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Stress-inducible phosphoprotein 1 has unique cochaperone activity during development and regulates cellular response to ischemia via the prion protein

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ABSTRACT Stress-inducible phosphoprotein 1 (STI1) is part of the chaperone machinery, but it also functions as an extracellular ligand for the prion protein. However, the physiological relevance of these STI1 activities in vivo is unknown. Here, we show that in the absence of embryonic STI1, several Hsp90 client proteins are decreased by 50%, although Hsp90 levels are unaffected. Mutant STI1 mice showed increased caspase-3 activation and 50% impairment in cellular proliferation. Moreover, placental disruption and lack of cellular viability were linked to embryonic death by E10.5 in STI1-mutant mice. Rescue of embryonic lethality in these mutants, by transgenic expression of the STI1 gene, supported a unique role for STI1 during embryonic development. The response of STI1 haploinsufficient mice to cellular stress seemed compromised, and mutant mice showed increased vulnerability to ischemic insult. At the cellular level, ischemia increased the secretion of STI1 from wild-type astrocytes by 3-fold, whereas STI1 haploinsufficient mice secreted half as much STI1. Interesting, extracellular STI1 prevented ischemia-mediated neuronal death in a prion protein-dependent way. Our study reveals essential roles for intracellular and extracellular STI1 in cellular resilience.—Beraldo, F. H., Soares, I. N., Goncalves, D. F., Fan, J., Thomas, A. A., Santos, T. G., Mohammad, A. H., Roffé, M., Calder, M. D., Nikolova, S., Hajj, G. N., Guimaraes, A. N., Massensini, A. R., Welch, I., Betts, D. H., Gros, R., Drangova, M., Watson, A. J., Bartha, R., Prado, V. F., Martins, V. R., and Prado, M. A. M. Stress-inducible phosphoprotein 1 has unique cochaperone activity during development and regulates cellular response to ischemia via the prion protein. FASEB J. 27, 000–000 (2013). www.fasebj.org

Key Words: Hsp90 ∙ stroke ∙ maternal-effect gene ∙ Hsp70 ∙ prion protein

Abbreviations: BAC, bacterial artificial chromosome; CM, conditioned medium; DIV, days in vitro; GRK2, G-protein-coupled receptor kinase 2; H&E, hematoxylin and eosin; HOP, heat-shock organizing protein; Hsp70, heat-shock protein 70; Hsp90, heat-shock protein 90; MCAO, middle cerebral artery occlusion; MEF, mouse embryonic fibroblast; micro-CT, microcomputed tomography; MRI, magnetic resonance imaging; OGD, oxygen and glucose deprivation; p53, protein 53; Piwi, P-element-induced wimpy testis; PrP, prion protein; STAT3, signal transducer and activator of transcription 3; STI1, stress-inducible phosphoprotein 1; Stip1, mouse stress-inducible phosphoprotein 1; TPR, tetratricopeptide repeat

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This article includes supplemental data. Please visit http://www.fasebj.org to obtain this information.
stability of many client proteins that are critical for cellular homeostasis (1, 2). Cochaperones are thought to play important roles in assisting Hsp70 and Hsp90 (3). The cochaperone stress-inducible phosphoprotein 1 [STI1; or heat-shock organizing protein (Hop)] is thought to participate in different aspects of cellular function (4). The protein contains 3 tetratricopeptide repeat (TPR)-containing domains (TPR1, TPR2A, and TPR2B), which allows simultaneous binding to Hsp70 and Hsp90 (5). These observations led to the hypothesis that STI1 acts as an adaptor protein to transfer client substrates between Hsp70 and Hsp90. Indeed, STI1 can regulate the ATPase activity of Hsp90 to help drive the sequential steps of the chaperone machinery (6). Recent structural work suggests that STI1 can function as a scaffolding protein, for example, by linking Hsp90 to P-element-induced wimpy testis (Piwi), a process that regulates phenotype canalization in Drosophila (8).

STI1, similar to heat-shock proteins (9, 10), can also be secreted by cells. The protein is secreted by astrocytes via microvesicles exerting extracellular effects on neurons and astrocytes (11–13). Extracellular STI1 can form a signaling complex with the prion protein (PrPC) in hippocampal neurons to activate cellular signaling via increasing intracellular calcium in hippocampal neurons to activate cellular signaling (7nAChRs (15). STI1/PrPc engagement can protect hippocampal neurons against staurosporine-mediated cell death and also increase their differentiation (4, 16, 17).

Elimination of STI1 does not affect growth in yeast, unless in the presence of Hsp90 mutants (18). In Caenorhabditis elegans, lack of STI1 is not lethal, but it decreases life span and increases sensitivity to stress (19). A number of cochaperones containing TPR domains, similar to those in STI1, interact with the MEEVD motif of Hsp90 (5); hence, it is possible that other cochaperones could substitute for STI1. In mammals, the in vivo roles of STI1 are not understood. Here, we targeted STI1 in mice and found that this protein is expressed early during development. Embryonic STI1 deficiency impairs survival of mice, and we show that several Hsp90 client proteins are reduced in STI1 mutant mice, suggesting that multiple Hsp90 clients may be affected in the absence of STI1. Early embryonic death of STI1-mutant mice could be rescued by transgenic expression of STI1, confirming that STI1 has unique cochaperone activity in mice. In adult mice, reduced levels of STI1 affected the response to cellular stress, demonstrated by the increased sensitivity of STI1 haploinsufficient mice to ischemic insult. We provide evidence that extracellular STI1 supports neuronal survival following ischemia and that this neuroprotective effect is lost in PrPc-null neurons. These data suggest that STI1 is a multifunctional protein required during development, and in the absence of STI1, cells have decreased resilience to stress.

MATERIALS AND METHODS

Mouse line generation

Genetically modified mice were generated using standard homologous recombination techniques (20), using C57BL/6J ES cells. Mice were generated by Ozgene (Perth, Australia). Construct design is shown in Supplemental Fig. S1A. Chimeric mice were bred to C57BL/6J mice, and germline transmission of the mutant STI1 allele was identified by Southern blot (not shown). F1 mice were then crossed to constitutive Cre mice to remove loxP-flanked regions. Cre-recombined mice were then crossed to C57BL/6J mice, and progeny bearing the recombined STI1 allele, but lacking the Cre transgene, was identified by Southern blot analysis (not shown). These mice were then used to expand the colony. STII+/− mice were then intercrossed to generate STII−/− mice. We have attempted to generate mice with a conditional STI1-floxed allele; however, we found that after removal of the neocassette, this particular floxed allele was null and also caused embryonic lethality (results not shown).

The BMQ-41A8 bacterial artificial chromosome (BAC), a 56,549-bp DNA fragment from mouse chromosome 19 containing the STII gene, was obtained from Source BioScience Life Sciences (Nottingham, UK). The BAC was digested and analyzed using restriction enzymes. This BAC was then used to generate transgenic mice using standard techniques at the Jackson Laboratories (Bar Harbor, ME, USA) transgenic facility in a C57BL/6J background. In all experiments that involved quantification between genotypes, the experimenters were blinded to the genotypes.

Ethics statement

Animals were maintained and handled by the University of Western Ontario Animal Care and Veterinarian Service, or in the A. C. Camargo Hospital vivarium. Procedures were conducted in accordance with approved animal use protocols at the University of Western Ontario (2008/127) and the A. C. Camargo Hospital (037/09), and they were in accordance with the Canadian Council of Animal Care (CCAC) and U.S. National Institutes of Health (NIH) guidelines.

Isolation of blastocysts and embryos

Heterozygous STI1 females, aged 4 to 5 wk, were superovulated (5 IU of pregnant mare serum gonadotropin followed 48 h later by 5 IU human chorionic gonadotropin) and mated with heterozygous STI1 males. Females were euthanized 3.5 d later, and the blastocysts were collected by uterine flushing using M2 medium (Sigma, Oakville, ON, Canada). Embryos were collected from timed pregnant females and were genotyped.

RT-PCR and qPCR

Samples [brain tissue, mouse embryos (E10) and astrocyte cultures] were homogenized in TRIzol, and total RNA was extracted using the Aurum Total RNA kit from Biocomp (Hercules, CA, USA). qRT-PCR and qPCR were performed as described previously (21, 22). A nontemplate reaction was used as a negative control for each experiment, and β-actin mRNA levels were used to normalize
the data, as described previously. Sequences of primers used are shown in Supplemental Table S1.

**Astrocyte primary culture**

Astrocyte primary cultures were prepared as described previously (11). Briefly, cortical tissue from each individual embryo was dissociated in 5 ml DMEM supplemented with 1% (v/v) penicillin/streptomycin and 10% FBS and plated in a 100-mm cell culture dish. Cultures were maintained in an incubator at 37°C, 5% CO₂ for 10–12 d, and the medium was replaced 1×/wk.

**Neuronal culture**

Primary cultures of hippocampal neurons from E17 pregnant females were obtained as described previously (17). Cultures from individual embryos were maintained separately. Neuronal cultures from control and PrPC-null mice were prepared as described previously (14).

**Mouse embryonic fibroblast (MEF) culture**

MEFs were prepared from E10.5 embryos as described previously (23). The cultures were obtained from each embryo, and the heads were used for genotyping. For survival curves, 2 × 10⁴ cells were plated in duplicate into 24-well plates; to one of them, we added DMEM supplemented with 20% FBS, and to the other, we added half of the volume of the same medium with 40% of FBS and half of 2× concentrated conditioned medium (CM) from STTI+/+ MEFs. The medium was supplemented with recombinant STTI (2 μM).

**Western blot analysis**

Samples were homogenized in ice-cold RIPA buffer (50 mM Tris-HCl, pH 8; 150 mM NaCl; 1% Nonidet P-40; 1% sodium deoxycholate; and 1% SDS), and the protein concentration was evaluated using protein assay reagent (Bio-Rad, Hercules, CA, USA). Protein (5–20 μg) was loaded on each gel lane and transferred to membrane for Western blot analysis, as described previously (21). All blots were quantified using FluorChem (Alpha Innotech; GE Healthcare, London, ON, Canada) or ImageJ software (NIH, Bethesda, MD, USA).

**Oxygen and glucose deprivation (OGD)**

Neurons were plated in P35 (5×10⁵ cells) dishes, and the cultures were kept under the conditions described above until the medium was changed after 7 days in vitro (DIV). Cells were submitted to OGD by using a chamber to control the levels of oxygen (0.5%) and CO₂ (5%) for 1 h, and the neurons were kept in glucose-deficient neurobasal medium just prior to the hypoxia treatment. After OGD, the glucose-free medium was replaced with regular medium with or without recombinant STTI (1 μM), and the cells were returned to normal oxygen conditions for 24 h.

Astrocytes were plated in P35 dishes. After 70% of confluence, the cells were deprived of FBS for 48 h, after which a medium change was performed. Astrocytes were submitted to OGD in glucose-deficient DMEM. For the secretion experiments, medium was collected after 9 h of OGD. In control cells, medium was collected after 9 h of incubation, as described below, and used in Western blots to detect secreted STTI. For the cell death assay, glucose-free medium was replaced with regular medium, and cells were returned to normal oxygen conditions for 48 h.

**Secretion of STTI in CM**

Astrocytes or fibroblasts were plated in P60 dishes as described above. After 90% of confluence, the cells were deprived of FBS for 48 h. Culture medium was then collected and centrifuged at 10,000 g for 20 min at 4°C, and the supernatant was used as CM.

**Micro-computed tomography (micro-CT)**

For micro-CT, embryos were harvested at E10.5 and fixed in 10% formalin in PBS (48 h at 4°C), then immersed in Lugol solution (10 g KI plus 5 g I in 400 ml H₂O) for 48 h (24). The Lugol solution acted as a micro-CT contrast agent. Scanning was performed using a GE Locus SP scanner (GE Healthcare) using the following scan parameters: 80 kVp with 0.508-ml AI filtration, 80 μA, 900 views over 360°; 4 frames averaged, with images reconstructed to yield 13-μm isotropic voxels (25).

**Hematoxylin and eosin (H&E) staining**

Embryos (E10.5) were harvested, formalin fixed (10% formalin for 48 h at 4°C), and paraffin embedded. Sections (3–5 μm) were deparaffinized and stained with H&E. Images were acquired with an Olympus DP72 camera using cellSens Dimension software (Olympus, Tokyo, Japan).

**Immunofluorescence**

Astrocytes (1×10⁵ cells) and fibroblasts (1×10⁵ cells) were grown on coverslips. Cells were fixed in 4% paraformaldehyde/PBS for 20 min and washed in PBS Triton X-100 (0.05%) solution followed by blocking in PBS with 0.05% TritonX-100 plus 10% of normal goat serum. Cells were incubated with anti-STTI (1:400), anti-Hop (1:100; Enzo Life Sciences, Farmingdale, NY, USA), anti-STTI (1:100; Sigma), anti-β-tubulin (1:100; Sigma), or anti-γ-H2AX (1:100; Cell Signaling, Boston, MA, USA) overnight at 4°C. The deciduae containing E6.5 embryos were dissected, fixed overnight at 4°C in 4% paraformaldehyde, and cryoprotected with 0.1 M phosphate buffer (pH 7.4) containing 30% sucrose. Whole deciduae were frozen with dry ice, and cryostat sections (10 μm) were obtained and mounted on silanized slides. E10.5 embryos were harvested, formalin fixed (10% formalin for 48 h at 4°C), and paraffin embedded. Sections (3–5 μm) were deparaffinized. For immunofluorescence, tissues were incubated with blocking solution (0.1 M phosphate buffer, pH 7.4, containing 0.2% Triton X-100 plus 20% goat serum) for 1 h at room temperature, followed by overnight incubation with anti-rabbit STTI antibody (1:200) or anti-rabbit caspase 3 activated (1:200). Tissues were washed 3× with PBS and anti-rabbit Alexa Fluor 488 plus TO-PRO3 iodide (1:1000) or Hoechst (1:1000) for nuclei staining for 1 h at room temperature. Slides were mounted on coverslips using FluoroSave (Calbiochem, La Jolla, CA, USA) and imaged using a Leica TCS SP5 II laser scanning confocal system or Zeiss LSM-510 confocal microscope (Carl Zeiss, Oberkochen, Germany).

**Cell death assay**

Neuronal and astrocyte cell death assays were performed using the Live/Dead viability/cytotoxicity kit for mammalian cells (Invitrogen, Oakville, ON, Canada) as described in the manual. Counting was done using ImageJ software and cal-
Middle cerebral artery occlusion (MCAO)

Anesthesia was induced by inhalation of 4% isoflurane (in O₂), and maintenance was done by inhalation of 1.5% isoflurane. A modification of a previously described method (26) for transient intraluminal MCAO was used. Under an operating microscope, a monofilament nylon suture (diameter 0.06-0.09 mm; Doccol Corp., Redlands, CA, USA) was inserted through the left common carotid artery into the internal carotid artery and then into the circle of Willis, effectively occluding the MCA for 60 min. Sham-treated animals underwent the same procedure, with the exception of the advancement of the filament to occlude the cerebral artery. After filament insertion, the mice were removed from anesthesia and kept at 37°C under a heat lamp freely moving during 1 h of occlusion. Mice were then anesthetized again, and the filament was quickly removed. At 1 h after surgery, mice were injected i.p. with saline to maintain hydration. This procedure was repeated again, if necessary, on the basis of weight recovery of the animal and clinical assessment score. Food pellets were wetted and kept on the floor of the cage for recovering mice. Neurological assessment was performed 1, 24, and 48 h after surgery following scores modified from the 5-point Bederson scale (27): 0, no deficit; 1, mild forelimb weakness; 2, severe forelimb weakness, consistent turns to the deficit side when lift by the tail; 3, compulsory circling; 4, unconscious; 5, dead. Only mice that scored 2 or 3 were used in the experiments. Heart rate data were obtained under baseline and during the MCAO period and analyzed for pH and glucose using a cardiac puncture under isoflurane anesthesia (Kent Scientific, Torrington, CT, USA). Rectal temperature was monitored by a homeothermic blanket control unit (Harvard Apparatus, Holliston, MA, USA). Arterial blood samples (obtained via cardiac puncture under isoflurane anesthesia) were taken at baseline or at the end of the MCAO period and analyzed for pH and glucose using a blood analyzer (ABL-725; Radiometer, Copenhagen, Denmark).

Magnetic resonance imaging (MRI)

An Agilent (Palo Alto, CA, USA) 9.4-T small-animal horizontal-bore MRI system was used to acquire images of the mouse brain 24 h after MCAO. Two imaging sequences were used to visualize tissue damage due to ischemia: a T₂-weighted 2-dimensional fast spin echo (FSE) sequence (T₁=35 ms, T₂=3000 ms, FOV 19.2×19.2 mm, 31 slices, slice thickness=500 μm, acquisition matrix 128×128) and a 3-dimensional balanced steady-state free precession (bSSFP) sequence (T₁=3.7 ms, T₂=7.4 ms, FOV 19×16×13 mm, acquisition matrix 154×132×102). Infarct volume was measured by manual tracing of the hyperintense tissue in each slice of the T₂ images using ImageJ by a single investigator blinded to the genotypes (D.G.).

Adhesive removal

Postschismic and sham-treated animals were subjected to behavioral tests 7 d after stroke. The adhesive removal test is sensitive to unilateral somatosensory dysfunction and was used to determine functional recovery (28). Three trials were conducted and averaged per day. Individual trials were separated by at least 15 min.

RESULTS

Mouse STI1 (Stip1) deficiency causes embryonic lethality

To uncover potential in vivo roles of STI1 in mammals, we generated a Stip1-knockout mouse line (Supplemental Fig. S1A). The strategy used the Cre/Lox system to remove exons 2 and 3 from a floxed Stip1 allele. This deletion truncated the STI1-coding region and created a stop codon close to the initial ATG, so that the protein expression was abolished. Sequencing of the targeted Stip1 allele (STI1⁻⁻) confirmed the genetic manipulation. We found that embryos containing 2 Sti1⁻⁻ alleles died around E9.5–E10.5 (Table 1). Further analysis of STI1⁺⁻/+ intercrosses revealed that recovery of STI1⁻⁻/- embryos did not follow Mendelian frequency. The reduced frequency observed (9 and 12% instead of 25% for E3.5 and E10.5, respectively; Table 1), suggested that STI1 deficiency affected the survival of embryos prior to implantation.

To test the possibility that STI1 is expressed early during development, we immunolabeled blastocysts (E3.5) with a STI1 antibody. Immunoreactivity for STI1 with genotypes (D.G.).

| TABLE 1. Viability analysis of STI1⁻⁻/- mice and embryos

<table>
<thead>
<tr>
<th>Stage</th>
<th>STI1⁺⁺/⁺⁺</th>
<th>STI1⁻⁻/⁻⁻</th>
<th>Reabsorbed</th>
<th>Total</th>
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<tr>
<td>Live birth</td>
<td>65</td>
<td>103</td>
<td>0***</td>
<td>0</td>
</tr>
<tr>
<td>E17.5</td>
<td>33</td>
<td>42</td>
<td>0***</td>
<td>21</td>
</tr>
<tr>
<td>E13.5</td>
<td>14</td>
<td>22</td>
<td>0***</td>
<td>16</td>
</tr>
<tr>
<td>E10.5</td>
<td>68</td>
<td>131</td>
<td>27 (12%) * ***</td>
<td>68</td>
</tr>
<tr>
<td>E3.5</td>
<td>47</td>
<td>57</td>
<td>16 (13%) * *</td>
<td>0</td>
</tr>
</tbody>
</table>

Genotypes were determined by PCR as described in Materials and Methods. Empty deciduas were scored as reabsorbed. *STI1⁻⁻/- ratio. Data were analyzed by χ² test, comparing the expected and observed frequencies for each genotype at different time points of embryonic development. *P ≤ 0.05, ***P ≤ 0.0005.
Figure 1. Expression of STI1, Oct4, and CDX2 in mouse blastocysts. A) STI1 labeling (green) in STI1+/+, STI1−/−, and STI1+/− blastocysts (representative images of 24 STI1+/+, 22 STI1−/−, and 8 STI1+/− blastocysts). Nucleus is shown in blue. Scale bars = 20 μm. B) Agarose gel showing an example of blastocyst genotyping. C) Control experiments for STI1 antibody specificity. Antibody was incubated (bottom panels) or not (upper panels) with recombinant STI1 and then used to label STI1+/+ blastocysts. D) Labeling for the pluripotency marker Oct4 (green) in STI1+/+, STI1+/−, and STI1−/− blastocysts (representative images of 13 STI1+/+, 23 STI1+/−, and 4 STI1−/−). E) Labeling for marker of trophoblast-committed cells, CDX2 (green), in STI1+/+, STI1+/−, and STI1−/− blastocysts (representative images of 13 STI1+/+, 25 STI1+/−, and 5 STI1−/−). Scale bars = 20 μm.

Although experiments showed that immunolabeling with the STI1 antibody was completely abolished by absorption with excess recombinant STI1 (Fig. 1), indicating that STI1 labeling in blastocysts was specific. Since there are no other STI1 homologs, these results suggest that maternal STI1, deposited either as protein or mRNA, is present in blastocysts and may play a role in early embryonic development.

We quantified STI1 mRNA in STI1−/− E10.5 embryos by RT-PCR and qPCR. Our results shown that no STI1 mRNA is present in STI1−/− E10.5 embryos (Fig. 2D), suggesting that the immunoreactivity that we detected in embryos might originate from extraembryonic sites.

Interestingly, we found high levels of STI1 present at extraembryonic sites in wild-type embryos, specifically at the trophoblast layer (Fig. 2E). Whether this extraembryonic STI1 may contribute for survival of mutant embryos during the early developmental stage is currently unknown. In addition, we observed the presence of acute inflammation in and around the labyrinth in the placenta from STI1−/− embryos (Fig. 2B), suggesting that their placenta has had a vascular disruption, as there was no evidence of infection. Hence, placental disruption might facilitate the transfer of maternal STI1 to embryos but could also contribute to embryonic dysfunction. STI1 has been shown to be secreted by distinct cell types (11, 29, 30), including ovarian cells (31, 32) and has also been found in plasma (31).

To understand the characteristics of embryonic development in STI1-mutant mice, we performed micro-CT analysis. This experiment demonstrated that neural tube and limb buds were poorly formed in STI1−/− embryos (Fig. 3A). In addition, histochemistry analysis showed that STI1−/− embryos presented a great number of cells that were undergoing apoptotic cell death, characterized by increased levels of activated caspase-3 (Fig. 3B), as well...
as pyknotic apoptotic bodies with a hypereosinophilic cytoplasm in H&E (Fig. 3C–H).

The early embryonic death with increased apoptosis in STI1-mutant mice is unexpected, given that elimination of STI1 in yeast (32) and C. elegans is not lethal (19). To test the possibility that the cochaperone activity of STI1 is required during development, we examined the expression levels of 3 distinct Hsp90 client proteins. Immunoblot analysis showed that Hsp90 levels were unchanged in STI1−/− mice (Fig. 4A, B). In contrast, the levels of G-protein-receptor kinase 2 (GRK2), signal transducer and activator of transcript...
tion 3 (STAT3), and protein 53 (p53), all Hsp90 client proteins (33–35) were significantly reduced to almost 50% (Fig. 4A, B).

To further understand the consequences of interference with Stip1 for cellular function, we cultured MEFs from E10.5 embryos. During the initial days in culture, we could not distinguish wild-type from STI1−/− cells using morphological criteria. However, after a few days, we noticed that STI1−/− MEFs were dying, whereas wild-type or STI1+/− MEFs thrived well in culture (Fig. 5A, B). Interestingly, most STI1−/− MEFs had died off following 8–10 d in culture (Fig. 5A, B). To understand potential causes of this increased lethality in STI1−/− MEFs, we labeled these cells during the first 2 d of culture with an antibody against γ-H2AX, a phosphorylated histone recruited to sites of DNA double-strand breaks in response to distinct cellular stresses (36). Wild-type MEFs showed no labeling for γ-H2AX (Fig. 5C); however, STI1−/− MEFs presented widespread nuclear foci labeling (Fig. 5C), suggestive of increased cellular stress levels. Interestingly, STI1+/− MEFs also presented γ-H2AX labeling (Fig. 5C). Since enhanced cellular stress levels increase DNA damage, which may affect cell cycle (37), we examined proliferation of STI1−/− MEFs and found decreased proliferation, as compared to STI1+/+ or STI1+/+ MEFs (Fig. 5D). Hence, STI1−/− MEFs were impaired in cellular proliferation and showed increased levels of cellular stress, which likely affected cellular viability.

We examined STI1 expression in MEFs and found that STI1−/− MEFs presented weak nuclear immunolabeling for STI1 (Fig. 5C). Immunolabeling in STI1−/− MEFs was specific because the same pattern of STI1 immunolabeling was observed with distinct antibodies (Supplemental Fig. S1B); STI1 immunolabeling in MEFs and other cells could be absorbed with excess recombinant STI1 (Supplemental Fig. S1C); and the main antibody that we used in these experiments recognizes only one band in immunoblots with the correct molecular mass in embryos or in brain extracts of adult STI1+/+ and STI1−/− mice (Fig. 2A, B and Supplemental Fig. S1D). Hence, it is likely that MEFs containing some maternal STI1 may have been selected to survive longer in these cultures.

The weak STI1 labeling was concentrated mostly in the nucleus in STI1−/− MEFs (Fig. 5C), as opposed to the predominantly cytoplasmic labeling observed in wild-type MEFs and in blastocysts (see Fig. 1A). Fibroblasts obtained from STI1−/− MEFs, which presented overall weaker labeling than wild-type MEFs, also showed increased nuclear labeling for STI1 (Fig. 5C). This increased level of nuclear STI1 is likely related to increased levels of cellular stress, as stress has been shown to increase nuclear accumulation of STI1 (38). STI1 has been previously shown to have a nuclear localization signal (NLS; refs. 38, 39). Given that wild-type MEFs secrete STI1 (Fig. 5A), we examined whether increasing levels of extracellular STI1 could perhaps attenuate the lethality observed in STI1−/− MEFs. We incubated STI1−/− MEFs with CM obtained from wild-type MEFs supplemented with 2 μM recombinant STI1 (Fig. 5E). Lethality of STI1−/− MEFs could not be rescued by increasing extracellular levels of STI1 or by other factors secreted by wild-type MEFs in this condition. This result suggests that extracellular STI1 is not sufficient to maintain mutant MEFs viability.

Rescue of lethality in STI1−/− mice

Given the presence of small amounts of extraembryonic STI1 in STI1-mutant mice, we decided to test further the requirement for embryonic STI1 during development. For this experiment, we initially generated STI1 transgenic mice, which were crossed with STI1−/−, and tested for rescue of lethality of STI1−/− mice. Since STI1 appears to be required prior to implantation for survival of blastocysts, we used BAC containing the STI1 gene to generate transgenic mouse lines and reproduce any required early expression of STI1 during embryogenesis. We generated two distinct lines of STI1 BAC transgenic mice (STI1-TgA and STI1-TgC) presenting 10 and 2 extra alleles of STI1, respectively, as determined by qPCR (Fig. 6A). STI1 mRNA levels were increased 5-fold in STI1-TgA line compared to wild-type littermates (Fig. 6B), and pro-
expression was sufficient to rescue expressing BAC-derived STI1. In contrast, STI1-TgA BAC (indetermining), supporting the notion that this line did not their control counterparts (Fig. 7A). In contrast, STI1-TgC line did not show line (Fig. 6C). Decreased levels of STI1 in astrocytes (Fig. 7C). Decreased levels of STI1 increased cellular stress and vulnerability of cells. To examine whether reduced levels of STI1 affects the capacity of mice to respond to cellular stress and injury, we used a model of ischemic insult. We submitted STI1−/+ and wild-type mice to the middle cerebral artery occlusion (MCAO, 60 min) technique to induce unilateral stroke. Sham-operated mice from both genotypes showed no signs of stroke and survived well after the surgery, independent of genotype (Fig. 8A). We detected no differences between genotypes in physiological parameters due to MCAO (Table 2). Whereas most wild-type mice survived after stroke injury (20% lethality rate), STI1−/+ mice, however, displayed increased mortality and nearly 50% of the mice died 5 d following surgery (Fig. 8A). STI1 mutant mice showed increased brain infarct volume when compared to wild-type mice 24 h following MCAO (Fig. 8B). We tested whether STI1−/+ mice that survived the stroke injury recovered similarly to wild-type mice. Both genotypes lost weight just after surgery, but recovered after 7 d (Fig. 8C). Interestingly, behavioral analysis demonstrated that 7 d after MCAO, STI1−/+ mice performed poorly compared to wild-type mice in the tape removal test used to evaluate fine sensorimotor function (28). Both genotypes showed a clear deficit to perceive and remove the tape in the contralateral (right paw, RP),
but not in the ipsilateral (left paw, LP) ischemic side (Fig. 8D), indicating a functional deficit due to ischemia. However, STI1−/− mice showed decreased dexterity in their RP compared to wild-type controls to remove the tape (Fig. 8E). These experiments indicate that decreased STI1 levels affect brain injury after ischemic stroke, survival of animals, and their functional recovery.

To establish potential mechanisms by which STI1 may affect neuronal function after stroke, we examined the response of astrocytes and neuronal cultures to OGD. We found that intracellular STI1 was reduced in STI1+/+ astrocytes after OGD (Fig. 9A). In STI1−/− astrocytes, the intracellular levels of STI1 were further reduced by OGD. Experiments using proteasome inhibitors indicated that reduced levels of STI1 were not due to increased protein degradation (not shown), suggesting the possibility of changes in secretion. Indeed, wild-type astrocytes increased STI1 secretion by close to 3-fold after OGD (Fig. 9B). Although astrocytes derived from STI1+/+ mice also showed increased secretion of STI1 following OGD, the levels of extracellular STI1 were, as expected, ~2-fold lower than those of wild-type astrocytes (Fig. 9B). Therefore, as a consequence of ischemia, astrocytes secrete significant amounts of STI1, (Fig. 9A, B); however, since STI1−/− astrocytes have 50% less protein, the amount of STI1 secreted by these mutated cells is lower. OGD did not increase cell death of astrocytes from either genotype (Fig. 9C).

Figure 6. Rescue of embryonic lethality of STI1−/− mice by transgenic expression of STI1. A) Number of copies of the STI1 gene in genomic DNA from STI1+/+ (n=8) and STI1−/− mice (n=4), as well as transgenic mouse lines STI1-TgA (n=5) and STI1-TgC (n=9) and transgenic lines STI1-TgA (n=4) and STI1-TgC mice (n=4). B) STI1 mRNA expression in the brain of STI1+/+ mice (n=9) and transgenic lines STI1-TgA (n=4) and STI1-TgC mice (n=4). C) Representative immunoblotting and STI1 protein expression quantification in the brain of STI1+/+ (n=4) and STI1-TgA mice (n=4). D) Representative immunoblotting and STI1 protein expression quantification in the brain of ST1+/+ (n=4) and STI1-TgC mice (n=4 mice). E) qPCR to determine the number of copies of the ST1−/− allele (which is absent in wild-type mice) in tissue from adult mice, showing that expression of BAC-STI1 allowed survival of STI1−/− mice (4 STI1+/+, 11 STI1−/−, and 5 TgASTI1−/−). Results are presented as means ± se; data were analyzed and compared by 1-way ANOVA and Newman-Keuls post hoc test (A, B), Student’s t test (C, D), and χ² test (P<0.05 from the expected distribution; E). **P < 0.001, ***P < 0.0001.

Figure 7. STI1 secretion, expression, and cellular localization in STI1+/+ and STI1−/− astrocyte cultures. A) STI1 expression in STI1+/+ and STI1−/− astrocytes (cultures obtained from 4 embryos/genotype). B) Detection of STI1 in CM from astrocytes (cultures obtained from 5 embryos/genotype). C) Immunofluorescence showing a reduction of STI1 labeling and localization of STI1 in STI1+/+ and STI1−/− astrocytes (top panel) and quantification of cells with nuclear STI1 (bottom panel). Scale bars = 15 μm. D) Top panel: immunofluorescence for STI1 expression (green) and labeling of γH2AX (red) in STI1+/+ and STI1−/− astrocytes. Scale bars = 30 μm. Bottom panel: quantification of number of foci per cell (cultures obtained from 7 embryos/genotype). Results are presented as means ± se. *P < 0.01; Student’s t test.
In cultured primary neurons, 1 h OGD caused increased cell death (Fig. 9D), an effect that was similar in both control and STI1/H11002/H11001 neurons. Hence, changes in intracellular levels of STI1 did not seem to affect the survival of neurons. To mimic the secretion of STI1 from astrocytes, we treated neurons with recombinant STI1, which reproduces the effects of astrocyte-secreted protein (40). Treatment of neurons from both genotypes with extracellular recombinant STI1 attenuated neuronal death in response to OGD (Fig. 9D), suggesting that rather than intracellular STI1, it is the extracellular protein that protects neurons from ischemic injury.

PrPC, an STI1-interacting protein, plays a role in ischemic injury and increased levels of PrP C seem to protect, whereas lack of PrP C exacerbates neuronal injury in response to ischemic insults in vivo (41–43). To investigate the possibility that extracellular STI1 protects neurons via a PrP C-dependent pathway in neurons, we repeated these experiments using neurons from STI1/H11001/H11001 and STI1/H11002/H11001 mice submitted to 60 min of unilateral ischemia (MCAO).

**Figure 8.** Regulation of functional recovery in ischemia by STI1. A) Survival curve of STI1+/+ and STI1−/− mice submitted to 60 min of unilateral ischemia (MCAO). B) Representative MR images and group analyses of infarct volume in brains of STI1+/+ (n=6) and STI1−/− mice (n=5). C) Weight of mice submitted to MCAO (n=6 Sham STI1+/+, n=5 Sham STI1−/−, n=9 STI1+/+, n=7 STI1−/−). D) Functional recovery of STI1+/+ and STI1−/− mice submitted to MCAO, determined at d 7 after stroke using the tape removal test. To perceive the tape in the right paw (RP) or left paw (LP) (n=9 STI1+/+, n=7 STI1−/−). E) Identical to D, but showing the time to remove the tape. Results are presented as means ± SE; data were analyzed and compared by 1-way ANOVA and Newman-Keuls post hoc test, Student’s t test, and Mantel-Cox log-rank test (P<0.0002; A). *P < 0.01 vs. control mice.

**Table 2. Physiological measurements before and during MCAO**

<table>
<thead>
<tr>
<th>Measurement</th>
<th>STI1+/+</th>
<th>STI1−/−</th>
</tr>
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<tbody>
<tr>
<td>Before MCAO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>697.4 ± 43.8 (n=9)</td>
<td>669.2 ± 34.8 (n=9)</td>
</tr>
<tr>
<td>Arterial blood pH</td>
<td>7.193 ± 0.003 (n=3)</td>
<td>7.233 ± 0.007 (n=3)</td>
</tr>
<tr>
<td>Oxygen saturation (%)</td>
<td>99.37 ± 3.74 (n=3)</td>
<td>97.80 ± 5.8 (n=3)</td>
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<tr>
<td>Temperature (°C)</td>
<td>35.90 ± 0.30 (n=3)</td>
<td>36.20 ± 0.36 (n=3)</td>
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<tr>
<td>Glucose (mM)</td>
<td>8.067 ± 0.633 (n=3)</td>
<td>8.833 ± 0.033 (n=3)</td>
</tr>
<tr>
<td>During MCAO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>693.1 ± 42.9 (n=6)</td>
<td>693.1 ± 55.9 (n=4)</td>
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<tr>
<td>Arterial blood pH</td>
<td>7.107 ± 0.0201 (n=6)</td>
<td>7.100 ± 0.044 (n=4)</td>
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<tr>
<td>Oxygen saturation (%)</td>
<td>99.32 ± 3.47 (n=6)</td>
<td>73.55 ± 10.31 (n=4)</td>
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<tr>
<td>Temperature (°C)</td>
<td>34.35 ± 0.43 (n=6)</td>
<td>34.65 ± 0.52 (n=4)</td>
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<tr>
<td>Glucose (mM)</td>
<td>10.03 ± 1.73 (n=6)</td>
<td>9.650 ± 1.707 (n=4)</td>
</tr>
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</table>

Values are expressed as means ± SE. MCAO, middle cerebral artery occlusion.
of PrP<sup>C</sup>-null mice (<i>Prnp<sup>0/0</sup></i>). The neuroprotective effect of STI1 was prevented in neurons from PrP<sup>C</sup>-null mice (Fig. 9E), suggesting that activation of PrP<sup>C</sup> by STI1 plays a role in protecting neurons against ischemic insult.

**DISCUSSION**

The present experiments demonstrate that Stip1 shows characteristics of a maternal (oogenetic)-effect gene, playing critical roles during development in mammals. STI1<sup>+/+</sup> blastocysts obtained from STI1<sup>+/−</sup> intercrosses were observed at 50% of the expected mendelian frequency, suggesting that blastocysts originating from zygotes lacking sufficient maternally inherited STI1 may not survive. Interestingly, surviving STI1<sup>+/−</sup> blastocysts showed immunostaining for STI1, suggesting the possibility that part of the blastocysts may have received sufficient maternally inherited STI1 to allow development of embryos. It would be of interest to define the precise mechanism by which maternally derived STI1 is transferred to blastocysts. Embryonically expressed STI1 is critical in later stages of development, as mutant embryos could not survive past E10.5. We show that maternal STI1 protein might be transferred to embryos in later stages of development, as we could detect small amounts of STI1 immunoreactivity in E10.5 embryos, likely due to placental disruption. Notably, we did not detect STI1 mRNA in STI1<sup>+/−</sup> E10.5 embryos, indicating lack of embryonic STI1 protein synthesis. The rescue of lethality by transgenic BAC expression supports the notion that STI1 has unique roles during development and that maternally transferred STI1 cannot support embryonic development. Embryonic lethality is commonly associated with placental dysfunction in the E9–E12 stage. Interestingly, Hsp90<sup>B</sup>-knockout mice (44), but not Hsp90<sup>A</sup>-knockout mice (45), present disrupted placenta. Hence, placental disruption may also contribute to embryonic lethality in STI1 mutant embryos.

Despite recent insights from structural models of STI1 interaction with Hsp70 and Hsp90 demonstrating STI1 regulation of client recruitment (7, 46–48), it is unknown whether STI1 has unique or overlapping roles in mammals in vivo. Our data suggest that loss of embryonic STI1 is not tolerated during development, and cells present decreased resilience to stress, showing increased DNA damage and cell death. Hence, other cochaperones do not seem to be able to compensate for the loss of STI1. These results contrast with those of mice deficient for p23, another Hsp90 chaperone, which present perinatal lethality specifically related to skin and lung development (49), suggesting distinct requirements for different cochaperones during development.

The early effects of STI1 in embryogenesis are compatible with its regulation of stem cell self-renewal (50). Interestingly, Hsp90 and, more recently, STI1 have
been shown to regulate epigenetic programs and transposon silencing via piRNAs in Drosophila (8, 51). Remarkably, maternal STI1 appears to influence canalization in Drosophila via the Piwi pathway (8). Future studies aimed at further defining these mechanisms may provide novel insight in mammalian embryonic development.

We show that STI1 has a critical role in cellular survival. This conclusion is supported by the increased cell death in embryos and the inability to maintain STI1−/− MEFs in culture. Because of the early death that we observed in blastocysts, as well as the inability of mutant cells to survive, we favor the possibility that the placental defect that we identified contributes to, but it is not the only cause of, cellular death in these mutant embryos. These effects of Stip1 seem to depend, at least in part, on intracellular STI1, as we were unable to rescue STI1−/− MEFs using recombinant extracellular STI1, but could rescue STI1−/− mice by transgenic expression of a BAC containing Stip1. The increase in foci number in STI1−/− MEFs indicates that lack of chaperone activity may affect DNA damage response. Interestingly, Hsp90, which is regulated by STI1, has been previously implicated in the DNA damage response (52–55). By targeting STI1, we likely affected the functions of a wide range of Hsp90/Hsp70 client proteins (56–58). Indeed, this seems to be the case, given that three known Hsp90 clients (59–61) show reduced levels in STI1-mutant mice, even though Hsp90 levels were unaffected. In contrast with Hsp90 and Hsp70 that have different isoforms, there are no known homologs for Stip1, which may explain why we observed such striking phenotype during development. Given the very large number of Hsp90 client proteins (5, 56, 57), it is unlikely that the phenotypes that we uncovered are related to one specific client. It is also unclear whether the remaining amounts of Hsp90 client proteins in STI1 mutant embryos are functional. Hence, inhibitors of STI1 may have more widespread effects in mammals than inhibitors of Hsp90. Indeed, a recent report demonstrates that inhibition of STI1 interaction with Hsp90 by drugs is effective to kill cancer cells (62).

Our experiment using a stroke model supports an important role for endogenous STI1 in the recovery from ischemic injury in vivo. PrP C expression has been shown to protect the brain following ischemic insults, and lack of PrP C leads to an increase in neuronal injury in stroke models (41, 42, 63). However, the exact mechanisms by which PrP C influences outcomes in stroke are unknown. We now show that OGD, used as an in vitro model of ischemic insult, can increase secretion of STI1 from astrocytes and that secretion from STI1−/− astrocytes is decreased in this condition. Our experiments using cultured neurons suggest that the increased sensitivity observed in vivo in STI1−/− mice may not be related to decreased intracellular levels of STI1 in neurons, as both control and STI1−/− neurons responded similarly to OGD. In these neuronal cultures, extracellular STI1 was able to provide neuroprotection for OGD-induced neuronal death. Moreover, this effect of STI1 was strictly dependent on the presence of PrP C in neurons. These results expand previous observations in which STI1 was shown to prevent neuronal death induced by staurosporine (14, 17) to demonstrate a role for this secreted cochaperone in a pathological relevant insult, ischemic injury. Our in vitro data with recombinant STI1 and in vivo with STI1−/− mice suggest that lack of STI1-mediated signaling may underlie the increased sensitivity of PrP C-null mice to ischemic injury.

Our experiments provide novel evidence that STI1 plays a unique and nonoverlapping role as a cochaperone during embryonic development and in cellular survival, suggesting that loss of STI1-regulated chaperone activity is not tolerated. Our data also support a role for STI1 in neuroprotection against ischemic insult, by a mechanism involving increased secretion from astrocytes and activation of PrP C. Hence, STI1 is a stress-response protein that presents multiple intracellular and extracellular roles with unique properties for protection of cells against stress.

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