Src Homology 2 domain–containing phosphotyrosine phosphatase 2 (Shp2) controls surface GluA1 protein in synaptic homeostasis

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Src Homology 2 domain–containing phosphotyrosine phosphatase 2 (Shp2) functions in synaptic plasticity, learning, and memory. However, the precise mechanisms by which this multifunctional protein contributes to synaptic function remains largely unknown. Homeostatic plasticity may be viewed as a process of bidirectional synaptic scaling, up or down. Through this process, neuronal circuitry stability is maintained so that changes in synaptic strength may be preserved under changing conditions. A better understanding of these processes is needed. In this regard, we report that phosphorylation of Shp2 at tyrosine 542 and its translocation to the postsynaptic compartment are integral processes in synaptic scaling. Furthermore, we show, using both pharmacological and genetic approaches, that Shp2 phosphatase activity is critical to the regulation of Ser(845) GluA1 and surface expression of this AMPA receptor subunit during synaptic scaling. Thus, Shp2 may contribute meaningfully to synaptic homeostasis.

Different types of stimuli elicit synapses of neurons to change their strength in a bidirectional manner, most studied as long-term potentiation (LTP) and long-term depression (LTD), the phenomenon well known as Hebbian-type synaptic plasticity (1). It is widely believed that the changes in relative synaptic strength via synaptic plasticity underpin the molecular and cellular basis of learning and memory (2, 3). However, LTP and LTD exert a vigorous long-term impact on neuronal circuits. Homeostatic plasticity, by synaptic scaling either up or down, maintains a stable neuronal circuit by adjusting synaptic properties to keep activity close to its optimal operating range, preserving network stability and, in turn, promoting learning and memory by offsetting deleterious effects induced by continuous LTP or LTD (4).

There is considerable evidence that changes in synaptic strength associated with synaptic plasticity or homeostatic plasticity are mediated by changes in the levels of AMPA receptors (1, 4). The majority of AMPA receptors in the hippocampus of rodents consists of GluA1/GluA2 or GluA2/GluA3 heterodimers, and homeostatic plasticity is tightly associated with changes in surface GluA1-containing AMPA receptors (1, 5). To bidirectionally regulate surface delivery of GluA1, phosphorylation of the GluA1 cytoplasmic C-terminal tail has been adopted in hippocampal neurons (5, 6). In particular, elevated phosphorylation of the PKA GluA1 Ser845 target site is widely speculated to facilitate forward surface delivery of GluA1 after LTP or synaptic upscaling (5, 7, 8). Recently we demonstrated that Shp2 contributes to the regulation of Ser(845) GluA1 during LTD. Specifically, inhibition of Shp2 phosphatase activity via pharmacological or genetic means impaired regulation of GluA1 phosphorylation during LTP induction in hippocampal neurons (9).

The ubiquitous tyrosine phosphatase Shp2 (encoded by PTPN11) has emerged as a critical regulator of synaptic plasticity, learning, and memory (9–13). In light of its structure, Shp2 contains two tandem N-terminal Src homology 2 (SH2) domains, a catalytic protein–tyrosine phosphatase (PTP) domain, and a C-terminal tail with tyrosyl phosphorylation sites and a proline-rich motif (14, 15). The N-terminal SH2 domain binds to the PTP domain, resulting in Shp2 autoinhibition, which can be relieved by phosphorylation of Tyr542 to disrupt the association of the SH2 and PTP domains (14, 16, 17). Hence, phosphorylation of Tyr542 correlates with activity of Shp2, which can be utilized as an indicative marker for the activity of Shp2. Germ line mutations in Shp2 cause Noonan syndrome, which may cause mental retardation and intellectual disability (18). Additionally, deregulation of Shp2 has been reported in various diseases, including juvenile myelomonocytic leukemia, breast cancer, and liver cancer (18–20). Thus, considerable effort has been directed toward the development of Shp2-specific inhibitors as potential drug therapies. Given this clinical relevance and the emerging role of Shp2 in synaptic plasticity, studies are warranted to further understand the role Shp2 plays in synaptic function and homeostasis.
In this study, we demonstrate that phosphorylation of Shp2, particularly at Tyr$^{542}$, is regulated by bidirectional homeostatic plasticity. Additionally, we provide convincing evidence that Shp2 is critical for elevated surface GluA1 expression in homeostatic plasticity. We show that inhibiting Shp2 phosphatase activity with its inhibitors, expressing a Shp2 phosphatase-inactive mutant, and adeno-associated virus (AAV)-mediated KO of Shp2 in hippocampal neurons prevented an increase in pS845 levels and surface GluA1. Therefore, our results reveal an undefined role of Shp2 in regulating GluA1 surface expression in homeostatic plasticity.

Results

The phosphorylation level of Shp2 Tyr$^{542}$ is regulated by synaptic scaling

To explore whether the phosphorylation level of Tyr$^{542}$ in Shp2 (hereafter referred to as the Tyr(P)$^{542}$ level) is regulated during synaptic scaling, we treated cultured rat hippocampal neurons with bicuculline (Bic) or tetrodotoxin (TTX) for 48 h to induce synaptic scaling down or scaling up, as reported previously (21). The phosphorylation level of Ser$^{845}$ (hereinafter referred to as the Ser(P)$^{845}$ level) in GluA1 and the total GluA1 level were simultaneously monitored by Western blotting to ascertain the success of synaptic scaling. As expected, TTX treatment elevated GluA1 and Ser(P)$^{845}$ levels, whereas bicuculline treatment reduced GluA1 and Ser(P)$^{845}$ levels (Fig. 1, A and B; for GluA1, TTX, 1.494 ± 0.069 and Bic, 0.664 ± 0.075; for Ser(P)$^{845}$, TTX, 1.760 ± 0.097 and Bic, 0.544 ± 0.040; n = 4), which is consistent with others reports (21, 22). Western blot analysis also showed an increased Tyr(P)$^{542}$ level in synaptic upscaling and a decreased Tyr(P)$^{542}$ level in synaptic downscaling (Fig. 1, A and B; TTX, 2.051 ± 0.094 and Bic, 0.369 ± 0.135), respectively. Interestingly, the total Shp2 level exhibited no significant difference among the control, the upscaling (TTX), and the downscaling (Bic) groups (Fig. 1, A and B). These results reveal that the activity of Shp2 is bidirectionally regulated during synaptic scaling.

Increased colocalization of Shp2 and PSD95 after synaptic upscaling

Our previous study suggested increased incorporation of Shp2 into excitatory postsynaptic sites after acute phase of chemical LTP (9). We wondered whether there was a similar process during synaptic upscaling. Therefore, we set out to examine the colocalization of endogenous Shp2 and PSD95 after 48-h TTX treatment. When the neurons were labeled with the secondary antibody but not the primary antibody against Shp2, no signal was detected under microscopy (supplemental Fig. S1). Afterward, colocalization analysis of Shp2 and PSD95 in dendrites was assessed by immunocytochemistry with or without 48-h TTX treatment in cultured rat hippocampal neurons (Fig. 2, A and B). We found that the proportion of Shp2 clusters merged with PSD95 clusters was increased after upscaling (Fig. 2, A and B). This result suggests that Shp2 translocates to postsynaptic sites after synaptic upscaling. To further confirm this result, the 48-h TTX-treated group and the control group were collected, followed by extraction of the postsynaptic density (PSD) fraction. Western blot analysis showed an increased Shp2 proportion in TTX-mediated synaptic upscaling against the control group in the PSD fraction (Fig. 2, C and D; GluA1, 2.088 ± 0.189, TTX versus control, n = 4; Shp2, 2.677 ± 0.237, TTX versus control, n = 4), indicating a translocation of Shp2 to postsynaptic sites after TTX-mediated upscaling. The results from confocal imaging and biochemistry suggest that Shp2 is subject to fine regulation by synaptic scaling in hippocampal neurons.

Inhibition of Shp2 phosphatase activity by specific inhibitors dampens surface GluA1 up-regulation in synaptic upscaling

Given our demonstration of the regulation of the Tyr(P)$^{542}$ level in synaptic scaling, we wondered whether activity of Shp2 was required for enhanced surface GluA1 after TTX-induced upscaling. To address this issue, we utilized two widely used inhibitors that block Shp2 activity. Our recent study revealed that 10 μM NSC87877 blocked chemical LTP-induced Tyr(P)$^{542}$ level elevation in cultured hippocampal neurons (9). Based on our previous data, we co-incubated 10 μM NSC87877 with TTX for 48 h. A Western blot analysis of cultured hippocampal neurons demonstrated that the level of Tyr(P)$^{542}$ was dramatically decreased in response to NSC87877 + TTX treatment compared with TTX alone, without altering the total Shp2 level (Fig. 3, A and B; Tyr(P)$^{542}$ level, TTX, 1.984 ± 0.216; NSC87877 + TTX, 1.028 ± 0.178; NSC87877, 0.974 ± 0.065; n = 3). Additionally, NSC87877 + TTX treatment blunted ele-
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Accumulating evidence illustrates that Shp2 is a protein–tyrosine phosphate implicated in activation of cell signaling and that mutation of C459S of Shp2 results in diminished Shp2 enzymatic activity (24, 25). Based on the above results, we predicted that the phosphatase-inactive Shp2 mutant, in which Cys459 was substituted with serine, would lead to the same phenomenon that was observed in Shp2 inhibitor experiments. To investigate this hypothesis, we transfected the Shp2 WT or C459S mutation together with GFP in DIV8 hippocampal neurons. The effect of TTX treatment (48 h) on surface expression of GluA1 in GFP-positive hippocampal neurons (DIV16) was assessed by immunostaining of cell surface receptors. As expected, hippocampal neurons transfected with Shp2 WT showed increased surface GluA1 expression after TTX treatment, whereas the increase was not observed in neurons transfected with the Shp2 C459S mutation after 48 h of TTX (Fig. 4, C and D), suggesting that the inhibitors of Shp2 have little effect on the translocation of Shp2 to the PSD fraction after synaptic upscaling. Together, our data suggest that inhibition of Shp2 activity by specific inhibitors dampens Ser(P)845 and GluA1 up-regulation in synaptic upscaling.

AAV-mediated KO of Shp2 blocks surface GluA1 up-regulation in synaptic upscaling

To rule out possible off-target effects of the Shp2 inhibitor, we utilized AAV-Cre–mediated KO of Shp2 in hippocampal neurons to evaluate the role of Shp2 in synaptic upscaling. As described in our previous study (9), we infected hippocampal neurons on DIV6–7. Seven days post-infection, TTX was
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A

MW (KDa)  Control  TTX  TTX + NSC
130  pS845-GluA1  GluA1  pY542-Shp2
130  Shp2  β-actin
70

B

Fold change (% Control)

pS845  GluA1  pY542  Shp2

C

Control  TTX  TTX + NSC
Surface GluA1  MAP2

D

Relative surface GluA1 (% Control)

E

MW (KDa)  Control  TTX  TTX + PHP51
130  pS845-GluA1  GluA1  pY542-Shp2
130  Shp2  β-actin
70

F

Fold change (% Control)

pS845  GluA1  pY542  Shp2

G

Control  TTX  TTX + PHP51
Surface GluA1  MAP2

H

Relative surface GluA1 (% Control)

I

PSD

MW (KDa)  Control  TTX  TTX + NSC  TTX + PHP51
130  GluA1  Shp2  PSD95
130

J

Fold change (% Control)

GluA1  Shp2

Control  TTX  NSC  TTX + NSC  PHP51
applied to the neurons for 48 h. Shp2, Tyr(P)542, GluA1, and Ser(P)845 levels were measured by Western blot analysis after-ward. Genetic ablation of Shp2 prevented an increase in GluA1 and Ser(P)845 levels elicited by 48-h TTX treatment (Fig. 4, C and D; GluA1: TTX for AAV-GFP, 1.632 ± 0.108; control for AAV-Cre, 1.187 ± 0.102; TTX for AAV-Cre, 1.077 ± 0.110; n = 4). In addition, surface staining of GluA1 was accessed on hippocam-pal neurons in the absence or presence of 48-h TTX treatment

Figure 3. Inhibition of Shp2 prevents elevation of the Ser(P)845 level and surface GluA1 after synaptic upscaling. A, hippocampal neurons were transfected with the Shp2 WT or Shp2 C459S mutant (phosphatase-inactive Shp2). After 48-h TTX treatment, surface GluA1 was analyzed. Scale bar = 5 μm. B, quantification of surface GluA1 levels (n = 15 neurons for each condition from three independent experiments). #, p ≤ 0.001 against control. C, hippocampal neurons from Shp2flox/flox mice were infected with AAV-GFP as a control or AAV-Cre to knock out Shp2. Seven days post-infection, 48-h TTX treatment was performed, followed by Western blot analysis with the indicated antibodies. MW, molecular weight. D, quantification of Ser(P)845, GluA1, Tyr(P)542, and Shp2 levels (n = 4 independent experiments). **, p ≤ 0.01; #, p ≤ 0.001 against the control; one-way ANOVA followed by Tukey’s post hoc test. E, hippocampal neurons were treated as in C. Surface GluA1 and MAP2 staining was performed after 48-h TTX treatment. Scale bar = 5 μm. F, quantification of surface GluA1 levels (n = 20 neurons for each condition from three independent experiments). #, p ≤ 0.001; one-way ANOVA followed by Tukey’s post hoc test.
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in WT or Shp2 KO hippocampal neurons. In line with our results from Shp2 inhibitors and the Shp2-inactive mutant, hippocampal neurons infected with AAV-GFP displayed increased surface GluA1 expression after 48-h TTX treatment, whereas surface GluA1 expression failed to elevate in neurons infected with AAV-Cre after 48 h of TTX (Fig. 4, E and F; TTX for AAV-GFP, 1.334 ± 0.039; control for AAV-Cre, 0.990 ± 0.038; TTX for AAV-Cre, 1.093 ± 0.046). These data suggest that loss of Shp2 function occludes surface GluA1 up-regulation during synaptic upscaling.

Discussion

Previously, we have shown that activity of Shp2 is critical for the increase in Ser(P)845 level in GluA1 as well as surface GluA1 after LTP (9). In this study, we focused on the molecular role of Shp2 in homeostatic plasticity and uncovered a novel role of Shp2 in synaptic upscaling-mediated changes of surface GluA1 by pharmacological and genetic approaches. We found that the phosphorylation level of Shp2 at Tyr442 was regulated by synaptic scaling. Additionally, increased colocalization of Shp2 and PSD95 and accumulated Shp2 sorting into the PSD fraction were observed after synaptic upscaling. The results suggest Shp2 translocation into the postsynaptic site to facilitate its function in the synapse during synaptic upscaling. Recently, we showed that Shp2 rapidly accumulated at the postsynaptic site after LTP stimuli (9). Given both synaptic upscaling and LTP-induced Shp2 translocation into the postsynaptic site, the shared phenomenon of accumulation of Shp2 at the postsynaptic site may contribute to modulating the phosphorylation of GluA1 in the two processes.

We provide evidence that phosphatase activity of Shp2 is critical for regulating GluA1 in synaptic upscaling. Considering that Shp2 constitutes two tandem SH2 domains (14), it is rational to hypothesize that Shp2 may also serve as a scaffolding protein to recruit other signaling proteins during synaptic upscaling for coordinated regulation of GluA1. However, expression of the phosphatase-inactive form of Shp2 in hippocampal neurons sufficed to negate increased surface expression of GluA1 after synaptic upscaling, implying that the phosphatase activity of Shp2 was critical for the process, whereas its scaffolding function exerted minimal effects on synaptic scaling up.

It is worth noting that both acute glycine-induced chemical LTP and chronic TTX treatment drive the accumulation of Shp2 at postsynaptic sites and elevate the Ser(P)845 level, which is Shp2-dependent. The signaling pathways are substantially complex with respect to synaptic plasticity (LTP) and synaptic upscaling, consisting of overlapping and separated signaling nodes for these two processes. Nevertheless, how these two distinct stimuli (chemical LTP and chronic TTX treatment) elicit similar phosphorylation outcomes remains elusive. Our results, demonstrating that Shp2 is activated after synaptic upscaling and that phosphatase activity of Shp2 is vital for elevated surface expression of GluA1, suggest that Shp2 is a common element in the regulation of GluA1 during the two processes. Recently, it has been suggested that Shp2 contributes to ERK1/2 pathway activation during LTP, which would agree with the results presented by us and others (9, 12). However, there is evidence showing that the activity of ERK1/2 was decreased at a time scale of dozens of minutes following blockade of neuronal activity by TTX and that ERK1/2 activity reached a stable level after 6–24 h of TTX treatment (26). The contradiction of activated Shp2 and inactivated ERK1/2 suggests dissociated roles of Shp2 and ERK1/2 during synaptic upscaling, adding complexity to the signaling pathway involved in synaptic scaling.

The downstream signaling mechanisms by which Shp2-dependent regulation of GluA1 is mediated during synaptic scaling remain unclear. Shp2 functions both as a protein phosphatase and scaffolding protein promoting assembly of protein complexes (14–16). Thus, depending on different circumstances, Shp2 may contribute to regulating the ERK1/2, Src family kinase, PI3K—Akt, and NF-κB pathways (27). More study is required to understand by which of the multiple functions and downstream pathways Shp2 regulates phosphorylation of GluA1 at Ser845 during synaptic scaling and the broader processes of synaptic plasticity.

Experimental procedures

Hippocampal neuron culture and transfection

The use and care of animals in this study were performed in strict accordance with Hangzhou Normal University Animal Experimentation Committee guidelines and completely followed the guidelines of the National Institutes of Health Animal Research Advisory Committee. We made maximal efforts to minimize animal numbers and to reduce animal suffering. Hippocampal primary neuronal cultures were harvested from embryonic day 18 Sprague-Dawley rats or newborn (postnatal day 1–2) Shp2lox/lox mice of either sex, as described previously (9, 29). Hippocampal tissue was quickly dissected in ice-cold Hanks’ balanced salt solution (HBSS) buffer and then digested in 0.5% trypsin for 15 min at 37 °C. Dissociated neurons were seeded on poly-L-lysine-coated (Sigma-Aldrich, St. Louis, MO) coverslips (Deckgläser, Carolina Biological, Burlington, NC) or dishes (Corning, Rochester, NY) at a proper density in Neurobasal medium containing 10% horse serum at 37 °C under 5% CO2. Four hours later, the primary medium was substituted with Neurobasal medium containing 0.5 mM GlutaMax supplemented with 2% B27 and 1% penicillin/streptomycin. Subsequently, the culture medium was replaced every 4 days. At days in vitro 4 (DIV4), cytosine arabinoside was added into the culture medium at a final concentration of 2.5 μM. All buffers were purchased from Gibco (Life Technologies) unless otherwise stated.

EGFP-N1 was co-transfected with Shp2 WT or mutant C459S at a mass ratio of 1:10 at DIV8 using the CalPhosTM mammalian
transfection kit (Clontech). Shp2floxflox neurons were infected with adenovirus-associated viruses at DIV7. All cell culture reagents were acquired from Gibco (Life Technologies) unless otherwise stated.

Synaptic scaling induction and drug treatment
To induce homeostatic scaling, hippocampal neurons at DIV14–16 were incubated for 48 h with bicuculline or TTX at a final concentration of 40 μM or 1 μM, respectively. For some experiments, TTX was used in combination with PHPS1 (5 μM) or NSC87877 (10 μM). Neurons treated with DMSO were considered the control group.

Immunocytochemistry
We followed a protocol described previously for surface staining of GluA1 (9). Briefly, hippocampal neurons were washed twice with 37 °C PBS. Immediately after washing, surface GluA1 was labeled with anti-GluA1 (MAB2263, Millipore, 1:50) for 15 min at 37 °C. Neurons were washed and fixed and then incubated with the respective secondary antibody for 15 min at 37 °C to visualize surface GluA1.

For staining of endogenous Shp2 and PSD95, neurons were fixed with 4% paraformaldehyde for 10 min and permeabilized and blocked simultaneously in PBS buffer containing 0.2% Triton X-100 and 5% BSA for 0.5 h. Then Shp2 was labeled with rabbit anti-Shp2 (SC-280, Santa Cruz Biotechnology, 1:50) and PSD95 was labeled with mouse anti-PSD95 (ab2723, Abcam, 1:500) primary antibodies for 1 h at room temperature. Neurons were washed and incubated with the respective secondary antibody for 1 h at room temperature or overnight at 4 °C. For colocalization of Shp2 and PSD95, donkey anti-rabbit Alexa 488–conjugated (A21206, Invitrogen, 1:500) and goat anti-mouse Alexa 546–conjugated (A11030, Invitrogen, 1:500) secondary antibodies were used. For the PHPS1 and NSC87877 treatment experiment, donkey anti-mouse Alexa 488–conjugated (A21202, Invitrogen, 1:500) antibody was used for surface GluA1 and goat anti-rabbit Alexa 633–conjugated (A21082, Invitrogen, 1:500) antibody was used for MAP2. For AAV-mediated Shp2 KO neurons or neurons co-transfected with EGFP-N1 and Shp2 or C459S, surface GluA1 was labeled with goat anti-mouse Alexa 546–conjugated (A11030, Invitrogen, 1:500) secondary antibody, and MAP2 was labeled with goat anti-rabbit Alexa 633–conjugated (A21082, Invitrogen, 1:500) secondary antibody.

Cells were imaged on a confocal microscope (Fluoview FV1000, Olympus). Manders coefficient was analyzed using the ImageJ plugin JACoP, and the percentage of Shp2 colocalized with PSD95 was analyzed by manually counting the overlapping cluster number of Shp2 and PSD95, which was divided by the total cluster number of PSD95. For surface AMPA receptor intensity, the integrated intensity of individual puncta of endogenous surface GluA1 per unit length of dendrite was measured, and the ratio of surface GluA1/MAP2 in other groups was normalized to the ratio of the control group. Experiments were repeated at least three times independently, and significance was analyzed using one-way ANOVA followed by Tukey’s post hoc test (n = number of cells).

PSD fractionation of cultured neurons
PSD fractionation was performed as described previously (9, 30).

Immunoblotting
Western blotting was adopted from previous publications with minor modifications (9, 30). After pharmacological treatments, neurons were washed with ice-cold PBS and lysed by radioimmunoprecipitation assay buffer containing 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholate (Beyotime, Shanghai, China). Then the cell lysate was clarified by centrifugation at 16,000 × g for 30 min. Subsequently, the supernatant was quantified using a BCA protein assay kit (Thermo Fisher Scientific, Pittsburgh, PA). Samples containing equal amounts of protein were denatured in loading buffer by boiling for 5 min, subjected to SDS-PAGE, transferred onto nitrocellulose membranes (Whatman, GE Healthcare), and subjected to immunoblot analysis. Blots were probed with ECL reagent (Thermo Fisher Scientific), and chemiluminescence was detected by photographic film. Protein bands were analyzed with Quantity One software (Bio-Rad).

Antibodies and reagents
The primary antibodies used in immunoblotting were rabbit anti-Shp2 (SC-280, Santa Cruz Biotechnology, 1:5000), rabbit anti-pShp2-Tyr542 (ab62322, Abcam, 1:5000), mouse anti-GluA1 (MAB2263, Millipore, 1:2000), rabbit anti-pGluA1-Ser845 (04-1073, Millipore, 1:1000), and mouse anti-β-actin (A5316, Sigma-Aldrich, 1:10,000). Rabbit anti-MAP2 antibody (4542, Cell Signaling Technology, 1:200) was used for immunocytochemistry. The secondary antibodies for immunoblotting were goat anti-rabbit IgG-HRP–conjugated secondary antibody (31422, Cell Signaling Technology, 1:200) and goat anti-mouse IgG-HRP–conjugated secondary antibody (31460, Pierce, 1:10,000) and goat anti-mouse IgG-HRP–conjugated secondary antibody (31460, Pierce, 1:10,000).

TTX (1078, Bic (2503), and NSC87877 (2613) were acquired from Tocris Bioscience (Minneapolis, MN), and PHPS1 (sc-253272) was purchased from Santa Cruz Biotechnology. AAVs, both AAV-CaMKII-GFP-Cre and AAV-CaMKII-GFP, were packaged by Obio Technology (Shanghai, China) and have been described previously (9).

Statistical analysis
All data were analyzed using GraphPad prism 6.0 software (GraphPad Software, La Jolla, CA) and are represented as mean ± S.E. Comparisons of two groups were done by Student’s t test. Data of multiple groups (three or more groups) were tested using one-way ANOVA followed by Tukey’s post hoc test. p ≤ 0.05 was considered significant. No significance (NS) indicates p > 0.05.

Author contributions—B. Z. designed and performed the biochemical and immunostaining experiments. W. L. designed and performed some biochemical experiments, analyzed the data, and conceived and coordinated the study. B. Z. and W. L. wrote the paper. Both authors reviewed the results and approved the final version of the manuscript.
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