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# Differential Roles of GSK-3 $\beta$ During Myocardial Ischemia and Ischemia/Reperfusion

Peiyong Zhai, Sebastiano Sciarretta, Jonathan Galeotti, Massimo Volpe, Junichi Sadoshima

**Rationale:** Inhibition of glycogen synthase kinase-3 (GSK-3) protects the heart during ischemia/reperfusion (I/R), yet the underlying mechanisms of cardioprotection afforded by beta isoform-specific inhibition GSK-3 remain to be elucidated.

**Objective:** We studied the molecular mechanism mediating the effect of GSK-3 $\beta$  activation/inhibition upon myocardial injury during prolonged ischemia and I/R.

**Methods and Results:** Beta isoform-specific inhibition of GSK-3 by dominant negative GSK-3 $\beta$  in transgenic mice (Tg-DnGSK-3 $\beta$ ) or in heterozygous GSK-3 $\beta$  knock-out mice (GSK-3 $\beta$ +/-) significantly increased, whereas activation of GSK-3 $\beta$  in constitutively active GSK-3 $\beta$  knock-in mice ( $\beta$ KI) significantly decreased, myocardial ischemic injury after prolonged ischemia. In contrast, inhibition of GSK-3 $\beta$  in Tg-DnGSK-3 $\beta$  or GSK-3 $\beta$ +/- significantly reduced, while activation of GSK-3 $\beta$  in  $\beta$ KI significantly enhanced, myocardial I/R injury. Inhibition of GSK-3 $\beta$  stimulated mTOR signaling and inhibited autophagy through a rapamycin-sensitive (mTOR dependent) mechanism. Rapamycin enhanced autophagy and, at the same time, abolished the effects of GSK-3 $\beta$  inhibition on both prolonged ischemic injury and I/R injury. Importantly, the influence of rapamycin over the effects of GSK-3 $\beta$  inhibition on myocardial injury was reversed by inhibition of autophagy.

**Conclusions:** Our results suggest that beta isoform-specific inhibition of GSK-3 exacerbates ischemic injury but protects against I/R injury by modulating mTOR and autophagy. (*Circ Res.* 2011;109:502-511.)

**Key Words:** ischemia ■ reperfusion ■ apoptosis ■ autophagy ■ GSK-3 ■ Beclin 1 ■ rapamycin ■ mTOR

Despite the recent progress in clinical interventions to facilitate early myocardial reperfusion for patients who suffer from acute myocardial infarction (AMI), the death rate during the acute phase of AMI is about 10% and the incidence of heart failure reaches 25% during the chronic phase. Restoring the blood flow to the ischemic myocardium paradoxically causes myocardial damage (termed *ischemia/reperfusion* [I/R] injury), which potentially explains why many patients develop heart failure despite successful reperfusion. Although many interventions have been shown to protect the heart from I/R injury in experimental animals, virtually no intervention has shown clearly protective effects against I/R injury in the clinical setting, indicating that reevaluation of existing or development of new modalities to protect the heart from I/R injury is urgently needed.

GSK-3 is a serine/threonine kinase, which is involved in many cellular functions in the heart, including gene expression, hypertrophy, and apoptosis. GSK-3 is a particularly important target of drug therapy for I/R injury because phosphorylation/inhibition of mitochondrial GSK-3 $\beta$  during

I/R is believed to be a final common mechanism mediating myocardial protection by many agents/interventions.<sup>1</sup> However, many important issues require clarification before inhibition of GSK-3 $\beta$  can be considered to be an option in clinical therapy for I/R injury. First, the precise mechanism through which inhibition of GSK-3 affords cardiac protection remains to be elucidated. Phosphorylation of GSK-3 $\beta$  suppresses opening of the mitochondrial permeability transition pore (mPTP) by binding to adenine nucleotide translocase (ANT) and thereby reducing the affinity of ANT for cyclophilin D.<sup>2</sup> GSK-3 inhibitors also reduce adenine nucleotide transport across the outer mitochondrial membrane by decreasing voltage-dependent anion channel phosphorylation,<sup>3</sup> which preserves ATP by blocking its consumption by mitochondria and prevents mitochondrial Ca<sup>2+</sup> overloading and oxidative stress.<sup>3</sup> However, judging from the fact that GSK-3 $\beta$  phosphorylates a wide variety of cellular proteins, other mechanisms, including the tuberous sclerosis complex 2 (TSC2)—mammalian target of rapamycin (mTOR) pathway, may also mediate the function of GSK-3 $\beta$ . Second, previous

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investigations into the role of GSK-3 in regulating I/R injury heavily relied on chemical inhibitors, which would equally affect both GSK-3 $\alpha$  and GSK-3 $\beta$ , the major isoforms of GSK-3 equally expressed in the heart. We have shown recently that these 2 isoforms have distinct subcellular localizations and functions in the heart.<sup>4</sup> Thus, the isoform-specific functions of GSK-3 should be reevaluated with genetically altered mouse models, in which the function of each isoform is specifically modulated. Third, activity and the significance of signaling molecules/biological responses could be different in the ischemic phase and the reperfusion phase. For example, we have shown recently that although activation of autophagy during prolonged ischemia is beneficial, it could be detrimental during reperfusion.<sup>5</sup> This issue is important because an effective treatment for I/R may not necessarily be ideal for prolonged ischemia. To our knowledge, the phase-specific roles of GSK-3 $\beta$  in regulating injury during prolonged ischemia and reperfusion have not been compared.

Thus, the purposes of this study were (1) to evaluate the activity of GSK-3 $\beta$  during prolonged ischemia and reperfusion, (2) to investigate the role of GSK-3 $\beta$  and downstream signaling mechanisms in regulating myocardial injury in response to prolonged ischemia and I/R, and (3) to evaluate the role of autophagy in mediating the effect of GSK-3 $\beta$  modulation on myocardial injury during prolonged ischemia and I/R. The  $\beta$ -isoform specific function of GSK-3 was evaluated with genetically altered mouse models in which activity of GSK-3 $\beta$  is specifically modified.

## Methods

An expanded Methods section is presented in the Online Data Supplement available at <http://circres.ahajournals.org>.

## Animals

Generation of transgenic mice with cardiac-specific overexpression of dominant negative GSK-3 $\beta$  (Tg-DnGSK-3 $\beta$ ) (FVB background) was previously reported.<sup>6</sup> Heterozygous GSK-3 $\beta$  knock-out (GSK-3 $\beta$ +/-) mice (C57BL/6J background) were purchased from Jackson Laboratories. GSK-3 $\beta$ <sup>S9A</sup> knock-in ( $\beta$ KI) mice (C57BL/6J background) were provided by Dr. D. R. Alessi (University of Dundee, Dundee, UK).<sup>4</sup> Disruption of the *TSC2* gene was achieved by a conventional gene targeting method. We used *TSC2* heterozygous knock-out mice bred on a C57BL/6J background. Heterozygous green fluorescent protein/light chain 3 (GFP-LC3) transgenic mice (RIKEN BioResource Center, C57BL/6J background) were provided by Dr. N. Mizushima (Tokyo Medical Dental University, Tokyo, Japan).<sup>7</sup> Heterozygous *beclin 1* knock-out mice (C57BL/6J background) were provided by Dr. B. Levine (University of Texas Southwestern, Dallas, TX).<sup>5</sup> All experiments involving animals were approved by the New Jersey Medical School's Institutional Animal Care and Use Committee.

## I/R

Myocardial I/R was achieved by temporarily occluding the left anterior descending coronary artery (LAD) and then releasing the occlusion.<sup>5</sup> The duration of ischemia was 45 minutes for mice on an FVB background and 20 minutes for mice on a C57BL/6J background. The reperfusion duration was 24 hours for mice used in determining MI and 2 hours for mice used in biochemical analyses, except for those used in a time course experiment. In some mice, rapamycin (1 mg/kg, i.p.) was administered 10 minutes before ischemia or 10 minutes before reperfusion. For myocardial ischemia

## Non-Standard Abbreviations and Acronyms

<b><math>\beta</math>KI</b>	GSK-3 $\beta$ <sup>S9A</sup> knock-in
<b>AAR</b>	area at risk
<b>Dn</b>	dominant negative
<b>GFP</b>	green fluorescent protein
<b>GSK-3</b>	glycogen synthase kinase-3
<b>I/R</b>	ischemia/reperfusion
<b>LAD</b>	left anterior descending coronary artery
<b>MI</b>	myocardial infarction
<b>mPTP</b>	mitochondrial permeability transition pore
<b>mTOR</b>	mammalian target of rapamycin
<b>NTg</b>	nontransgenic
<b>S6K</b>	p70 S6 kinase
<b>TCS2</b>	tuberous sclerosis complex 2
<b>Tg</b>	transgenic
<b>WT</b>	wild type

studies, the LAD was ligated and occluded for 2 hours, with the exception of a time course experiment.

## Statistics

All values are expressed as mean $\pm$ SEM. Statistical analyses were performed using an analysis of variance (ANOVA) and Tukey-Kramer posthoc test or the *t* test, with *P*<0.05 considered significant.

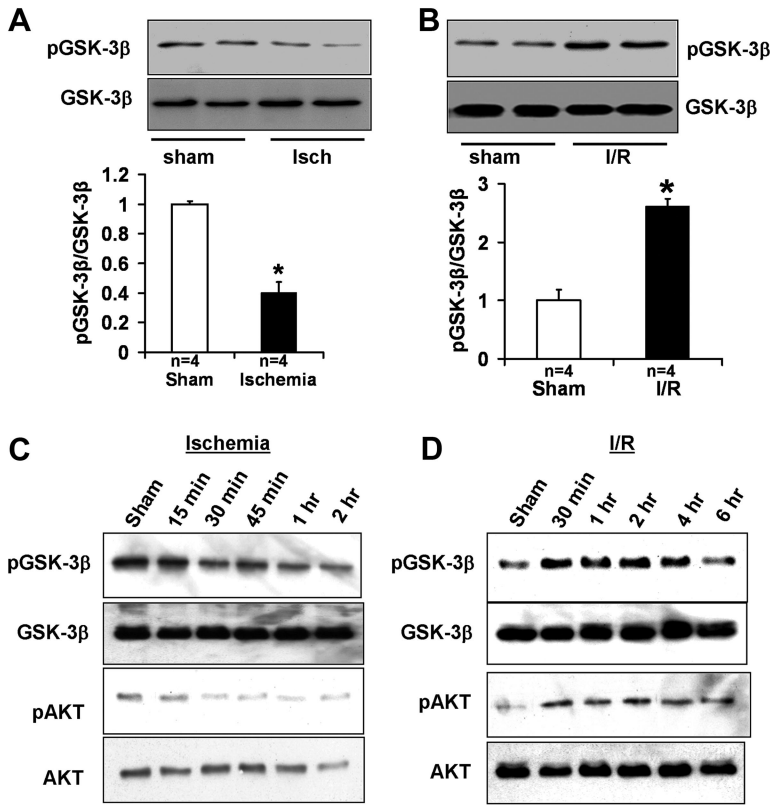
## Results

### GSK-3 $\beta$ Is Activated During Ischemia but Inhibited During Reperfusion

In order to examine the functional status of GSK-3 $\beta$  during ischemia and reperfusion, C57BL/6J mice were subjected to either prolonged ischemia (2 hours) or I/R (20 minutes/2 hours). Ischemia alone significantly reduced S9 phosphorylation of GSK-3 $\beta$ , but I/R increased S9 phosphorylation of GSK-3 $\beta$  in the mouse heart (Figure 1A and 1B). Similar results were obtained when FVB mice were subjected to either prolonged ischemia (2 hours) or I/R (45 minutes/2 hours) (Figure 1C and 1D). The progressive reduction in S9 phosphorylation of GSK-3 $\beta$  during ischemia started within 30 minutes of ischemia and persisted for the 2-hour duration (Figure 1C and Online Figure IA). The S473 phosphorylation of AKT, an upstream kinase of GSK-3 $\beta$ , was also decreased during ischemia, from 30 minutes onward (Figure 1C). The increase in S9 phosphorylation of GSK-3 $\beta$  during reperfusion started within 30 minutes of reperfusion, persisted for 4 hours, and returned to baseline by 6 hours of reperfusion (Figure 1D and Online Figure IB). The S473 phosphorylation of AKT was increased within 30 minutes of reperfusion and remained increased for up to 6 hours of reperfusion (Figure 1D). These results suggest that the activity of GSK-3 $\beta$  is differentially regulated by ischemia and I/R. Because the activity of GSK-3 $\beta$  is negatively regulated by S9 phosphorylation, GSK-3 $\beta$  is activated by prolonged ischemia but is inhibited by I/R.

### Inhibition of GSK-3 $\beta$ Exacerbates, While Activation of GSK-3 $\beta$ Diminishes Ischemic Injury

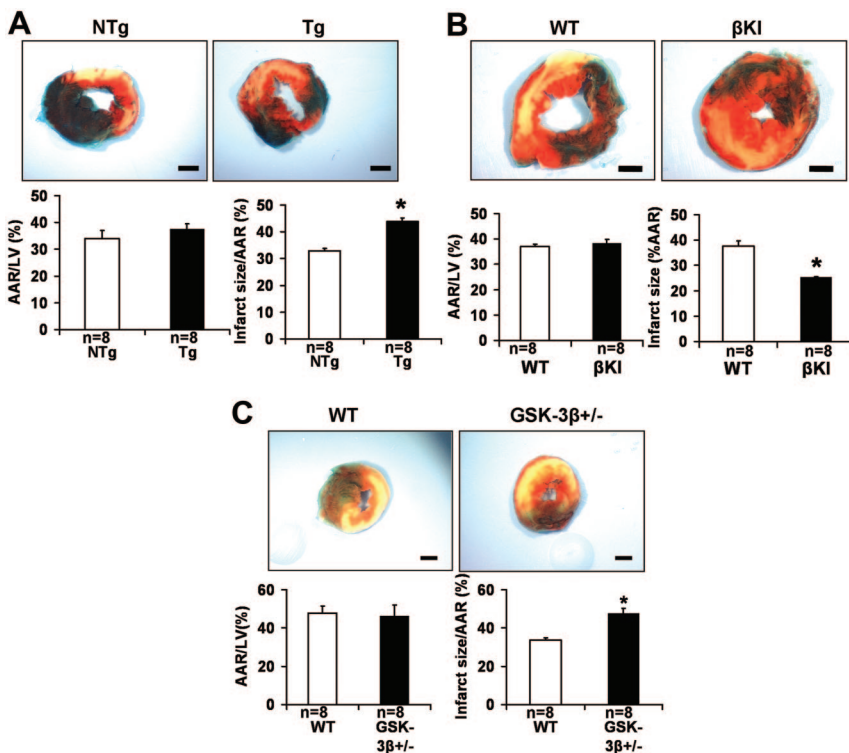
In order to study the role of GSK-3 $\beta$  in prolonged ischemia, a 2-hour ischemia protocol was carried out. After 2 hours of



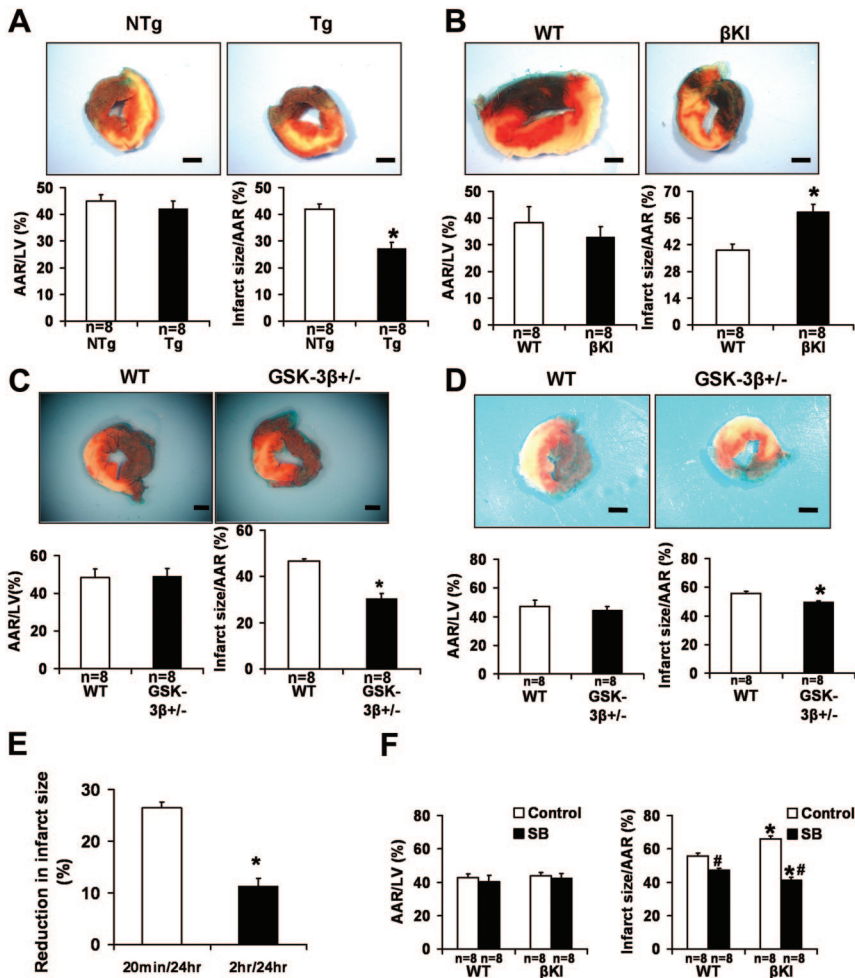
**Figure 1.** Phosphorylation of GSK-3 $\beta$  after 2 hours of ischemia (A) and after 20 minutes of ischemia and 2 hours of reperfusion (B) in C57BL/6J mice. \* $P < 0.01$  versus respective sham. Data are mean  $\pm$  SEM. Phosphorylation of GSK-3 $\beta$  and AKT at various time points during prolonged ischemia (C) and at various time points during reperfusion after 45 minutes ischemia (D) in FVB mice.

ischemia without reperfusion, the infarct size/area at risk (AAR) in Tg-DnGSK-3 $\beta$  mice, which have diminished GSK-3 $\beta$  activity,<sup>6</sup> was significantly increased (Figure 2A). After ischemia, the infarct size/AAR in constitutively active GSK-3 $\beta$ <sup>S9A</sup> knock-in ( $\beta$ KI) mice was significantly de-

creased, but that in GSK-3 $\beta$ <sup>+/-</sup> mice was significantly increased (Figure 2B and 2C). Because the use of TTC staining at an early point during ischemia without reperfusion could have underestimated the infarct size, we performed Hairpin-2 staining, a method of directly detecting necrosis.<sup>8</sup>



**Figure 2.** The role of GSK-3 $\beta$  in modulating myocardial injury caused by prolonged ischemia. In A–C, animals were subjected to 2 hours of ischemia. Representative images of left ventricular (LV) slices with Alcian blue and TTC staining are shown. Area at risk (AAR)/LV (left) and infarct size/AAR (right) are shown. Data are mean  $\pm$  SEM. Scale bar = 1 mm. **A**, Tg-DnGSK-3 $\beta$  (Tg) and littermate NTg mice were used. \* $P < 0.05$  versus NTg. **B**,  $\beta$ KI and wild type (WT) were used. \* $P < 0.05$  versus WT. **C**, GSK-3 $\beta$ <sup>+/-</sup> and WT mice were used. \* $P < 0.05$  versus WT.



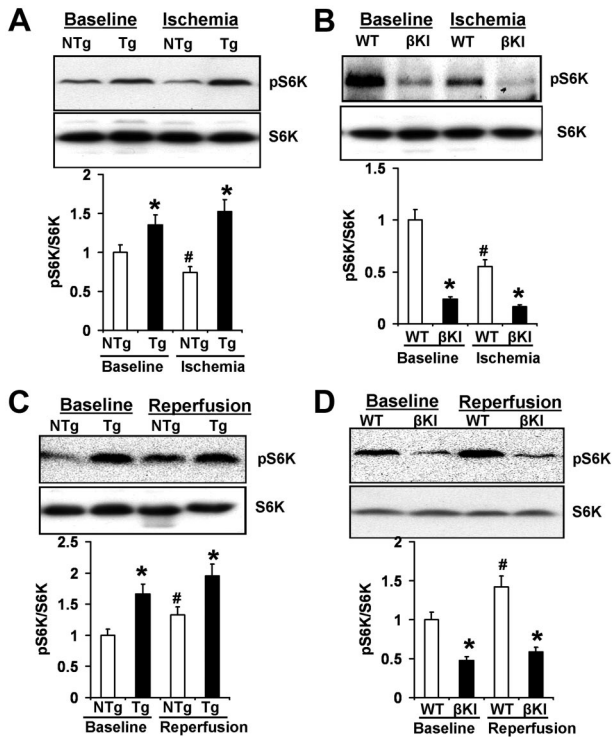
**Figure 3.** The role of GSK-3 $\beta$  in modulating myocardial injury caused by ischemia/reperfusion (I/R). In **A–D** and **F**, animals were subjected to either 45 minutes (**A**), 20 minutes (**B**, **C**), or 2 hours (**D**, **F**) of ischemia, followed by 24 hours of reperfusion. In **A–D**, representative images of left ventricular (LV) slices with Alcian blue and TTC staining are shown. The area at risk (AAR)/LV (left) and infarct size/AAR (right) are shown (**A–D**, **F**). Data are mean  $\pm$  SEM. Scale bar = 1 mm. **A**, Tg-DnGSK-3 $\beta$  (Tg) and littermate NTg mice were used. \* $P < 0.05$  versus NTg. **B**, The  $\beta$ KI and wild-type (WT) mice were used. \* $P < 0.05$  versus WT. **C**, **D**, GSK-3 $\beta$ +/- and WT mice were used. \* $P < 0.05$  versus WT. **E**, Reduction in infarct size in GSK-3 $\beta$ +/- mice after 20 minutes or 2 hours of ischemia followed by 24 hours of reperfusion, in comparison with the average infarct size of respective WT mice. \* $P < 0.01$  versus 20 minutes/24 hours I/R. **F**, GSK-3 inhibitor SB216763 (SB) was given to  $\beta$ KI mice at the time of reperfusion. \* $P < 0.05$  versus respective WT. # $P < 0.05$  versus vehicle control.

The percentage of Hairpin-2 positive nuclei after ischemia was significantly higher in Tg-DnGSK-3 $\beta$  than in nontransgenic (NTg) mice (Online Figure IIA). The percentage of Hairpin-2 positive nuclei after ischemia was significantly lower in  $\beta$ KI mice, but was significantly higher in GSK-3 $\beta$ +/- mice, than in wild-type (WT) mice (Online Figure IIB and IIC). These results suggest that activation of GSK-3 $\beta$  during ischemia ameliorates ischemic myocardial injury, whereas interventions to inhibit GSK-3 $\beta$  during prolonged ischemia enhance ischemic injury.

### Inhibition of GSK-3 $\beta$ Attenuates, While Activation of GSK-3 $\beta$ Enhances Reperfusion Injury

In order to study the role of GSK-3 in I/R injury, we used Tg-DnGSK-3 $\beta$ , GSK-3 $\beta$ +/-, and  $\beta$ KI mice. After I/R, the infarct size/AAR in Tg-DnGSK-3 $\beta$  was significantly reduced in comparison with that in NTg (Figure 3A). On the other hand, infarct size/AAR after I/R was significantly greater in  $\beta$ KI than in WT mice (Figure 3B). The number of TUNEL positive nuclei after I/R was significantly greater in  $\beta$ KI mice than in WT mice (Online Figure III). After 20 minutes of ischemia and 24 hours of reperfusion, the infarct size/AAR was significantly smaller in GSK-3 $\beta$ +/- mice than in WT mice, although AAR/LV was not significantly different between them (Figure 3C). To test whether the difference in

ischemic duration between the ischemic protocol and I/R protocol makes inhibition of GSK-3 $\beta$  appear to have different effects, we carried out a protocol of 2 hours ischemia followed by 24 hours reperfusion in GSK-3 $\beta$ +/- mice. In this experimental condition, the infarct size/AAR was still significantly smaller in GSK-3 $\beta$ +/- mice than in WT mice (Figure 3D). This result suggests that GSK-3 $\beta$  inhibition is protective during reperfusion even after prolonged ischemia. It should be noted that the extent of protection (percentage reduction in the MI size versus control mice) was significantly smaller after 2 hours ischemia/24 hours reperfusion than after 20 minutes ischemia/24 hours reperfusion (Figure 3E). Thus, it is possible that the overall benefit of inhibiting GSK-3 $\beta$  during the entire period of I/R model may be attenuated if the period of ischemia is prolonged. In order to manipulate the activity of GSK-3 $\beta$  in a phase-specific manner, a protocol of 2 hours ischemia followed by 24 hours reperfusion was carried out in  $\beta$ KI mice with or without SB216763, a specific inhibitor of GSK-3, given at the time of reperfusion. Without SB216763,  $\beta$ KI mice exhibited a significantly greater infarct size than did WT mice (Figure 3F). SB216763 significantly decreased infarct size in both WT mice and  $\beta$ KI mice. Importantly, with SB216763,  $\beta$ KI mice had a significantly smaller infarct size than did WT mice (Figure 3F). These results further support the idea that



**Figure 4. GSK-3 $\beta$  inhibits mTOR signaling.** **A**, Phospho-p70 S6 kinase (pS6K) and total S6K in the ischemic myocardium of Tg-DnGSK-3 $\beta$  (Tg) and NTg mice at baseline and after 2 hours of ischemia. \* $P$ <0.05 versus NTg. # $P$ <0.05 versus baseline. **B**, The pS6K and total S6K in ischemic myocardium of  $\beta$ KI and wild-type (WT) mice at baseline and after 2 hours of ischemia. \* $P$ <0.05 versus WT. # $P$ <0.05 versus baseline. **C**, The pS6K and total S6K in Tg and NTg at baseline and after 45 minutes of ischemia and 2 hours of reperfusion. \* $P$ <0.05 versus NTg. # $P$ <0.05 versus baseline. **D**, The pS6K and total S6K in  $\beta$ KI and WT at baseline and after 20 minutes of ischemia and 2 hours of reperfusion. \* $P$ <0.05 versus WT. # $P$ <0.05 versus baseline.

activation of GSK-3 $\beta$  is detrimental during reperfusion but protective during ischemia.

### Inhibition of GSK-3 $\beta$ Increases, While Activation of GSK-3 $\beta$ Inhibits mTOR Activity During Both Ischemia and Reperfusion Phases

Increasing lines of evidence suggest that mTOR regulates fundamental cellular functions relevant to hypoxia/ischemia,<sup>9</sup> thereby serving as a critical regulator of cell survival/death under conditions of hypoxia/ischemia. In order to study the molecular mechanisms mediating the effects of GSK-3 on ischemic and I/R injury, we studied the activity of mTOR after prolonged ischemia and I/R. Both at baseline and after 2 hours of ischemia, the phosphorylation level of p70 S6 kinase (S6K), a downstream target phosphorylated by mTOR, in Tg-DnGSK-3 $\beta$  mice (Figure 4A) was increased, but that in  $\beta$ KI mice was decreased (Figure 4B), in comparison with that of WT mice, indicating that inhibition of GSK-3 $\beta$  increased, but activation of GSK-3 $\beta$  inhibited mTOR activity after prolonged ischemia. Importantly, ischemia decreased the phosphorylation level of S6K in NTg and WT mice, indicating that dephosphorylation of GSK-3 $\beta$  by ischemia might inhibit mTOR activity. I/R increased the phosphorylation level of S6K in NTg (Figure 4C) and WT mice (Figure 4D),

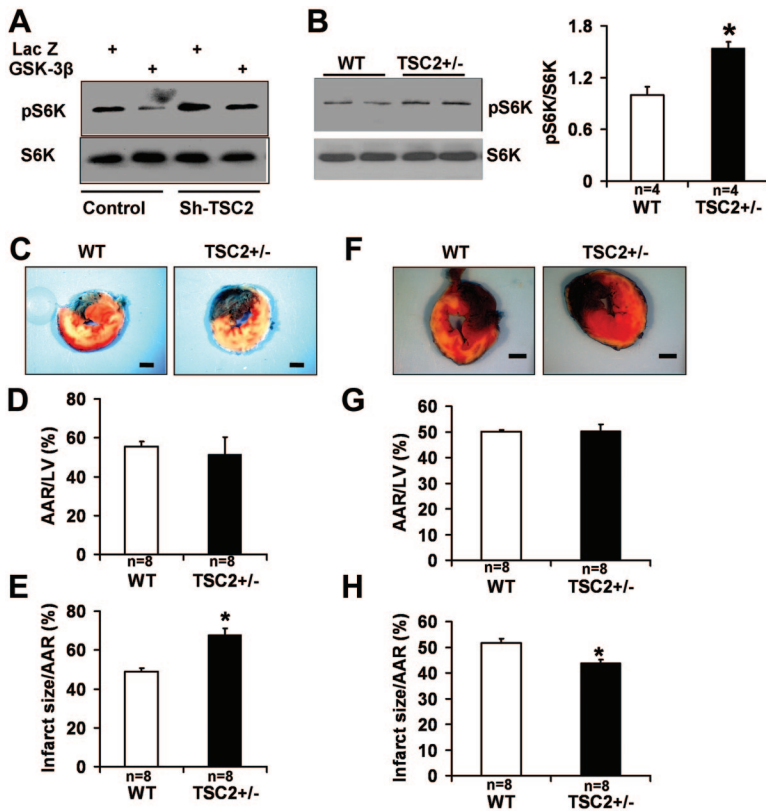
indicating that I/R elevated mTOR activity. Both at baseline and after I/R, the phosphorylation level of S6K in Tg-DnGSK-3 $\beta$  mice was markedly increased in comparison with that of NTg mice (Figure 4C). In contrast, both at baseline and after I/R, the phosphorylation level of S6K in  $\beta$ KI mice was significantly lower than that of WT mice (Figure 4D). These results suggest that inhibition of GSK-3 $\beta$  increases mTOR activity, whereas activation of GSK-3 $\beta$  decreases mTOR activity during I/R.

### Down-Regulation of TSC2 Stimulates Ischemic Injury but Protects Against Reperfusion Injury

Increasing lines of evidence suggest that mTOR is controlled by ischemia through TSC2 as an essential mechanism of adaptation.<sup>10</sup> Importantly, GSK-3 $\beta$  regulates mTOR through phosphorylation of TSC2 in cancer cell lines.<sup>11</sup> In order to study whether GSK-3 $\beta$  regulates mTOR in cardiomyocytes, experiments were carried out in neonatal rat cardiomyocytes. Overexpression of GSK-3 $\beta$  reduced the phosphorylation of S6K in cardiomyocytes, and knocking down TSC2 abolished the inhibitory effect of GSK-3 $\beta$  overexpression on S6K phosphorylation (Figure 5A). These results suggest that GSK-3 $\beta$  down-regulates mTOR through TSC2-dependent mechanisms in cardiomyocytes. In order to study the role of mTOR activation in mediating myocardial injury caused by prolonged ischemia and I/R, experiments were conducted in TSC2+/- mice. In TSC2+/- hearts, the phosphorylation level of S6K was increased (Figure 5B) in comparison with that in WT mouse hearts, indicating that mTOR activity was stimulated by down-regulation of TSC2. After 2 hours of ischemia without reperfusion, the infarct size/AAR was significantly larger in TSC2+/- mice than in WT mice, although the AAR was not significantly different between them (Figure 5C through 5E). The percentage of Hairpin-2 positive nuclei was significantly higher in TSC2+/- mice than in WT mice after 2 hours of ischemia (Online Figure IV). In contrast, after 45 minutes of ischemia and 24 hours of reperfusion, the infarct size/AAR was significantly smaller in TSC2+/- mice than in WT mice, although the AAR was similar between them (Figure 5F through 5H). These results indicate that activation of mTOR is protective during I/R, but detrimental during prolonged ischemia. In the absence of TSC2 knock-out, the protein level of TSC2 does not change during prolonged ischemia but is decreased during reperfusion in the mouse heart in vivo. Neither constitutively active GSK-3 $\beta$  nor heterozygous deletion of GSK-3 $\beta$  significantly affected baseline expression of TSC2 (Online Figure V). Thus, down-regulation of endogenous TSC2 may also contribute to mTOR activation during I/R.

### Inhibition of GSK-3 $\beta$ Prevents Autophagy Through mTOR and Is Detrimental During Ischemia

Autophagy is activated by nutrient starvation, and it is protective during the ischemic phase.<sup>5</sup> In order to study the effect of GSK-3 inhibition on autophagy after prolonged ischemia, DnGSK-3 $\beta$ /GFP-LC3 bigenic mice were subjected to 2 hours of ischemia, and the number of GFP-LC3 dots in the ischemic area was counted. The number of GFP-LC3 dots



**Figure 5. The mTOR signaling and ischemic and ischemia/reperfusion injury.** **A**, Phospho-p70 S6 kinase (pS6K) and total S6K in neonatal rat cardiomyocytes transfected with Lac Z or GSK-3 $\beta$ . GSK-3 $\beta$  decreased pS6K level. Knocking down TSC2 restored the pS6K level in GSK-3 $\beta$ -transfected myocytes. **B**, The pS6K and total S6K in TSC2+/- and wild-type (WT) mice. \* $P$ <0.01 versus WT. **C–E**, MI in TSC2+/- and WT mice after 2 hours of ischemia. Representative images of left ventricular (LV) slices with Alcian blue and TTC staining (**C**), area at risk (AAR)/LV (**D**), and infarct size/AAR (**E**) are shown. Scale bar=1 mm. \* $P$ <0.05 versus WT. **F–H**, MI in TSC2+/- and WT mice after 45 minutes of ischemia and 24 hours of reperfusion. Representative images of LV slices with Alcian blue and TTC staining (**F**), AAR/LV (**G**), and infarct size/AAR (**H**) are shown. Scale bar=1 mm. \* $P$ <0.05 versus WT.

per microscopic field in DnGSK-3 $\beta$ /GFP-LC3 bigenic mice was significantly lower than in GFP-LC3 transgenic mice (Figure 6A). Moreover, there was more p62, a protein degraded by autophagy, in the ischemic area of Tg-DnGSK-3 $\beta$  than in that of NTg mice (Figure 6B). These results suggest that inhibition of GSK-3 $\beta$  by overexpression of dominant negative GSK-3 $\beta$  resulted in less autophagy in the heart after prolonged ischemia. Rapamycin abolished the inhibitory effects of dominant negative GSK-3 $\beta$  on the number of GFP-LC3 dots after prolonged ischemia (Figure 6C), and reduced the infarct size/AAR and the percentage of Hairpin-2 positive nuclei in Tg-DnGSK-3 $\beta$  to the levels of those in NTg mice (Figure 6D), indicating that GSK-3 $\beta$  inhibition prevents autophagy through an mTOR-dependent mechanism and that GSK-3 $\beta$  inhibition may enhance ischemic injury via mTOR-dependent prevention of autophagy. Rapamycin also abolished the increase in infarct size caused by SB216763 (Figure 6E). However, in *beclin 1* heterozygous knock-out mice, which have a decreased level of autophagy, as well as a larger infarct/AAR and a higher percentage of Hairpin-2 positive nuclei than do control WT mice, rapamycin failed to abolish the infarct-enhancing effect of SB216763 after ischemia (Figure 6E). These results indicate that autophagy is one of the targets of mTOR inhibition that mediates its protective effect during prolonged ischemia.

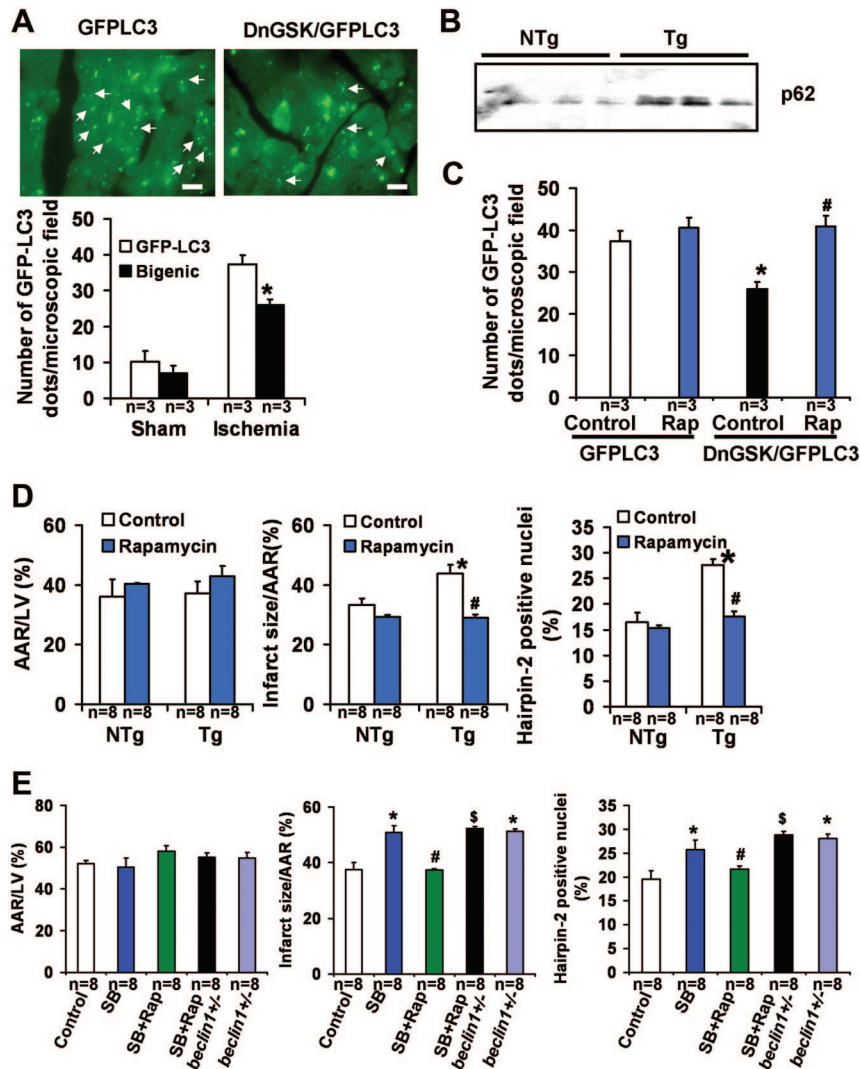
### Inhibition of GSK-3 $\beta$ Is Protective Against Reperfusion Injury Through mTOR-Dependent Down-Regulation of Autophagy

In order to study the involvement of mTOR in the effects of GSK-3 $\beta$  on reperfusion injury, rapamycin was used to inhibit

mTOR. Rapamycin abolished the infarct-limiting effect observed in Tg-DnGSK-3 $\beta$  (Figure 7A). Rapamycin also abolished the decrease in infarct size caused by SB216763 (Figure 7B). These results suggest that stimulation of mTOR plays an important role in mediating the protective effects of GSK-3 $\beta$  inhibition during reperfusion. Importantly, the number of GFP-LC3 dots per microscopic field was significantly decreased after I/R in DnGSK-3 $\beta$ /GFP-LC3 bigenic mice in comparison with that in GFP-LC3 transgenic mice (Figure 7C), indicating that reperfusion-induced autophagy was attenuated by GSK-3 $\beta$  inhibition. Rapamycin treatment restored the number of GFP-LC3 dots after I/R in DnGSK-3 $\beta$ /GFP-LC3 bigenic mice (Figure 7D), indicating that inhibition of mTOR enhances autophagy in Tg-DnGSK-3 $\beta$  mice. However, in *beclin 1* heterozygous knock-out mice, which have a decreased level of autophagy, rapamycin failed to abolish the infarct-limiting effect of SB216763 after I/R (Figure 7B). These results suggest that GSK-3 inhibition protects against reperfusion injury through mTOR-dependent prevention of autophagy.

### Discussion

We here investigated the  $\beta$  isoform-specific function of GSK-3 in the heart subjected to either prolonged ischemia alone or a short period of ischemia followed by reperfusion, using genetically engineered mouse models of GSK-3 $\beta$  inhibition (Tg-DnGSK-3 $\beta$  and GSK-3 $\beta$ +/-) and activation ( $\beta$ KI). The main findings in this study are (1) that GSK-3 $\beta$  is dephosphorylated and activated during prolonged ischemia, whereas it is phosphorylated and inhibited during reperfusion, (2) that activation of GSK-3 $\beta$  during ischemia and inactivation



**Figure 6. GSK-3 $\beta$  inhibition and myocardial autophagy after 2 hours of ischemia.** **A**, GFP-LC3 dots (arrow) in GFP-LC3 mice (GFP-LC3) and DnGSK-3 $\beta$ /GFP-LC3 mice (Bigenic, DnGSK/GFP-LC3). Scale bar=100  $\mu$ m. The bar graph shows the number of GFP-LC3 dots per microscopic field. \* $P$ <0.05 versus GFP-LC3 ischemia. **B**, Immunoblotting results of p62 in Tg-DnGSK-3 $\beta$  (Tg) and NTg mice. **C**, The number of GFP-LC3 dots per microscopic field in Tg-GFP-LC3 and DnGSK-3 $\beta$ /GFP-LC3 bigenic mice with or without rapamycin. \* $P$ <0.05 versus GFP-LC3 control. # $P$ <0.05 versus DnGSK-3 $\beta$ /GFP-LC3 control. **D**, The area at risk (AAR)/left ventricular (LV) (left), infarct size/AAR (middle), and percentage Hairpin-2 positive nuclei (right) 2 hours after ischemia in Tg and NTg with or without rapamycin. \* $P$ <0.05 versus NTg Control. # $P$ <0.05 versus Tg Control. **E**, AAR/LV (left), infarct size/AAR (middle), and percentage Hairpin-2 positive nuclei (right) in wild-type (WT) mice and *beclin1*<sup>+/-</sup> mice after 2 hours of ischemia. SB=SB216763. Rap, rapamycin. \* $P$ <0.05 versus control. # $P$ <0.05 versus SB. \$ $P$ <0.05 versus SB+Rap.

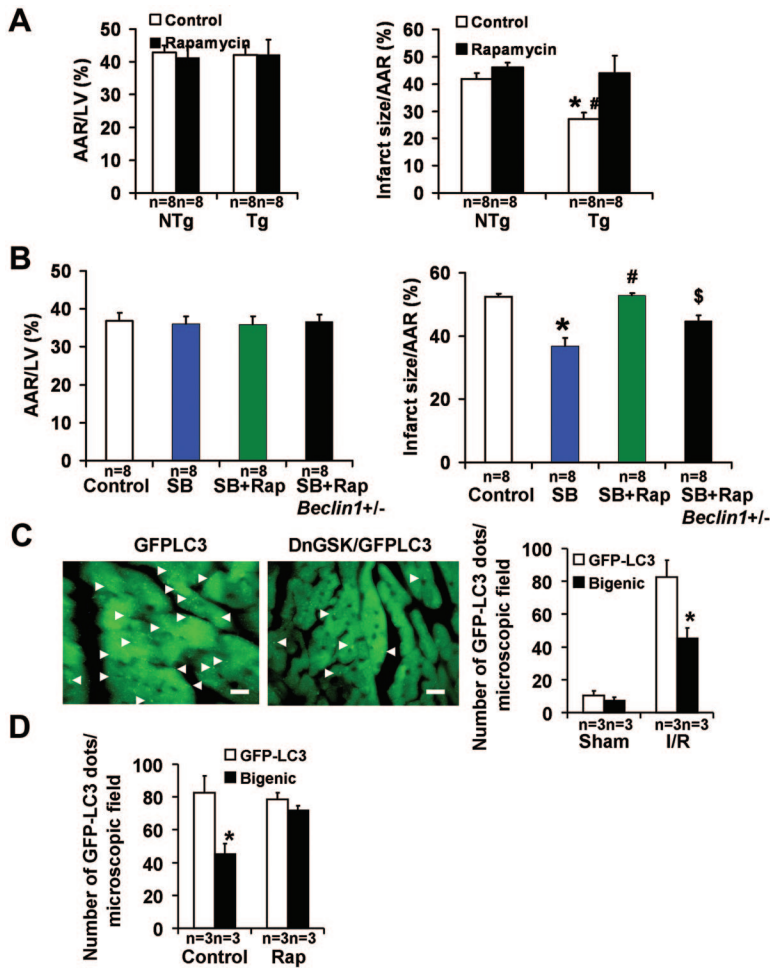
of GSK-3 $\beta$  during reperfusion are both compensatory for the heart, and (3) that the compensatory role of GSK-3 $\beta$  activation and inactivation during ischemia and reperfusion, respectively, is mediated through regulation of mTOR and autophagy (Figure 8).

The fact that phosphorylation of the S9 residue in endogenous GSK-3 $\beta$  is regulated by ischemia and reperfusion differentially but in a way that promotes cardioprotection during both phases is intriguing. The status of S9 phosphorylation in GSK-3 $\beta$  coincided with that of Akt phosphorylation, suggesting that Akt may control the activity of GSK-3 $\beta$  during ischemia and reperfusion. At present, however, phosphatases or kinases mediating either dephosphorylation during ischemia or phosphorylation during reperfusion remain to be identified.<sup>1</sup> Interestingly, although activation of GSK-3 $\beta$  through dephosphorylation during prolonged ischemia is protective, inactivation of GSK-3 $\beta$  by phosphorylation during the reperfusion phase is also protective. To our knowledge, phase-dependent changes in modulation and function of GSK-3 $\beta$  between ischemia and reperfusion have not been demonstrated previously.

Hypoxia inhibits mTOR through multiple signaling mechanisms, most of which utilize TSC1/2-dependent mecha-

nisms. For examples, brief exposures to modest hypoxia prevent insulin-mediated stimulation of mTORC1 through AMP-activated protein kinase (AMPK)-dependent activation of TSC1–TSC2.<sup>12</sup> Hypoxia induces expression of REDD1, which in turn reactivates TSC1–TSC2 by releasing TSC2 from 14-3-3,<sup>10,13</sup> which could also lead to inactivation of mTOR. GSK-3 $\beta$  phosphorylates/activates TSC2, which in turn inhibits mTOR,<sup>11</sup> for which GSK-3 $\beta$  is required for mTOR regulation by energy starvation.<sup>11</sup> Our results suggest that GSK-3 $\beta$  is activated during prolonged ischemia, which in turn inhibits mTOR in the heart. Suppression of GSK-3 $\beta$  activation significantly attenuated suppression of mTOR during ischemia, indicating a causative role of GSK-3 $\beta$  activation in mediating mTOR inhibition. Suppression of mTOR by GSK-3 $\beta$  was reversed when TSC2 was down-regulated, indicating that GSK-3 $\beta$  regulates mTOR through TSC2 in cardiomyocytes. Constitutive activation of GSK-3 $\beta$  inhibited activation of mTOR during reperfusion, suggesting that phosphorylation/suppression of GSK-3 $\beta$  plays an essential role in mediating mTOR activation during reperfusion as well. Taken together, our results suggest that GSK-3 $\beta$  is a critical upstream regulator of mTOR during both prolonged ischemia and reperfusion in the heart.





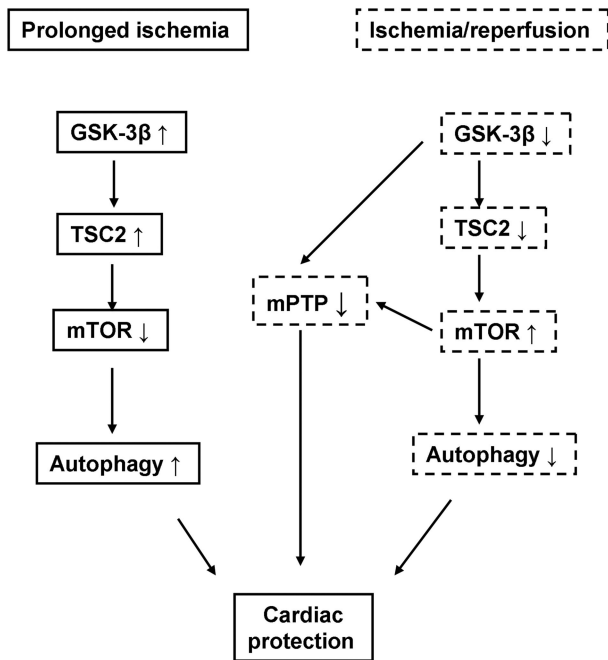
**Figure 7. GSK-3 inhibition, mTOR signaling, and autophagy after ischemia/reperfusion. A,** Area at risk (AAR)/left ventricular (LV) (left) and infarct size/AAR (right) in Tg-DnGSK-3β mice (Tg) after 45 minutes of ischemia and 24 hours of reperfusion, with or without rapamycin. \**P*<0.05 versus NTg. #*P*<0.05 versus Tg Rapamycin. **B,** AAR/LV (left) and infarct size/AAR (right) in wild-type (WT) mice and *beclin1*<sup>+/-</sup> mice after ischemia/reperfusion (I/R). SB, SB216763; Rap, rapamycin. \**P*<0.05 versus control. #*P*<0.05 versus SB. \$*P*<0.05 versus SB+Rap. **C,** GFP-LC3 dots in Tg-GFP-LC3 and DnGSK-3β/GFP-LC3 bigenic mice (Bigenic, DnGSK/GFP-LC3) after 45 minutes of ischemia and 2 hours of reperfusion. Representative images are shown. Scale bar=100 μm. The bar graph shows the number of GFP-LC3 dots per microscopic field. \**P*<0.05 versus GFP-LC3. **D,** The number of GFP-LC3 dots per microscopic field in GFP-LC3 mice and DnGSK/GFP-LC3 bigenic mice, after 45 minutes of ischemia and 2 hours of reperfusion, with or without rapamycin. \**P*<0.05 versus GFP-LC3 control.

GSK-3β has many downstream targets, which can mediate both survival and death of cardiomyocytes. For example, suppression of GSK-3β stabilizes Mcl-1, a Bcl-2 family protein, thereby promoting survival.<sup>6</sup> However, perhaps one of the most important mechanisms by which suppression of GSK-3β mediates cardioprotection during I/R is regulation of mPTP opening and other mitochondrial proteins.<sup>1-3</sup> GSK-3β regulates components of the mPTP through direct phosphorylation, thereby inducing mitochondrial depolarization, release of cytochrome c, and eventual cell death.<sup>1</sup> Interestingly, the enhancement of myocardial injury in response to prolonged ischemia by suppression of GSK-3β was reversed in the presence of rapamycin. Furthermore, the suppression of reperfusion injury by inhibition of GSK-3β was also completely reversed by rapamycin. These results suggest that, besides regulation of the mPTP, modulation of mTOR also plays a significant role in mediating the cardioprotective effects of GSK-3β activation and inactivation during prolonged ischemia and reperfusion, respectively.

Accumulating lines of evidence suggest that modulation of mTOR has a significant influence on I/R injury in the heart. However, the effect of mTOR modulation on myocardial injury appears to be model dependent. The infarct-reducing effects of insulin and ischemic preconditioning are abolished by rapamycin given before the onset of reperfusion in Langendorff-perfused rat hearts.<sup>14</sup> In addition, rapamycin

given prior to preconditioning abolished the second window of ischemic preconditioning in rabbits.<sup>15</sup> On the other hand, rapamycin given to mice 1 hour before ischemia conferred infarct-limiting effects in Langendorff-perfused mouse hearts.<sup>16</sup> In the present study, increased mTOR signaling in *TSC2*<sup>+/-</sup> mice induced a smaller MI after I/R, whereas it induced a greater MI after prolonged ischemia. Interestingly, although the detrimental effect of GSK-3β inhibition during prolonged ischemia was reversed by rapamycin, the cardioprotective effect of GSK-3β inhibition during I/R was also attenuated by rapamycin. Our results clearly suggest that the function of mTOR is distinct between prolonged ischemia and reperfusion.

What is the underlying mechanism mediating the effect of mTOR regulation on myocardial injury during ischemia and reperfusion? The mTOR is an essential regulator of protein synthesis and cardiac hypertrophy.<sup>17</sup> Inhibition of protein synthesis during ischemia is beneficial because protein synthesis not only consumes energy, which is undesirable during ischemia, but also promotes ER stress.<sup>18</sup> On the other hand, activation of mTOR during the reperfusion phase may up-regulate cell survival signaling mechanisms<sup>19</sup> and mitochondrial biogenesis through peroxisome proliferator-activated receptor gamma coactivator-1 alpha and Ying Yang 1, which may facilitate recovery from myocardial ischemia during reperfusion.<sup>20</sup> Importantly, mTOR inhibits autophagy by



**Figure 8. A schematic showing distinct roles of GSK-3 $\beta$  during ischemia and reperfusion.** Solid line boxes show the role of GSK-3 $\beta$  during ischemia. Dotted line boxes show the role of GSK-3 $\beta$  during reperfusion.

negatively regulating the association between autophagy-related gene 1 (ATG1) and ATG13,<sup>21</sup> which affects survival and death of cardiomyocytes during ischemia and reperfusion in a phase-dependent manner.<sup>5</sup>

Autophagy is an intracellular degradation process through which long-lived cytosolic proteins and organelles are degraded by lysosomes and recycled.<sup>22</sup> Hypoxia/reoxygenation and I/R are among the stresses known to prominently activate autophagy in the heart.<sup>5</sup> Autophagy appears to be protective when it is activated during transient ischemia,<sup>5</sup> ischemic preconditioning,<sup>23</sup> and myocardial hibernation,<sup>24</sup> possibly by regenerating ATP and removing protein aggregates and damaged organelles, including mitochondria. On the other hand, excessive activation of autophagy during reperfusion may lead to cell death,<sup>5</sup> although autophagy also plays a salutary role during myocardial reperfusion in some experimental conditions.<sup>25</sup> Our results suggest that GSK-3 $\beta$  inhibition and the resultant mTOR-dependent attenuation of autophagy are detrimental during prolonged ischemia, whereas they are protective during reperfusion. Furthermore, inhibition of mTOR signaling by rapamycin reversed the inhibitory effect of GSK-3 $\beta$  inhibition on autophagy and, at the same time, abolished the effect of GSK-3 $\beta$  inhibition on myocardial ischemic injury and reperfusion injury. These findings suggest that GSK-3 $\beta$  modulates autophagy through mTOR-dependent mechanisms, thereby mediating survival and death of cardiomyocytes in a phase-dependent manner. The fact that heterozygous *beclin 1* knock-out, which inhibits autophagy, canceled the effect of rapamycin on GSK-3 $\beta$  inhibition during prolonged ischemia and I/R further indicates that GSK-3 $\beta$  inhibition indeed modulates myocardial injury due to prolonged ischemia and I/R through mTOR-

dependent regulation of autophagy. Regulation of mTOR and that of mPTP opening may not be mutually exclusive as mechanisms mediating the effect of GSK-3 $\beta$  on myocardial injury/protection. For example, mTOR-mediated regulation of mitochondrial genes<sup>20</sup> may indirectly affect mPTP opening, with mPTP opening being a critical mediator of mitochondrial autophagy.<sup>26</sup>

In summary, GSK-3 $\beta$  plays an important role in modulating mTOR during both prolonged ischemia and reperfusion in the heart. Changes in autophagy contribute to the differential effects of GSK-3 $\beta$  on myocardial injury during prolonged ischemia and I/R. Inhibition of GSK-3 $\beta$  by a small molecule inhibitor may be considered to reduce reperfusion injury due to its inhibitory effects on mPTP opening. However, if ischemia is prolonged, GSK-3 $\beta$  inhibition may exacerbate myocardial injury before the heart is reperfused. In this case, an alternative strategy might be to limit the timing of GSK-3 $\beta$  inhibition to the time of reperfusion only.

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### Disclosures

None.

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## Novelty and Significance

### What Is Known?

- Inhibition of glycogen synthase kinase-3 (GSK-3) attenuates ischemia/reperfusion (I/R) injury by inhibiting mitochondrial permeability transition pore (mPTP) opening.
- Autophagy is activated by both myocardial ischemia and reperfusion and affects survival and death of cardiac myocytes.

### What New Information Does This Article Contribute?

- Activation of the  $\beta$ -isoform of GSK-3, GSK-3 $\beta$ , is protective during ischemia, whereas it is detrimental during reperfusion.
- GSK-3 $\beta$  modulates autophagy through inhibition of mTOR, which significantly affects survival and death of cardiac myocytes during both prolonged ischemia and reperfusion.

Although inhibition of GSK-3 during I/R protects the heart by inhibiting mPTP opening, the function of endogenous GSK-3 $\beta$  during prolonged ischemia and during reperfusion had not been addressed separately. In this study, using genetically altered

mouse models, in which the activity of the  $\beta$ -isoform of GSK-3 is modulated, we investigated the effect of GSK-3 $\beta$  on survival and death of cardiac myocytes during ischemia and reperfusion. Endogenous GSK-3 $\beta$  is activated during prolonged ischemia, whereas it is inhibited during reperfusion. These phase-dependent responses of endogenous GSK-3 $\beta$  appear adaptive because GSK-3 $\beta$  activation during ischemia stimulates autophagy, whereas GSK-3 $\beta$  inhibition during reperfusion inhibits autophagy through modulation of mTOR, both of which significantly reduce myocardial injury. These results suggest that the function of GSK-3 $\beta$  during ischemia and reperfusion is phase dependent and that regulation of autophagy through modulation of mTOR significantly contributes to the effect of GSK-3 $\beta$  on survival and death of cardiac myocytes. Inhibition of GSK-3 $\beta$  by a small molecule inhibitor has been considered to reduce reperfusion injury. However, if ischemia is prolonged, GSK-3 $\beta$  inhibition may exacerbate myocardial injury before the heart is reperfused. Thus, the timing of GSK-3 $\beta$  inhibition should be limited to the time of reperfusion only.

## 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 <sup>1</sup>.

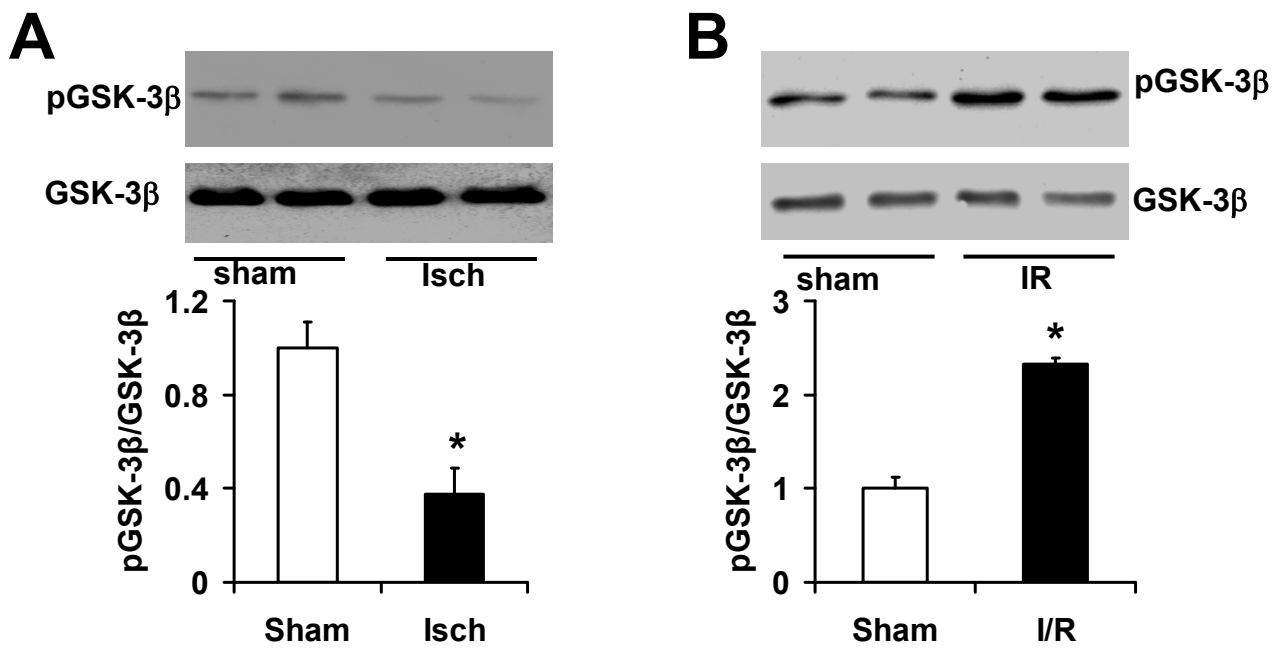
**Hairpin-2 staining** A double stranded DNA fragment with blunt ends was prepared as previously described<sup>2-4</sup>. Polymerase chain reaction (PCR) with Pfu Ultra polymerase was performed with 16.6 µmol/L Texas Red-12-dUTP (Molecular Probes), 16.6 µmol/L dTTP, 50 µmol/L dATP, 50 µmol/L dCTP and 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<sup>5-7</sup>. 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<sub>2</sub>, 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 <sup>8</sup>.

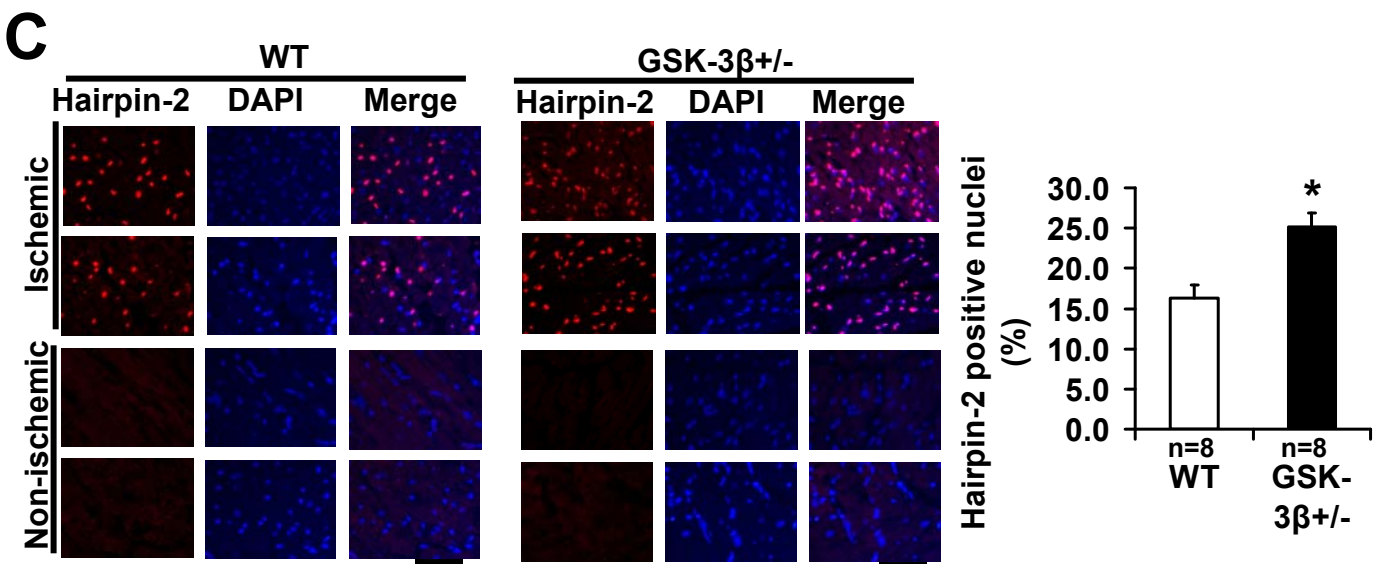
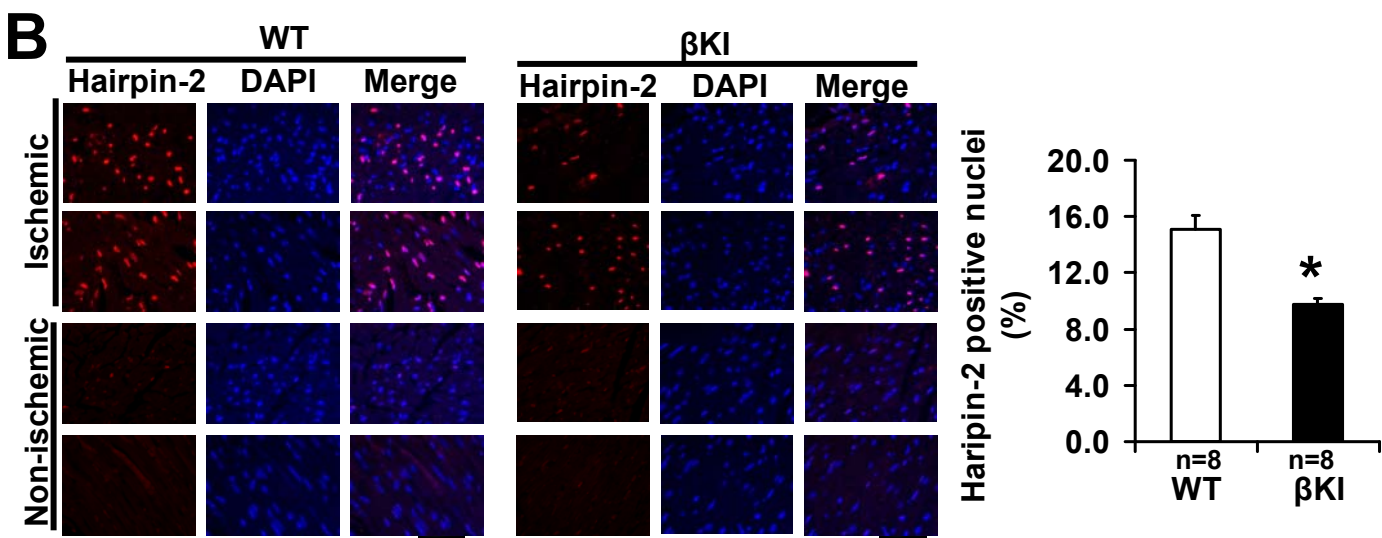
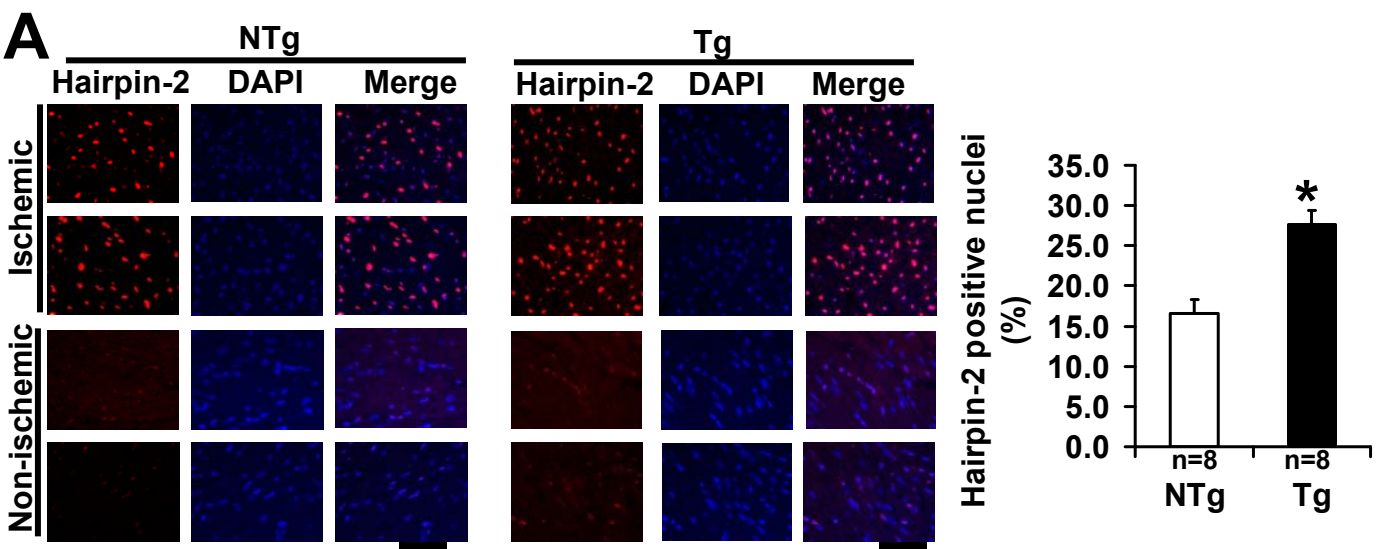
**Immunoblotting** The ischemic myocardium was isolated and homogenized in RIPA buffer. Immunoblotting using phospho-p70 S6 kinase (S6K), total S6K, and p62 primary antibodies was performed as previously described <sup>1</sup>.

### Supplemental References

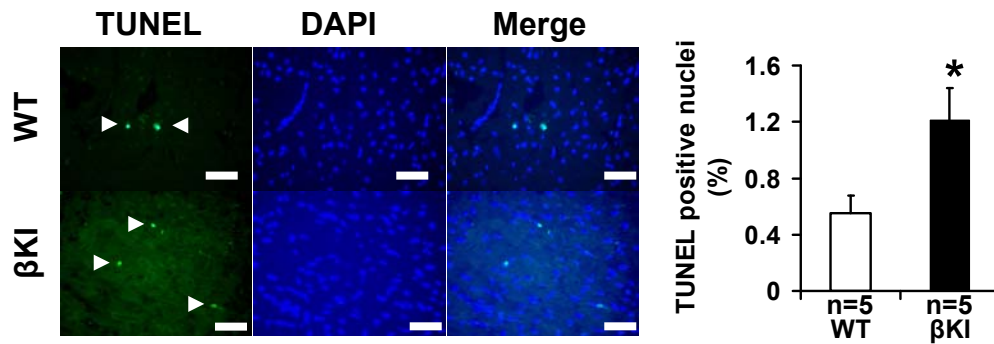
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**Online Figure I.** Phosphorylation of GSK-3 $\beta$  after 30 min of ischemia (A) and after 20 min of ischemia and 30 min of reperfusion (B) in C57BL/6J mice. \*P<0.01 vs. respective Sham. Data are mean  $\pm$  SEM.

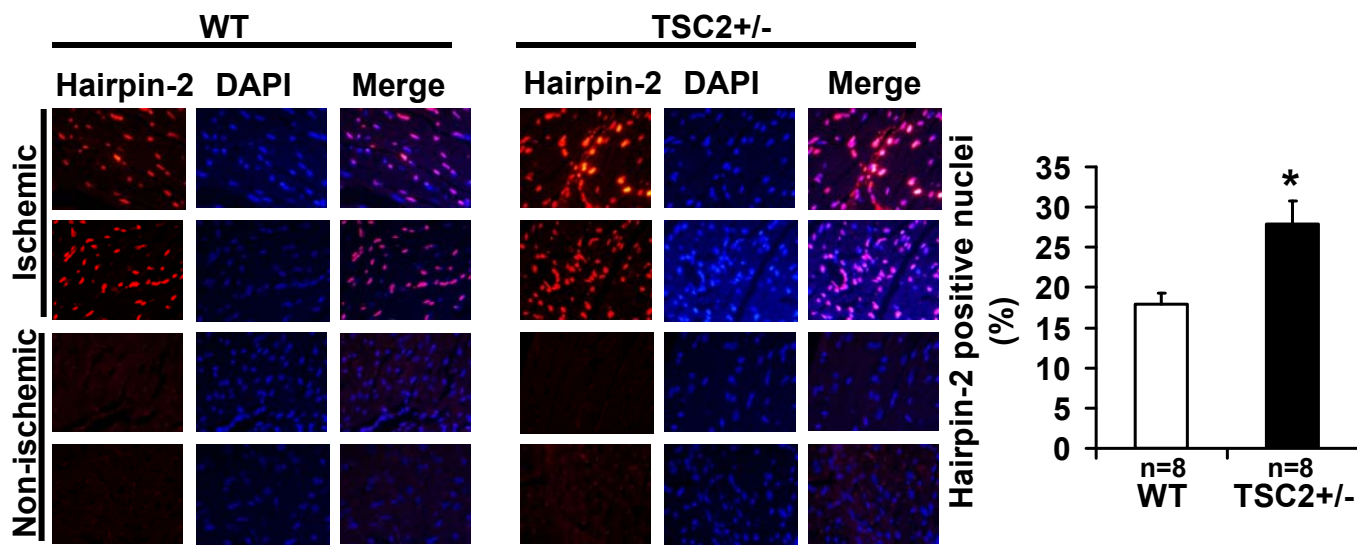


**Online Figure II.** The role of GSK-3 $\beta$  in modulating myocardial injury caused by prolonged ischemia. In A-C, images of Hairpin-2 staining of cardiac tissue sections from animals 2 hours after myocardial ischemia are shown. Scale bar = 100  $\mu$ m. The percentages of Hairpin-2 positive nuclei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

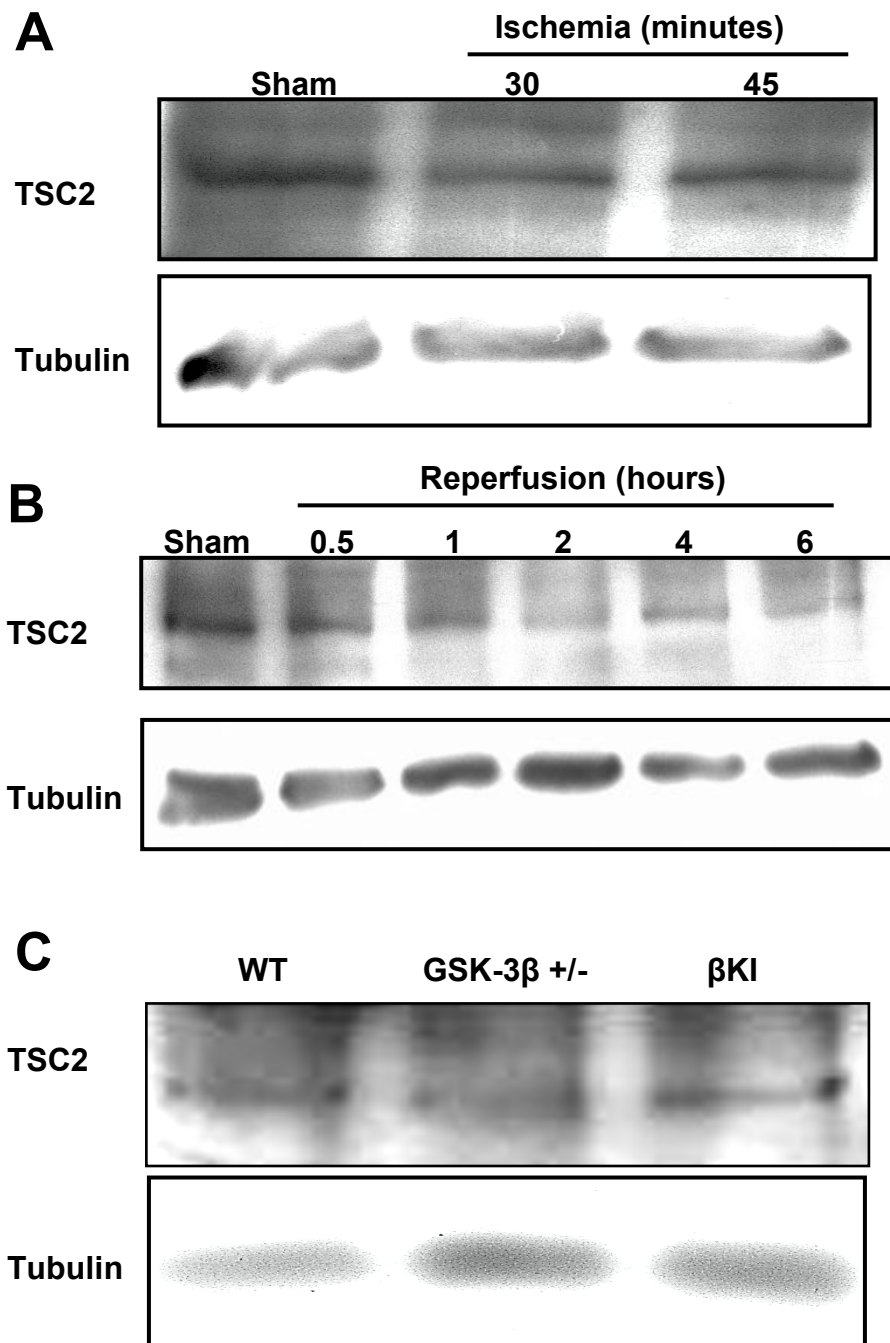


**Online Figure III.** Apoptosis in constitutively active GSK-3 $\beta^{S9A}$  knock-in mice ( $\beta$ KI). Images were taken from TUNEL and DAPI stained cardiac sections. Scale bar = 50  $\mu$ m. The bar graph shows the percentage of TUNEL positive nuclei. Data are mean  $\pm$  SEM. \*P<0.05 vs. WT.





**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  $\mu$ m. The percentages of Hairpin-2 positive nuclei are shown in the bar graph. Data are mean  $\pm$  SEM. \*P<0.01 vs. WT.



**Online Figure V.** TSC2 expression. A. During ischemia. B. During reperfusion. C. At baseline, in GSK-3 $\beta$  heterozygous knock-out mouse heart (GSK-3 $\beta$  +/-) and constitutively active GSK-3 $\beta$ <sup>S9A</sup> knock-in mouse heart ( $\beta$ KI).