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Tet1-mediated DNA demethylation regulates neuronal cell death induced by oxidative stress

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Epigenetic regulations including DNA methylation and demethylation play critical roles in neural development. However, whether DNA methylation and demethylation may play a role in neuronal cell death remains largely unclear. Here we report that the blockade of DNA methyltransferase inhibits apoptosis of cerebellar granule cells and cortical neurons in response to oxidative stress. We found that knockdown of ten-eleven translocation methylcytosine dioxygenase (Tet1), a critical enzyme for DNA demethylation, significantly increase apoptosis of cerebellar granule cells induced by hydrogen peroxide. Moreover, cerebellar granule cells from *tet1^{null}* mice appeared to be more sensitive to oxidative stress, suggesting the critical role of Tet1 in neuronal cell death. We further showed that the expression of Klotho, an antiaging protein, in cerebellar granule cells is tightly regulated by DNA methylation. Finally, we found that knockdown of Klotho diminished the rescue effects of DNA methyltransferase inhibitors and Tet1 on neuronal cell death induced by oxidative stress. Our work revealed the role of Tet1-mediated DNA demethylation on neuronal protection against oxidative stress and provided the molecular mechanisms underlying the epigenetic regulation of neuronal cell death, suggesting the role of Klotho in regulating neuronal cell death in response to oxidative stress.

Oxidative stress play critical roles in neuronal cell death in various neurodegenerative disorders[1]. Transcription-related programs are involved in cellular responses to oxidative stress[2]. Neuronal cell death in responses to oxidative stress usually exhibits destructive functions for the nervous system during disease conditions[3]. Cerebellar granule neurons (CGNs) serve a robust system for studying molecular and cellular mechanism underlying neuronal cell death[4]. During brain development, the survival of CGNs is regulated by various factors, including growth factors, neuronal activity and oxidative stress[5]. However, whether epigenetic regulations such as DNA methylation and demthylation contribute to the survival of CGNs in response to oxidative stress remains largely unclear.

DNA methylation and demethylation plays crucial roles in many biological processes, including regulation of gene expression, maintenance of genomic stability and integrity. Studies in cancer biology revealed that DNA methylation play a determinant role in silencing of oncogenes during cancer development, suggesting that DNA methylation and demethylation may play important roles in various physiological processes other than house-keeping-like functions such as maintaining genome stability.

Programmed cell death plays a vital role in the maintenance of cellular homeostasis, especially for the balance between cell proliferation and cell death[6]. It is not clear whether DNA methylation and demethylation play roles in neuronal cell death in responses to fatal stimuli such as oxidative stress. It is reported recently that forced expression of DNA methyltransferase 3a (Dnmt3a) drove apoptosis of motor neurons, and elevated levels of Dnmts protein and 5mC (5-methylcytosine) in human amyotrophic lateral sclerosis (ALS) patients samples were detected, suggesting that aberrant regulation of DNA methylation in the pathobiology of ALS[7]. Thus the molecular mechanisms underlying DNA methylation-mediated neuronal cell death are critical for us to understand the potential neuropathology of neurodegenerative diseases, given the fact that Dnmts appears to have high expression levels in adult rodent brain[8].

Recent studies showed that the Tet methylcytosine dioxygenase (TET1) protein could catalyze the conversion of 5-methylcytosine (5mC) of DNA to 5-hydroxymethylcytosine (5hmC), raising the possibility that DNA demethylation may be a TET1-mediated event[9]. TET1 depletion diminishes 5hmC levels at transcription start

sites (TSS)[10] and after TET1 activation, 5hmC levels increase significantly during reprogramming to human iPSCs and this hydroxymethylation changes is critical for optimal epigenetic reprogramming[11]. However, whether TET family proteins may be involved in neuronal cell death is not clear.

Despite the abundance of 5hmC and TET family proteins in the brain, little is known about their neuronal functions. Tet1 knockout mice exhibited abnormal hippocampal long-term depression, impaired memory extinction, as well as deficiency in adult hippocampal neurogenesis, indicating the critical role of Tet1 in the central nervous system[12,13].

In this work, we report that the role of Tet1-mediated DNA methylation in neuronal cell death of cerebellar granule cells in response to oxidative stress. We found that knockdown of Tet1, a critical enzyme for DNA demethylation, significantly increase apoptosis of cerebellar granule cells induced by hydrogen peroxide. Whereas, overexpression of the catalytically active domain of Tet1 (Tet1-CD) remarkably decreases neuronal apoptosis induced by oxidative stress. Importantly, we found that cerebellar granule cells from *tet1*^{null} mice appeared to be more sensitive to oxidative stress, suggesting the critical role of Tet1 in neuronal cell death. We further showed that the expression of Klotho, an antiaging protein, in cerebellar granule cells is tightly regulated by DNA methylation and demethylation. Oxidative stress decrease the expression level of Klotho protein, which is rescued by DNA methyltransferase inhibitors. Finally, we found that knockdown of Klotho compromised the rescue effects of DNA methyltransferase inhibitors and Tet1 on neuronal cell death induced by oxidative stress. Our work revealed the role of DNA demethylation on neuronal cell death induced by oxidative stress and provided the molecular mechanisms underlying the epigenetic regulation of neuronal cell death, suggesting the role of DNA demethylation-dependent Klotho expression in neuronal cell death.

Results

The role of DNA methylation and demethylation in neuronal cell death induced by oxidative stress. DNA methyltransferase inhibitors are commonly used as the treatment of tumors in clinical trials[14], we would like to test whether it may have effects in neuronal survival in response to oxidative stress. We used mouse cerebellar granule cells (CGNs) as the main cellular model for studying neuronal cell death, with a standard apoptosis-inducing protocol using hydrogen peroxide as an oxidative stress[15,16]. Mouse cerebellar granule cells were dissected from new postnatal mouse brain, plated, and transfected with GFP-expression plasmids as marker. Neuronal apoptosis were induced by applying 100 μM hydrogen peroxide for 10–12 h. Condensed or shrinking nuclei were counted as apoptotic cells. We found that hydrogen peroxide treatment strongly induced neuronal cell death (Figure 1a,b). However, pre-treatment of Dnmts inhibitor 5-aza-2′-deoxycitydine (5-aza-2-dC) significantly inhibits the neuronal cell death induced by hydrogen peroxide, suggesting that DNA methylation may play a role in promoting neuronal cell death induced by oxidative stress (Figure 1a,b).

The ten-eleven translocation (Tet) family of methylcytosine dioxygenases, including Tet1, Tet2, and Tet3, has been recently implicated in the DNA demethylation cascade. Thus we set out to examine whether Tet1, the primary Tet family protein in the brain, may participate in neuronal cell death. We first designed specific short hairpin RNA (shRNA) against mouse Tet1 gene and validated its efficiency of down-regulating the endogenous Tet1 protein level in mouse neurons (Figure 1c)[17]. After we transfected Tet1 shRNA in cultured mouse cerebellar granule cells, we found that Tet1 knockdown increased the apoptotic ratio induced by hydrogen peroxide, indicating that Tet1-mediated DNA demethylation play a positive role in cerebellar granule cells survival in response to the oxidative stress (Figure 1d,e).

Tet1 is critical for neuronal cell death induced by hydrogen peroxide. As Tet1 is a critical factor in the oxidation-deamination reaction underlying active DNA demethylation in mammals, Tet1 has been shown to be crucial for regulating DNA demethylation in the central nervous system. Genetic deletion of Tet1 seems not to interfere with embryonic development[18]. However various defects in brains of *tet1* null mice have been identified including adult hippocampal neurogenesis and cognitive functions, suggesting the remarkable role of Tet1-mediated DNA demethylation in the postnatal brain[12,13].

Thus we use Tet1 null mice with deletion of the exons 11–13 encoding the critical catalytic domain in dioxygenases to investigate whether Tet1 plays a role in cerebellar granule cell death in response to oxidative stress[13]. We cultured cerebellar granule cell from Tet1 knockout mice and wild-type littermates at postnatal 6 day and stimulated cells with H_2O_2 in various concentrations for 10–12 h. Surprisingly, we found that CGNs with Tet1 deletion appear to be more sensitive to hydrogen peroxide and exhibit more apoptosis comparing to cells from wild-type mice (Figure 2a,b).

To further confirm whether the enzymatic activity of Tet1 is responsible for effects we identified, we further used hemagglutinin (HA)-tagged human Tet1 catalytic domain (a.a. 1418–2136, referred to Tet1-CD) and a catalytically inactive Tet1 mutant (H1671Y/D1673A, referred to Tet1-CDm)[19]. We co-transfected Tet1-CD and Tet1-CDm with the GFP-expressing plasmid in cultured cerebellar granule cells, and stimulated cells with H_2O_2 as described above. We found that Tet1-CD significantly reduce the apoptotic cell ratio, on the contrary, Tet1-CDm did not has similar effect, further indicating that Tet1-mediated DNA demethylation promotes neuronal survival in response to oxidative stress (Figure 2c,d).

The expression of Klotho is regulated by oxidative stress and DNA methylation. Klotho was previously reported as an ageing-related gene and also plays a critical role in neurodegenerative diseases[20]. Klotho deficient mice show growth retardation and die prematurely at 8–9 weeks of age. Interestingly, it is reported that Klotho may be regulated by DNA methylation events associated with uremic toxins injection[21]. Thus we would like to examine whether expression of Klotho is regulated by oxidative stress or DNA methylation and demethylation in the central nervous system.

First we investigated whether the expression of Klotho was regulated by DNA methylation in mouse neurons. We examined the mRNA levels of Klotho and brain derived neurotrophin factor gene (BDNF) after 5-aza-2-dC treatment for 6–12 h and with various concentrations. We found that treatment of 5-aza-2-dC for 12 h significantly increased the mRNA level of Klotho in cortical neurons, similar with BDNF, which is well-known to be regulated by DNA methylation (Figure 3a,b). Consistently with this observation, mRNA levels of Klotho and BDNF were remarkably increased when cortical neurons were treated with various concentration of 5-aza-2-dC (higher than 0.1 μM) (Supplemental Figure 1a,b). The protein level of Klotho is also increased by 5-aza-2-dC treatment for 12 h in mouse cortical neurons (Supplemental Figure 1c).

Next we treated the CGNs with 5-aza-2-dC for a time course from 0 to 48 h and examined the protein level of Klotho. We found that the blockade of Dnmts with 5-aza-2-dC greatly increased the Klotho level 12 h after treatment, suggesting that expression of Klotho is regulated by DNA methylation (Figure 3c). We then treated CGNs with various concentration of H_2O_2 (0–400 μM) for 12 h and measured the protein level of Klotho in CGNs. We found that H_2O_2 treatment significantly decreased the protein level of Klotho in CGNs (Figure 3d), suggesting that expression of Klotho is sensitive for oxidative stress. Thus we asked whether inhibition of DNA methylation may affect Klotho protein level in response to oxidative responses. Indeed, we found that pre-treatment of 5-aza-2dC 6 h prior to oxidative stress significantly rescue the Klotho protein level

Figure 1 | DNA demethylation inhibits neuronal cell death induced by oxidative stress. (a) CGNs transfected with GFP vector with or without pretreated with 5-aza-2-dC, a Dnmt inhibitor, and treated with H_2O_2. Yellow arrowhead stands for the healthy cells and red arrowhead indicates apoptotic cells. Chromatin condensation was monitored by Hoechst staining. Scale bar = 50 µm. (b) Quantitation of (a). (ANOVA followed by Tukey's test, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, $n = 4$). Error bar represents s.e.m. Each bar represents at least 600 cells of each condition. (c) Validation for Tet1 shRNA efficacy. Cortical neurons were electroporated with control and Tet1 shRNA expressing plasmids and lysated for Western blot analysis. (d) CGNs transfected with GFP-expressing plasmid with or without Tet1 shRNA plasmid, were treated with H_2O_2. Yellow arrowhead stands for the healthy cells and red arrowhead indicates apoptotic cells. Scale bar = 50 µm. (e) Quantitation of (d). (ANOVA followed by Tukey's test, ***$p < 0.001$, $n = 4$). Error bar represents s.e.m. ANOVA, analysis of variance; CGNs, cerebellar granule neurons; GFP, green fluorescent protein; shRNA, short hairpin RNA.

down-regulated by H_2O_2 stimulation (Figure 3e). To ask whether Tet1 has play a role in regulating Klotho expression, we examined the protein level of Klotho in Tet1 knocked down cortical neurons. Indeed, we found that Klotho protein level significantly decreased when Tet1 was knocked down by shRNA (Figure 3f). This data indicate that Klotho expression is tightly regulated by Tet1-depend-

Figure 2 | Tet1 is involved in neuronal cell death induced by oxidative stress. (a) Tet1-null neurons showed higher sensitivity to oxidative stress. CGNs were cultured from Tet1 KO mice and control littermates, treated with H_2O_2 of a gradient 0, 100 μM, 200 μM. Cell condensation was monitored by Hoechst staining. Yellow arrowhead stands for the healthy cells and red arrowhead indicates apoptotic cells. (b) Quantitation of (a). (student t test, $^{**}p < 0.01$, $^{*}p < 0.05$, $n = 3$. Error bar represents s.e.m. Scale bar = 20 μm. WT, wild type; KO, Tet1 knockout). (c) CGNs transfected with HA-tagged Tet1CD and Tet1CDm together with GFP were treated with H_2O_2. Yellow arrowhead stands for the healthy cells and red arrowhead indicates apoptotic cells. (d) Quantitation of (c). (ANOVA followed by Tukey's test, $^{***}p < 0.001$, $n = 4$). Error bar represents s.e.m. Scale bar = 50 μm.

ent DNA methylation and demethylation mechanisms in mouse neurons.

We would like to further test whether the Klotho protein level is regulated in the middle cerebral artery occlusion model in mouse (MCAO protocol see Materials and Methods), for examining oxidative stress *in vivo*[22]. Briefly, mouse focal cerebral ischemia (I) was induced by blood vessels suture for 2 h, and after reperfusion (R) for different periods from 0 h, 6 h to 12 h, Klotho protein levels were examined in protein extracts from the contralateral (c) or ipsilateral (i) cortex. Interestingly, we found that the protein level of Klotho significantly decreased in the experiment side–ipsilateral cortices, comparing to contralateral cortices as control, after re-opening the suture for 12 h (Figure 3g), suggesting that the protein level of Klotho in the brain is regulated by oxidative stress *in vivo*.

Klotho plays a crucial role in DNA demethylation-mediated neuronal survival in response to oxidative stress. Next we want to examine whether Klotho contribute to neuronal cell death induced by oxidative stress. We first designed a specific short hairpin RNA against mouse Klotho (shKlotho) so we could inhibit the endogenous expression of Klotho in CGNs (Figure 4a). We then transfected shKlotho into CGNs and followed with or without H_2O_2 stimulation. Surprisingly, we found that knockdown of Klotho led to neuronal cell death to a similar extent with H_2O_2 treatment, whereas H_2O_2 treatment on Klotho-knockdowned CGNs would lead to a greater extent of neuronal death, comparing with H_2O_2 treated condition (Figure 4b,c). These results suggest that Klotho is critical for neuronal survival, particularly under oxidative stress.

Next, we want to examine whether Klotho is involved in DNA methylation and Tet1-mediated neuronal responses in oxidative stress. We transfected shKlotho into CGNs and treated with H_2O_2 and 5-aza-2-dC as described above (Figure 5a,b). Consistently, 5-aza-2-dC showed a strong effect in promoting neuronal survival in response to H_2O_2. Strikingly, we found that knockdown of Klotho neutralized the effect of 5-aza-2-dC and led to neuronal cell death to a greater extent (Figure 5a,b). Moreover, we expressed Tet1-CD into CGNs with or without shKlotho and treated CGNs with H_2O_2 (Figure 6a,b). We found that knockdown of Klotho also diminished

Figure 3 | Expression of Klotho is regulated by oxidative stress and DNA methylation. (a–b) Expression of Klotho in cortical neurons is regulated by DNA methylation. Cortical neurons were treated with 5-aza-2-dC for 6–12 h and collected for analyzing mRNA levels of Klotho and BDNF. Quantitative PCR were performed. All data points are normalized to untreated samples. (c) Klotho protein expression was up-regulated with 5-aza-2-dC treatment. CGNs were treated with 5-aza-2-dC for 12–48 h and collected for examination protein level of Klotho. Lower panel: Quantification of western blot results. (d) Klotho protein expression was down-regulated with H_2O_2 treatment. CGNs were treated with various concentrations of H_2O_2 for 12 h and collected for examination protein level of Klotho. Antibodies against Klotho and Tubulin were used. Lower panel: Quantification of western blot results. (e) H_2O_2-induced Klotho downregulation is rescued by 5-aza-2-dC treatment. CGNs were treated with 5-aza-2-dC 6 h prior to H_2O_2 stimulation for 12 h and collected for examination protein level of Klotho. Lower panel: Quantification of western blot results. (f) Klotho protein level was down-regulated by knockdown of Tet1. Lentivirus harboring Tet1 shRNA were seed to mouse cortical neurons and cell samples were harvested 4–5 days after viral infection. Western blots with specific antibody were performed to examine protein level of Klotho. Right panel: quantitation of (f). (Student t test, $n = 4$, *$P < 0.05$, Error bar represents s.e.m.) (g) Klotho in the neurons was down-regulated in the MCAO model. Mouse focal cerebral ischemia (I) was induced for 2 h, and after reperfusion (R) for different periods 0 h, 6 h and 12 h, immunoblots were done of the extracts from the contralateral (c) or ipsilateral (i) cortex. Tubulin served as a loading control. Lower panel: quantification of the normalized klotho protein levels (Student t test, $n = 3–4$ mice per time point, *$P < 0.05$ versus contralateral, Error bar represents s.e.m. I, ischemia; R, reperfusion). All data were collected from 3–6 independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$; one-way ANOVA with Tukey's test.

Figure 4 | Klotho is involved in neuronal protection in response to oxidative stress. (a) Validation of Klotho shRNA. CGNs were electroporated with plasmids containing control and Klotho shRNA and collected for Western blot analysis. (b) CGNs transfected with GFP-expressing plasmids along with control and Klotho shRNA plasmids, treated with H_2O_2 and immunostained for GFP and Hoechst for measure neuronal cell death. Yellow arrowhead stands for the healthy cells and red arrowhead indicates apoptotic cells. (c) Quantitation of (b). (ANOVA followed by Tukey's test, ***$p < 0.001$, $n = 4$). Error bar represents s.e.m. Scale bar = 50 μm.

the effect of Tet1-CD on neuronal survival (Figure 6a,b). This evidence indicate that Klotho is critical for DNA demethylation and Tet1-mediated neuronal protection in response to oxidative stress.

In the last set of experiment, we further tested our model in mouse primary cortical neurons using different stress induction methods. We used staurosporine, a PKC inhibitor to induce apoptosis in mouse cortical neurons[23]. First, we found that pre-treated with staurosporine, mouse cortical neurons undergo neuronal cell death similar to cerebellar granule cells (Supplemental Figure 2 a,b). Importantly, we found that either blocking DNA methylation by 5-aza-2-dC or promoting DNA demethylation with expression of Tet1-CD significantly promote neuronal survival (Supplemental Figure 2,3). Moreover, knockdown of Klotho diminishes the protective effects of 5-aza-2-dC and Tet1-CD (Supplemental Figure 2,3), indicating that stress-induced cell death of cortical neurons also depends on DNA methylation-associated mechanisms and Klotho plays a critical role in DNA methylation-dependent neuronal cell death.

Discussion

Our results reveal that block DNA methylation with DNMTs' inhibitors, especially 5-aza-2-dC, inhibits hydrogen peroxide induced neuronal cell death. Loss of Tet1 mediated DNA demethylation increase the sensitivity of neuronal cell death to oxidative stress, suggesting that DNA demethylation may have protection effect in neuronal system. As DNA methylation and demethylation may affect gene expression during neuronal development, we want to investigate the molecular mechanism underlying DNA demethylation-mediated neuronal protection in response to oxidative stress. Previous work found that 5-aza-2-dC treatment increased the expression level of Klotho, an anti-aging gene. Thus our hypothesis is that klotho may be involved in the methylation and demethylation mediated neuronal cell death. We expressed klotho shRNA in 5-aza-2-dC and Tet1-CD cells and found that it could reduce the protection effect from oxidative stress, suggesting that klotho is involved in DNA demethylation mediated neuronal protection.

Figure 5 | Klotho is critical for DNA demethylation-mediated neuronal protection in response to oxidative stress. (a) CGNs were transfected with shKlotho, pretreated with 5-aza-2-dC 6 h prior to H_2O_2 stimulation for 12 h. Cell condensation was monitored by Hoechst staining. Yellow arrowhead stands for the healthy cells and red arrowhead indicates apoptotic cells. (b) Quantitation of (a). (ANOVA followed by Tukey's test, ***$p < 0.001$, **$p < 0.01$, $n = 4$). Error bar represents s.e.m. Scale bar = 50 μm.

Although it remains to be determined whether klotho is directly or indirectly regulated by Tet1, the expression of Klotho is correlated with CpG hypermethylation of its promoter region[21]. Inhibiting klotho expression by klotho shRNA up-regulated the p53/p21 pathway and induce premature senescence of human cells[24]. Consequently, DNA methylation and demethylation mediate neuronal protection may be mediated by Klotho and p53 pathway, which can be potential molecular therapy for neurodegenerative disorder and ageing.

Methods

All experimental protocols using animals and cell samples were approved by Shanghai Institutes for Biological Science, Chinese Academy of Sciences.

Genetic modified mice. Tet1 KO mice were a gift from Dr. Guoliang Xu at Shanghai Institute of Biochemistry and Cell Biology, CAS. The use and care of animals were carried out in accordance with approved guideline of the Biomedical Research Ethics Committee at the Shanghai Institutes for Biological Science, Chinese Academy of Sciences.

Figure 6 | Klotho is critical for Tet1-mediated neuronal protection in response to oxidative stress. (a) CGNs were transfected with either Klotho shRNA plasmid or HA-tagged Tet1-CD and both plasmids, treated with H_2O_2 and immunostained for HA, GFP and Hoechst for examining neuronal cell death. Yellow arrowhead stands for the healthy cells and red arrowhead indicates apoptotic cells. (b) Quantitation of (a). (ANOVA followed by Tukey's test, ***$p < 0.001$, **$p < 0.01$, $n = 4$). Error bar represents s.e.m. Scale bar = 50 μm.

Plasmids and constructs. The FUGW-H1 expression vector (#25870) and Tet1-CD (#39454) and Tet1-CDm (#39455) expression vector[19] were purchased from Addgene. The RNAi sequences targeting mouse Tet1 (Tet1 shRNA-1: 5′-GCAGATGGCCGTGACAC AAAT-3′)[17], mouse Klotho (Klotho shRNA-3: 5′-GGAGATGT GGCCAGCGATA-3′) were synthesized and cloned into FUGW-H1 by inserting target sequence between the XbaI and BamHI sites following the instructions.

Cell culture, transfection and hydrogen peroxide treatment. Mouse cerebellar granule neurons (CGNs) were obtained from 6–7 days old pups as described[15]. Cells were digested with 0.25% trypsin and plated at appropriate density in plates, pre-coated with poly-D-lysine and laminin. Neurobasal-A with B27 supplement were used for culturing CGNs. Cultures were kept at 37°C and 5% CO2 until use. At 48 h after initial plating, transfections with GFP-tagged control and shRNA plasmid were

performed using a lipofectamine 2000 (Invitrogen, 11668019) follow the user's instruction manual. On Div6-7, cells were treated only or together with 5-aza-2-dC (1 μM) (Merck/Calbiochem 189825) and H_2O_2 (100 μM) (ACROS, 202465000). Apoptotic cell death ratio was evaluated 10–14 h after treatment.

Cell death assay and Immunocytochemistry. Cerebellum granular cells were grown on Lab-tek chamber slides (Nunc, #177445) or cover slice (Carolina, 63-3009) in 24 well plate at a density of 20 w cells/well. On Div7 cells were treated with hydrogen peroxide to induce the apoptotic cell death. After treatment, the cells were fixed in 4% paraformalehyde for 20 min at room temperature. Fixed cells were washed three times with PBS, followed by block with 3% BSA with 0.1% Triton x-100 for 30 min, RT. Then cells were stained with anti-GFP (invitrogen, #A-11122) or HA (Covance,MMS-101R) for 1 h at RT, anti-488 or anti-555 for 40 min at RT. At last cells were stained with 1 μg/ml Hoechst33258 (beyotime, #C1011) in PBS for 10 min at RT. The cells were washed three times with PBS and mounted using the medium for fluorescence, followed by observation under a laser scanning confocal microscope. Data are presented as the ratio of chromosomal condensation and morphological change as a percentage of total cells. Cell counts were carried out in a blinded manner and analyzed for statistical significance by ANOVA followed by Tukey's test. Approximately 600 cells were counted per group. Unless stated otherwise.

Immunoblotting. Cultured cells were extracted by 1xSDS lysis buffer (APPLYGEN, 000792). The protein concentration was analyzed by a protein assay kit based on the Bradford method (Bio-rad Laboratiories). Cellular lysates were resolved in an 6% or 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto hybond-P (Amersham biosciences, Uppsala, Sweden) polyvinylidene difluoride membranes. Western blots were blocked with 5% BSA in TBS containing 0.1% Tween 20 and incubated 1 h RT and then with 3% BSA in TBST as blocking buffer containing primary antibodies against Tubulin (Sigma,T5168,1 : 5000), Kl (R&D, MAB1819,1 : 500), HA (Covance,MMS-101R,1 : 5000) and Tet1 (Santa Cruz, sc-163443,1 : 100). After washing, blots were then incubated with horseradish peroxidase-conjugated secondary antibodies diluted (1 : 5000) in blocking buffer and developed using the Super signal West Pico Chemiluminescent Substrate method (Pierce).

Quantitative Real-Time PCR Assays. For quantitative real-time PCR assays, total RNA was extracted from mouse cortical neurons using Trizol (Invitrogen, 15596018) follow the user's instructions. And cDNA was synthesized by Premix Taq (Takara, RR003A). SYBR premix Ex Taq from Takara (RR82LR) was used in this study. Quantitative real-time PCR was performed with the Rotor-Gene Q machine (QIAGEN). Results were normalized to GAPDH, and data analysis was done by using the comparative CT method in software by QIAGEN. Primers used in quantitative real-time PCR assays were as follows: Klotho-forward: CAAAGTCTTCGGCCT-TGTTC; Klotho-reverse: CTCCCCAAGC AAAGTCACA; BDNF-forward: GGCT-GGTGCAGAAAAGCAACAA BDNF -reverse: TCGCCAGGTAAGAAACCCTT-CG.

Mouse middle cerebral artery occlusion (MCAO). Male mice (25–30 g) with congenic C57BL6 background were anesthetized with 10% chloral hydrate (body temperature 37°C), and focal cerebral ischemia was induced by MCAO (the suture method) for 120 min. After reperfusion for different periods 0 h, 6 h and 12 h, the animals were killed and mouse brains were rapidly removed. RNAs and proteins were collected following the manufacturer's protocol. All of the animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Institute of Neuroscience, SIBS, CAS, China.

Statistical analysis. Statistical analysis of the data was performed with a one-way ANOVA followed by Tukey's test or two-tailed Student's t test using GraphPad Prism (Version 5). Data are presented as the mean + SEM and the number of experiments are indicated in each figure. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ denotes statistical significance.

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