interaction. These findings provided new insights into the role of PSAP in motor neuron death and axon degeneration in ALS, highlighting the potential of PSAP as a novel therapeutic target for ALS treatment and prevention.

ALS is a neurodegenerative disease characterized by motor neuron death and axon degeneration that rapidly

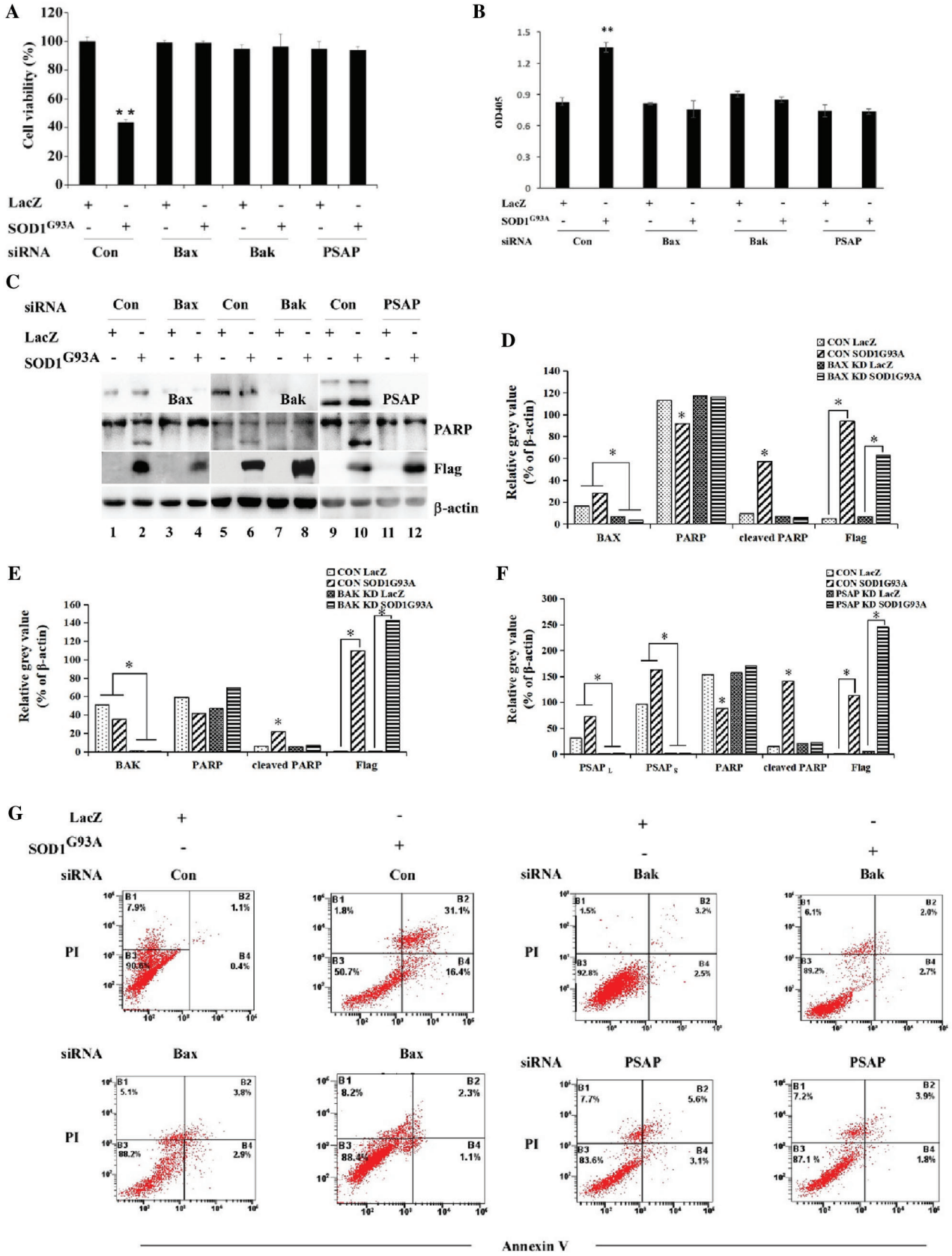

FIGURE 3. Silencing of Bax, Bak, and PSAP attenuated SOD1^{G93A}-induced apoptosis. As indicated, NSC-34 cells were transiently transfected with LacZ, wtSOD1, or SOD1^{G93A}, respectively, 48 h after the transfection of scrambled Bax-, Bak-, and PSAP-specific siRNAs. (A) The cells were harvested for CCK-8 assays. Experiments were performed in triplicate. Data represent the mean ± standard deviation. $^{**}P < 0.01$. (B) Caspase-3 activity in cells treated with specific siRNAs to Bax, Bak, or PSAP on SOD1^{G93A} transfection using caspase-3 activity detection kits. (C) Top panel: knockdown efficiency of Bax, Bak, and PSAP. Second panel: probed with anti-PARP antibodies. Third panel: transfection efficiency of SOD1^{G93A} using anti-flag antibodies. Bottom panel: β-actin as a loading control. (D), (E), and (F) Data are presented as mean ± SEM. ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control. (D) for Bax, (E) for Bak, (F) for PSAP. (G) Percentage of apoptotic cells determined following LacZ, SOD1, and SOD1^{G93A} transfection on Bax, Bak, or PSAP silencing using Annexin V/PI staining and flow cytometry analysis.

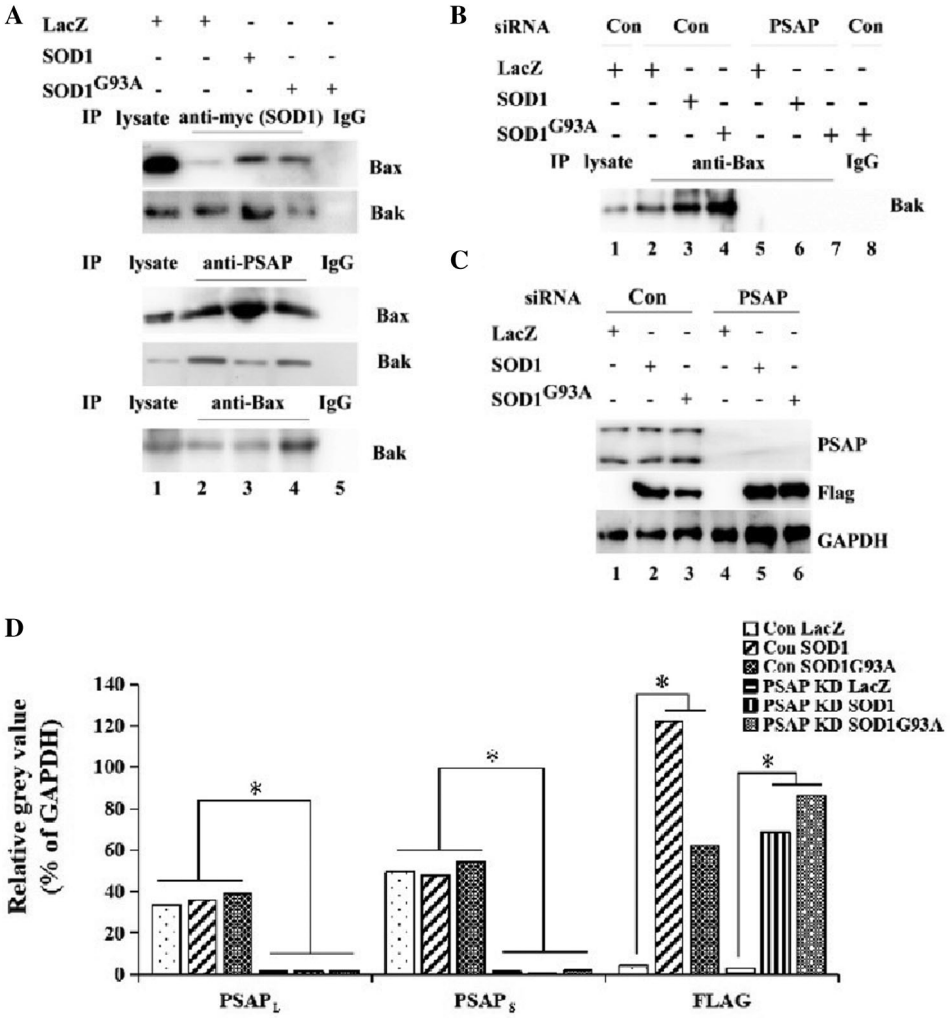

FIGURE 4. PSAP was involved in SOD1^{G93A}-induced Bax–Bak complex formation required for SOD1^{G93A}-induced apoptosis. (A) Both wtSOD1 and SOD1^{G93A} complexed with Bax and Bak and induced the formation of PSAP-Bax, PSAP-Bak, or Bax-Bak complexes. The cell lysates were immunoprecipitated after transfection with LacZ (lane 2), wtSOD1 (lane 3), or SOD1^{G93A} (lane 4) using anti-Myc antibodies. Immunoprecipitations were probed with anti-Bax (top panel) or anti-Bak (Panel 2) antibodies. The cell lysates were also immunoprecipitated with anti-PSAP antibodies and probed with anti-Bax (Panel 3) and anti-Bak antibodies (Panel 4). Finally, the cell lysates were immunoprecipitated with anti-Bax antibodies and probed for Bak (bottom panel). Lane 1: positive control. Lane 7: SOD1^{G93A}-transfected cells were immunoprecipitated with IgG as a negative control. (B) As indicated, NSC-34 cells were transiently transfected with LacZ (lanes 2 and 5), wtSOD1 (lanes 3 and 6), or SOD1^{G93A}(lanes 4 and 7), respectively 48 h after the transfection of scrambled PSAP-specific siRNAs (lanes 5, 6, and 7).The cell lysates were immunoprecipitated using anti-Bax antibodies. Immunoprecipitations were probed with anti-Bak antibodies. Lane 1: positive control. Lane 8: SOD1^{G93A}-transfected cells were immunoprecipitated with IgG as a negative control. (C) Top panel: knockdown efficiency of PSAP. Second panel: transfection efficiency of SOD1^{G93A} using anti-flag antibodies. Bottom panel: GAPDH as a loading control. (D) Datapresented as mean ± SEM. ANOVA, $^{*}P < 0.05$ compared with control.

progresses after onset (Rowland and Shneider, 2001). Statistically, the average survival time of patients with ALS is ~18 months (Bonafede *et al.*, 2016). Studies have shown that oxidative stress, excitotoxicity, inflammation, and apoptosis can induce ALS (Krishnaraj *et al.*, 2016). The mechanisms of motor neuron death remain unclear, and hence current therapeutic strategies are limited. Riluzole and edaravone are approved by the Food and Drug Administration for treating ALS (Dash *et al.*, 2018; Jaiswal, 2019). Although they delay the development of ALS and prolong the life of patients by months, the benefits are negligible. Huang *et al.* (2013) demonstrated that DR6 was involved in the etiology of motor neuron death and axon degeneration, and that blocking DR6 with antibodies promoted motor neuron survival, improved motor neuron function, and protected neuromuscular junction integrity. Further evidence indicated that DR6 played a pivotal role in neuronal cell death and induced apoptosis through its interaction with Bax via a unique mitochondrial pathway (Zeng *et al.*, 2012).

PSAP, also known as mitochondrial carrier homolog 1 (MTCH1) due to its homology with a mitochondrial carrier protein, was identified as a specific mitochondrial resident protein and shown to induce apoptosis via Apaf-1 and Smac (Li *et al.*, 2013). Another mitochondrial carrier homolog, mitochondrial carrier homolog 2 (MTCH2), also known as met-induced mitochondrial protein, has been reported to interact with tBid in the mitochondria, forming a protein complex containing Bax (Lamarca *et al.*, 2008). PSAP is the key to enhancing the function of the permeability transition

pore (MOMP), which is a target of mitochondrial pro-apoptotic proteins or other related proteins. However, the mechanism(s) by which PSAP regulates mitochondrial apoptosis remains elusive (Lamarca, *et al.*, 2008). Previous data demonstrated that PSAP -Bax complex formation was detected on the overexpression of DR6 or following Tumor Necrosis Factor α (TNFα) (Zhang *et al.*, 2020). More interestingly, PSAP was crucial for DR6-induced apoptosis. DR6 triggered PARP cleavage, caspase activation, Cyt C release, and Bax translocation following PSAP silencing in vitro. Transgenic mice carrying mutant human SOD1, particularly human SOD1^{G93A}, were similar to patients with ALS in the clinic and hence could be used as reliable fALS models to dissect the mechanisms of cell death (Xie *et al.*, 2015). Thus, NSC-34 cells were transfected with SOD1^{G93A} to serve as an ALS cell model in vitro. PSAP specifically localized on the mitochondria, and Bcl-2 family members, including Bax and Puma, were associated with the pathology of ALS (Gould *et al.*, 2006; Kieran *et al.*, 2007). The study first examined the role of Bcl-2 family members in SOD1^{G93A}-induced apoptosis. CCK-8, PARP cleavage, and Annexin V/PI double staining showed that both Bax and Bak played important roles in SOD1-induced apoptosis, as SOD1^{G93A}-induced cell death was inhibited by Bax or Bak silencing. The study assessed cell activity via CCK-8 assay, PARP cleavage via Western blot analysis, and Annexin V/PI staining via flow cytometry using PSAP siRNA to determine the effects of PSAP on ALS. Similar to DR6, the silencing of PSAP attenuated SOD1^{G93A}-induced apoptosis.

Oxidative stress, excitotoxicity, inflammation, loss of growth factors, and apoptosis induced ALS. Interestingly, these processes were either directly or indirectly related to mitochondrial function. Mitochondria are crucial for ATP production through oxidative phosphorylation and are critical for neuronal function. Mitochondrial dysfunction results in the loss of ATP production and reactive oxygen species (ROS) generation, promoting cell death. Mitochondrial damage therefore leads to neurodegenerative diseases (Xie *et al.*, 2015). Mutant SOD1 was first identified by Rosen et al. in 1993 and was shown to be involved in ALS. To date, ≥130 mut SODl cell and animal models have been established for clinical research and drug development. Despite the initial promise, SOD1 gene therapy for patients with ALS has proved ineffective. SOD1^{G93A} and other mutation models still hold an important research value because ALS is not a simple motor neuron disease, as changes in non-motor neuron cells also contribute to disease pathogenesis (Reyes *et al.*, 2010). ALS-related genes, including wtSOD1 and mutSOD1, are associated with mitochondrial dysfunction (Cozzolino *et al.*, 2013; Xie, *et al.*, 2015). The key mitochondrial regulatory factors, the Bcl-2 family, also play an important role in SOD1^{G93A}-induced apoptosis, including Bax and Bak, which regulate the intrinsic mitochondrial apoptosis pathway. Bax and Bak shuttle between the cytosol and the mitochondria outer membrane (MOM) in healthy cells (Todt *et al.*, 2015). In response to an apoptotic stimulus, Bax and Bak are activated and accumulate on the MOM, triggering MOMP, leading to Cyt C release, capsase activation, and apoptosis (Pena-Blanco and Garcia-Saez, 2018). In addition, the mitochondrial apoptosis -induced channel (MAC) was identified as an outer membrane channel associated with apoptosis, although the role of Bax and Bak in MAC regulation remains undefined (Kroemer *et al.*, 2007).

IP was performed to determine the relationship between SOD1, PSAP, Bax, and Bak so as to further explore the mechanism of PSAP regulating SOD1^{G93A}-induced apoptosis. A higher number of SOD1 -Bax complexes were evident after the overexpression of wtSOD1, while SOD1 -Bax complex formation decreased in response to SOD1^{G93A} overexpression. Bax translocation from the cytosol into the mitochondria was also observed in SOD1^{G93A} but not wtSOD1-overexpressing cells. Generally, SOD1 localized to the cytosol, and SOD1 -Bax complexes were abundant following wtSOD1 transfection. Higher levels of Bax translocated into the mitochondria; SOD1^{G93A} transfection explained the decreased SOD1-Bax interaction observed in cells expressing this mutant. The overexpression of SOD1 could disrupt Bax-Bak, PSAP-Bax, and PSAP-Bak complex formation in wtSOD1-transfected cells. In contrast, only slight differences in PSAP-Bax or PSAP-Bak complex formation were observed on SOD1^{G93A} overexpression. However, SOD1^{G93A} dramatically increased the number of Bax-Bak complexes, thereby promoting apoptosis. Furthermore, the knockdown of PSAP inhibited SOD1^{G93A}-induced Bax-Bak complex formation. These data suggested that PSAP regulated the formation of the Bax-Bak complex, either directly or indirectly, and mediated its effects on apoptotic induction through MOMP.

In summary, the results indicated that the mitochondrial protein PSAP was required for SOD1^{G93A}-induced apoptosis mediated through mitochondrial apoptosis-induced channels. PSAP is pivotal for Bax-Bak interaction, leading to caspase activation and apoptosis. Inhibiting these activities of PSAP therefore represents a potential approach for treating ALS.

Abbreviations: ALS, Amyotrophic lateral syndrome; Cyt C, cytochrome c; DR6, death receptor 6; PS1, presenilin 1; PSAP, PS1-associated protein; SOD1, superoxide dismutase 1; Bax, Bcl2-Associated X; Puma, P53 up-regulated modulator of apoptosis.

Availability of Data and Material: All data generated or analyzed during this study are included in this published article.

Author Contribution: Study conception and design: Linlin Zeng, Fuqiang Zhang. Performed the experiments: Han Niu, Xin Chen, Jingtian Zhang, Yuxiang Wang, Jiayue Song, Yu Wang. Data collection: Han Niu, Xin Chen, Jingtian Zhang. Analysis and interpretation of results: Han Niu, Xin Chen, Xueting Ma, Xueqi Fu, Chen Hu, Xuemin Xu. Funding acquisition: Guodong Li, Fuqiang Zhang, Linlin Zeng. Writing-Original draft preparation: Linlin Zeng, Han Niu. Writing-Reviewing and editing: Fuqiang Zhang, Xueqi Fu, Xuemin Xu.

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