Peroxiredoxin 2 Battles Poly(ADP-Ribose) Polymerase 1- and p53-Dependent Prodeath Pathways After Ischemic Injury

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Background and Purpose—Ischemic/reperfusion neuronal injury is characterized by accumulation of reactive oxygen species and oxidative DNA damage, which can trigger cell death by various signaling pathways. Two of these modes of death include poly(ADP-ribose) polymerase 1–mediated death or p53- and Bax-mediated apoptosis. The present study tested the hypothesis that peroxiredoxin 2 (PRX2) attenuates DNA damage–mediated prodeath signaling using in vitro and in vivo models of ischemic injury. The impact of this peroxide scavenger on p53- and poly(ADP-ribose) polymerase 1–mediated ischemic death is unknown.

Methods—Neuronal PRX2 overexpression in primary cortical cultures and transgenic mice was combined with the poly(ADP-ribose) polymerase 1 inhibitor AG14361. AG14361 was also applied to p53 and Bax knockout cultures and mice and combined with the JNK inhibitor SP600125. DCF fluorescence, apurinic/apyrimidinic sites, single-strand breaks, Comet tail-length, nicotinamide adenine dinucleotide depletion, and viability were assessed in response to oxygen-glucose deprivation in cultures or transient focal cerebral ischemia in mice.

Results—PRX2 attenuated reactive oxygen species, DNA damage, nicotinamide adenine dinucleotide depletion, and cell death. PRX2 knockdown exacerbated neuronal death after oxygen and glucose deprivation. PRX2 ameliorated poly(ADP-ribose) polymerase 1, p53, Bax, and caspase activation after ischemia. AG14361 reduced ischemic cell death in wild-type and p53 or Bax knockout cultures and animals but had no additional effect in PRX2-overexpressing mice. AG14361 and p53 knockou elicited additive effects with SP600125 on viability in vitro. Our findings support the existence of multiple parallel prodeath pathways with some crosstalk.

Conclusions—The promising therapeutic candidate PRX2 can clamp upstream DNA damage and efficiently inhibit multiple prodeath cascades operating in both parallel and interactive fashions. (Stroke. 2013;44:1124-1134.)

Key Words: apoptosis ■ Bax ■ necrosis ■ p53 ■ PARP1 ■ PRX2 ■ stroke

Stroke is a major cause of long-term disability across the world. The damaging events that unfold after stroke are manifold, with excitotoxicity and reactive oxygen species (ROS) driving cell death.1 ROS can elicit oxidation of the bases and deoxyribose phosphate backbone of DNA.2 One major mediator of this genotoxic damage is H2O2, partly because it is converted to peroxynitrites and hydroxyl radicals in the presence of nitric oxide and transition metals.3 For example, H2O2 or its derivates can damage DNA and trigger prodeath signaling when applied directly to cultures.4 DNA damage after ischemia involves apurinic/apyrimidinic (AP) sites (apurinic/apyrimidinic sites formed by hydrolysis of the bond between the base and deoxyribose sugar) as well as single and double-strand breaks and is an important trigger of ischemic death.2 Some forms of DNA damage, especially single-strand breaks, can activate poly(ADP-ribose) polymerase 1 (PARP1), leading to nicotinamide adenine dinucleotide (NAD+) depletion and energy failure.2 Thus, exogenous NAD+ replenishment protects against DNA damage–induced and ischemic cell death.5,6 Similarly, PARP1 knockout or inhibition protects against apoptosis-inducing factor translocation and ischemic neuronal injury.7,8 However, DNA damage also activates PARP1-independent forms of cell death through ataxia telangiectasia mutated (ATM) and ATM-Rad 3 Related (ATR), which in turn activate H2AX and p53 stress sensors.10 Among many other roles, p53 causes Bax to translocate to the mitochondrion to elicit cytochrome c release and trigger caspase-mediated apoptosis.11 Thus, p53 knockout or inhibition protects against ischemia- or excitotoxicity-induced neuronal death.12-15 However, once DNA is damaged beyond repair, neuronal cell death ensues.2 Given these lethal sequelae, it is imperative to clamp ischemic injury upstream of oxidative DNA damage, such as directly at the level of H2O2.
One strategy to effectively control $\text{H}_2\text{O}_2$ is through the peroxiredoxins (PRXs). PRXs are a newly characterized family of antioxidant enzymes that scavenge peroxides, including $\text{H}_2\text{O}_2$, lipid peroxides, and peroxynitrites through redox reactions at cysteine residues.\textsuperscript{16} Of the PRX family members, PRX2 is an abundant neuronal form.\textsuperscript{17} We previously described the neuroprotective effect of PRX2 overexpression in models of ischemia and Parkinson disease. In those studies, PRX2 overexpression modulated the redox status of thioredoxin to inhibit its dissociation from apoptosis signal–regulating kinase 1 (ASK1).\textsuperscript{18,19} Endogenous PRXs also appear to combat ischemic injury because knockout animals are more susceptible to ischemia,\textsuperscript{20,21} and PRXs are upregulated in preconditioned and ischemic tissue.\textsuperscript{22}

Although it is known that ROS elicit DNA damage and that PRX2 scavenges $\text{H}_2\text{O}_2$, it is not known whether PRX2 effectively controls the oxidative DNA damage from ischemic insults. This is important to discern because a critical component of neuroprotection against stroke is the preservation of DNA integrity. The present study examined this important question in both cellular and animal models of stroke. We hypothesized that PRX2 protects against ischemic injury by inhibiting both PARP1- and p53-dependent death pathways. The impact of PRX2 on these 2 parallel forms of cell death in ischemia has never been investigated. Finally, we tested for the impact of PRX2 on these 2 forms of cell death in ischemia has never been investigated. Finally, we tested for the impact of PRX2 on these 2 forms of cell death in ischemia has never been investigated. Finally, we tested for the impact of PRX2 on these 2 forms of cell death in ischemia has never been investigated. Finally, we tested for the impact of PRX2 on these 2 forms of cell death in ischemia has never been investigated. Finally, we tested for the impact of PRX2 on these 2 forms of cell death in ischemia has never been investigated. Finally, we tested for the impact of PRX2 on these 2 forms of cell death in ischemia has never been investigated. Finally, we tested for the impact of PRX2 on these 2 forms of cell death in ischemia has never been investigated. Finally, we tested for the impact of PRX2 on these 2 forms of cell death.

### Methods

Descriptions beyond what are provided below can be found in Methods in the online-only Data Supplement. All assessments were performed by investigators blinded to the experimental group.

#### PRX2 Transgenic Mice

Experiments were approved by the Institutional Animal Care and Use Committee of Capital Medical University and performed in accordance with the National Institutes of Health Guide. The chimeric transgene used to create PRX2 overexpressors contained human Prx2 or mutant Prx2 (Cys51Ala and Cys172Ala) under control of the synapsin-I promoter, as described previously.\textsuperscript{18} Plasmids were purified and microinjected into eggs of C57BL/6J×SJL/Ji mice. Founders to establish transgenic lines were bred to wild-type F1 hybrid mice. All lines were backcrossed to the C57BL/6j background for ≥7 generations.

#### Primary Neuronal Cultures, Lentiviral Vectors, and OGD-Induced Cell Death

Primary cortical cultures (>97% neuronal) were prepared from embryonic day 17 Sprague-Dawley rats as before and experiments begun on days in vitro 10 to 12.\textsuperscript{23} Oxygen and glucose deprivation (OGD) was used to model ischemia as before.\textsuperscript{24} Lentiviral vectors with human Prx1 or Prx2 genes were generated as described.\textsuperscript{10} Experiments were begun 2 to 3 days after infection.

Alamar Blue fluorescence was used to indicate metabolic activity 24 hours after OGD.\textsuperscript{10} Loss of membrane integrity was quantified by measuring lactate dehydrogenase (LDH) release according to manufacturer instructions (Sigma, St. Louis, MO). Cells with ≥2 Hoechst 33258-stained condensed nuclear fragments were designated as apoptotic.

#### Transient Focal Cerebral Ischemia

Intraluminal occlusion of the left middle cerebral artery was used to model transient focal cerebral ischemia (tFCI). Silicone-coated 8.0 monofilament sutures were inserted into the internal carotid of 1.5% isoflurane-anesthetized 8- to 10-week-old male mice for 90 minutes. Additional laser Doppler flowmetry methods, randomization, and exclusion criteria are described in Methods in the online-only Data Supplement. Blood pH, gases, and glucose were also monitored and were not affected by PRX2 transgene expression and p53 or Bax knockout (Tables in the online-only Data Supplement).

#### Infarct Volume

Two days after tFCI, brains were removed and sliced into 1 mm thick coronal sections for 2% 2,3,5-triphenyltetrazolium chloride staining. Seven days after tFCI, separate animals were perfused for microtubule-associated protein-2 immunofluorescence (Methods in the online-only Data Supplement). Infarct volumes were measured by MCID software (St. Catherine’s, Ontario, Canada) and corrected for brain edema.

#### Statistics

Results are reported as mean±SEM. In vitro data were collected in triplicate in 3 independent experiments. In vivo data were collected from 6 to 8 animals per group. Student $t$ test or ANOVAs followed by Bonferroni/Dunn post hoc tests were used for single and multiple comparisons, respectively. $P \leq 0.05$ was deemed statistically significant.

#### Results

**PRX1 and PRX2 Scavenge H$_2$O$_2$ and Preserve DNA Integrity, NAD$^+$, and Viability in Primary Neurons Exposed to OGD**

Cortical neurons were infected with empty lentiviral particles or lentiviruses bearing Prx1 or Prx2 genes. PRX1 and PRX2 were heavily expressed in infected cultures, respectively (Figure 1A), resulting in ≥5-fold increases in peroxidase activity (not shown). Within 0.5 to 2 hours after OGD (90 minutes), a rise in DCF was attenuated by PRX1 and PRX2, reflecting the $\text{H}_2\text{O}_2$ scavenging capacity of both proteins (Figure 1A). We determined whether various types of DNA damage could be ameliorated by PRXs. AP sites were indeed decreased by PRX1 and PRX2 for ≥6 hours after OGD (Figure 1B). PANT labeling revealed that the rise in single-strand breaks after OGD was ameliorated by PRX1 and PRX2 (Figure 1C). PRX1 and PRX2 also reduced DNA double-strand breaks as measured by tail-length in the Comet assay from 0.5 to 24 hours (Figure 1C). These results consistently show that PRX1 and PRX2 can reduce OGD-induced DNA damage.

DNA damage can trigger PARP1 activation, which depletes NAD$^+$ and leads to energy failure.\textsuperscript{25,6} Because PRX1 and PRX2 preserved DNA integrity, we tested whether they also attenuate NAD$^+$ depletion. As expected, NAD$^+$ depletion was ameliorated by PRX1 and PRX2 (Figure 1D). Finally, PRX1 and PRX2 also preserved cellular viability by the Alamar Blue assay and membrane integrity by the LDH assay in cultures...
challenged with either 90 or 60 minutes OGD (Figure 1D).

Although PRX2 overexpression attenuated \( \text{H}_2\text{O}_2 \) toxicity, it did not prevent staurosporine or camptothecin-induced death (Figure I in the online-only Data Supplement), suggesting that PRX2 could not inhibit apoptosis elicited by a nonoxidative inducer. Taken together, these findings reveal a potent protective role for PRXs in preventing ROS-mediated DNA damage and neuronal death.
Loss of PRX2, but not PRX1, Exacerbates OGD-Induced ROS, DNA Damage, NAD⁺ Depletion, and Increases Neuronal Death in a PARP1-Dependent Manner

RNA interference with PRX1 or PRX2 expression was used to examine the protective role of the endogenous proteins in primary cortical neurons. PRX1 and PRX2 were reduced 2 to 3 days after short hairpin RNA-bearing lentiviral infection (Figure 2A). PRX2, but not PRX1, knockdown increased DCF fluorescence after OGD (Figure 2A). The experiments in Figure 1 involved 90 minutes of OGD. However, to resolve an increase in damage with PRX knockdown, the OGD duration was reduced from 90 minutes to 60 minutes for experiments in Figure 2. The DCF findings suggest that the natural role of PRX2, but not PRX1, in neurons includes peroxide scavenging. Although both PRXs were protective when overexpressed, our results are consistent with previous observations that endogenous PRX2 is neuronal, whereas PRX1 is predominantly expressed in oligodendrocytes and microglia.17 Our findings suggested that loss of endogenous PRX2, but not PRX1, might also exacerbate oxidative DNA damage. As expected, OGD-induced AP sites, PANT⁺ cells, and comet tail-length were all increased by PRX2, but not PRX1 knockdown (Figure 2B). Similarly, NAD⁺ depletion was also exacerbated only by PRX2 knockdown (Figure 2C). Furthermore, only PRX2 knockdown enhanced LDH release and loss of viability in cultures challenged with either 60 or 45 minutes OGD (Figure 2C). Because PARP1 activation can deplete NAD⁺ and cause energy failure,2,5,6 the potent cell membrane-permeable PARP1 inhibitor AG14361 was used to assess the role of PARP1 activation after OGD. First, 1 μmol/L AG14361 was found to be maximally effective in preventing OGD-induced PARP1 activation (Figure II in the online-only Data Supplement). AG14361 almost completely prevented OGD-induced loss of NAD⁺ both under scrambled short hairpin RNA and PRX2 knockdown conditions (Figure 2D). AG14361 also attenuated loss of viability and membrane integrity under scrambled short hairpin RNA and PRX2 knockdown conditions (Figure 2D). Notably, LDH release was almost completely prevented by AG14361. These results are consistent with the hypothesis that PARP activation is the dominant means by which OGD causes cell death, especially when PRX2 is deficient.

PRX2, but not PARP1 Inhibition, prevents OGD-Induced Activation of Proapoptotic Molecules in Neurons

We hypothesized that when PRX2 fails to scavenge peroxides, ROS can freely damage DNA and activate PARP1-dependent and p53-dependent death pathways. DNA damage can elicit apoptosis by phosphorylation of ATM, H2AX, and p53.10 As expected, OGD (90 minutes) increased levels of phospho-ATM, phospho-H2AX, and phospho-p53 (Figure 3A). Whereas PRX2 attenuated these responses, AG14361 had no significant impact on these proapoptotic players. Sirtuin 1 is an NAD⁺-dependent protein that is reduced after energy failure.24 A fall in sirtuin 1 with OGD was prevented either with PRX2 overexpression or AG14361 (Figure 3A), suggesting that AG14631 was indeed effective in preserving NAD⁺.

p53 activation elicits mitochondrial Bax translocation.11 As expected, Bax entered the mitochondrial fraction after OGD, and this was prevented by PRX2 but not AG14361 (Figure 3B). Interestingly, PARP1 inhibition with OGD caused a large enough drop in cytoplasmic Bax to be resolved by immunoblotting, unlike OGD alone. This may reflect an increase in the number of cells dying by apoptosis when PARP1-mediated death is inhibited, and is examined further below.

OGD Elicits p53- and PARP1-Dependent Cell Death in Primary Neurons

To examine the contribution of p53 and Bax to OGD-induced apoptosis, we used cultures from p53 and Bax knockout animals. A slight but significant protection against 90 minutes OGD was elicited when p53 and Bax were absent (Figure 3C), suggesting that a minority of cells die by p53 and Bax-mediated apoptosis. We also combined PARP1 inhibition with p53 or Bax knockout. AG14361 robustly protected wild-type cultures and cultures lacking p53 or Bax, reflecting the larger contribution of PARP1-mediated death after 90 minutes OGD. To test the hypothesis that excitotoxicity also plays a role, the N-Methyl-D-aspartate receptor antagonist MK801 was applied. MK801 protected robustly against OGD in wild-type and p53 or Bax knockout cultures. Notably, p53 or Bax knockout did not impact OGD-induced LDH release, suggesting that p53 and Bax do not affect loss of membrane integrity (Figure 3C). In contrast, AG14361 and MK801 were robustly protective against LDH release, suggesting that excitotoxicity and PARP1-dependent pathways were both prominent causes of necrosis.

To dissect out the small fraction of apoptotic cells, we counted the number of condensed nuclei with ≥2 fragments in p53 or Bax knockout cultures treated with AG1463 or MK801. Approximately 12% of cells died by apoptosis in response to OGD (Figure 3C), at least twice the basal level in control non-OGD cultures. As expected, this number was reduced in cultures lacking p53 or Bax. Notably, AG14361 and MK801 increased the number of fragmented nuclei in wild-type cultures after OGD, despite the finding that either treatment markedly reduced overall cell death, suggesting again that apoptosis could proceed more readily when PARP1 and excitotoxicity were inhibited. These apoptotic responses were abolished in the absence of p53 or Bax. Images of apoptotic fragments (arrowheads) are illustrated in Figure 3D for select groups.

Interactions Between the JNK and PARP1 or p53 Pathways In Vitro

Previously, we showed that PRX2 prevented ASK1/JNK signaling.18,19 In studies in the online-only Data Supplement, we examined crosstalk between the PARP1 and ASK1/JNK pathways. JNK signaling was measured by phosphorylation of c-Jun. ASK1 and c-Jun were activated more after 60 minutes OGD than 90 minutes OGD in primary neurons, but AG14361 did not change these responses (Figure IIIA and IIIB in the online-only Data Supplement). Thus, ASK1 and JNK are not downstream of PARP1 in this model, although PARP1 may activate JNK in other paradigms.25 In contrast, the JNK inhibitor...
SP600125 partially attenuated NAD⁺ depletion and the rise in poly(ADP-ribosylated) proteins (PARS), particularly after 60 minutes OGD (PARS; Figure IIIC–IIIE in the online-only Data Supplement). PARS are long negatively charged poly(ADP-ribosylated) polymers attached to various proteins, a reaction catalyzed by PARP1, and induce ischemic and excitotoxic cell death.26 SP600125 did not impact PANT⁺ cells, suggesting that the effect of JNK lies downstream of DNA damage (Figure IIIF in the online-only Data Supplement). Thus, JNK may activate PARP1, particularly under milder injury conditions, consistent with previous reports that JNK may promote PARP1 activity by directly phosphorylating PARP1.27

Next, we examined whether SP600125 would also impact ATM and p53 phosphorylation after OGD, but found no effect (Figure IVA in the online-only Data Supplement). However, SP600125 partially attenuated mitochondrial Bax translocation after 60 minutes OGD (Figure IVB in the online-only Data Supplement), suggesting that JNK can activate Bax when the ischemia duration is relatively short. Next, we examined the impact of JNK inhibition on viability in p53 knockout or wild-type cultures (Figure IVC in the online-only Data Supplement). After 60 minutes OGD, SP600125 protected viability, but had no further impact on the protection elicited by p53 knockout. However, after 90 minutes OGD, SP600125 protected wild-type as well p53 knockout cultures, suggesting that JNK and p53 pathways operate independently after severe injury. Inhibiting JNK and p53 simultaneously was not additive after 60 minutes OGD, perhaps because

Figure 2. Loss of peroxiredoxin 2 (PRX2), but not PRX1, exacerbates oxygen and glucose deprivation (OGD)–induced reactive oxygen species (ROS), DNA damage, and nicotinamide adenine dinucleotide (NAD⁺) depletion, and increases cell death in primary neurons in a poly(ADP-ribose) polymerase 1 (PARP1)–dependent manner. A, Neurons were infected with lentiviruses bearing scrambled (Sc) sequences or short hairpin RNA against PRX1 or PRX2 (t, target sequence). PRX expression was reduced 2 to 3 days after infection. DCF fluorescence reveals that PRX2, but not PRX1 knockdown, enhanced ROS formation after 60 minutes OGD. B, apurinic/apyrimidinic (AP) sites, PANT labeling, and comet tail-length were all increased by PRX2 knockdown after 60 minutes OGD. C, PRX2 knockdown worsened NAD⁺ depletion after 60 minutes OGD and reduced viability and membrane integrity after 60 or 45 minutes OGD. D, PARP1 inhibition with AG14361 (1 μmol/L) prevented NAD⁺ depletion after 60 minutes OGD and loss of viability and membrane integrity after 60 or 45 minutes OGD in both scrambled and PRX2-targeted sequence groups. *P≤0.05, **P≤0.01, ***P≤0.001 vs scrambled sequence or vs indicated groups.
JNK could also activate Bax under these conditions (Figure IVB in the online-only Data Supplement). Thus, JNK and p53 pathways are parallel, but not completely independent. Finally, we found that PARP1 inhibition further protected wild-type cultures against 90 minutes OGD when JNK was inhibited (Figure IVD in the online-only Data Supplement). However, the impact of AG14361 and SP600125 was not additive after 60 minutes OGD, suggesting that JNK and PARP1 interact more when the injury is less severe. This is consistent with previous observations that JNK increased PARS levels and attenuated NAD⁺ depletion more after 60 than 90 minutes OGD (Figure IIIC–IIIE in the online-only Data Supplement). These data suggest that the JNK and PARP1 pathways are largely independent but that there is some crosstalk, particularly if the injury is less severe.

PRX2 Reduces Infarct Volume, DNA Damage, PARS Levels, and NAD⁺ Depletion After Ischemia/Reperfusion Injury In Vivo

For animal studies, we generated 2 types of transgenics: PRX2 neuronal overexpressors and overexpressors of neuronal PRX2 with alanine mutations in 2 cysteine residues (PRX2M).
First, we measured infarct volume after 90 minutes tFCI. Two days after ischemia, PRX2 reduced infarct volume, an effect that was absent in mutant PRX2 overexpressors (Figure 4A). Similar results were achieved with MAP2 staining 7 days after tFCI. PRX2, but not PRX2M, also protected against neurological deficits after tFCI (Figure 4A). We then assessed DNA damage in the cortical ischemic territory at 0.5 to 24 hours after injury (Figure 4B). AP sites were reduced in PRX2 transgenics but not in PRX2 mutants. 3’OH and 3’ blocked PANT-labeled cells were both reduced in PRX2 but not PRX2M animals. To assess PARP1 activation, PARS were assessed (Figure 4C). PRX2 reduced the ischemia-induced rise in PARS, an effect that was lost in PRX2M mice. Dual-immunofluorescent staining for neuronal nuclear antigen and PARS revealed an increase in PARS+ neurons after tFCI (Figure 4C). Again, this was attenuated by PRX2 but not PRX2M. Consistent with the PARS data, we found that PRX2, but not PRX2M, transgenics exhibited less NAD+ depletion than wild-type animals after tFCI (Figure 4D). These results suggest that PRX2 reduces DNA damage and PARP1 activation and protects against ischemia/reperfusion in vivo.

**PRX2 Attenuates Both PARP1-Dependent and p53-Dependent Death Pathways In Vivo**

To confirm the in vitro observations that p53-dependent pathways proceed more readily when PARP1 is inhibited, we probed for nuclear phospho-p53 and mitochondrial Bax in animals exposed to tFCI in the presence or absence of AG14361 (maximally effective dose of 3 mg/kg; Figure V in the online-only Data Supplement). The data reveal an ischemia-induced rise in nuclear phospho-p53 and mitochondrial Bax and a loss in cytosolic Bax in ischemic brain tissue harvested 6 or 12 hours after 90 minutes tFCI (Figure 5A). These responses were all enhanced with AG14361, paralleling the in vitro findings that more cells undergo apoptosis when PARP1-dependent death is inhibited and NAD+ content is preserved.

We then assessed whether PRX2 would reduce p53 activation and mitochondrial Bax translocation in vivo (Figure 5B). As

**Figure 4.** Peroxiredoxin 2 (PRX2) reduces infarct volume, DNA damage, PARS levels, and nicotinamide adenine dinucleotide (NAD+) depletion after ischemia/reperfusion injury in vivo. **A,** 2,3,5-triphenyltetrazolium chloride (TTC; 2 days) and microtubule-associated protein-2 (MAP2; 7 days) staining of infarct after transient focal cerebral ischemia (tFCI) in wild-type (Wt) mice or transgenic animals overexpressing human PRX2 (Tg-PRX2) or PRX2 with Cys51Ala and Cys172Ala mutations (Tg-PRX2M). Infarct volume and neurological deficits were reduced in Tg-PRX2 but not in Tg-PRX2M animals. **B,** apurinic/apyrimidinic (AP) sites, 3’OH, and 3’ blocked PANT-labeled cells were reduced by PRX2, but not PRX2M overexpression. **C,** PARS levels in ischemic brain tissue rose in response to tFCI and were attenuated by PRX2, but not PRX2M. Immunofluorescent staining for neuronal nuclear antigen (green) and PARS (red) revealed numerous PARS+ neurons after tFCI in Wt and Tg-PRX2M animals, but not PRX2 overexpressors. **D,** NAD+ depletion after tFCI was ameliorated in PRX2 but not in PRX2M overexpressors. *P<0.05, **P<0.01 vs WT animals or indicated groups. on the left in C, *P<0.05, **P<0.01 vs sham levels and #P<0.05 vs WT animals.
expected, PRX2 overexpressors exhibited less phospho-p53 and decreased Bax translocation in response to tFCI. PARP1 inhibition with AG14361 was then combined with PRX2 overexpression (Figure 5C). AG14361 led to a significant reduction in infarct volume in wild-type but not in PRX2 transgenics. These data were corroborated by dual-labeling studies showing an increase in tFCI-induced phospho-p53+ neurons in the presence of AG14361, an effect that was also blocked by PRX2 overexpression (Figure VI in the online-only Data Supplement). These findings are consistent with the operation of 2 largely parallel modes of cell death and support the hypothesis that additional cells have the energy to die along the more adaptive apoptotic route when PARP1 is inhibited and NAD+ is preserved. Furthermore, these 2 types of death probably do not coexist in the same cells because immunofluorescence studies reveal that PARs are present in a different group of cells than phospho-p53 (Figure VIB in the online-only Data Supplement).

Next, we tested the hypothesis that PRX2 overexpression would reduce caspase activation after tFCI (Figure VII in the online-only Data Supplement). As hypothesized, the cleavage of caspases 9 and 3 and their activity levels were significantly reduced in PRX2 overexpressors. We then measured dying cells by stereologically counting terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL+) cells in the infarct zone (Figure 5D). PRX2 reduced the number of dying profiles in the core and infarct inner border regions, and AG14361 had no additional impact.

Figure 5. Peroxiredoxin 2 (PRX2) reduces both poly(ADP-ribose) polymerase 1 (PARP1)–dependent and p53-dependent death pathways in vivo. Transient focal cerebral ischemia (tFCI; 90 minutes) increased nuclear phospho-p53 and mitochondrial translocation of Bax in ischemic brain, and these effects were increased with PARP1 inhibition (A) but decreased by transgenic PRX2 overexpression (B). Animals were exposed to tFCI or sham surgery (S) and treated with vehicle or 3 mg/kg AG14362 at the onset of reperfusion (A). Wild-type (Wt) or PRX2 transgenics (Tg-PRX2) were subjected to tFCI or sham surgery in B. Ischemic brain tissue was harvested 6 and 12 hours after reperfusion. *P≤0.05, **P≤0.01 vs vehicle in A and vs Wt in B. C, AG14361 (3 mg/kg) had no additional impact on infarct volume as measured 2 days later by 2,3,5-triphenyltetrazolium chloride (TTC) in PRX2 transgenics but reduced infarct volume in p53 or Bax knockout animals. D, AG14361 reduced dying, TUNEL+ profiles in Wt and p53 or Bax knockout animals but had no additional impact in Tg-PRX2 mice. *P≤0.05, **P≤0.01 vs Wt animals in C and vs vehicle in D. #P≤0.05, ##P≤0.01 vs indicated groups in C and D. n.s. indicates not significant.
This parallels the infarct volume data in Figure 5C and suggests that PARP1 inhibition may already be maximal in PRX2 overexpressors. In contrast, AG14361 significantly reduced cell death in p53 or Bax knockout animals, again supporting 2 largely independent modes of death. However, as shown previously, cell death may proceed along the p53-dependent route more easily when PARP1 is inhibited. This is illustrated by an increase in punctate Bax+ profiles in the presence of AG14361 after tFCI (Figure VIII in the online-only Data Supplement).

Interactions Between JNK and PARP1 or p53
Pathways In Vivo
To confirm the in vitro interactions between JNK and PARP1, we performed studies in the online-only Data Supplement examining the impact of SP600125 on PARS levels after 90 minutes tFCI. SP600125 abolished c-Jun phosphorylation and partially reduced PARS levels after tFCI (Figure IXA in the online-only Data Supplement). Furthermore, SP600125 was more protective in combination with AG14361 than alone (Figure IXB in the online-only Data Supplement). However, there was only a trend for AG14361 and SP600125 to reduce infarct volume more than AG14361 alone. Next, we examined the impact of p53 knockout on ASK1 and c-Jun. p53 knockout did not affect ASK1 phosphorylation after 90 minutes tFCI (Figure IXC in the online-only Data Supplement). However, loss of p53 reduced the ischemia-induced rise in phosphorylated c-Jun, pointing to crosstalk between p53 and JNK. Finally, infarct volume was greater in SP600125-treated p53 knockout animals than both vehicle-treated knockout animals or wild-type, SP600125-treated animals (Figure IXD in the online-only Data Supplement). Taken together, these data reveal that the JNK and p53 pathways operate in parallel with some interactions.

Discussion
The present study reports that at least 2 largely independent death pathways are activated in stroke models and attenuated by the peroxide scavenger PRX2. One pathway is dependent on PARP1 and the other on p53 and Bax. Our data support the presence of largely parallel pathways with some crosstalk with JNK signaling (Figure 6). Our findings expand current knowledge of the protective impact of PRX2. A close examination

![Figure 6](http://stroke.ahajournals.org/)

**Figure 6.** Upstream role of peroxiredoxin 2 (PRX2) in combating H\(_2\)O\(_2\)-mediated DNA damage and inhibiting JNK-, poly(ADP-ribose) polymerase 1 (PARP1)-, and p53-dependent pathways.
of PRX2 as a rational target for stroke therapy is warranted because PRXs work upstream of DNA damage. This is beneficial because once DNA is damaged beyond repair, cells may activate death pathways as an adaptive but irreversible response against potential mutagenicity or against overt cellular dysfunction to protect the organism as a whole.

Although both PRXs were protective in the present study, our knockdown results are consistent with PRX2 being the predominant functional form within neurons. As one might expect from its role in peroxide scavenging, PRX2 prevented ROS accumulation. ROS elicit AP sites and single- and double-strand breaks in DNA. Thus, it is not surprising that PRX2 also prevented multiple forms of DNA damage in our models. PRX2 prevented downstream NAD⁺ depletion and reduced the robust LDH release after OGD. PRX2 also prevented the ischemia-induced rise in PARs. PARs expression by immunofluorescence was abundant in the infarct zone (Figure VIB in the online-only Data Supplement). Our results suggest that the majority of neurons die by PARP1-mediated loss of membrane integrity in response to severe ischemic injury. A smaller fraction appears to die by nuclear fragmentation in a p53- and Bax-dependent manner under these conditions. PRX2 prevented p53 activation and mitochondrial Bax translocation in vivo and in vitro. Interestingly, we observed that more cells die by the p53-dependent route when PARP1 is not allowed to function. This alternative type of cell death may be related to the prevention of energy failure in ischemic neurons. We speculate that preservation of NAD⁺ is not allowed to function. This alternative type of cell death may be related to the prevention of energy failure in ischemic neurons. We speculate that preservation of NAD⁺ and its downstream utilization is important in maintaining mitochondrial function and cell survival. Our results support the hypothesis that PRX2 has a protective role in ischemic injury by preserving NAD⁺ levels and preventing PARP1-mediated cell death.

The present study, therefore, examined interactions between JNK and PARP1 or p53 pathways and found that although PARP1 did not affect ASK1 signaling, JNK enhanced PARP1 activity of DNA damage, particularly after less severe injury (Figure 6). JNK also increased mitochondrial Bax translocation after 60 minutes OGD, although it had no impact on p53 or ATM phosphorylation. Conversely, p53 appeared to increase c-Jun activation, reflecting enhanced JNK signaling. Combining JNK inhibition with PARP1 suppression or p53 knockdown increased protection against 90 minutes OGD more than when given alone, supporting the interpretation that these pathways operate largely in parallel after severe injury. Combining JNK inhibition with p53 knockdown also had an additive impact in vivo. However, JNK and PARP1 inhibition in vivo were not additive, perhaps reflecting greater crosstalk between JNK and PARP1 in animals.

If PARP1 and JNK signal in parallel after severe injury, PRX2 overexpression should have a greater impact than either SP600125 or AG14361 alone because both PARP1 and JNK are decreased simultaneously. Indeed, PRX2 overexpression was more protective than SP600125 against 90 minutes OGD (compare Figure ID with Figure IVC and IVD in the online-only Data Supplement). However, AG14361 alone was quite protective after 90 minutes OGD and tFCI, suggesting that PARP1 signaling predominates after severe injury. JNK inhibition was more protective after 60 than 90 minutes OGD, and the impact of JNK and PARP1 inhibition was not additive after this mild injury. Thus, this and our previous study reveal that ASK1/JNK signaling predominates when the injury is milder, whereas PARP1 predominates with severe injury. Even though one predominates over the other, the parallel pathways are co-activated (for example, see Figure IIIA versus IIB and Figure IIIC versus IIID in the online-only Data Supplement) and exhibit interactions. These interactions and the domination of one pathway both preclude the appearance of potent cumulative effects of inhibiting both pathways simultaneously with PRX2. Parallel but interacting layers of damage control by PRX2 may allow superior titration of proapoptotic and prolife signaling decisions before ischemic cell fate is finally met.

Because PRX2 engages in multiple layers of damage control, it is a promising target to help clamp the injury from stroke at a level upstream of DNA damage and prodeath signaling cascades. Brain levels of PRXs could potentially be raised by chemical inducers or carrier-based protein delivery in the clinic. In this respect, it is noteworthy that PRXs are naturally induced in response to injury, even in human stroke victims. These stress responses may be speculated to protect against stroke-induced oxidative stress and inhibit prodeath pathways. We conclude that PRX upregulation may be useful as a future stroke therapy.

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**Disclosures**

None.

**References**


Peroxisome 2 Battles Poly(ADP-Ribose) Polymerase 1- and p53-Dependent Prodeath Pathways After Ischemic Injury

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Peroxiredoxin 2 battles PARP1- and p53-dependent pro-death pathways following ischemic injury

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Supplemental Methods

Transient focal cerebral ischemia (tFCI)
Transgenic/knockout mice and wild-type mice were randomly assigned to vehicle or drug treatment groups and to tFCI or sham surgery with a lottery-drawing box. Regional cerebral blood flow was measured in all stroke animals using laser Doppler flowmetry (PeriFlux System 5000, Perimed, Sweden). Animals that did not show a regional cerebral blood flow reduction to <30% of pre-ischemia baseline levels during tFCI were excluded from further experimentation, as were animals that died during post-ischemic reperfusion. Sham-operated animals underwent the same anesthesia and surgical procedures except tFCI.

Neurobehavioral tests
Neurological scores were measured at 24 and 72 hours after tFCI approximately 4 hours after light onset using the scoring method by Wacker and colleagues with slight modifications. This was done by an investigator who was blinded to experimental groups.

Neurological deficit scoring scale

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td>0.5</td>
<td>Discontinuous mild contralateral paw flexion</td>
</tr>
<tr>
<td>1.0</td>
<td>Paw flexion</td>
</tr>
<tr>
<td>1.25</td>
<td>Severe paw flexion, coat not ruffled</td>
</tr>
<tr>
<td>1.5</td>
<td>Paw flexion, ruffled coat</td>
</tr>
<tr>
<td>1.75</td>
<td>Directional turning preference</td>
</tr>
<tr>
<td>2.0</td>
<td>Continuous circling</td>
</tr>
<tr>
<td>2.25</td>
<td>Tight circling</td>
</tr>
<tr>
<td>2.5</td>
<td>Collapsed circling, near rolling over</td>
</tr>
<tr>
<td>3.0</td>
<td>Rolling</td>
</tr>
<tr>
<td>3.5</td>
<td>Continuous rolling</td>
</tr>
<tr>
<td>4.0</td>
<td>Morbid, appears comatose</td>
</tr>
</tbody>
</table>

DCF fluorescence
Primary cortical neurons were incubated with 10 μM CM-H2DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester, Life Technologies, Carlsbad, CA). This is a chloromethyl derivative of H2DCFDA that exhibits better retention in cells and is a relatively specific sensor for H2O2. Cells were incubated at 37°C in the dark for 30 min beginning 1.5 hours after 90 min OGD. Fluorescence was read on a microplate plate at 485 nm excitation and 530 nm emission.

PARP1 and JNK inhibitor treatments of animals
AG14361 was dissolved in dimethyl sulfoxide (DMSO) to create a stock solution of 62.5 mM and then diluted using saline upon injection. Animals were injected intraperitoneally with vehicle (2% DMSO in saline) or 0.3, 1, 3, 6, and 10 mg/kg AG14361 at the onset of reperfusion in order to determine the maximally effective dose for reducing infarct volume. AG14361 treated animals were sacrificed 2 days after tFCI. Coronal sections were stained with TTC and infarct volume was measured with correction for edema by an investigator who was blinded to experimental groups.

The anthrapyrazolone JNK inhibitor SP600125 was dissolved in DMSO to generate a stock solution of 20 mmol/L. This was further diluted in artificial CSF. A dose of 3 mg/kg was injected intravenously into the tail vein 15 minutes prior to middle cerebral artery occlusion and 3 h into reperfusion, based on the half-life of SP600125. This dose of SP600125 achieved the optimal neuroprotective effect against tFCI in our previous dose-response efficacy studies.
Lentiviral vectors for PRX1 and PRX2 knockdown

To construct lentiviral vectors expressing short hairpin interfering RNA (shRNA) for PRX1 and PRX2 gene knockdown, the gene-specific targeting sequence (PRX1: 5' -GGATTCTCACTTCTGTTCATCT-3'; PRX2: 5' -GGATGGTGCCCTCAAGGAAAT-3') or its counterpart scramble sequence was inserted into the vector FSW under control of the U6 promoter. These transfer vectors were transformed into recombinase free Stbl3 E. coli and isolated with an Endofree Plasmid Maxi kit (Qiagen, Valencia, CA). Virus production was scaled up as described before. Briefly, a plasmid mixture with 435 mg of pCMVΔR8.9 (packaging construct), 237 mg of pVSVG (envelope plasmid) and 675 mg of FSW (transfer vector) were suspended in 34.2 mL of CaCl2 (250 mM) and added volume for volume into 2x BES buffer (pH 6.95). The DNA-CaCl2 precipitate was added to human kidney 293 FT cells and incubated for 12 hours until fresh medium was added. The supernatant was harvested 3 days after transfection, filtered through 0.45 mm pores and centrifuged at 21,000 rpm for 2 hours. The pellet was resuspended in 3 mL PBS, loaded on top of 2 mL of 20% sucrose, and centrifuged for 2 hours at 22,000 rpm. The pellet was resuspended in 200 ml of DMEM and stored at -80°C. The titer of the vector stock was measured with a lentivirus-associated p24 ELISA kit in accordance with manufacturer’s instructions (Cell Biolabs, San Diego, CA).

AP site and DNA strand break measurements

A colorimetric assay to measure AP sites in isolated nuclear DNA was performed as before. The DNA polymerase I-mediated biotin-dATP nick-translation (PANT) assay was also performed as previously described. In this procedure, a two-step method was used to detect single-strand breaks with either a 3'OH or a 3'blocked group. PANT-positive cells were quantified with MCID software. The Comet assay to detect DNA damage in individual cells was performed as described. This assay uses alkaline electrophoresis so that DNA strands migrate towards the anode, with the extent of migration depending on the number of strand breaks. The sizes of comet tail-lengths signify DNA strand breaks and were quantified with MCID software. All the above assays were performed by investigators who were blinded to experimental groups.

TUNEL staining

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining was performed as before and counted blindly by stereology using the Bioquant image analysis software. Grid squares of 50 × 50 µm were generated over the cortical infarct regions. A 25 × 25 µm counting frame with a focus depth of 25 µm in a 40 µm section were used as a dissector, with a mean of 9 dissectors per section. Every 5th section was counted using the optical dissector, or the quotient of the total number of neurons counted and the product of the fractions for sampling section frequency (SSF, fraction of sections counted), area section frequency (ASF, sampling area/area between dissectors, and thickness section frequency (TSF, dissector depth/section thickness). SSF = ¼ sections, ASF = 25 × 25 µm/50 × 50 µm, and TSF = 25 µm/40 µm.

Caspase activity measurements

Tissue lysates for caspase activity measurements were prepared as described previously. Protein (150 µg) was incubated for 1 hour at 37°C with reaction buffer containing 25 mmol/L HEPES (pH 7.5), 10% sucrose, 0.1% 3-[3-cholamidopropyl]-dimethylammonio]-1-propane sulfonate, 5-mmol/L chloroform, and 5-mmol/L ethylic acid in a total volume of 150 µL. This mixture also contained 25 µmol/L fluorogenic peptide cleavage substrate (Medical and Biological Laboratories, Watertown, MA). Ac-DEVD-AFC was used as the substrate for caspase-3-like activity and Ac-LEHD-AFC was used for caspase-9-like activity. For background subtraction, protein extracts were incubated in reaction mixture as above, but in the presence of inhibitors for caspase 3 (DEVD-CHO) and caspase 9 (LEHD-CHO) at 5 µmol/L each.

NAD content measurements

NAD content was measured in the MCAO territory with an in situ, liquid nitrogen-based freezing technique to preserve metabolites, as previously described. In this assay, NAD is converted to NADH by alcohol dehydrogenase and the reduction of thiazolyl blue to purple formazan is measured at 556 nm.

Bax and p53 knockout animals
The Bax and p53 knockout mice were purchased from the Jacksons Laboratory and Taconic, respectively. The Bax knockout colony (on the C57BL/6J background) is maintained at the University of Pittsburgh Animal Facility through mating of heterozygous females with heterozygous males. The homozygous p53 knockout colony is maintained at Taconic at N4 through mating of heterozygous females with homozygous males, and the male homozygous p53 knockout mice were purchased at the age of 5-8 weeks. Because these mice are prone to the development of spontaneous tumors at the age beyond 10 weeks, stroke experimentation was performed at 8-9 weeks of age in all animals.

**Immunofluorescence and Western blotting**

Animals were perfused with 4% paraformaldehyde and brains were cut into coronal sections for standard immunofluorescence protocols. Double label fluorescence was viewed with confocal microscopy (Olympus Fluoview, Olympus, Center Valley, PA). Sections were incubated with anti-NeuN (1:500, EMD Millipore, Billerica, MA), anti-poly(ADP-ribosylated) proteins (PARS, 1:100, BIOMOL Research Laboratories, Plymouth Meeting, PA), cleaved caspase 3 (5A1E, 1:250, Cell Signaling), anti-phospho-p53 (Ser46, 1:50, Cell Signaling, Danvers, MA), anti-MAP2 (1:200, Santa Cruz Antibodies H-300, Santa Cruz, CA) and anti-Bax (1:200, EMD Millipore). This was followed by incubations with fluorescent DyLight488 and Cy3 secondary antibodies (1:1000, Jackson ImmunoResearch, West Grove, PA).

For standard SDS-Page and Western immunoblotting, nuclear, cytosolic, and mitochondrial fractions or total fractions were isolated as previously described. For immunoblotting, antibodies against the following proteins were used: HA (1:1000, Santa Cruz Antibodies, Santa Cruz, CA), PRX2 (1:1000, AbFrontier, Korea), PRX1 (1:1000, AbFrontier, Korea), phospho-ATM (1:500, Abcam, Cambridge, MA), total-ATM (1:500, Abcam), phospho-H2AX (1:500, Abcam), total-H2AX (1:2500, Abcam), phospho-p53 (1:500, Cell Signaling), sirtuin 1 (SIRT1, 1:1000, Abcam), cleaved caspase 9 (1:50, Cell Signaling), anti-phospho-ASK1 (Thr845, 1:1000, Cell Signaling), anti-phospho-c-Jun (Ser63, 1:1000, Cell Signaling), β-actin (1:2000, Abcam), Bax (1:500, EMD Millipore), COXIV (1:1000, Cell Signaling), PARS (1:500, BIOMOL Research Laboratories, Plymouth Meeting, PA), and histone H4 (1:1000, Abcam).
Supplemental References


Supplemental Fig.1. PRX2 overexpression protects against neuronal cell death induced by oxidative stress but not by staurosporine or camptothecin. Cell viability and apoptosis were measured at 24 hours after the insults using Alamar blue and Hoechst-33258 staining, respectively. Each experiment was done in triplicate, and data are based on 3 independent experiments. *p<0.05, **p<0.01 for comparison between the indicated groups.
Supplemental Fig. 2. AG14361 inhibits PARP1 activity in cultured cortical neurons. A: PARP1 activation in the form of $^3$H-NAD incorporation was measured at the indicated timepoints after initiation of OGD in cortical neuronal cultures. OGD caused a time-dependent rise in $^3$H-NAD incorporation that peaked at 120 minutes. B: $^3$H-NAD incorporation was measured as a function of PARP1 inhibition with indicated concentrations of AG14361 at 120 min after 90 min OGD. PARP1 activity was maximally inhibited by 1 µM AG1461, the concentration chosen for subsequent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus sham, non-OGD control.
Supplemental Fig.3. PARP1 inhibition has no impact on ASK1 or c-Jun phosphorylation but JNK inhibition attenuates poly(ADP-ribosylated) proteins (PARS) and NAD⁺ depletion following OGD. A and B: Cortical neuronal cultures were exposed to OGD conditions for 60 (A) or 90 min (B) and treated with vehicle or 1 µM AG14361. Phospho-ASK1 and phospho-c-Jun levels were quantified by Western immunoblotting 2 and 6 hours later. C and D: Cultures were exposed to 60 (C) or 90 min (D) OGD and treated with vehicle or the JNK inhibitor SP600125 (0.3 µM). Two and 6 hours later lysates were assessed for PARS levels by immunoblotting. E: Cultures were exposed to OGD and treated with SP600125 or vehicle for 60 or 90 min. NAD⁺ content was measured 6 hours later. F: PANT-positive cortical neurons were quantified 2 hours after 60 and 90 min OGD in the presence or absence of SP600125. Each experiment was done in triplicate, and data are based on 3 independent experiments. *p<0.05, **p<0.01 versus vehicle. n.s. not significant.
Supplemental Fig. 4. JNK inhibition has no impact on phosphorylation of p53 or ATM after OGD, but decreases the mitochondrial translocation of Bax and enhances cell viability in combination with p53 knockout or PARP1 inhibition. A and B: Cortical neuronal cultures were exposed to 60 or 90 min OGD and treated with 0.3 µM SP600125 or vehicle. Lysates were harvested 2 hours (A) or 6 hours (B) after OGD and assessed for phospho-ATM, phospho-p53, and mitochondrial Bax levels. C: Cultures from wild-type or p53−/− mice were exposed to OGD for 60 or 90 min, treated with SP600125 or vehicle, and assessed for cell viability by the Alamar Blue assay 24 hours later. D: Cultured neurons were exposed to 60 or 90 min OGD and treated with vehicle, SP600125 (0.3 µM), AG14361 (1 µM), or the combination of the two inhibitors. Viability was measured 24 hours later with the Alamar Blue assay. Each experiment was done in triplicate, and data are based on 3 independent experiments. *p<0.05 versus vehicle. @p<0.05 for comparison between indicated groups. n.s. not significant.
Supplemental Fig.5. AG14361 dose-dependently reduces infarct volume after transient focal cerebral ischemia (tFCI). AG14361 was injected (i.p.) at the onset of reperfusion after tFCI, and infarct volume was measured 48 hours after tFCI on TTC-stained coronal sections with edema correction. *p<0.05, **p<0.01 vs vehicle controls. N=6-8/group.
Supplemental Fig. 6. PRX2 overexpression attenuates p53 activation after tFCI but has no additional impact when PARP1 is inhibited. 

A: Double-label immunofluorescent staining for phospho-p53 (p-53 Ser46) and NeuN was performed at 6 hours after tFCI (90 min) in Wt or Tg-PRX2 mice that had been treated with vehicle or with the PARP1 inhibitor AG14361 (3 mg/kg). Arrows point to representative p-p53+ neurons. 

B: Double-label immunofluorescent staining for p-p53 and PARS was performed 6 hours after tFCI (90 min) in Wt mice that had been treated with vehicle or with AG14361 (3 mg/kg). Note that PARS and p-p53 staining occurs in different cells and that PARP1 inhibition does not attenuate p53 activation, but increases it. 

C: Coronal sections taken from a mouse at 6 hours after tFCI and stained for MAP2. The boxes indicate the penumbra regions in the cortex where the immunofluorescent images were taken and analyzed. Note that at this time point, MAP2 staining was lost in the striatum but largely preserved in the cortex. 

D-F: Cell counting of p-p53 positive cells (data are expressed as the percentage of NeuN-positive cells) in the cortical regions marked in C. *p<0.05, **p<0.01, ***p<0.001 vs sham controls. #p<0.05 between the indicated groups. N=6/group.
Supplemental Fig. 7. PRX2 overexpression reduces caspase 3 and 9 activation following tFCI. A and B: Wt or Tg-PRX2 mice were exposed to tFCI and ischemic brain tissue was harvested 6 and 12 hours later for immunoblotting for cleaved caspase 9 or cleaved caspase 3 (A) and for activity levels of these two proteases (B). C: Separate Wt and Tg-PRX2 animals were perfused at the same timepoints after tFCI for immunofluorescent staining for active caspase 3 (red) and neuronal marker NeuN (green). *p<0.05, **p<0.01 versus Wt. N=5/group.
Supplemental Fig. 8. PRX2 overexpression attenuates Bax translocation after tFCI but has no additional impact when PARP1 is inhibited. A: Double-label immunofluorescent staining for Bax and NeuN was performed 6 hours after tFCI (90 min). Note that Bax immunofluorescence is converted in neurons from a diffuse pattern to a punctate pattern. B: Double-label immunofluorescent staining for Bax and NeuN was performed at 6 hours after tFCI (90 min) in Wt or Tg-PRX2 mice that had been treated with vehicle or the PARP1 inhibitor AG14361 (3 mg/kg). Arrows point to representative neurons that contain punctate Bax immunofluorescence. C: Coronal sections taken from a mouse 6 hours after tFCI and stained for MAP2. The boxes indicate the penumbra regions in the cortex where the immunofluorescent images were taken and analyzed. Note that at this time point, MAP2 staining was lost in the striatum but largely preserved in the cortex. D-F: Number of neurons that showed punctate Bax immunofluorescence (data are expressed as the percentage of NeuN-positive cells) in the cortical regions marked in C. *p<0.05, **p<0.01 versus sham controls. #p<0.05 between the groups indicated. N=6/group.
Supplemental Fig. 9. JNK inhibition attenuates PARS levels after tFCI and decreases infarct volume in p53 knockout animals. A: Animals were exposed to 90 min tFCI or sham surgery and injected intravenously with vehicle or SP600125 (3 mg/kg × 2). Ischemic brain tissue was harvested 6 hours later and probed for PARS and phospho-c-Jun. B: Infarct volume was measured on TTC-stained coronal sections at 48 hours after 90 min tFCI in animals injected with vehicle, SP600125 (3 mg/kg × 2), AG15361 (3 mg/kg), or the combination of both inhibitors. C: Wt (p53+/+) or p53 knockout (p53−/−) mice were exposed to 90 min tFCI or sham surgery and ischemic brain tissue was harvested 3 and 6 hours later for phospho-ASK1 and phospho-c-Jun immunoblotting. D: Infarct volume was measured on TTC-stained coronal sections at 48 hours after 90 min tFCI in p53+/+ and p53−/− animals treated with vehicle or SP600125. *p<0.05, **p<0.01 for the comparison between the indicated groups. n.s. not significant. N=6/group.