# Elevating sestrin2 attenuates endoplasmic reticulum stress and improves functional recovery through autophagy activation after spinal cord injury

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**Research**

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# Abstract

Background Spinal cord injury (SCI) is a devastating central neurological trauma that causes a loss of motor and sensory function. Sestrin2, also known as hypoxia inducible gene 95 (Hi95), is emerging as a critical determinant of cell homeostasis in response to cellular stress. However, the role of sestrin2 in neuron response to endoplasmic reticulum (ER) stress and potential mechanism remains undefined. In this study, we investigated the effects of sestrin2 on ER stress and delineated a underlying molecular mechanism after SCI. Methods The induction of sestrin2 after traumatic injury and ER stress insult were investigated in vitro and in vivo. West blot and immunofluorescence were used to analyze the potential mechanisms of sestrin2 on autophagy and ER stress after SCI. Behavior assessment were used to evaluated the effect of sestrin2 on function recovery in vivo. Results Elevated sestrin2 is a protective process in neurons against chemical ER stress induced by tunicamycin (TM) or traumatic invasion. While treatment with PERK inhibitor or knockdown of ATF4 reduces sestrin2 expression upon ER stress. In addition, overexpression of sestrin2 limits ER stress, promotes the survival of neuron and improves functional recovery after SCI, which is associated with activation of autophagy and restoration of autophagic flux mediated by sestrin2. Meanwhile, sestrin2 activates autophagy dependent on AMPK-mTOR signaling pathway. Consistently, inhibition of AMPK abrogates the effect of sestrin2 on activation of autophagy, and blockage of autophagic flux abolishes the effect of sestrin2 on limiting ER stress and neural death. Conclusion Upregulation of sestrin2 as an important resistance mechanism of neuron to ER stress, and its potential role as a therapeutic target for SCI.


# Background

Traumatic spinal cord injury (SCI) is a devastating central neurological disorder that leads to sensory deficits and physical disabilities and affects thousands of individuals worldwide [1]. The physiological progression of SCI is characterized by a series of secondary molecular events including inflammatory response, mitochondrial dysfunction and oxidative stress, axonal demyelination, which cause neuron death and injury beyond the initial mechanical damage [2]. Despite there is yet no completely satisfactory therapy for the treatment of SCI in clinical trials, separately or combined targeting these secondary reactions have been regarded as proper therapeutic strategies and promoted functional recovery in almost animal cases [3-6].

As a stress inducible protein induced upon various stresses, sestrin2 is essential for maintaining cell metabolism and homeostasis by regulating a series of kinases and pathways, and loss of sestrin2 can result in metabolic disturbance and mitochondrial dysfunction[7-9]. Sestrin2 has been shown to be capable against oxidative stress and apoptosis by regulating mammalian target of rapamycin complex (mTOR) by activating AMP-activated protein kinase (AMPK). Importantly, sestrin2 is also known as hypoxia inducible gene 95 (Hi95) and originally discovered in hypoxia condition, and the responsive induction of sestrin2 is associated with HIF-1α stabilization [7, 10]. Extensive evidences confirmed that upregulated sestrin2 protect heart against ischemic heart injury, alleviates insulin resistance, and delays the process of neurodegenerative diseases [11-13].

In addition, numerous evidences implicated that sestrin2 provides beneficial effects on acute central nervous system injury. As previous study reported, sestrin2 was upregulated in cortical region in an acute stroke model in rats [14]. Further studies revealed that silence of sestrin2 aggravates cerebral infarct but overexpression of sestrin2 enhanced angiogenesis and attenuates focal cerebral ischemic injury under ischemic condition, the potential mechanisms are related to the phosphorylation of AMPK and activation of nuclear factor erythroid 2 related factor 2 (Nrf2) [15, 16]. Previous studies have indicated that neuron is vulnerable to the various stresses stimulation induced by the fierce damage of microcirculation and mechanical destruction, maintaining neuron homeostasis is a feasible method for functional recovery[17, 18]. As the versatile function of sestrin2 exhibited above, we hypothesized that sestrin2 is highly advantageous to neuron homeostasis in spinal cord. However, the role of sestrin2 in neuron response to traumatic SCI is not well-elucidated.

The endoplasmic reticulum (ER) serves the major site for protein synthesis and controls the quality of newly synthesized [19]. The perturbation of ER environment homeostasis lead to overabundance of misfolded protein and ER stress [20, 21]. Though unfolded protein response (UPR) provides effect on attenuating misfolded proteins, durative ER stress without treatment ultimately exceeds the compensation and turns UPR into pro-death [22, 23]. Accordingly, maintaining ER homeostasis is available for neuron regeneration and function [24, 25]. In particular, a study reported by park et al [26] showed that ER stress suppressed by sestrin2 is important for hepatic rehabilitation. Therefore, we reasoned that sestrin2 plays a critical role in regulating neural ER stress. Numerous evidences indicated that a link between UPR and autophagy, in which the initiated of ER stress often stimulates the autophagic activity and autophagy acts as a compensatory protection for eliminating aggregated misfolded proteins [27-29]. Meanwhile, it has been found that sestrin2 can activate autophagy under various pathological stresses and deficient of sestrin2 results in impairment of autophagy [9, 30]. As described previously, ER stress limited by sestrin2 may related to the activation of autophagy. However, the relationship of sestrin2, autophagy and ER stress in neural injury remains elusive.

Given that sestrin2 performs such critical and versatile physiological functions and prevents the progression of diverse pathologies, we choose to determine the role of sestrin2 in ER stress and autophagy in neuron and therapeutic effect on SCI. In the current study, we hypothesized that ER stress is involved in sestrin2 regulation, the induction of sestrin2 would activate autophagy, enhance ER homeostasis and reduce neurons apoptosis in a mouse model of acute SCI for decreasing damage size, enhancing neural regeneration and function recovery.

# Materials And Methods

### Reagents and antibodies

Reagents including tert-Butyl hydroperoxide solution (TBHP), GSK2606414, KIRA6, bafilomycin-A1, tunicamycin (TM), chloroquine (CQ) and dorsomorphin (Compound c, BML-275) were purchased from MCE (Monmouth Junction, NJ, USA) and dissolved in DMSO. Antibodies against sestrin2, NeuN, Tuj1,

PERK, ATF4, ATF6, GRP78, PDI, caspase12, p62, Lamp2, p-ULK1 were purchased from Abcam (Cambridge, MA, USA), antibodies against p-PERK, CHOP, CTSB, p-AMPK, AMPK, were obtained from Cell Signaling Technologies (Danvers, MA, USA), antibodies against p62, Lamp2, Bax, Bcl2, GAPDH and ULK1 were acquired from Proteintech (Chicago, IL, USA), an antibody against cleaved caspase 3 was purchased from Affinity (Cincinnati, OH, USA), an antibody against LC3 was purchased from Novus (Littleton, Co, USA), antibodies against IRE1α, p-mTOR, mTOR and ubiquitinated proteins were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Alexa Fluor 488-labeled and Alexa Fluor 647-labeled goat anti-rabbit/mouse/rat secondary antibodies were purchased from Abcam (Cambridge, MA, USA). 4,6-Diami- dino-2-phenylindole (DAPI) was obtained from Beyotime (Shanghai, China). The reagents for PC12 cells and neuron cultures were obtained from Gibco (Grand Island, NY, USA) and Sigma-Aldrich (St. Louis, MO, USA).

### Mice spinal cord injury model and drug administration

Briefly, adult C57BL/6 mice (20-25g) were anesthetized with 4% (w/v) chloral hydrate (3.5 mL/kg, i.p.), a laminectomy was performed at T9 level after incision of skin and muscle adjacent to the spinous processes. For SCI group, the exposed spinal cord was subjected to a moderate crush injury using a 10 g weight from a height of 20 mm using a MASCIS impactor [31]. After muscle and skin was sutured, mice were received 0.5 mL normal saline and returned to a warm blanket for postoperative recovery. For the control group, a T9 laminectomy was performed without crush injury.

To induce chemical ER stress in the spinal cord of mice, tunicamycin saline (1mg/kg/day) was applied to mice by intraperitoneal injection for 2 days [32]. To inhibit lysosomal function, bafilomycin-A1 (0.3 mg/kg) was injected intraperitoneally daily from the 7th day after AAV injection to end of transfection [31]. Chloroquine (50mg/kg) was administered intraperitoneally daily starting from 3 days before SCI and continued until the mice were euthanized [33]. Compound c (10mg/kg/day, i.p.) was applied to mice from the 7th day after AAV injection to end of transfection [34].

### Adeno-associated viral (AAV) and intrathecal injection.

AAV vectors containing the genes for sestrin2 and empty vectors were constructed by GeneChem (GOSV0196538, Shanghai, China).Virus delivery by intrathecal injection was performed in adult mice using a method as described previously [35]. Following anesthetized with chloral hydrate by intraperitoneal injection, the lamina of L5 and L6 region was exposed and a small laminectomy was made for exposure of spinal dura mater. AAV particles at $5x10^{12}$ were injected in 2ul of viral solution per site using a 10 μL Hamilton syringe with a 32-gauge needle at rate of 0.2ul/min. After injection and suture finished, mice were put on a warm blanket for postoperative recovery. Any mice exhibiting hind limb paralysis or paresis after surgery were excluded from the study.

### Cell culture and drug administration

PC12 cells and primary cortical neurons were applied in this study. PC12 cells (Cell Storage Center of Wuhan University, Wuhan, China) were cultured and maintained in RPMI1640 medium with 10% foetal bovine serum (FBS) and 1% antibiotic antimycotic at 37°Cwith 5% $CO_2$. Primary cortical neurons were postnatal Day 0 (P0) mouse pups by dissecting the cerebral cortex as described previously[3]. Neurons were seeded on poly-D-lysine coated 12-well plate at a density of 3-5×$10^5$ cells per well and incubated in neurobasal medium supplemented with 2% B27 and 1% glutamine at 37 °C in a humidified 5% $CO_2$-containing atmosphere. Half of the culture media was replaced with fresh on day 2 and every 3 days and neurons were cultured for additional seven days before use. For injury stimulation and chemical ER stress in vitro, cells were respectively treated with TBHP (50μM) and tunicamycin (2μg/mL) in a time dependent manner as designed.

### Small interfering RNA (siRNA) transfection

The siRNA targeting mouse ATF4, ATF6 and the negative control siRNA were obtained from Genepharma (A10001; Shanghai, China). Cultured PC12 cells were transfected following manufacturer's instructions. In brief, cells were seeded on a 6-well plate and cultured to 60% confluence, and reverse transfected 25 nM negative control or siRNA duplexes using Lipofectamine 2000 siRNA transfection reagent (Thermo Fisher, UT, USA). After 48 h of incubation, cells were treated with TM.

### Behavior assessment

*Basso mouse scale*. The basso mouse scale (BMS) score was used to evaluate the hind limb motor function on the coordination in movement at the following time points: baseline, 2, 7, 14, 21, 28, 35, and 42 days after injury [36]. The mice were placed in an open field and allowed to move freely for 5 min and observed for a period of 2 min. The average of the scores was measured when the scores of the right and left hind limbs were different.

*Balance beam test*. The balance beam test was used to assess the motor coordination and balance during walking on the beam with slight modification from previous report[37]. The beam is designed to be 110 cm long with gradually reduced width and suspended 15 cm high from the ground. Particularly, the width of beam is 12 mm at the beginning and 5 mm at the end with a platform. The measurement of the test is the time that mice reach the platform from the beginning. The maximum allowable walking time is 60 s and mice will be recorded as 60 s if failed to reach destination.

*Inclined plane test*. The inclined plane test was performed for evaluation of the strength of hind limbs as described previously [3]. In brief, the mice were put on a board with a rubber surface. With a continuous increase of the board angle, the maximum angle was recorded and defined as the value that the mouse could not maintain its position for 5 s without falling. Each mouse was assessed three trials and given 1 min for break.

### Tissue preparation

Mice were euthanized at specific time points and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Spinal cords from 10-mm longitudinal sections centered around the contusion epicenter were dissected out, post-fixed in 4% PFA for 6 hours and embedded in paraffin before being cut into (0.5-μm) slides, or stored at -80°C immediately without 4% PFA fixing for western blot.

### Western blot assay

For in vivo analysis, 5-mm tissue of spinal cord around the injury center from SCI mice or the same area of control. Cell lysates were prepared by lysing the PC12 cells and primary cortical neurons in 6-well plates. Proteins was quantified by BCA reagents (Thermo, Rockford, IL, USA), and equivalent amounts of protein (80 μg in vivo, 40 μg in vitro) were separated on SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% nonfat milk in TBST (Tris-buffered saline with 0.1% Tween-20), and then incubated overnight at 4°C with primary antibodies. The titer of primary antibodies as followed: sestrin2 (1:1000), GRP78 (1:1000), PDI (1:1000), CHOP (1:1000), p-PERK (1:500), PERK (1:1000), IRE1α (1:1000), ATF6 (1:1000), ATF4 (1:1000), caspase12 (1:1000), ubiquitinated proteins (1:200), Bax (1:500), Bcl2 (1:500), cleaved caspase3 (1:1000), p62 (1:1000), LC3 (1:1000), CTSB (1:1000), Lamp2 (1:1000), p-AMPK (1:1000), AMPK (1:1000), p-mTOR (1:200), mTOR (1:200), p-ULK1 (1:500), ULK1 (1:1000), GAPDH (1:1000). The membranes were washed with TBST and incubated with secondary antibodies for 90 min at room temperature. Protein bands were detected using ChemiDocXRS+ Imaging System (Bio-Rad), and the bands were quantified using densitometric measurement by Quantity-One software.

### Immunofluorescence staining

Longitudinal and transverse sections of 5-μm thickness were cut, deparaffinized, rehydrated and washed in PBS. Primary cortical neurons were seed on cover slips in 12-well plates, fixed for 15 min in 4% PFA and washed in PBS three times for 2 min each. And the sections were incubated with 5% bovine serum albumin (BSA) for 30 min, then incubated with the primary antibodies overnight at 4 °C in the same buffer. The titer of primary antibodies as followed: sestrin2 (1:500), NeuN (1:1000), Tuj1 (1:1000), GRP78 (1:1000), CHOP (1:500), cleaved caspase 3 (1:500), LC3 (1:1000), Lapm2 (1:500). After primary antibodies incubation, sections were washed and then incubated in secondary antibodies (AlexaFluor 488 or AlexaFluor 647 donkey anti rabbit/mouse) for 60 min at 37°C. Sections were rinsed in PBS, labeled with DAPI for 7 min and sealed with a cover slip. Images were observed by a Nikon ECLIPSE Ti microscope (Nikon, Tokyo, Japan).

### TUNEL staining

A terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was used according to the manufacturer's instructions (Yeasen Biochemical, Shanghai, China). Briefly, sections were cut from the area 4-5 mm rostral and caudal from contusion center of the mice tissue, incubated in 0.1% Triton X-

100 for 30 min and incubated with 50 μL TUNEL reaction mixture. Finally, the sections were stained with DAPI and observed by a Nikon ECLIPSE Ti microscope.

### Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using Graphpad prism (version 8). Parametric data (normality and equal variance passed) were analyzed using one-way analysis of variance (ANOVA) and followed by Dunnett test for between-group comparisons. Equal variance failed data were analyzed using Kruskal-Wallis ANOVA based on ranks followed by Dunn's post-hoc test. Comparisons at multiple time points were analyzed with a repeated measures ANOVA and followed by LSD test for between-group comparisons. Results of only two groups were analyzed using unpaired Student t-test for equal variance passed data and Mann-Whitney Rank Sum Test for equal variance failed data. A P value < 0.05 was considered being significant.

## Results

### Expression of sestrin2 after spinal cord injury

To determine expression changes of sestrin2 in neurons of mice after SCI, we evaluated the expression levels of this protein in a time dependent manner by western blot and immunostaining. Compared with control group, an increased protein level of sestrin2 at days 1 and 3 after injury, and decreased level at days 7 and 14 after injury were observed (Figure 1A-B). In addition, the immunofluorescence staining result also indicated that the up-regulated sestrin2 is mainly co-localized in neurons (Figure 1C-D). Furthermore, the sestrin2 expression in PC12 cells and primary cortical neurons were evaluated when subject to TBHP stimulation. The results of western blot shown that sestrin2 is increased in response to TBHP insult (Figures 1E-F). With regarding to primary cortical neurons, the immunofluorescence staining co-localization of sestrin2 with Tuj1 indicated that treatment with TBHP in a time dependent manner not only enhances sestrin2 expression, but also causes unhealthy neurites growth (Figure 1G-H). Overall, these data suggested that sestrin2 is upregulated after SCI and may provide a protective effect in response to neural injury.

### TM treatment induces sestrin2 expression

ER stress is a series processes charactered by the perturbation in ER homeostasis with misfolded/unfolded protein accumulation, which extensive exists in SCI and aggravates neural injury[21]. We reasoned that ER stress may relate to the sestrin2 induction and firstly monitored the expression level of sestrin2 with the treatment of chemical ER stress inducers in vivo. The results indicated that Tm treatment was significantly increased the expression of GRP78, PDI, CHOP and sestrin2 in spinal cord compared with control group (Figures S1A-B). The immunofluorescence staining also indicated sestrin2 associated with higher intensity and mainly co-localized with NeuN in Tm treatment group (Figure S1C-D). In vitro, primary cortical neurons treated with Tm presented an increased intensity of sestrin2 and

unhealthy growth (Figure S1E-F). Thus, these results demonstrated that ER stress could induce sestrin2 expression in vivo and vitro.

### Sestrin2 induced by TM depends on PERK-ATF4 axis

Unfolded protein response (UPR) is a well-known adaptive mechanism for cell against ER stress in the early stage [22]. To determine whether unfolded protein response is responsible for sestrin2 induction, we determine the contribution of three transmembrane ER stress sensor proteins in vitro. As the results of western blot indicated, the level of sestrin2 was remarkably downregulated by administration of PERK inhibitor (GSK2606414), specially when PC12 cells treated with or without to TM (Figure S2A-B). In contrast, inhibition of IRE1α and ATF6 were not involved in sestrin2 expression both in control and Tm treatment circumstances (Figure S2C-F). In addition, as a well-established downstream of transcription factor of PERK [38], ATF4 knockdown using siRNA presented a significant reduction of sestrin2 expression compared to PC12 cells transfected with NC siRNA (Figure S2G-H). To gain further insights into the mechanism of sestrin2 induction upon ER stress, we utilized PERK inhibitor for treating primary cortical neurons. The results of immunofluorescence staining in primary cortical neurons showed that the presence of PERK inhibitor results in lower sestrin2 expression than that without inhibitor treatment (Figure S2I-J). Collectively, these results indicated that PERK-ATF4 plays a critical role in sestrin2 induction in response to ER stress.

### Sestrin2 overexpression promotes functional recovery from SCI

To evaluate the role of sestrin2 in spinal cord after injury, we utilized AAV9 vectors for overexpression of sestrin2 in vivo. Introduction of a construct expressing AAV of sestrin2 resulted in a significantly increased sestrin2 protein level in spinal cord (Figure 2A-D). To evaluate the effect of sestrin2 on tissue rehabilitation, we examined the lesion volume of spinal cord after injury. Spinal cord lesion volume was smaller in the AAV-sestrin2 treated mice in comparison to the mice that received the vehicle (Figure 2E-F). Furthermore, to determine whether sestrin2 contributes to functional recovery in SCI mice, a 6-week period of behavioral tests including BMS, balance beam and inclined plane test were performed in a blinded manner (Figure 2G-L). As the results showed, all mice presented similar severities of injury at 2 days after SCI and progressive recovery at several weeks after SCI. It should be note that mice received AAV-sestrin2 display better functional recovery and coordination improvement than mice treated with AAV-control after SCI, especially at the later stages of injury. Specifically, results of BMS indicated that the SCI mice treated with AAV-sestrin2 acquired higher score than that treated with vehicle AAV starting from the 4 weeks after injury (Figure 2G-H). Moreover, assessment of balance beam (Figure 2I-J) and inclined plane test (Figure 2K-L) suggested that SCI mice injected with AAV-sestrin2 performed better in the motor coordination and balance and strength of hind limbs in comparison to corresponding injury controls from 3 weeks to 6 weeks. These results demonstrated that upregulation of sestrin2 is responsible for functional recovery after SCI.

### Sestrin2 overexpression maintains ER homeostasis after SCI

Several evidences have indicated that adaptive sestrin2 induction protects cell against ER stress[8, 26]. GRP78 and PDI were recognized as ER chaperones and increased when misfolded proteins exceptional accumulation. Using western blot assay, sestrin2 overexpression was sufficient to reduce the expression of GRP78 and PDI compared with that with empty vectors after SCI (Figure 3A-B). Meanwhile, sestrin2 overexpression also attenuated the expression of ubiquitinated proteins, which is a group of polypeptides and label in misfolded proteins (Figure 3C-D). Thus, sestrin2 acts as a critical role in alleviating ER stress after SCI. Meanwhile, sestrin2 overexpression decreased the level of CHOP and caspase12, which were regarded as the downstream of transcription factors and execution for ER stress induced apoptosis (Figure 3A-B). In line with this notion, immunofluorescence staining showed that the effect of sestrin2 on reducing GRP78 and CHOP mainly occurs in neurons (Figure 3E-H). Taken together, these results suggested that sestrin2 maintains ER homeostasis and promotes recovery from ER stress in neurons.

### Sestrin2 protects neurons from apoptotic death

Prolonged ER stress eventually over runs the compensatory capacity of ER lumen for misfolded proteins aggregation and switches ER stress to pro-death process[39]. Since sestrin2 is beneficial to ER homeostasis, here we focused on examination of the functional importance of sestrin2 to neural death. Remarkably, administration of AAV-sestrin2 reduces Bax, cleaved caspase3 and increases Bcl2 expression (Figure 4A-B), also significantly decreased the number of TUNEL-positive cells in spinal cord (Figure 4C-D). Consistently, immunofluorescence staining co-localization of NeuN and cleaved caspase3 was basically in agreement with the result of western blot (Figure 4E-F). Thus, the above data demonstrated that sestrin2 is a beneficial factor for protecting neurons from apoptosis.

### Sestrin2 activates autophagy in spinal cord

Despite several evidences identified sestrin2 as a regulator of the autophagy lysosome pathway, its function in neuron is still unclear and examined here[9, 12]. Western blot showed that overexpression of sestrin2 in spinal cord of mice markedly upregulates the level of p62, LC3-II/I, CTSB and Lamp2, which are corresponding proteins regulating autophagosome formation and lysosomal biogenesis (Figure 5A-B). Meanwhile, higher intensity of LC3 (Figure 5C-D) and Lamp2 (Figure 5E-F) were detected in neuron with sestrin2 overexpression. Considering autophagy is a dynamic process and LC3-II level may decrease followed with autolysosomes degeneration, the expression level of LC3-II in mice treated with bafilomycin-A1 was proposed to quantize the gross product. Results showed that the lysosomal inhibitor successfully caused the accumulation of LC3-II and the AAV-sestrin2 group treated with bafilomycin-A1 is associated with higher level of LC3-II (Figure 5G-H). Thus, autophagy activity was increased following sestrin2 upregulation.

### Upregulation of sestrin2 ameliorates the autophagic flux dysfunction after SCI

Subsequently, we investigated whether sestrin2 affect autophagic flux after SCI. Using western blot analysis, we found that significant increase of p62 and LC3-II expression, decrease of CTSB and Lamp2 expression in the spinal cord after injury (Figure 6A-C). These data suggested that lysosomes are damage

and autophagosomes are abnormal accumulation. In line with above notion, we selected third day after injury as the time point for the following assessment. As the results presented, an increased protein level of LC3-II, CTSB and Lamp2, and a decreased protein level of p62 were observed in overexpression of sestrin2 mice in comparison to vehicle mice after injury in spinal cord (Figure 6D-F). Consistently, immunofluorescence analysis also showed that the fusion of LC3 (autophagosome marker) and Lamp2 (lysosome marker) was decreased in injured group, which was reversed in AAV-sestrin2 group after SCI (Figure 6G-I). These assessments indicated a beneficial effect of sestrin2 during the autophagic flux restoration in mice after SCI.

### Inhibition of autophgaic flux abrogates sestrin2 induced protective effects after SCI

To further evaluate the beneficial role of sestrin2 for neural survival and ER homeostasis is associated with activation of autophagy, we treated AAV-sestrin2 mice with CQ, which blocks autophagic flux by alkalizinglysosomal cavity. Followed with CQ treatment, obviously increased level of ER stress was not observed in the intact AAV-sestrin2 mice, while significant upregulation of GRP78, PDI, CHOP and ubiquitinated proteins were detected in the injuerd AAV-sestrin2 mice (Figure 7A-B). Immunofluorescence staining also revealed that neurons are associated with higher signal of GRP78 in AAV-sestrin2 mice after SCI (Figure 7C-D). Meanwhile, CQ treatment group exhibited remarkably increased TUNEL-positive cells in spinal cord compared with control group (Figure 7E-F). Results of western blot also revealed that CQ treatment did not result in significant alteration of apoptotic proteins in AAV-sestrin2 mice without injury, but marked increase of cleaved caspase3 and Bax, decrease of bcl-2 in AAV-sestrin2 mice with SCI (Figure 7G-H) . Therefore, blockage of autophagic flux by CQ treatment could significantly abolish sestrin2 induced protective effects on neural survival and ER homeostasis after SCI.

### AMPK-mTOR axis is involved in sestrin2 mediated autophagy

Since previous studies suggested that sestrin2 is involved in stress dependent mTOR regulation, which induced by AMPK phosphorylation, we evaluated whether sestrin2 enhanced autophagy is related to modulation of AMPK-mTOR axis [7]. As the results presented (Figure 8A-B), sestrin2 overexpression led to significant upregulation of AMPK phosphorylation and inhibition of mTOR in spinal cord compared with vehicle group. As a protein kinase that is responsible for the initiation of autophagy, ULK1 was also activated in AAV-sestrin2 mice. In addition, we applied Compound c to inhibit AMPK for the further assessment of the mechanism of sestrin2 induced autophagy. While mice treated with Compound c, the effect of sestrin2 mediated increase of LC3-II and decrease of p62 was abolished (Figure 8C-D). Consistent with the western blot data, immunofluorescence analysis displayed lower level of LC3 in neurons of Compound c treated AAV-sestrin2 mice as compared AAV-sestrin2 vehicle mice (Figure 8E-F). Taken together, our findings indicated that sestrin2 activated autophagy through regulation of the AMPK-mTOR signaling pathway.

## Discussion

Different from other cells, neuron exhibits a poor capacity for intrinsic regeneration owing to they hold no potential in replication. Therefore, promoting neuron survival and enhancing the surviving neuronal function are considered as the critical strategy for SCI therapy. This study examined the neuroprotective effect of sestrin2 in a mouse model of contusive SCI. The main result of our study is that sestrin2 is upregulated after SCI or pharmacological endoplasmic reticulum (ER) stress induced by TM treatment, and this regulation is associated with Perk-ATF4 dependent pathway. Meanwhile, overexpression of sestrin2 contributes to maintaining ER homeostasis, limiting the damage of spinal cord and promoting function recovery. The underlying mechanism for the recovery of SCI by sestrin2 is an activation of autophagy and restoration of autophagic flux mediated by AMPK-mTOR axis. Thus, our results suggested that manipulating sestrin2 may form a potential therapeutic strategy, or become a part of combinatorial therapy for enhancing recovery after SCI.

As a highly conserved of stress inducible protein, sestrin2 appears to regard as a potential candidate for various pathologies therapy [8, 9]. Sestrin2 is a critical component in response to ischemia/reperfusion injury in heart, and sestrin2 deficiency hearts display exacerbated myocardial infarction and impaired cardiac function [13]. Kim et al [40] indicated that maintaining mitochondrial homeostasis and inhibiting the activation of the NLRP3 inflammasome by sestrin2 in macrophage noticeably protects the host from sepsis. Additionally, induction of sestrin2 represents an endogenous protective mechanism in response to amyloid beta-peptide neurotoxicity in primary cortical neurons [11]. More importantly, sestrin2 also provides a beneficial role in amelioration of acute ischemic brain injury [16]. The results presented previously indicated that treatment with rh-sestrin2 not only reduces the disruption of blood-brain barrier, but also protects hippocampal neurons against ischemia induced apoptosis [41]. Similar results were observed in the current study, we found that overexpression of sestrin2 promotes neuronal survival and function recovery after SCI.

Apparently, the molecular mechanism of how sestrin2 provides the protective role for above diseases is an important issue. Previous studies indicated that sestrin2 is one of the several antioxidant defenses and exhibits similar functional sequence with alkylhydroperoxidase D, an antioxidant protein in mycobacterium[9]. Under genotoxic damage or oxidative stress, adaptive sestrin2 expression in a p53 dependent manner prevents reactive oxygen species (ROS) induced cytotoxicity and cell death [7]. Specifically, Nrf2 and antioxidant response element (ARE) response axis is also required for the upregulation of sestrin2 [42]. As a matter of fact, sestrin2 has another name called hypoxia inducible gene 95 (Hi95) and originally discovered in the cell under hypoxia environment. The mechanism of sestrin2 induction in response to hypoxia mainly depends on hypoxia inducible factor-1(HIF-1) activation. On the other hand, as the consequence of energy deprivation secondary to continued hypoxia, sestrin2 could be induced in a HIF-1 independent mechanism [7].

ER stress and unfolded protein response (UPR) is a common and crucial molecular event in CNS trauma. To the best of our knowledge, the regulation of sestrin2 in neuron upon ER stress has not been thorough explicated. ER stress induced by palmitic acid could be activate the sestrin2 expression in hepatocytes [26]. In the current study, we found that there is a significant increase in sestrin2 expression in spinal cord

after injury and primary neuron treatment with tunicamycin in vitro. Moreover, it is well-characterized that UPR is an adaptive process activated by ER sensors and lead to cells induce numerous transcriptional processes in the face of impaired proteostasis. The upregulation of ATF4 induces expression of the stress response protein sestrin2 during amino acid deprivation [43]. Moreover, PERK and c/EBPβ is responsible for the expression of sestrin2 in response to ER stress in hepatocytes [26]. Consistence with previous studies, the results of this study indicated that the activation of PERK and ATF4 is involved in sestrin2 induction under ER stress in neuron after SCI.

Previous studies demonstrated that restoring ER function is one of the available strategies for acute CNS injury, as it helps neuron against ER stress induced apoptosis [24, 25]. In the present study, we also identified that upregulation of sestrin2 by AAV provides beneficial effects on reducing ER stress and apoptosis. However, the mechanism regarding sestrin2 contribution to ER homeostasis has not been well-identified. As previous reported, sestrin2 can enhance autophagy during diverse environmental stresses, which is responsible for the clearance of damaged mitochondria, attenuating insulin resistance [12, 40]. Here we hypothesized that the lessened ER stress by sestrin2 overexpression is associated with activation of autophagy after SCI.

Subsequently, we assessed the status of autophagy and the link between sestrin2 and autophagy in neuron. In the current study, though the ratio of LC3-II/I appear increased, which may implicate as the result of dysfunction of lysosome or failed autolysosome formation. Previous study have mentioned that autophagosome is activated in spinal cord after acute traumatic injury [44]. However, accumulating evidences implicated that lysosomal is impaired and could not effective combine with autophagosome when neurons undergo the alteration of intracellular and the extracellular microenvironment in neural injury [27, 28]. The inhibited autophagic flux leads to exacerbation of ER stress owing to the autophagy is one of the processes for the elimination of misfolded protein. Present study showed the restoration of autophagic flux mediated by sestrin2 overexpression contributes to alleviation of ER stress and apoptosis.

Results presented here showed that the mechanism of increased autophagy in the spinal cord of AAV-sestrin2 mice is a result of the significant regulation of AMPK-mTOR axis, which evidenced by treated with AMPK inhibitor. As a functional regulator in maintaining cell survival and homeostasis, AMPK phosphorylation is modulated by sestrin2 induction which has been identified currently. The mechanism of sestrin2 regulating AMPK was mentioned that sestrin2 can bind to AMPK and forms a complex, increase AMPK phosphorylation at Thr172 [45]. Moreover, sestrin2 mediated AMPK activity has shown to be important for inhibition of mTOR and phosphorylation of ULK1, which has been proposed to be a crucial positive regulator for autophagy induction [9]. On the other hand, the amelioration of lysosomal function is essential for autophagic flux restoration. Previous studies indicated that the lysosome is the signaling hub of mTOR regulation, and mTOR regulates lysosomal function through modulating localization of transcription factor EB (TFEB), which is a major factor for regulating lysosomal proteins activity and lysosomal biogenesis [46]. We regarded mTOR as the main regulator in modulating Lamp2

and CTSB, two lysosomal markers, though there is no definite mechanism to reveal the effect of sestrin2 on lysosomal function.

Certainly, there are several limitations with regarding to the use of sestrin2 as a therapy target for SCI and underlying molecular mechanism, and it still requires further study and investigation. For instance, in view of the complexity of and various cell structured found in spinal cord, primary cortical neurons cultured alone in vitro are informative. In the current study, we also found that the change of sestrin2 expression not only located in neurons, but also exists other cells. Therefore, we merely focus on the effect of sestrin2 on neuron may be one-sided to the current study. Additionally, we note that employing plasmid or gene knockdown instead of molecular inhibitors would be more persuasive. Nevertheless, the effect of sestrin2 on function recovery after SCI is confirmed and it is available to clarify the properties and underlying mechanisms in future researches.

# Conclusion

The current study demonstrated that the regulation of sestrin2 is a stress inducible process in the adaptive response to SCI and pharmacological ER stress, which mainly activated by the Perk-ATF4 axis of the UPR. Moreover, the results of our study also suggested that sestrin2 promotes function recovery and neuron survival after SCI through attenuation of ER stress that is mediated by activation of autophagy and restoration of autophagic flux as the consequence of AMPK-mTOR axis regulation (Figure 9). Therefore, above evidences suggested that sestrin2 could exhibit neuroprotection and appear to be a feasible therapeutic target for acute traumatic SCI.

# List Of Abbreviations

SCI, spinal cord injury; ER, endoplasmic reticulum; TM, tunicamycin; PERK, protein kinase like ER kinase; IRE1, inositol requiring enzyme 1; ATF6, activating transcription factor 6; ATF4, activating transcription factor 4; UPR, unfolded protein response; mTOR, mammalian target of rapamycin complex; AMPK, AMP-activated protein kinase; Nrf-2, nuclear factorerythroid2 related factor 2; TBHP, tert-Butyl hydroperoxide solution; CQ, chloroquine; Comp c, Compound c; AAV, Adeno-associated viral; siRNA, Small interfering RNA; BMS, basso mouse scale; PVDF, polyvinylidene fluoride; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; CNS, central neural system

# Declarations

### Ethics approval and consent to participate

All protocols and animal experiments were conducted in strict accordance with the Animal Care and Use Committee of Wenzhou Medical University (No. wydw-2018-0043).

### Consent for publication

All contributing authors agree to the publication of this article.

### Competing interests

The authors declare that they have no competing interests.


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### Author contributions

Yao Li designed research and wrote the paper, Jing Zhang and Kailiang Zhou assisted in performing the experiment in vivo and in vitro; Guangheng Xiang and Mingqiao Fang guided experiment and picture performing; Ling Xie and Wen Han modified the syntax of paper; Xiangyang Wang and Xiao Jian assisted in designing research and approved the final version and submitted. All authors read and approved the final manuscript.

### Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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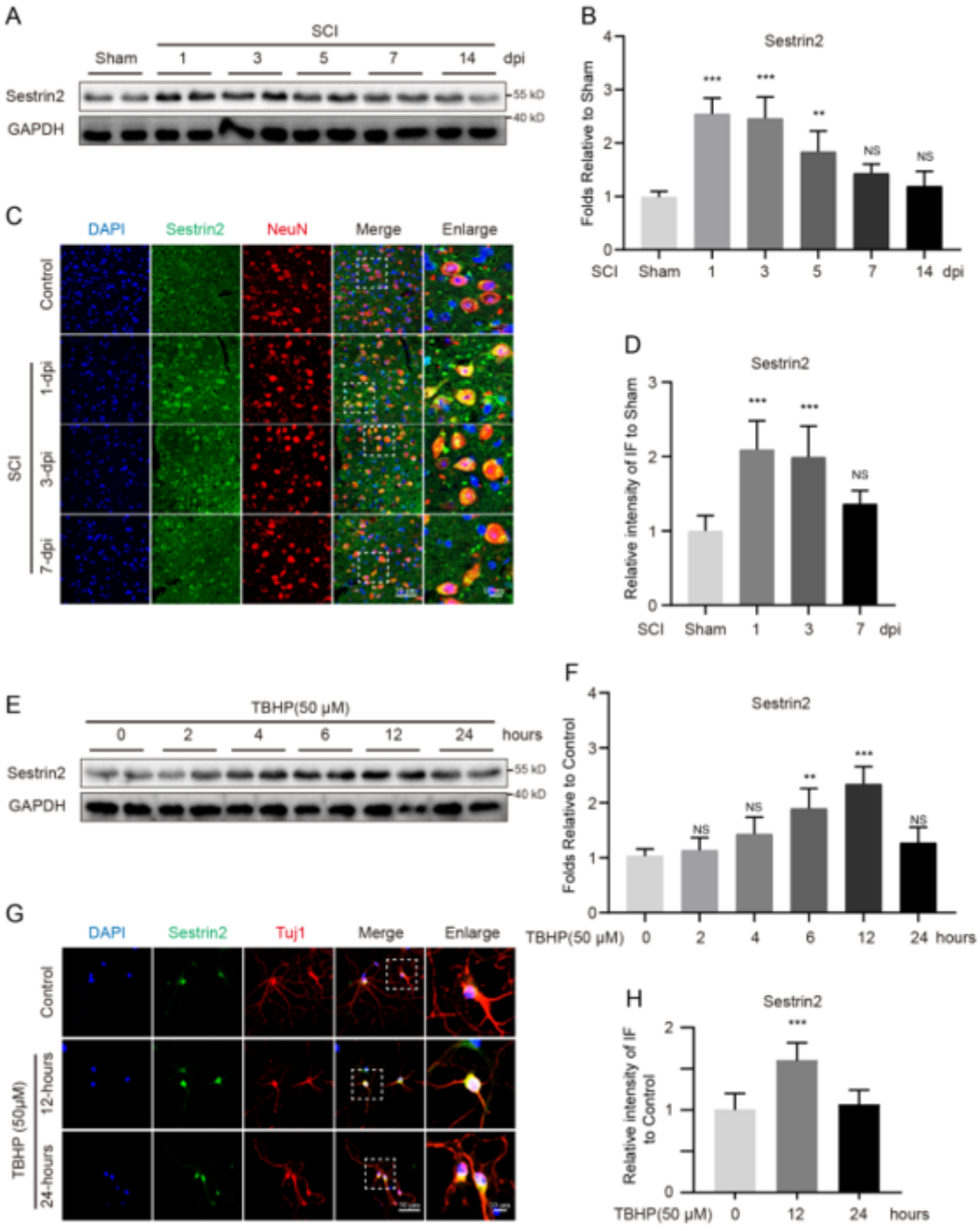
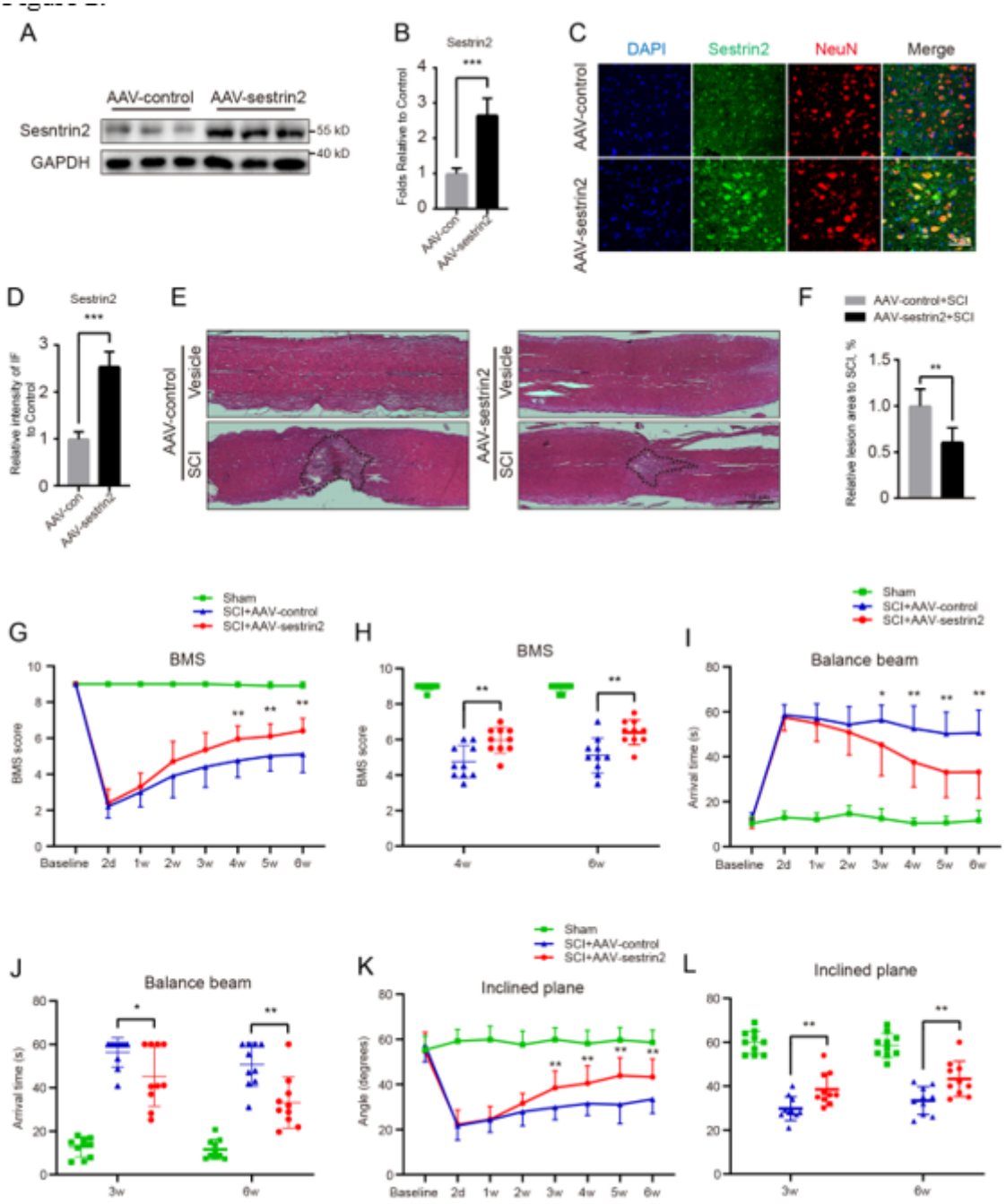
# Figures




## Figure 1

Injury stimulation induces sestrin2 upregulation in vivo and vitro. (A and B) Western blot and quantification of the time dependent expression level of sestrin2 in spinal cord of mice after SCI. (C and D) Immunofluorescence of sestrin2 puncta in neuron and quantitative analysis of sestrin2 intensity in mice at different time points after SCI. Scale bar = 50 μm; scale bar (enlarged) = 10 μm. (E and F) The protein expression of sestrin2 in PC12 cells subjected to TBHP (50 μM) in a time dependent manner. (G and H) Immunofluorescence of sestrin2 in primary cortical neurons treated with TBHP (50 μM) for 12 and

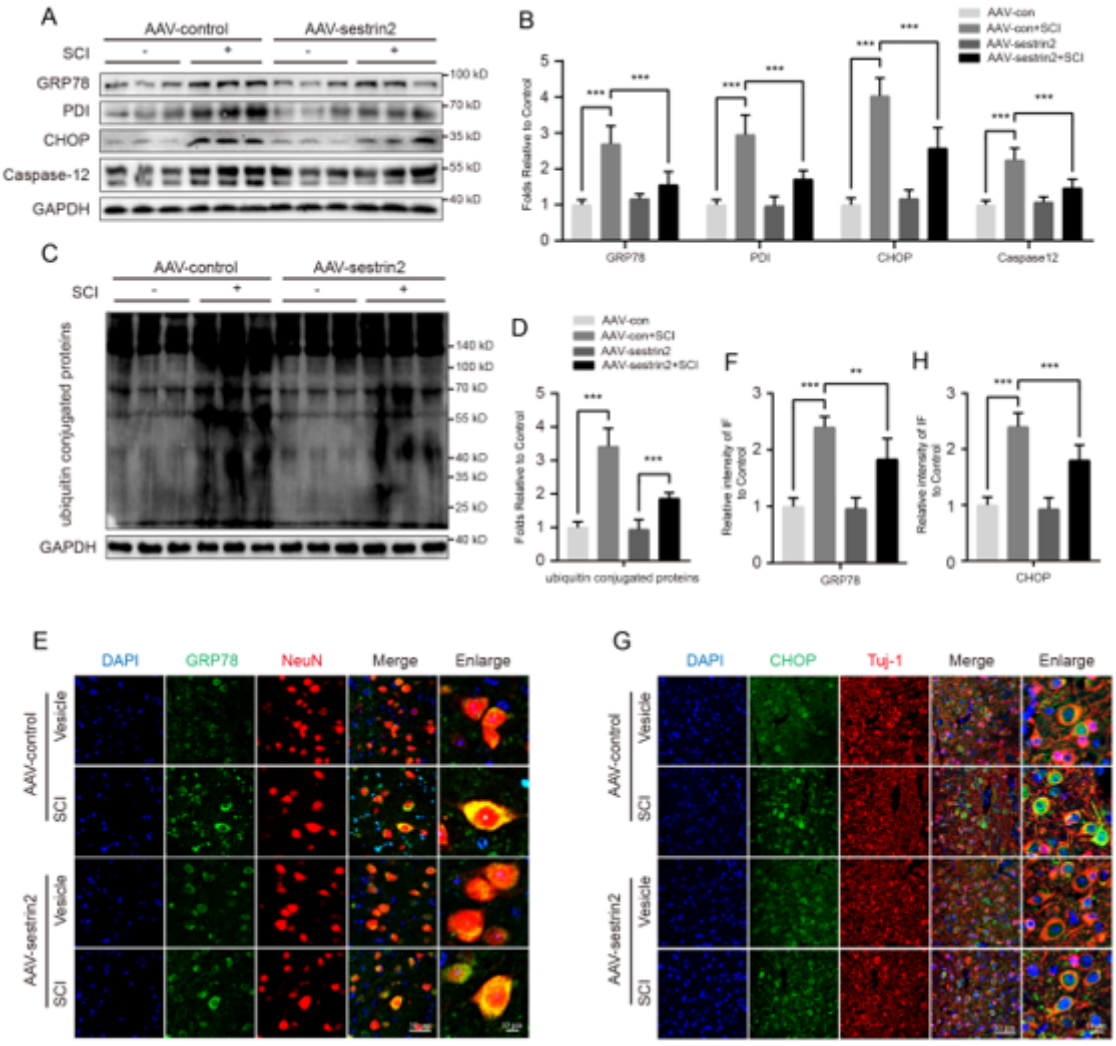
24 h. Scale bar = 50 μm; scale bar (enlarged) = 10 μm. GAPDH was the loading control. Data represent mean ± S.D. (n = 6). *P<0.05, **P<0.01, ***P<0.001, NS, not significant.




## Figure 2

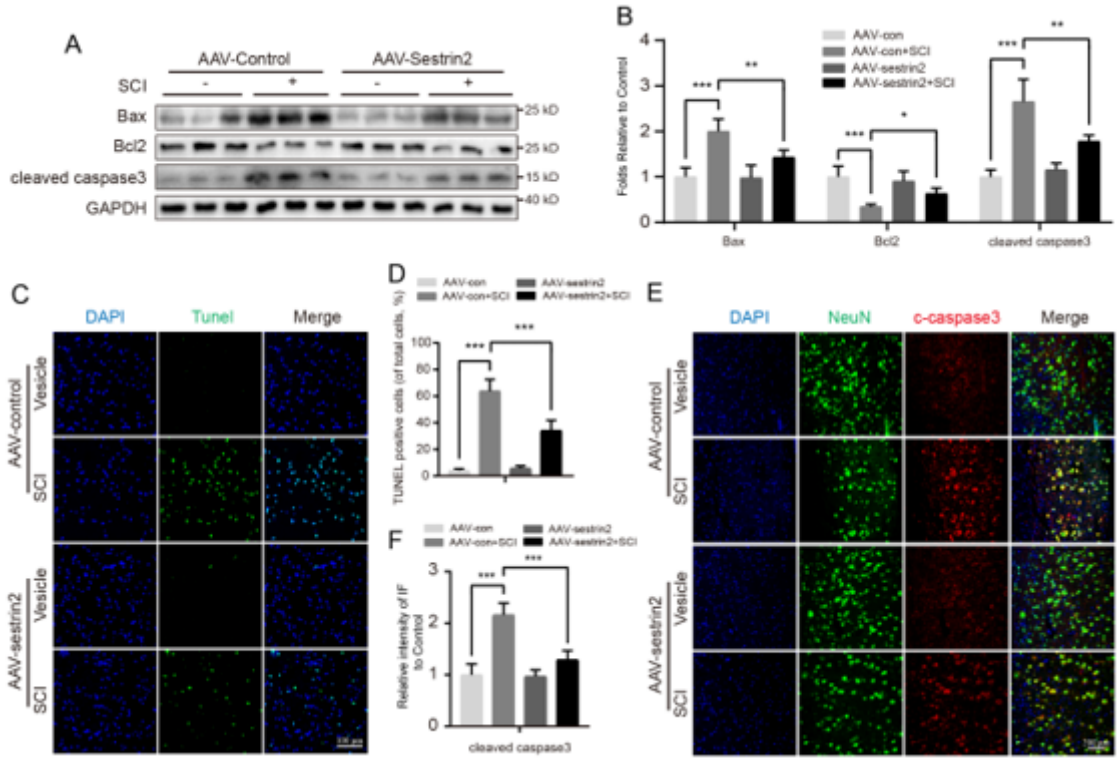
Sestrin2 improves functional recovery after SCI. (A-D) Western blot and immunofluorescence of sestrin2 in spinal cord of mice after injected with AAV-control or AAV-sestrin2 for 14 days. (E) Representative images from H&E staining at 28 days post-injury, scale bar = 750 μm. (F) Quantification of the lesion area of the spinal cord from H&E staining. (G and H) Graphs of the score of BMS at different time points after SCI in mice treated with or without AAV-sestrin2 injection. (I and J) Graphs of arrival time of balance beam at different time points after SCI in mice treated with or without AAV-sestrin2 injection. (K and L)

Graphs of angle of inclined plane test at different time points after SCI in mice treated with or without AAV-sestrin2 injection. GAPDH was the loading control. *P<0.05, **P<0.01, ***P<0.001.



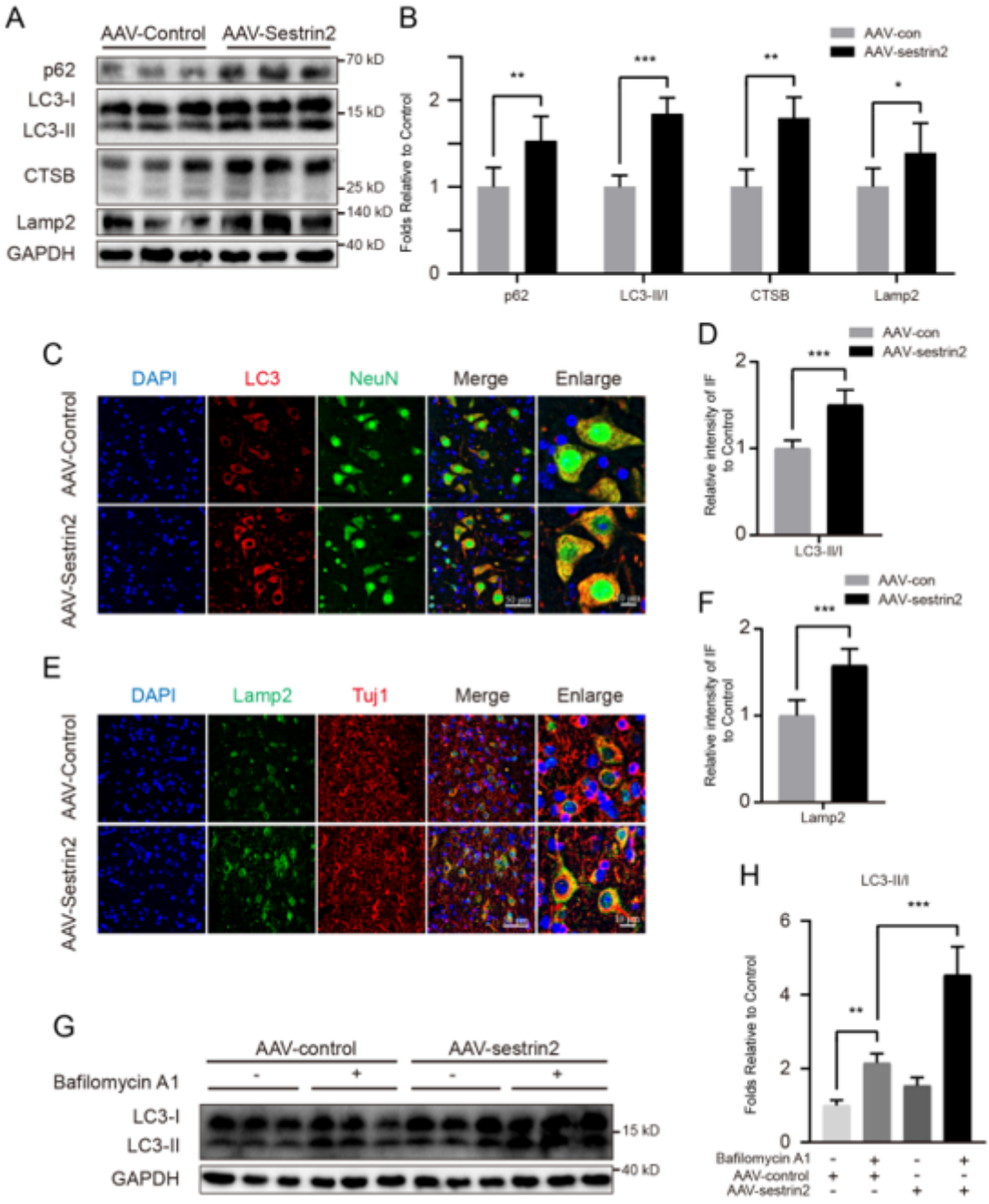

## Figure 3

Upregulation of sestrin2 contributes to ER homeostasis after SCI. (A and B) Western blot and quantification of multiple ER stress markers in spinal cord of mice in each group at 7 days after SCI. (C and D) Western blot and quantification of ubiquitin conjugated proteins in each group at 7 days after SCI. (E and F) Double immunofluorescence of GRP78 and NeuN in sections from tissue in each group at 7 days after SCI. (G and H) Double immunofluorescence of CHOP and Tuj1 in sections from tissue in each group at 7 days after SCI. Scale bar = 50μm; scale bar (enlarged) = 10μm. GAPDH was the loading control. Data represent mean ± S.D. (n = 6). *P<0.05, **P<0.01, ***P<0.001.



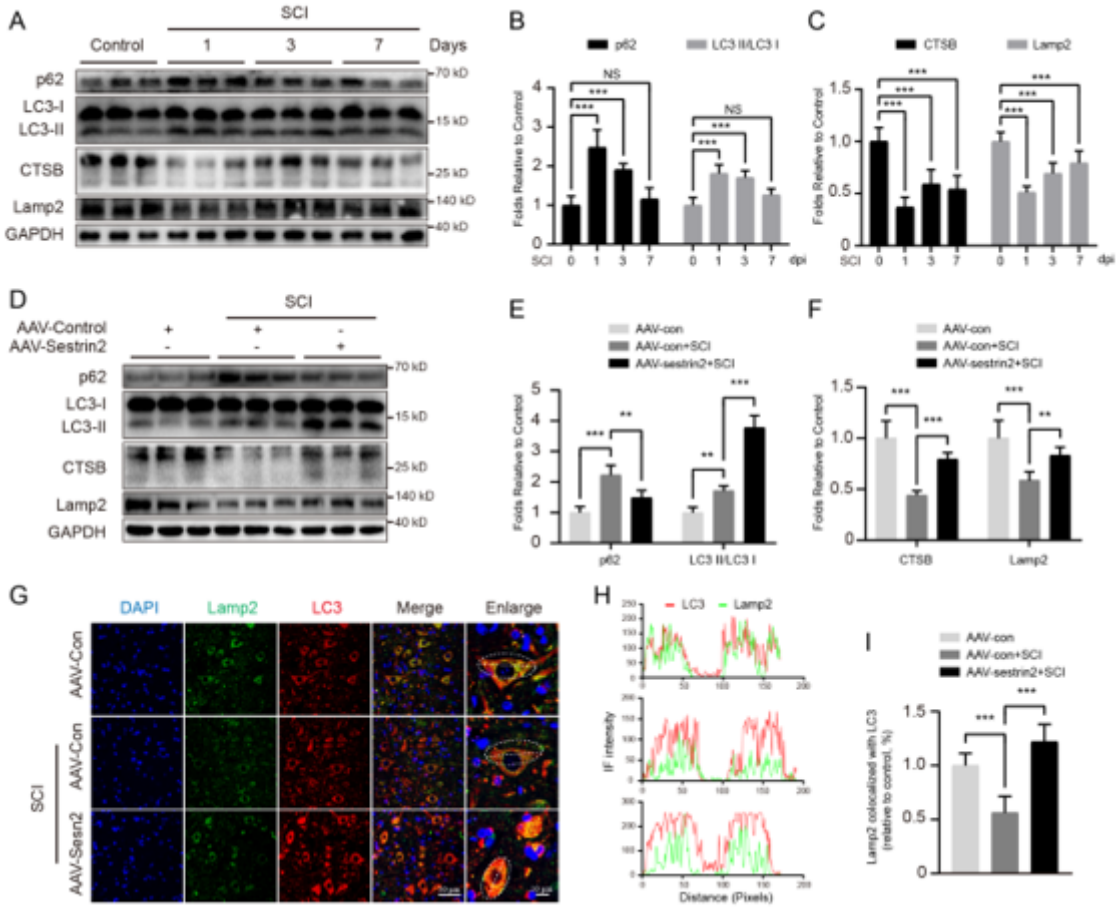

## Figure 4

Upregulation of sestrin2 reduces neural apoptosis after SCI. (A and B) Western blot and quantification of Bax, Bcl2 and cleaved caspase3 in spinal cord of mice in each group at 7 days after SCI. (C) Immunofluorescence staining for TUNEL (green) of sections from the spinal cord in each group 7 days after surgery, scale bar = 100 μm. (D) Quantitative assessment of TUNEL-positive cells from 6 independent sections from the area within 5 mm above and below the injury epicenter. (E and F) Double immunofluorescence of cleaved caspase3 and NeuN in sections from tissue in each group at 7 days after SCI, scale bar = 200 μm. GAPDH was the loading control. Data represent mean ± S.D. (n = 6). *P<0.05, **P<0.01, ***P<0.001.



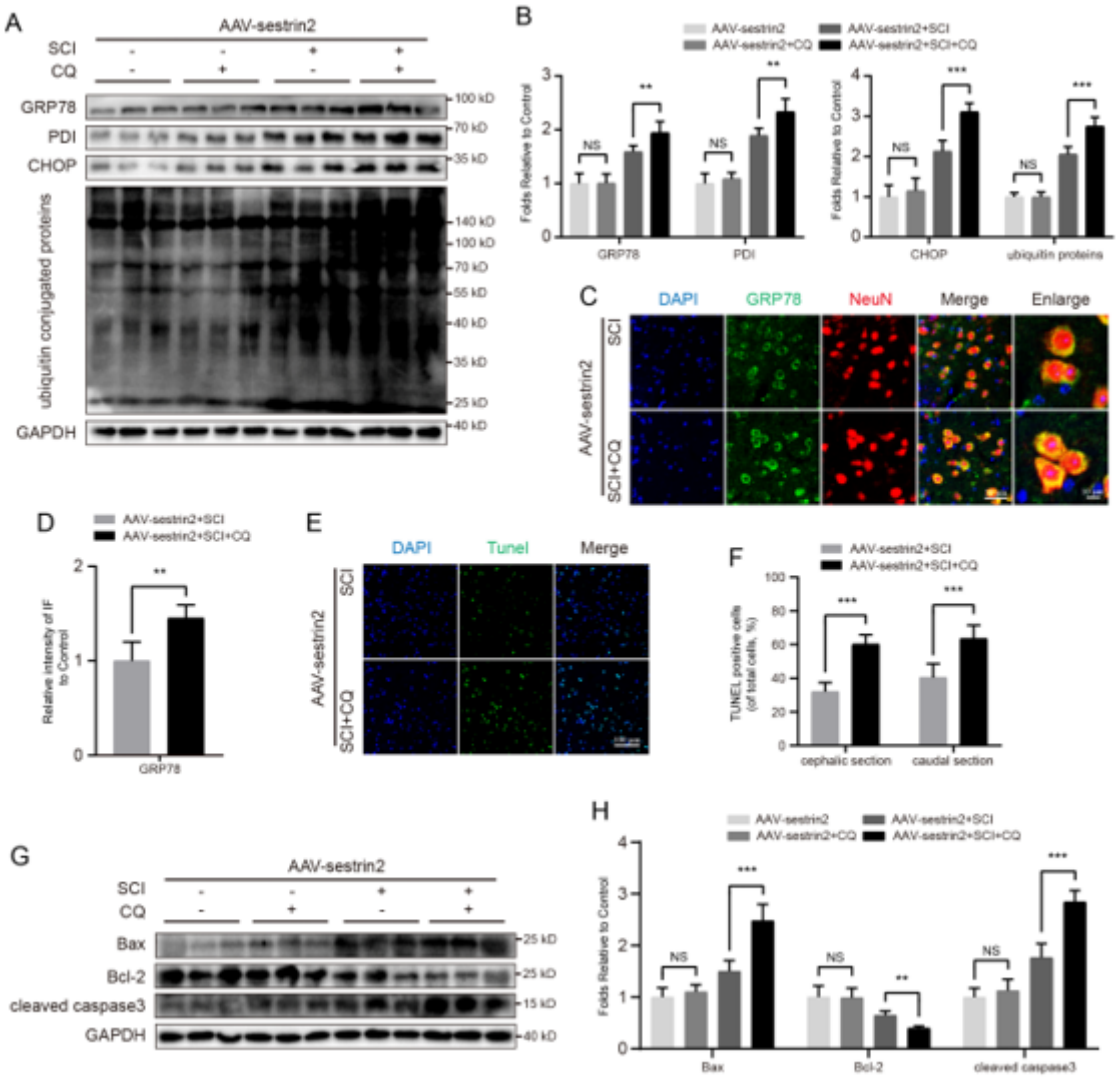

## Figure 5

Sestrin2 activates autophagy in neurons in vivo. (A and B) Western blot and quantification of p62, LC3, CTSB and Lamp2 in spinal cord of mice after injected with AAV-control or AAV-sestrin2 for 14 days. (C and D) Double staining of LC3 and NeuN in spinal cord of mice in each group, scale bar = 50μm; scale bar (enlarged) = 10μm. (E and F) Double staining of Lamp2 and Tuj1 in spinal cord of mice in each group, scale bar = 50μm; scale bar (enlarged) = 10μm. (G and H) Mice were transfected with AAV-control or AAV-sestrin2 for 14 days, and then treated with Bafilomycin A1 (0.3 mg/kg), level of LC3 was analyzed by western blot. GAPDH was the loading control. Data represent mean ± S.D. (n = 6). *P<0.05, **P<0.01, ***P<0.001.



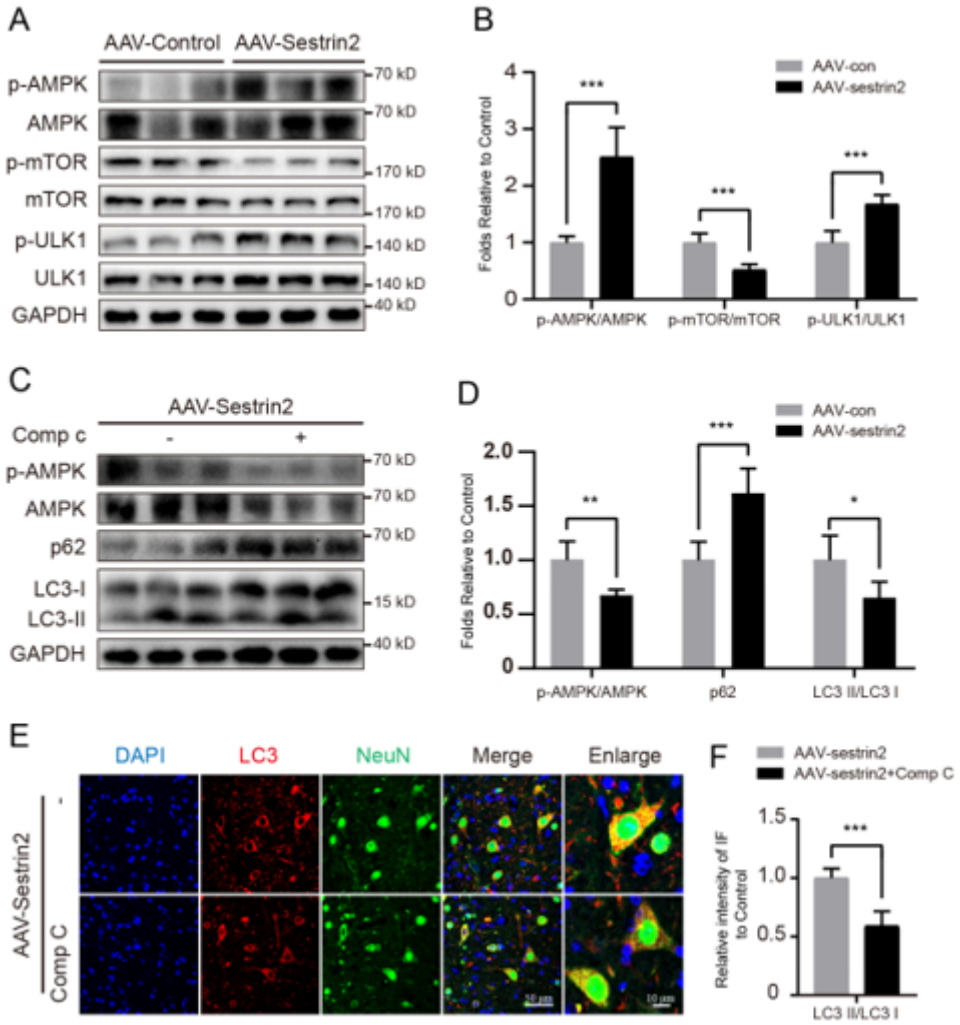

## Figure 6

Sestrin2 restores the impairment of autophagic flux after SCI. (A-C) Western blot and quantification of the time dependent expression level of autophagy related proteins in spinal cord of mice after SCI. (D-F) Western blot and quantification of autophagy related proteins in spinal cord of mice in each group at 3 days after SCI. (G) Double staining of LC3 and Lamp2 in each group at 3 days after SCI. (H) Immunofluorescence intensity of LC3 and Lamp2 across the white dotted lines in each group, scale bar = 50μm; scale bar (enlarged) = 10μm. (I) Quantification of LC3 puncta in Lamp2 in each group. GAPDH was the loading control. Data represent mean ± S.D. (n = 6). *P<0.05, **P<0.01, ***P<0.001.




## Figure 7

Sestrin2 attenuates ER stress and apoptosis by activation of autophagy. (A and B) AAV-sestrin2 mice were pretreated with CQ (50mg/kg) or saline at 3 days before SCI, the ER related proteins were analyzed by western blot at 3 days after SCI. (C and D) Double staining of GRP78 and NeuN in each group, scale bar = 50μm; scale bar (enlarged) = 10μm. (E) Staining for TUNEL (green) of sections from the spinal cord in each group, scale bar = 100 μm. (F) Quantitative assessment of TUNEL-positive cells from 6 independent sections from the area within 5 mm above and below the injury epicenter in each group. (G and H) Western blot and quantification of apoptotic proteins in each group. GAPDH was the loading control. Data represent mean ± S.D. (n = 6). *P<0.05, **P<0.01, ***P<0.001.




## Figure 8

AMPK-mTOR axis is responsible for sestrin2 induced autophagy. (A and B) Western blot and quantification of phosphorylation and total of AMPK, mTOR and ULK1 mice injected with AAV-control or AAV-sestrin2 for 14 days. (C and D) Western blot and quantification of phosphorylation and total of AMPK, p62 and LC3 in mice treated with BML-275 (10 mg/kg) or saline that parallels with AAV injection. (E and F) Double staining of LC3 and Lamp2 in each group as above, scale bar = 50μm; scale bar (enlarged) = 10μm. GAPDH was the loading control. Data represent mean ± S.D. (n = 6). *P<0.05, **P<0.01, ***P<0.001.

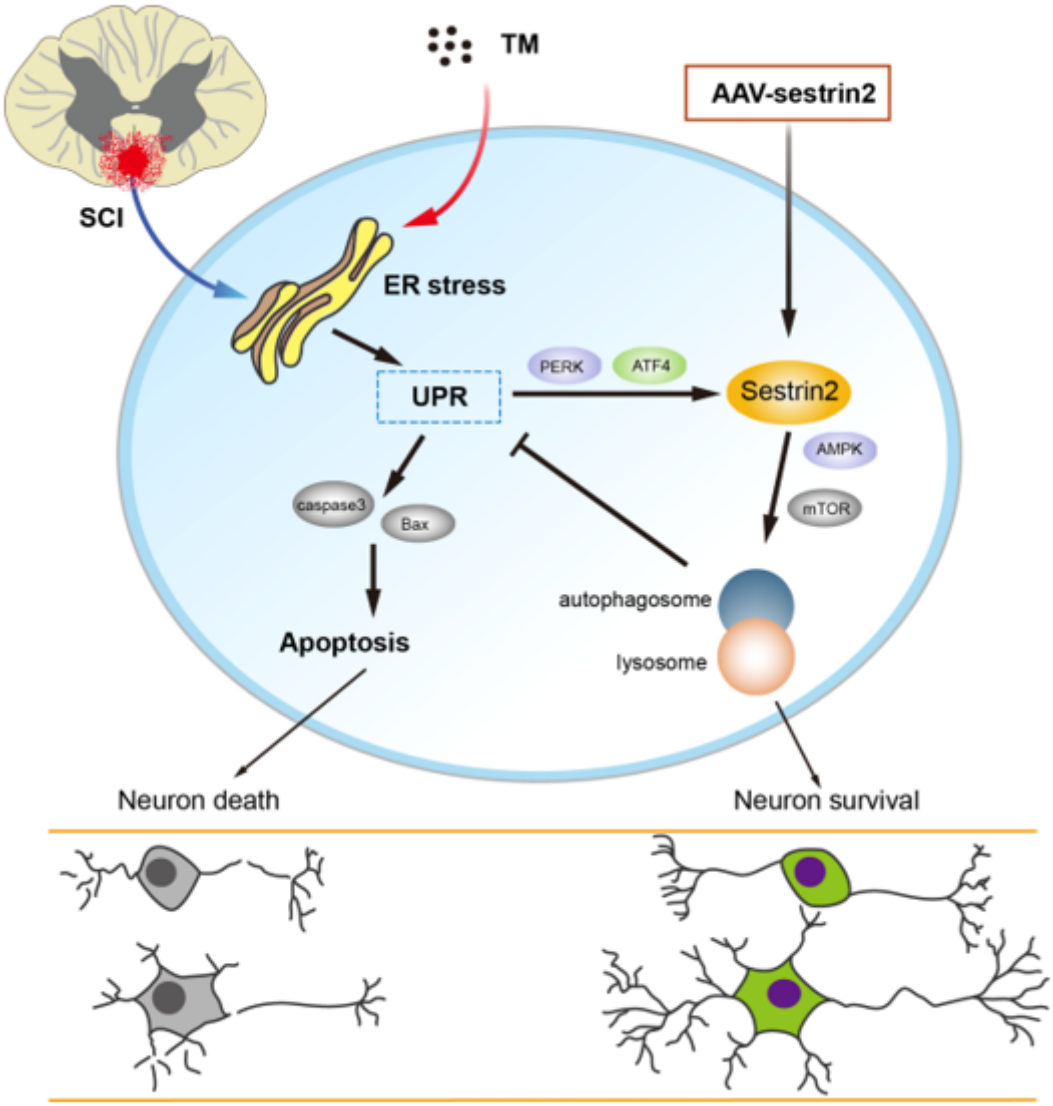



**Figure 9**

A potential mechanism of how ER stress induces sestrin2 and a proposed role of sestrin2 protects neuron from injury. Sestrin2 was adaptively upregulated in a PERK-ATF4 dependent manner after SCI and ER stress insult, which maintains neuron homeostasis against ER stress and apoptosis through activation of autophagy.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementFiguresandFigureLegends.pdf