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. $Emx1^{Cre/Cre}$ 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'-GATACTCGAGCTATGTCGGAAGAGAAGCCCAA-3' (forward) and 5'-ACCTTGAAGCGCATGAACTC-3' (reverse) for the SUMO transgene, and 5'-GGTCGATGCAACGAGTGATGAGG-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.[1] 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 After 10 minutes of ischemia, the carotid arteries were deoccluded and withdrawn blood was reinfused.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.[2] 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.[3] 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), GADPH (#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 NaCl2, 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, (http://www.genome.duke.edu/cores/proteomics/sample-preparation/), adapted from a reference protocol.[4] 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/MeCN/water for analysis.

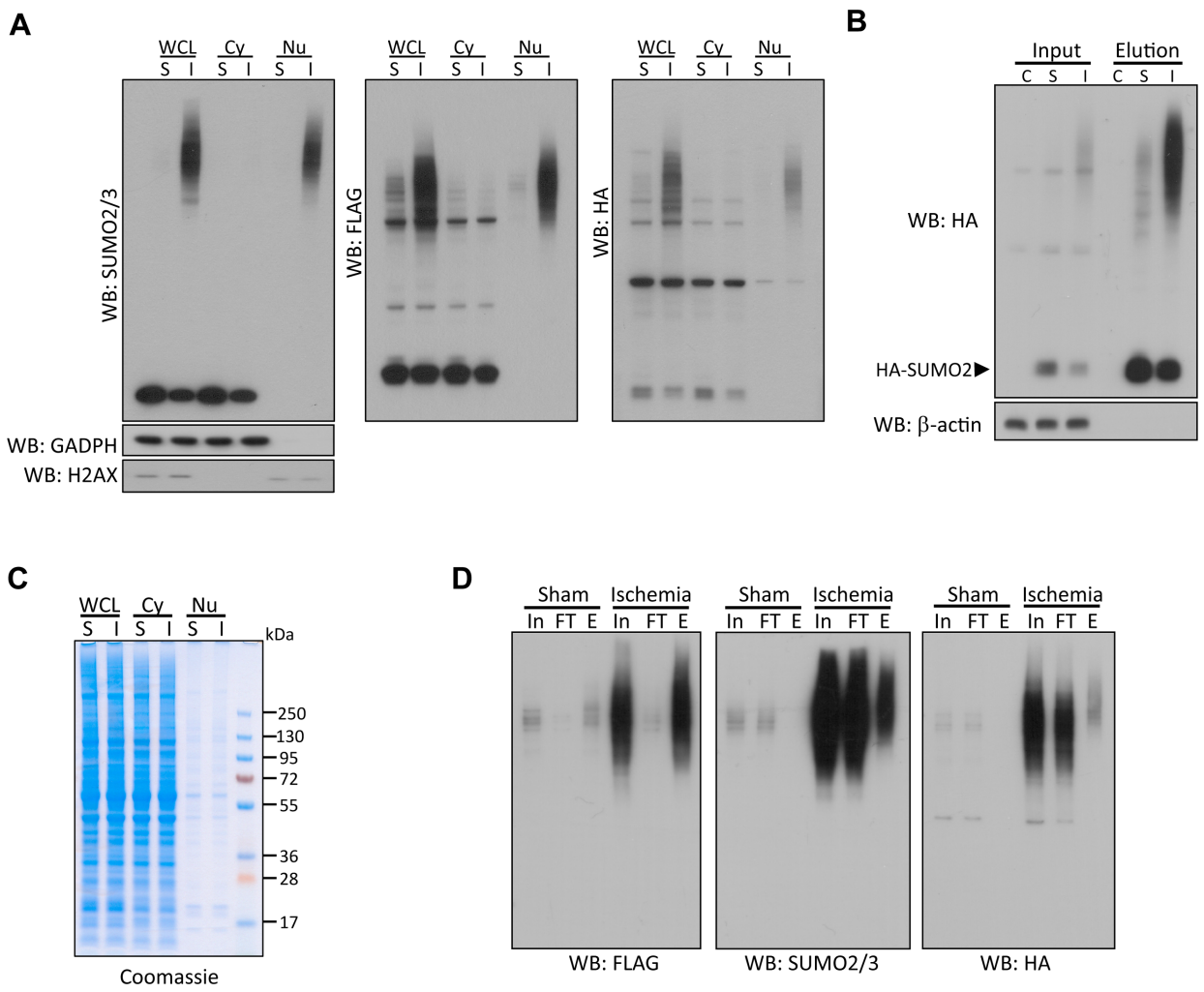
The in-gel digested peptide samples from each slice were analyzed by LC-MS/MS as follows. Five µL of each sample were injected onto a 75 µm x 250 mm BEH C18 column (Waters, Milford, MA) and separated using a gradient of 5% to 40% acetonitrile with 0.1% formic acid, with a flow rate of 0.4 µL/min, in 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. Carbamidomethylation (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.[5,6] 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 *).

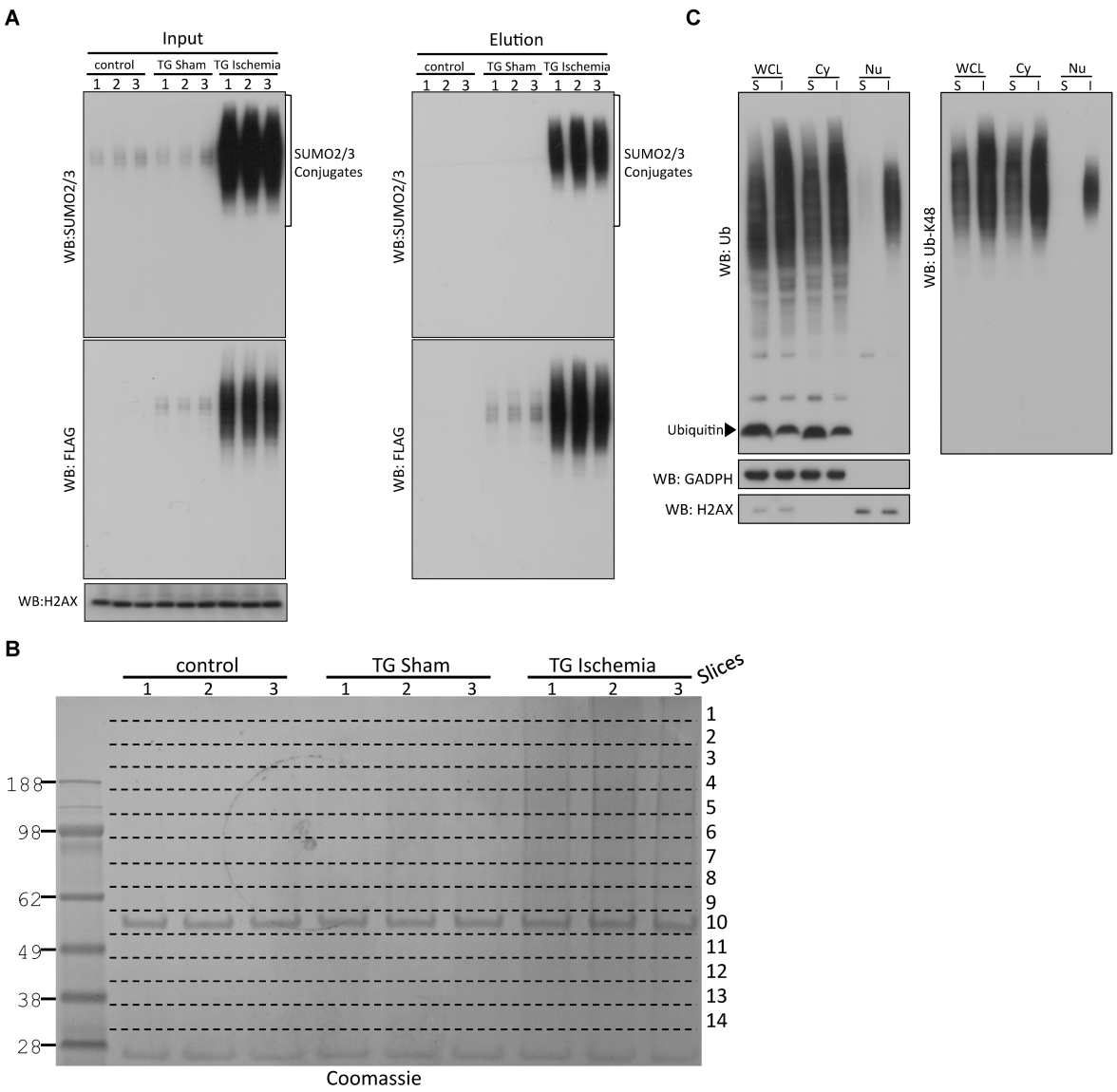
Bioinformatics analysis. The PANTHER (Protein Analysis Through Evolutionary Relationships; http://www.pantherdb.org/) program was used to categorize proteins according to cellular component and molecular function based on the Gene Ontology (GO) database. IPA (Ingenuity Pathway Analysis; www.ingenuity.com) 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/). 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_ShamVIschemia_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.

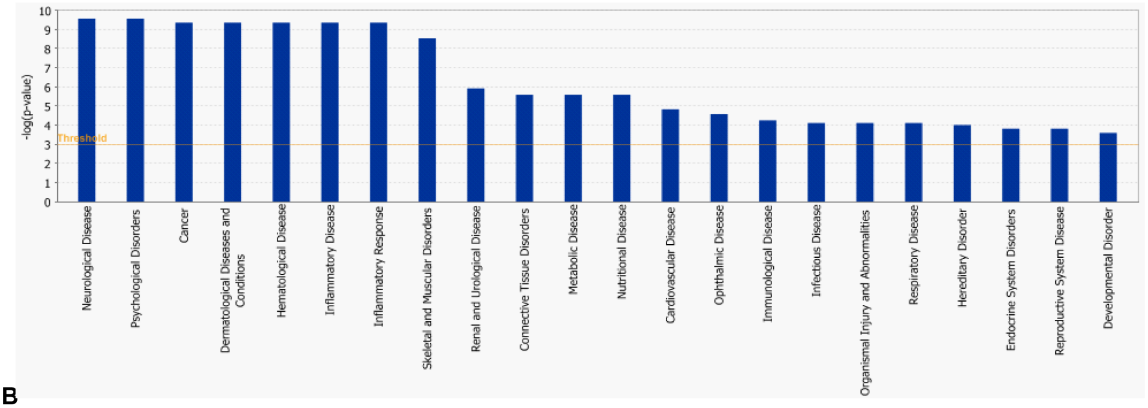


Supplemental Figure I. Analysis of SUMOylation in the brain using CAG-SUMO/Emx1-Cre mice. **A**, CAG-SUMO/Emx1-Cre mice were subjected to sham surgery (S) or 10 minutes forebrain ischemia and 1 hour reperfusion (I). Whole cell lysates (WCL), cytoplasmic (Cy), and nuclear (Nu) fractions were prepared 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). $Emx1^{Cre/+}$ 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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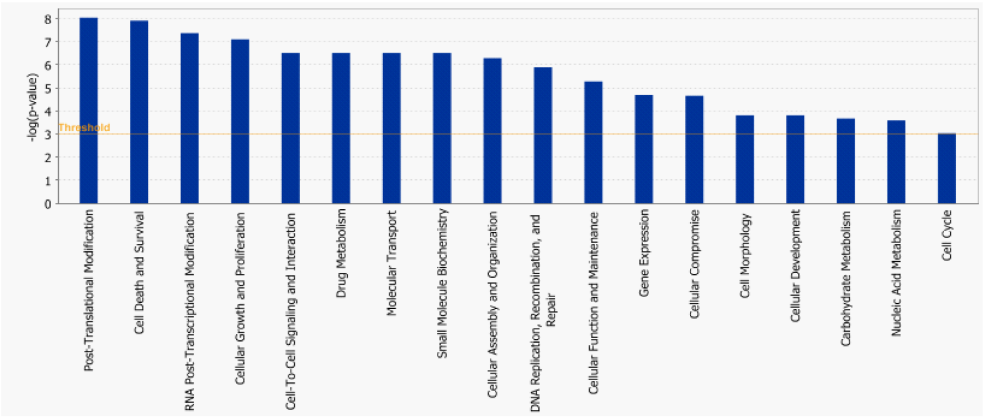
Threshold
Neurological Disease
Psychological Disorders
Cancer
Dermatological Diseases and Conditions
Hematological Disease
Inflammatory Disease
Inflammatory Response
Skeletal and Muscular Disorders
Renal and Urological Disease
Connective Tissue Disorders
Metabolic Disease
Nutritional Disease
Cardiovascular Disease
Ophthalmic Disease
Immunological Disease
Infectious Disease
Organismal Injury and Abnormalities
Respiratory Disease
Hereditary Disorder
Endocrine System Disorders
Reproductive System Disease
Developmental Disorder

B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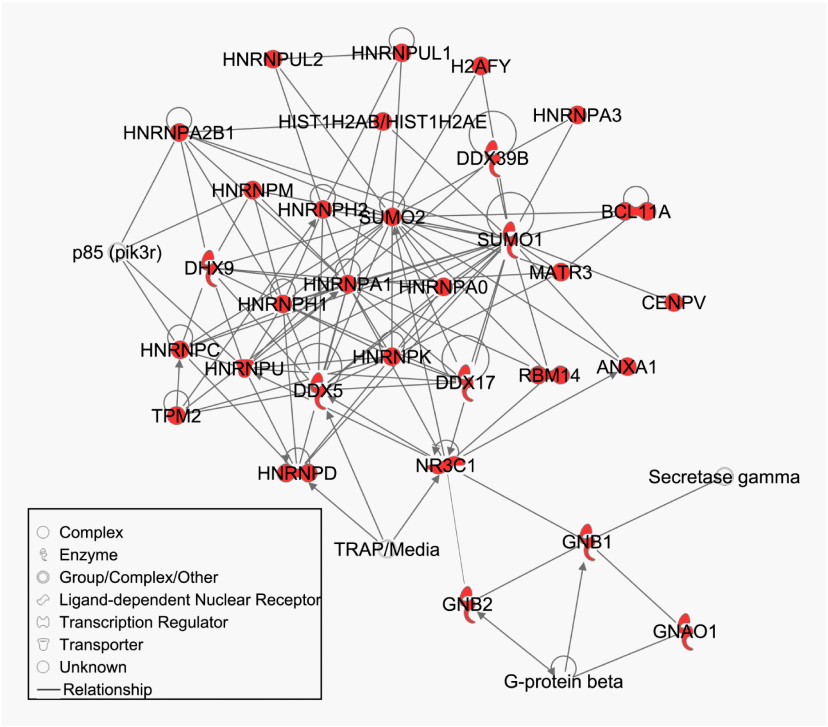
Threshold
Post-Translational Modification
Cell Death and Survival
RNA Post-Transcriptional Modification
Cellular Growth and Proliferation
Cell-To-Cell Signaling and Interaction
Drug Metabolism
Molecular Transport
Small Molecule Biochemistry
Cellular Assembly and Organization
DNA Replication, Recombination, and Repair
Cellular Function and Maintenance
Gene Expression
Cellular Compromise
Cell Morphology
Cellular Development
Carbohydrate Metabolism
Nucleic Acid Metabolism
Cell Cycle

C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HNRNPUL1
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DDX39B
HNRNPM
HNRNPH3
SUMO2
BCL11A
SUMO1
p85 (pik3r)
DHX9
MATR3
HNRNPA1
HNRNPA0
CENPV
HNRNPH1
HNRNPC
HNRNPK
HNRNPU
DDX17
RBM14
ANXA1
DDX5
TPM2
NR3C1
HNRNPD
Secretase gamma
TRAP/Media
GNB1
GNB2
GNAO1
G-protein beta
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

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