SUPPLEMENTAL MATERIAL

Detailed Methods

TTC staining The mouse heart was cut into 6 1-mm thick slices which were submerged in 1% TTC and incubated at 37°C for 10-15 min. After aspiration of TTC, the slices were fixed in 10% formalin and then images were captured to calculate infarct size and size of area at risk (AAR) as previously reported [1].

Hairpin-2 staining A double stranded DNA fragment with blunt ends was prepared as previously described[2-4]. Polymerase chain reaction (PCR) with Pfu Ultra polymerase was performed with 16.6 μmol/L Texas Red-12-dUTP (Molecular Probes), 16.6 μmol/L dTTP, 50 μmol/L dATP, 50 μmol/L dCTP and 50 μmol/L dGTP. Pfu probe recognizes a form of DNA damage characterized by cleavage of multiple DNA fragments with blunt ends, typically observed in necrotic cell death [5-7]. Heart sections were deparaffinized with xylene, rehydrated in graded alcohol concentrations, briefly washed in water, and then treated with proteinase K (50 g/ml) in PBS for 45 minutes at 37°C. After washing with PBS, a mix of 50 mmol/L Tris-HCl, pH 7.8, 10 mmol/L $MgCl_2$, 10 mmol/L DTT, 1 mmol/L ATP, 25 μg/mL BSA, 15% polyethylene glycol (8,000 mol wt, Sigma), 1 μg/mL Texas red-labeled DNA fragment and 250 U/mL DNA T4 ligase (Boehringer Mannheim) was added. Sections were then placed in a humidified box for 16 h. The sections were thoroughly washed in 70°C water and then were observed under a fluorescent microscope immediately after counterstaining with 10 μg/mL 4,6-diamidino-2-phenylindole (DAPI).

TUNEL staining The mouse heart was harvested after 24 hours of reperfusion and fixed in 10% formalin. TUNEL staining was carried out and TUNEL positive nuclei were counted as previously reported [8].

Immunoblotting The ischemic myocardium was isolated and homogenized in RIPA buffer. Immunoblotting using phospho-p70 S6 kinase (S6K), total S6K, and p62 primary antibodies was performed as previously described [1].

Supplemental References

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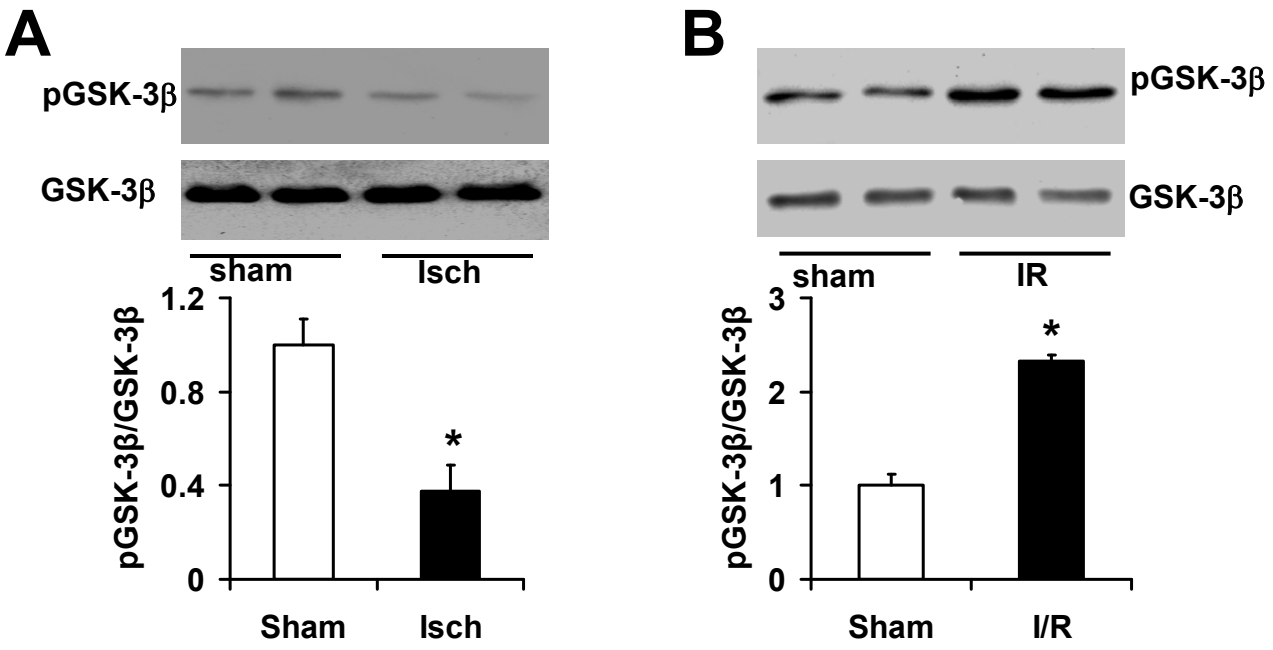
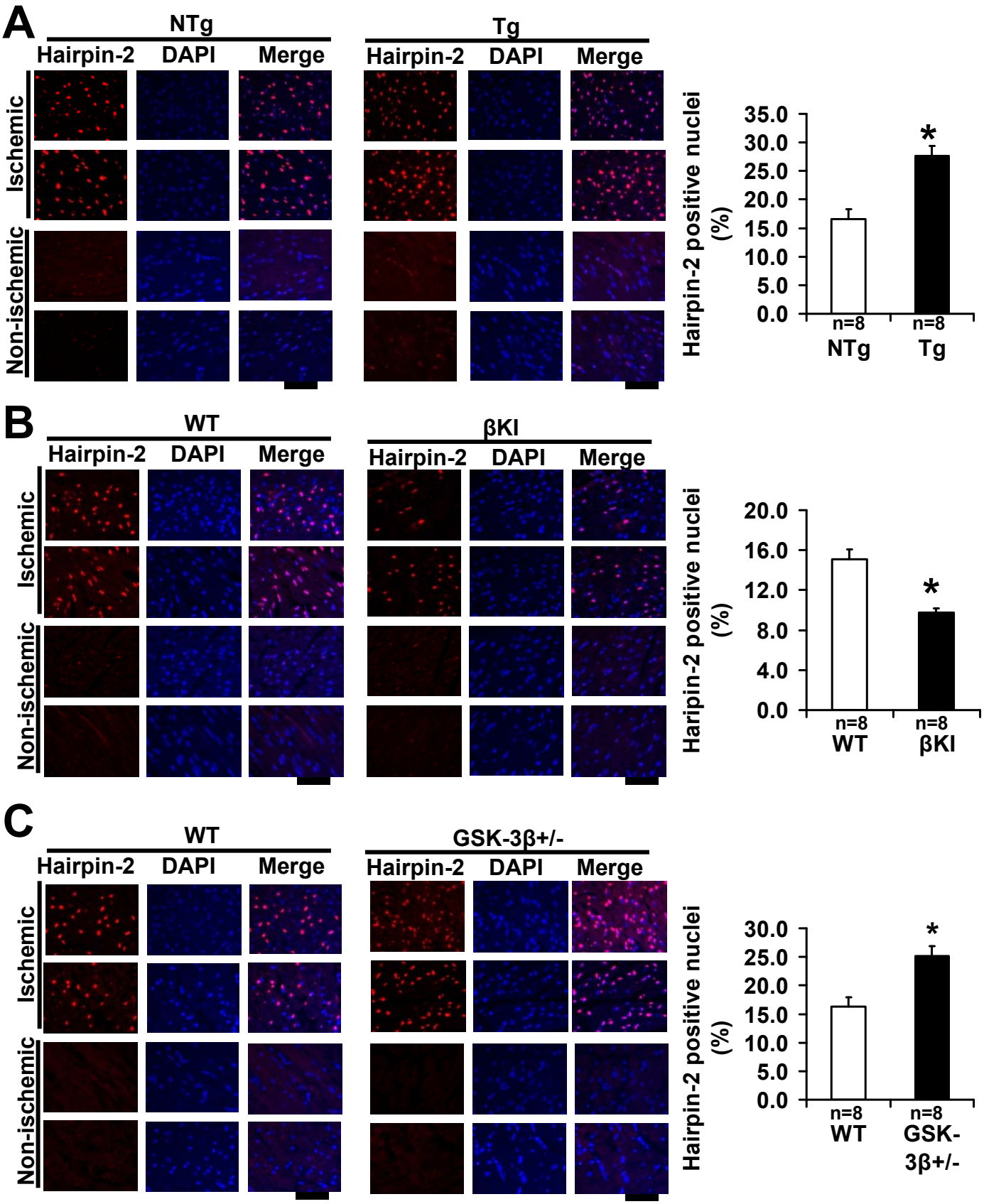
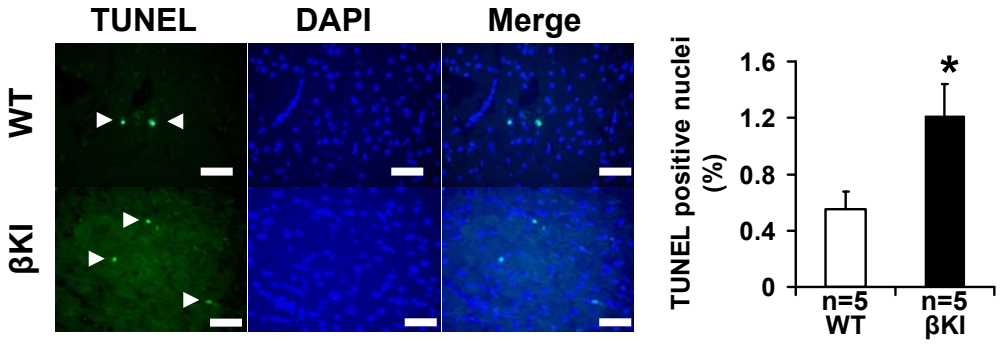


Online Figure I. Phosphorylation of GSK-3β after 30 min of ischemia (A) and after 20 min of ischemia and 30 min of reperfusion (B) in C57BL/6J mice. *P<0.01 vs. respective Sham. Data are mean ± SEM.

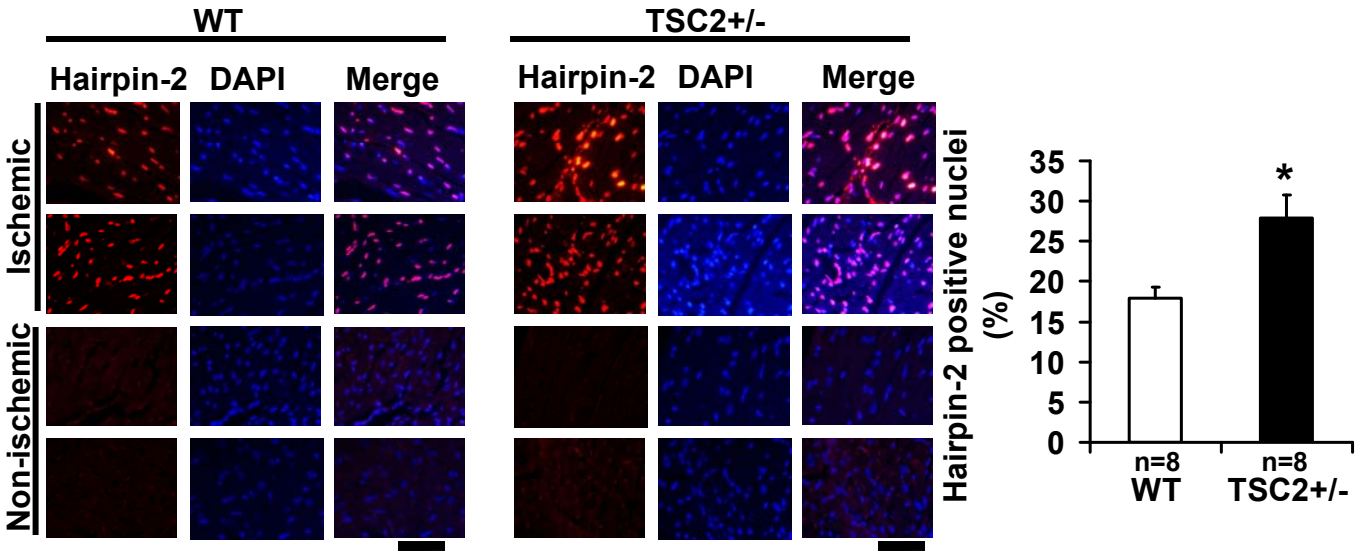


Online Figure II

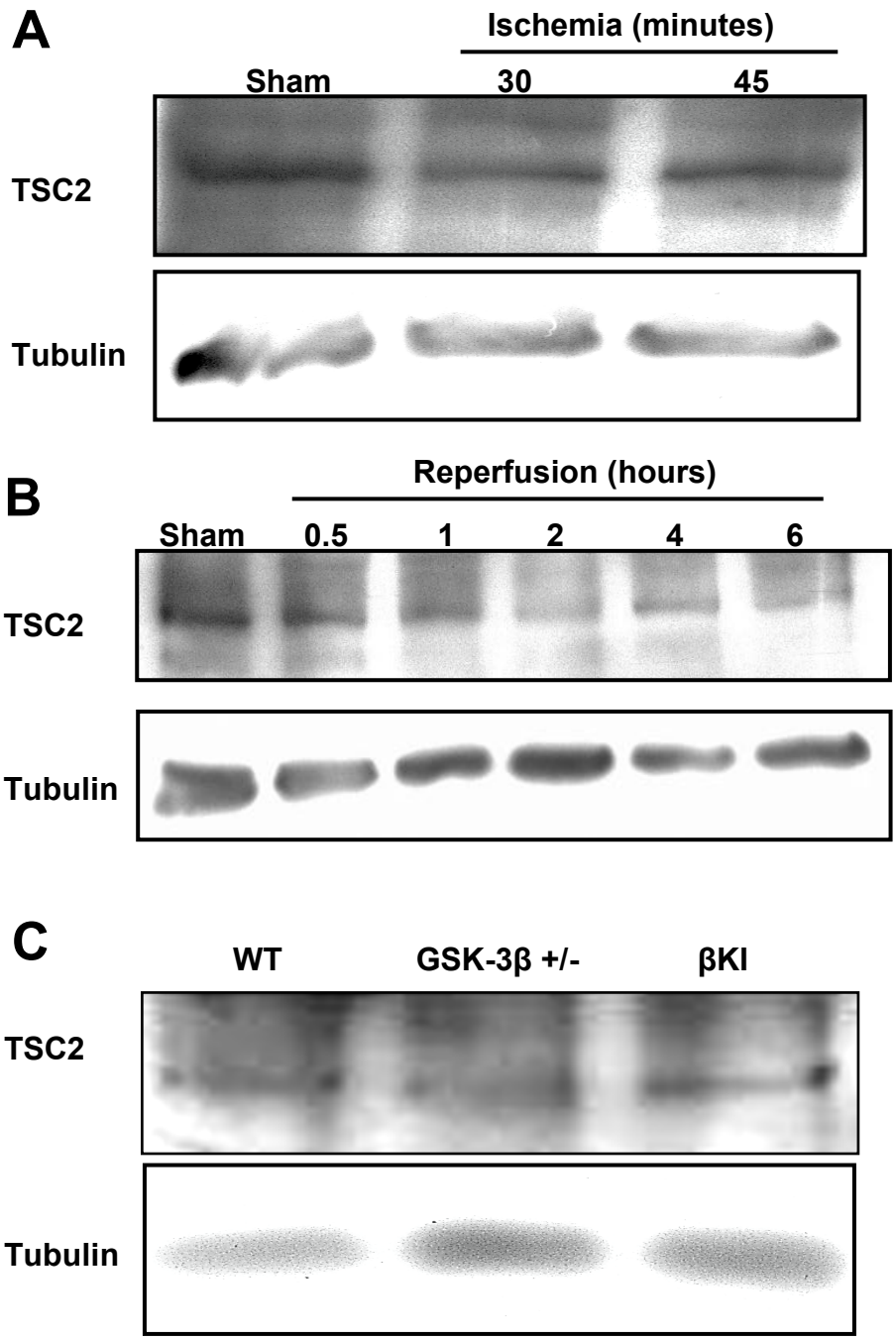
Online Figure II. The role of GSK-3β in modulating myocardial injury caused by prolonged ischemia. In A-C, images of Hairpin-2 staining of cardiac tissue sections from animals 2 hours after myocardial ischemia are shown. Scale bar = 100 μm. The percentages of Hairpin-2 positive nuclei are shown (bar graphs). Data are mean ± SEM. A. Cardiac-specific dominant negative GSK-3β transgenic mice (Tg) and their littermate non-transgenic mice (NTg) were used. *P<0.01 vs. NTg. B. Constitutively active GSK-3β^{S9A} knock-in mice (βKI) and wild type mice (WT) were used. *P<0.01 vs. WT. C. Heterozygous GSK-3β knock-out mice (GSK-3β +/-) were used. *P<0.01 vs. WT.



Online Figure III. Apoptosis in constitutively active GSK-3β^{S9A} knock-in mice (βKI). Images were taken from TUNEL and DAPI stained cardiac sections. Scale bar = 50 μm. The bar graph shows the percentage of TUNEL positive nuclei. Data are mean ± SEM. *P<0.05 vs. WT.



Online Figure IV. Myocardial necrosis in heterozygous TSC2 knock-out mice (TSC2+/-) and wild type mice (WT) after 2 hours of ischemia. Images were taken from Hairpin-2 stained cardiac sections. Scale bar = 100 μm. The percentages of Hairpin-2 positive nuclei are shown in the bar graph. Data are mean ± SEM. *P<0.01 vs. WT.



Online Figure V. TSC2 expression. A. During ischemia. B. During reperfusion. C. At baseline, in GSK-3β heterozygous knock-out mouse heart (GSK-3β +/-) and constitutively active GSK-$3\beta^{S9A}$ knock-in mouse heart (βKI).

Online Figure V