Small Ubiquitin-Like Modifier 3–Modified Proteome Regulated by Brain Ischemia in Novel Small Ubiquitin-Like Modifier Transgenic Mice
Putative Protective Proteins/Pathways

Wei Yang, PhD; Huaxin Sheng, MD; J. Will Thompson, PhD; Shengli Zhao, PhD; Liangli Wang, PhD; Pei Miao, BS; Xiaozhi Liu, MS; M. Arthur Moseley, PhD; Wulf Paschen, PhD

Background and Purpose—Small ubiquitin-like modifier (SUMO) conjugation is a post-translational modification associated with many human diseases. Characterization of the SUMO-modified proteome is pivotal to define the mechanistic link between SUMO conjugation and such diseases. This is particularly evident for SUMO2/3 conjugation, which is massively activated after brain ischemia/stroke, and is believed to be a protective response. The purpose of this study was to perform a comprehensive analysis of the SUMO3-modified proteome regulated by brain ischemia using a novel SUMO transgenic mouse.

Methods—To enable SUMO proteomics analysis in vivo, we generated transgenic mice conditionally expressing tagged SUMO1-3 paralogues. Transgenic mice were subjected to 10 minutes forebrain ischemia and 1 hour of reperfusion. SUMO3-conjugated proteins were enriched by anti-FLAG affinity purification and analyzed by liquid chromatography–tandem mass spectrometry.

Results—Characterization of SUMO transgenic mice demonstrated that all 3 tagged SUMO paralogues were functionally active, and expression of exogenous SUMOs did not modify the endogenous SUMOylation machinery. Proteomics analysis identified 112 putative SUMO3 substrates of which 91 candidates were more abundant in the ischemia group than the sham group. Data analysis revealed processes/pathways with putative neuroprotective functions, including glucocorticoid receptor signaling, RNA processing, and SUMOylation-dependent ubiquitin conjugation.

Conclusions—The identified proteins/pathways modulated by SUMOylation could be the key to understand the mechanisms linking SUMOylation to neuroprotection, and thus provide new promising targets for therapeutic interventions. The new transgenic mouse will be an invaluable platform for analyzing the SUMO-modified proteome in models of human disorders and thereby help to mechanistically link SUMOylation to the pathological processes. (Stroke. 2014;45:1115-1122.)

Key Words: brain ischemia ■ proteomics ■ SUMO proteins ■ transgenic mice

Small ubiquitin-like modifiers (SUMOs) are covalently conjugated to lysine residues of target proteins, and thereby modulate their function, stability, and localization.1,2 SUMO1, SUMO2, and SUMO3 are widely expressed in mammalian tissues. SUMO2 and SUMO3 are almost identical and are often referred to as SUMO2/3. They are distinct from SUMO1, however, with about 50% homology. The SUMO conjugation (SUMOylation) is an energy-dependent process catalyzed by activating enzyme (SAE1/SAE2), conjugating enzyme (Ubc9), and ligating enzyme.1,2 SUMOylation is a dynamic and reversible reaction, as SUMOylated proteins can be readily deconjugated by SUMO proteases.3

SUMOylation modulates many cellular functions including DNA repair, genome maintenance, gene transcription, and protein degradation control4–6 and plays key roles in many human diseases such as cerebral ischemia/stroke, cancer, and heart failure.1,7–9 It is, therefore, of tremendous clinical interest to characterize the SUMO-modified proteome in these disorders. This is particularly evident for cerebral ischemia/stroke because transient cerebral ischemia dramatically activates SUMOylation, and this is believed to be a neuroprotective stress response. It is, therefore, of key interest to identify the proteins SUMOylated after brain ischemia to uncover the mechanisms linking SUMOylation to neuroprotection. However, SUMO proteomics analysis is hampered...
by the low levels of SUMOylated proteins. A common strategy is, therefore, to perform proteomics analysis on purified SUMO-conjugated proteins from cells stably expressing tagged SUMOs. Recently, a His$_6$-HA-SUMO1 knockin mouse was generated, which allowed researchers, for the first time, to characterize the SUMO1-modified proteome in brains. In the present study, we generated a transgenic mouse (CAG-loxP-STOP-loxP-SUMO, hereafter referred to as CAG-SUMO) in which His-SUMO1, HA-SUMO2, and FLAG-SUMO3 are expressed in a Cre-dependent manner. Using this new mouse line, we report here the first profile of the SUMO3-modified proteome in mouse brain after ischemia, a pathological state associated with a dramatic activation of SUMO2/3 conjugation.

**Methods**

Full details of the methods are provided in the online-only Data Supplement.

**Transgenic Mice**

Animal experiments were approved by the Duke University Animal Care and Use Committee. CAG-SUMO transgenic mice were generated by pronuclear injection of the transgene vector illustrated in Figure 1A.

**Animal Surgery**

Transient forebrain ischemia was performed as described previously with minor modifications.

**Sample Preparation for Proteomic Analysis**

Each sample for proteomic analysis was generated by FLAG pull-down of nuclear fraction from cortical tissues pooled from 4 mice. Three biological replicates were used for each group.

**Results**

**Generation and Characterization of CAG-SUMO Mice**

We designed a transgene vector based on Cre/lox recombination and 2A-mediated cotranslational cleavage to express His-SUMO1, HA-SUMO2, and FLAG-SUMO3 from a single multicistronic transgene in a conditional manner (Figure 1A). We used the cDNAs encoding precursor SUMO1-3 that allows the endogenous SUMO proteases to remove the extra 2A peptides and expose the C-terminal di-glycine motif of SUMOs. Green fluorescent protein (GFP) and mCherry were used as indicators of transgene expression before and after Cre recombination. We obtained 8 founder lines with varying levels and patterns of GFP expression. Because of ubiquitous expression of GFP, line 10 was chosen for the present study. To examine the global pattern of Cre-mediated transgene expression, mice were cross-bred with hemizygous β-actin-Cre mice to generate double transgenic CAG-SUMO/β-actin-Cre mice. CAG-SUMO line 10 exhibited strong GFP fluorescence in all organs examined including brain, heart, lung, kidney, and liver; those organs showed mCherry expression in CAG-SUMO/β-actin-Cre mice. Expression of FLAG-SUMO3 was also confirmed in those organs (data not shown).

![Figure 1. Generation of CAG-small ubiquitin-like modifier (SUMO) transgenic mice. A, Scheme of the transgene construct. The transgene consists of the cytomegalovirus early enhancer/chicken β-actin promoter (CAG), a fragment containing GFP and a transcriptional/translational STOP cassette (STOP) flanked by loxP sites, 3 tagged SUMOs linked by 2A sequences, mCherry, and a polyadenylation signal (pA). B, Expression patterns of native GFP fluorescence in a CAG-SUMO line 10 mouse brain. A sagittal brain section indicated widespread GFP expression (top). Bottom, Confocal images of different areas of the brain. C, A sagittal brain section of a double transgenic CAG-SUMO/Emx1-Cre mouse showing the mCherry expression, indicative of expression of tagged SUMOs, restricted to forebrain regions. CA1 indicates hippocampal CA1 subfield; Cb, cerebellum; Ct, cortex; HI, hippocampus; OB, olfactory bulb; SC, superior colliculus; St, striatum; and Th, thalamus (scale bars, 50 μm).](http://stroke.ahajournals.org/issue/4/4/1116/001116-fig1.jpg)
In the brain of CAG-SUMO line 10, GFP was ubiquitously expressed with strong signals in hippocampus and cerebellum (Figure 1B). Because we were particularly interested in the SUMO-modified proteome regulated by forebrain ischemia, we mated hemizygous CAG-SUMO mice with homozygous Emx1Cre/+ mice to generate double transgenic CAG-SUMO/Emx1-Cre as tagged SUMO-expressing mice and littermates Emx1Cre/+ as control mice. In line with a previous report,12 CAG-SUMO/Emx1-Cre mice showed strong mCherry expression in the cerebral cortex and hippocampus (Figure 1C). For both CAG-SUMO and CAG-SUMO/Emx1-Cre mice, we did not find any obvious physical or behavioral abnormalities.

Postischemic SUMOylation in the Brain of Transgenic Mice

To extend our previous findings8 and also demonstrate the suitability of CAG-SUMO/Emx1-Cre mice for in vivo SUMO studies, we investigated the temporal and spatial profiles of postischemic SUMOylation by endogenous and exogenous SUMOs in brains of Emx1Cre/+ control and CAG-SUMO/Emx1-Cre mice. First, the temporal profile studies indicated that SUMO1-3 conjugation was decreased dramatically during and rapidly activated after ischemia (Figure 2A). Similar pattern was found for FLAG-SUMO3 (data not shown). Then, we carefully compared SUMOylation response to brain ischemia in Emx1Cre+ and CAG-SUMO/Emx1-Cre mice to check for possible off-target effects that might be caused by overexpressing SUMOs. In both sham and ischemia groups, levels of SUMO1 and SUMO2/3 conjugates in the high-molecular-weight regions were comparable in controls and double transgenic mice, although there were substantially higher levels of unconjugated SUMOs in CAG-SUMO/Emx1-Cre mice because of expression of tagged SUMOs (Figure 2B–2D, data not shown). Finally, transient forebrain ischemia induced nuclear accumulation of SUMO2/3-conjugated proteins, and the same pattern was also observed for HA-SUMO2 and FLAG-SUMO3 (Figure 3 and Figure IA in the online-only Data Supplement).

SUMO3-Modified Proteome Regulated by Transient Forebrain Ischemia

To compare results to our previous SUMO3 proteomics analysis using an in vitro ischemia model,13 we focused on the SUMO3-modified proteome in this study. We chose 1 hour of reperfusion when SUMO2/3 conjugation was maximally activated (Figure 2A). Furthermore, we used cortical tissues because we were interested in the neuroprotective role of SUMOylation, and the cortex is spared from damage in this ischemia model.14 For future studies, we also performed a small-scale HA pull-down to confirm enrichment of HA-SUMO2–conjugated proteins (Figure IB in the online-only Data Supplement).

First, we optimized the FLAG pull-down procedure by using nuclear fractions as input for FLAG pull-down. This greatly enhanced specificity because nuclear fractions were devoid of unconjugated FLAG-SUMO3, had markedly less unspecific bands on Western blots (Figure IA in the online-only Data Supplement), and exhibited dramatically lower total protein levels (Figure IC in the online-only Data Supplement).

Indeed, FLAG-SUMO3–conjugated proteins were immunoprecipitated effectively from nuclear fractions (Figure ID in the online-only Data Supplement). Interestingly, we did not notice a marked decrease in SUMO2/3 and HA signals in flow-through samples (Figure ID in the online-only Data Supplement). This suggested that FLAG-SUMO3 represented only a small fraction of the total SUMO2/3 pool. We also found HA-SUMO2 in FLAG-SUMO3 pull-down eluates, and, notably, there was a shift toward higher molecular weights in the ischemic sample, implying increased length of SUMO2/3 chains (Figure ID in the online-only Data Supplement).

For the large-scale SUMO3 proteomics study, 3 groups of mice were used: Emx1Cre+ without surgery (control, to account for background binding to anti-FLAG beads) and CAG-SUMO/Emx1-Cre double transgenic mice with sham (transgenic sham) or ischemia surgery (transgenic ischemia; Figure 4A). All 9 FLAG pull-down samples (n=3/group) were confirmed by Western blotting (Figure IIA in the online-only Data Supplement) and then separated on an SDS-PAGE gel (Figure IIB in the online-only Data Supplement). Fourteen gel slices per lane were cut for liquid chromatography–tandem mass spectrometric analysis (Figure IIB in the online-only Data Supplement).

Proteomics data showed that SUMO2/3 and ubiquitin shared a similar distribution of spectral counts (Figure 4B), suggesting a marked postischemic activation of the cross talk between these 2 post-translational modifications. Indeed, we found ubiquitin conjugation to be activated after ischemia, particularly pronounced in nuclei (Figure IIC in the online-only Data Supplement). Based on selection criteria described in online-only Data Supplement Methods, 112 proteins were considered as putative SUMO3 substrates (Table I in the online-only Data Supplement), and 91 proteins (Table I in the online-only Data Supplement, asterisks) were considered as ischemia-upregulated candidates of which 46 candidates were found only in ischemia samples (Table I in the online-only Data Supplement, triangles), including the general transcription factor IIi (TFI-I/GTF2I), tripartite motif containing 33 (TRIM33), glucocorticoid receptor (GR/GCR), and B-cell lymphoma/leukemia 11B (CTIP2/BCL11B).

Gene ontology annotation analysis by the PANTHER program indicated that 38.5% and 47.3% of the 91 candidates were predicted to have nuclear and cytoplasmic localization, respectively (Figure 4C). We did not expect that most of the identified proteins would have predicted cytoplasmic localization, considering that nuclear fractions were used for proteomics analyses. A plausible explanation is that cytoplasmic SUMO3 target proteins were translocated to the nucleus after ischemia (eg, see below for GR). PANTHER analysis also revealed that most of the 91 proteins belonged to a group of binding proteins of which nucleic acid binding accounted for 55.1% (Figure 4D).

Ingenuity Pathway Analysis core analysis of the 91 candidates revealed significant enrichment in the categories of neurological disease (46 targets) and cell death and survival (47 targets; Figure IIIA and IIIB in the online-only Data Supplement). Strikingly, 27 proteins were grouped with functions in RNA processing with high confidence (Ingenuity Pathway Analysis score=72; Figure IIIIC in the online-only Data Supplement).

To verify proteomics analysis findings independently, a separate large-scale FLAG pull-down was performed using 3 CAG-SUMO/Emx1-Cre mice per group (sham and ischemia).
First, we treated FLAG pull-down samples with SUMO/sentrin-specific protease 2 catalytic fragment (SENP2c) for de-SUMOylation. Western blot analysis confirmed that the strong high-molecular-weight smear of bands indeed represented SUMO-conjugated proteins (Figure 5A). The presence of SUMO1 is consistent with the report that SUMO1-3 can form mixed chains. Interestingly, ubiquitinated proteins slightly shifted to lower molecular weight after SENP2c treatment (Figure 5A). These data suggest that ubiquitin and SUMO conjugated to different lysine residues for a subset of

Figure 2. Effect of transient forebrain ischemia on small ubiquitin-like modifier (SUMO)ylation. A, CAG-SUMO/Emx1-Cre mice were subjected to 10 minutes forebrain ischemia and 0, 1, 3, or 6 hours reperfusion (n=3 per group). Sham-operated mice were used as control. SUMO conjugates in high-molecular-weight regions are marked by brackets. B–D, Comparison of SUMO2/3 conjugation in brains of Emx1Cre/+ (control) and CAG-SUMO/Emx1-Cre (transgenic [TG]) mice. Emx1Cre/+ and CAG-SUMO/Emx1-Cre mice were subjected to sham surgery or 10 minutes forebrain ischemia and 1 hour reperfusion (n=3 per group). Protein samples were prepared from the cortex (B) and the hippocampus (C) and analyzed by Western blotting using indicated antibodies. D, The high-molecular-weight regions marked by brackets were used to quantify SUMO2/3 conjugates in the cortex and hippocampus. Films with shorter exposure times were used to measure intensities. Data were normalized to β-actin and presented as means±SD. NS indicates not significant; and WB, Western blot.
postischemic SUMO3 conjugates. Similar findings were also reported in cells exposed to ischemia-like conditions or proteasome inhibitor MG132.13,16

Then, we selected 5 candidate SUMO substrates for further verification, GR, TFIH-I, TRIM33, transcriptional intermediary factor 1β (TIF1β), and CTIP2. The unmodified forms

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**Figure 3.** Nuclear accumulation of small ubiquitin-like modifier (SUMO)2/3–conjugated proteins after ischemia. A and B, CAG-SUMO/Emx1-Cre mice were subjected to sham surgery or 10 minutes forebrain ischemia and 1 hour reperfusion. Brain sections were stained with antibodies against SUMO2/3, HA, and microtubule-associated protein 2 (MAP2) as neuron-specific marker. Ischemia-induced nuclear accumulation of SUMO2/3- and HA-SUMO2–conjugated proteins was found both in cortex and hippocampus neurons (scale bars, 20 μm).

**Figure 4.** Proteomics analysis of small ubiquitin-like modifier (SUMO)3–conjugated proteins in postischemic mouse brains. A, Overview of the workflow to identify FLAG-SUMO3-conjugates in the postischemic cerebral cortex. Coronal brain sections of Emx1Cre/+ (control; 4',6-diamidino-2-phenylindole staining, blue) and CAG-SUMO/Emx1-Cre double transgenic (transgenic [TG]; mCherry fluorescence, red) mice were shown to indicate cortical regions used in the study. B, Distribution of total spectral counts for SUMO1, SUMO2/3, and ubiquitin for each gel slice of ischemia samples from high (slice 1) to low (slice 14) molecular weights. Subcellular localization (C) and molecular functions (D) of the 91 putative SUMO3 substrates with upregulated SUMO3 conjugation state after ischemia were grouped by the PANTHER program. LC-MS/MS indicates liquid chromatography–tandem mass spectrometry.
of all 5 proteins were detected in sham and ischemia input samples (Figure 5B). In contrast, only slower migrating bands were present in FLAG pull-down samples, with much stronger signals in the ischemia sample. After SENP2c treatment, almost all slower migrating bands disappeared, and bands representing unmodified forms appeared (Figure 5B). These experiments convincingly confirmed that these 5 proteins are authentic SUMO3 substrates and that SUMOylation of these proteins was markedly increased after ischemia (Figure 5B).

We further determined the quantitative trend across molecular weight for 4 of these proteins (GR, TFII-I, TRIM33, and TIF1β) by performing a targeted data extraction from the liquid chromatography–tandem mass spectrometric analysis of each gel band (Figure 5C). TFII-I and TRIM33 were virtually undetectable in sham samples, whereas GR and TIF1β were detectable in sham but highly upregulated in ischemia samples. All 4 proteins showed the highest quantity in molecular weight regions that are significantly higher than the unmodified protein molecular weight (Figure 5C), consistent with multiple SUMO modifications.

Finally, we further characterized GR SUMOylation. SUMO3-conjugated GR was already detected in postischemic nuclear fractions without immunoprecipitation (Figure 5B, input), a remarkable observation considering that only a small fraction of a given protein is usually SUMOylated. This suggests a dramatic postischemic activation of GR SUMOylation. Indeed, we found that ischemia/reperfusion triggered massive SUMO conjugation and nuclear accumulation of GR (Figure 5D).
The new SUMO transgenic mouse model has several advantages compared with previously published approaches to investigate the SUMO2/3-modified proteome using tissue samples. Protein sequences of SUMO2 and SUMO3 are almost identical and cannot be differentiated by available antibodies. Because SUMO2 and SUMO3 are expressed with different tags in this new transgenic mouse, it is possible for future studies to identify individually the SUMO2- and SUMO3-modified proteome. Furthermore, because tagged SUMOs are expressed in a conditional manner, they can be expressed in any cell/organ type for which the respective Cre mouse is available, thus making this new mouse model a universal tool for characterizing the SUMO-modified proteome.

Increased SUMOylation is thought to be a protective response that shields neurons from ischemia-induced damage. Transient forebrain ischemia triggered a massive increase in levels of SUMO2/3-conjugated proteins in neurons of the resistant cortex and vulnerable hippocampal CA1 subfield (Figures 2 and 3). Whether this postischemic activation of SUMO2/3 conjugation is a stress response protecting neurons in both regions needs to be established in future studies. Recently, we have generated a novel SUMO knockdown transgenic mouse that will be well suited for these studies. It also needs to be verified whether transient cerebral ischemia may activate SUMOylation in non-neuronal cells such as astrocytes and endothelial cells. Notably, SUMOylation of the liver X receptor in brain astrocytes blocks inflammatory responses, and inflammation is a major contributing factor to ischemia-induced brain damage. Crossing our CAG-SUMO mice with mice expressing Cre in non-neuronal brain cells will help to identify the postischemic SUMO-modified proteome in these cells and thereby clarify this important aspect.

We have identified 91 protein candidates that exhibit a postischemic upregulated SUMO3 conjugation state in cortex, a brain region relatively resistant to a short interruption of blood supply (Table I in the online-only Data Supplement, asterisks). This high stringency list includes many known SUMO substrates, such as TIF1β, heterogeneous nuclear ribonucleoproteins (hnRNPs), TFII-I, and GR. When we compared the in vivo data presented here with a previous in vitro study, we found that 34 of the 91 candidates (37%) were identified in both studies. Furthermore, we confidently confirmed 5 in vivo SUMO3 substrates regulated by ischemia (Figure 5). Taken together, these analyses confirm the validity of the approach and new SUMO mouse model and establish the credibility of our SUMO substrate list.

Our data revealed several potentially important processes modulated by SUMO3 conjugation that may play neuroprotective roles during reperfusion. First, we provided evidence that the cross talk between ubiquitylation and SUMOylation is a protective stress response. We report here the first proteomics analysis of SUMO3-conjugated proteins in tissue samples from a pathological state, brain ischemia. We identified several pathways modulated by SUMOylation in the postischemic brain that warrant future investigations because they could be therapeutic targets for neuroprotection in brain ischemia. Because a large portion of identified SUMO targets were nuclear proteins involved in gene expression, genome stability, and RNA processing, we expect activation of SUMOylation to have long-lasting effects on postischemic neurons. The new conditional SUMO transgenic mouse and the highly stringent purification approach developed in the present study provide an invaluable platform for in-depth analysis of the SUMO-modified proteome in vivo in physiological or pathological states under investigation.

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Disclosures
None.

References


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SUPPLEMENTAL MATERIAL

Supplemental Methods

Generation and breeding of transgenic mice. Animal experiments were approved by the Duke University Animal Care and Use Committee. The fragment for generating the CAG-SUMO transgenic mice contained a CMV early enhancer/chicken β-actin promoter (CAG) promoter with loxP sites flanking both the GFP coding sequence and a transcriptional/translational STOP cassette (STOP), followed by tagged SUMO1-3 and mCherry coding sequences, and a polyadenylation signal (pA). (Fig. 1A). Three different 2A peptide sequences were used to link SUMOs and mCherry. The transgene fragment was purified and injected into fertilized oocytes of FVB/N mice at the Duke Transgenic Mouse Facility. After founders were identified by genotyping and their GFP expression patterns were characterized, mouse line 10 was chosen for the present study. CAG-SUMO mouse line 10 was then backcrossed to C57BL/6 mice for 3 generations. Eml1^{Cre/C}re mice (JAX stock #005628; The Jackson Laboratory, Bar Harbor, ME) and β-Actin-Cre mice (JAX stock #003376; The Jackson Laboratory, Bar Harbor, ME) were used to crossbreed with CAG-SUMO mice to generate double transgenic mice. For genotyping, we used the following primers: 5’-GATACTCGAGTCTGGAAGAGCACCA-3’ (forward) and 5’-ACCTTGAAGCCATGAACTC-3’ (reverse) for the SUMO transgene, and 5’-GGTCGAATCGAGGTAGAAG-3’ (forward) and 5’-GCCAGATTACGTATATCCTGGCAG-3’ (reverse) for the Cre transgene.

Animal surgery. Transient global cerebral ischemia was performed in male mice as described previously with minor modifications. Briefly, male mice (22-28 g) were anesthetized with 5% isoflurane in 40% oxygen balanced with nitrogen. Then, mice were orally intubated and mechanically ventilated with 1.8% isoflurane. The right femoral artery and the right internal jugular vein were cannulated (PE 10, Becton-Dickson, Sparks, MD) for monitoring arterial blood pressure and withdrawing blood, respectively. Cerebral ischemia was induced by a combination of 10 minutes of bilateral common carotid artery occlusion, and hypotension to a mean arterial blood pressure of 30 mm Hg induced by blood withdrawal. During the surgical procedure, the rectal temperature was maintained at 37°C using a heating pad. After 10 minutes of ischemia, the carotid arteries were deoccluded and withdrawn blood was reinfused. Sham-operated mice underwent the same procedures except for carotid artery occlusion and blood withdrawal. At the end of the experiments, animals were deeply anesthetized with 5% isoflurane, and decapitated. The hippocampus and cortex were quickly isolated on ice, and the samples were snap frozen in liquid nitrogen and stored at -80°C.

Western blot and immunohistochemistry. Western blot analysis was performed using a standard protocol. For quantification of SUMO conjugates, the higher-molecular-weight area of each lane, as indicated in the respective figures, was measured and normalized to β-actin using ImageJ software (NIH, Bethesda, MD). Immunohistochemistry was performed as described previously. In short, brains were fixed with 4% paraformaldehyde and then paraffin-embedded. After deparaffinization and antigen retrieval, sections were incubated with the primary antibodies at 4°C overnight. After extensive washing, the sections were incubated with fluorescent secondary antibodies for 1 hour at room temperature. The fol-
lowing antibodies were used for Western blotting and immunohistochemistry: SUMO2/3 and HA.11 (16B12) from Covance (Princeton, NJ); SUMO1 (a gift from Dr. Matunis); FLAG M2 and β-actin (AC-15) from Sigma (St. Louis, MO); MAP2 (PA1-4742) from Thermo Scientific (Rockford, IL); mCherry (1C51) from Novus Biologicals (Littleton, CO); HA (#3724), His (#2366), GAPDH (#5174), H2AX (#2595), ubiquitin (#3936), poly ubiquitin K48 (#8081), TRIM33 (#8972), CTIP2 (#12120), TIF-1β (#4123), GR (#3660), TFII-I (#4562), and GST (#2625) from Cell Signaling (Danvers, MA).

Microscopy. Overview images of brain sections were generated on an Axio Observer Z1 motorized fluorescence microscope (Carl Zeiss MicroImaging). Confocal images were captured on a Leica SP5 confocal microscope (Leica Microsystems).

Subcellular fractionation. Frozen brain tissue samples were homogenized by 30 strokes with pestle B in a Dounce homogenizer in hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM NaCl, 10 mM KCl, 0.5 mM EDTA, 0.5 mM DTT) supplemented with 0.5% NP40, 1 mM PMSF, 20 mM N-ethylmaleimide (NEM), and 1X protease inhibitor cocktail (Sigma, St. Louis, MO). Homogenates were filtered through 4-ply sponges to remove cell debris, and were then centrifuged to pellet nuclei. Supernatants constituted the cytosolic fractions. Nuclear pellets were washed 3 times with hypotonic buffer, and were then resuspended in lysis buffer (50 mM Tris pH 7.5, 250 mM NaCl, 0.5% sodium deoxycholate, 1 mM EDTA, 0.5% Triton X 100) supplemented with 1% SDS, 10 mM NEM, and 1X protease inhibitor cocktail. After brief sonication and centrifugation, supernatants were collected as nuclear fractions.

FLAG pulldown samples for proteomic analysis. Brain tissue samples were collected from 3 experimental groups (control, TG Sham, and TG Ischemia). For each group, samples were prepared in triplicate. To minimize the variation in biological replicates, affinity purification was performed under the identical conditions for all triplicate samples in the 3 groups. For each sample, cortices from 4 mouse brains were pooled and used to prepare nuclear fractions as described above. Nuclear pellets from all 9 samples were resuspended in 1.4 mL lysis buffer containing 1% SDS to inactivate the deconjugating enzymes and disrupt protein-protein interactions.

Protein concentration was measured by BCA assay (Thermo Scientific, Rockford, IL). For each sample, 5 mg of nuclear proteins were diluted 1:10 with lysis buffer without SDS. After centrifugation and filtration through 0.45-µm PVDF syringe filters (Fisher Scientific, Pittsburgh, PA), the clarified nuclear fractions were incubated with 100 µL mouse IgG agarose beads (Sigma, St. Louis, MO) for 2 hours at 4°C to reduce non-specific binding. The cleared supernatants were then incubated with 60 µL anti-FLAG M2 agarose beads (Sigma, St. Louis, MO) on a rotary mixer at 4°C for 18 hours. Beads were washed 6 times with 1 mL lysis buffer containing 0.1% SDS. Proteins were then eluted twice with 250 µL of TBS buffer (50 mM Tris pH 8.0, 250 mM NaCl) plus 200 µg/mL 3XFLAG peptide for 1 hour at 4°C. Finally, beads were washed with 200 µL TBS buffer, and this wash was combined with the two elutes as final FLAG pulldown samples.

LC-MS/MS and data analysis. Proteins from the 9 FLAG pulldown samples were precipitated with 4 volumes of cold acetone and resuspended in 50 µL SDS sample buffer. Proteins were separated on a NuPAGE 4%-12% Bis/Tris gel (Life Technologies, Grand Island, NY),
and stained with colloidal Coomassie (Life Technologies, Grand Island, NY). Each lane was then dissected into 14 slices. In-gel tryptic digestion was performed according to a standard protocol, adapted from a reference protocol. Briefly, gel pieces were repeatedly shrunken and swelled in MeCN and 50 mM ammonium bicarbonate (AmBic). Proteins were then reduced using a solution of 10 mM dithiothreitol, alkylated with 20 mM iodoacetamide, and digested in-gel with approximately 200 ng trypsin per band at 37°C overnight. Peptides were extracted under acidic conditions, dried, and resuspended in 12 μL 0.2/2/97.8 v/v/v formic acid/McCN/water for analysis.

The in-gel digested peptide samples from each slice were analyzed by LC-MS/MS as follows. Five μL of each sample were injected onto a 75 μm x 250 mm BEH C18 column (Waters, Milford, MA) and separated using a gradient of 5% to 40% acetonitrile with 0.1% formic acid, with a flow rate of 0.4 μL/min, in 30 minutes on a nanoAcquity liquid chromatograph (Waters). Electrospray ionization was used to introduce the sample in real-time to a Q-Tof Synapt G2 mass spectrometer in sensitivity mode, resolution 17,000 (Waters, Milford, MA). Data were collected in data-dependent acquisition (DDA) mode with 0.6-second survey scans and three 0.6-second MS/MS scans in CID mode of the top 3 most abundant multiply-charged precursor ions. Raw data were processed in Mascot Distiller (v2.3) and searched in Mascot v2.2 (Matrix Science) against the Swissprot 2013x database with *mus musculus* taxonomy (16,611 entries). Search tolerances in Mascot were 10 ppm on precursor and 0.04 Da product ion tolerance, requiring full trypsin specificity and allowing at most 2 missed cleavages. Carboxymethylation (C) was included as a fixed modification, and deamidation (N and Q) and oxidation (M) were allowed as variable modifications. Scaffold (v4.0.3, Proteome Software Inc.) was used to validate MS/MS-based peptide and protein identifications. Protein and peptide identifications were validated using the Peptide Prophet algorithm with decoy database validation, and the final dataset was curated to 1% false-positive rate (FDR) at the protein level and 0.07% FDR at the spectrum level. MS/MS data and all identifications can be downloaded directly by accessing the Scaffold file at the following link: https://discovery.genome.duke.edu/express/resources/3320/3320_lanewalking_spmouse_041213_withGOannotations.sf3.

The LC-MS/MS database search identifications were used to define a list of putative SUMO3 candidates and SUMO3 candidates upregulated by ischemia/reperfusion (Supplemental Table I) according to the following selection criteria. The FDR was set to 1% at the protein level and 0.1% at the peptide level. All keratins and immunoglobulins were regarded as contaminants and excluded from the list. Proteins that were detected in at least 2 control samples were excluded as non-specific binding proteins. Proteins were required to be detected in 2 or more FLAG-SUMO3 pulldown samples (TG Sham and TG Ischemia) in order to qualify as putative SUMO3 substrates (Supplemental Table I). Finally, those proteins that were only identified in the ischemia samples, or showed more than 2 times the spectral count in ischemia vs sham group, were defined as the SUMO3 candidates with upregulated SUMO3 conjugation state following ischemia/reperfusion (Supplemental Table I, marked with *).
**Bioinformatics analysis.** The PANTHER (Protein Analysis Through Evolutionary Relationships; [http://www.pantherdb.org/](http://www.pantherdb.org/)) program was used to categorize proteins according to cellular component and molecular function based on the Gene Ontology (GO) database. IPA (Ingenuity Pathway Analysis; [www.ingenuity.com](http://www.ingenuity.com)) core analyses were performed to identify biological functions enriched in our protein list.

Targeted extracted ion chromatogram (XIC) quantitation was performed from LC-MS/MS data using Skyline v2.1 ([http://skyline.gs.washington.edu/](http://skyline.gs.washington.edu/)). The raw data analysis in Skyline has been made accessible for public download at the following link: [https://discovery.genome.duke.edu/express/resources/3320/3320_v2p1_ShamVIIschemia_2013-10-03_12-23-44.zip](https://discovery.genome.duke.edu/express/resources/3320/3320_v2p1_ShamVIIschemia_2013-10-03_12-23-44.zip). Briefly, Mascot (v2.2) search results from the LC-MS/MS analyses were imported in Skyline to create a spectral library, and the best 2 peptides per protein were selected based on their intensity and S/N in the gel slices in which those proteins were identified. MS1-based quantitation for these peptides was then performed across all gel slices with the M, M+1, and M+2 ions at 12,000 resolution. Finally, only the gel slices that contained a detectable signal in one or more samples were retained, such that the final quantitative analysis included 5 gel bands from each sample, encompassing the molecular weight region between approximately 65 and 205 kDa.

**Statistical analysis.** Quantitative Western blotting data are presented as means ± SD (n = 3/group). Statistical significance between groups was evaluated with Student’s t-test, and P values <0.05 were considered significant.
Supplemental Figure I. Analysis of SUMOylation in the brain using CAG-SUMO/Emx1-Cre mice. A, CAG-SUMO/Emx1-Cre mice were subjected to sham surgery (S) or 10 minutes forebrain ischemia and 1 hour reperfusion (I). Whole cell lysates (WCL), cytoplasmic (Cy), and nuclear (Nu) fractions were prepared from cortical tissues and analyzed by Western blotting with the indicated antibodies. GADPH and H2AX were used as cytoplasmic and nuclear markers, respectively. B, CAG-SUMO/Emx1-Cre mice were subjected to sham surgery (S) or 10 minutes forebrain ischemia and 1 hour reperfusion (I). Emx1<sup>Cre/+</sup> mice without surgery were used as control (C). Whole cell lysates were prepared from the cortical tissues and used for HA pulldown. Enrichment of free HA-SUMO2 and its conjugates in the eluates was effective. C-D, CAG-SUMO/Emx1-Cre mice were subjected to sham surgery (S) or 10 minutes forebrain ischemia and 1 hour reperfusion (I). C, Whole cell lysates (WCL), and cytoplasmic (Cy) and nuclear (Nu) fractions were prepared from the cortical tissues and analyzed by SDS-PAGE with colloidal Coomassie staining. D, Nuclear fractions from the cortical tissues (Sham and Ischemia) were used for FLAG pulldown. Input (In), flow-through (FT), and eluates (E) were analyzed by Western blotting with the indicated antibodies. WB, Western blot.
Supplemental Figure II. The large-scale proteomics analysis of FLAG-SUMO3-conjugated proteins in post-ischemic mouse brains. 
A, Enriched FLAG-SUMO3 conjugates prepared for the large-scale proteomic analysis were verified. Western blot analysis was used to evaluate all inputs and eluates of 3 samples from each of 3 experimental groups (control, TG Sham, and TG Ischemia). The nuclear marker H2AX indicated that equal amounts of proteins were used for FLAG pulldown. 
B, All 9 FLAG pulldown samples were concentrated and resolved on an SDS-PAGE gel and stained with colloidal Coomassie. Each gel lane was cut into 14 slices for proteomic analysis. 
C, Nuclear accumulation of ubiquitinated proteins after ischemia revealed by the large-scale SUMO proteomics analysis was confirmed by Western blotting. CAG-SUMO/Emx1-Cre mice were subjected to sham surgery (S) or 10 minutes forebrain ischemia and 1 hour reperfusion (I). Whole cell lysates (WCL), and cytoplasmic (Cy) and nuclear (Nu) fractions were prepared from cortical tissues and analyzed by Western blotting with ubiquitin and ubiquitin K48 antibodies. GADPH and H2AX were used as cytoplasmic and nuclear markers, respectively. Ub, ubiquitin; WB, Western blot.
A

B

C

- Complex
- Enzyme
- Group/Complex/Other
- Ligand-dependent Nuclear Receptor
- Transcription Regulator
- Transporter
- Unknown
- Relationship
Supplemental Figure III. IPA core analysis of the 91 putative SUMO3 substrates with up-regulated SUMO3 conjugation state after ischemia. A-B. The significantly enriched categories of Diseases and Disorders (A) and Molecular and Cellular Functions (B) identified by IPA core analysis are shown. The y-axis indicates the significance score with a threshold set at 0.001 (Fisher’s exact test, orange line). C. The graph shows the network with the highest score (IPA score = 72).

Supplemental References


