Sestrin2 promotes LKB1-mediated AMPK activation in the ischemic heart

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ABSTRACT **The regulation of AMPK in the ischemic heart remains incompletely understood. Recent evidence implicates the role of Sestrin2 in the AMPK signaling pathway, and it is hypothesized that Sestrin2 plays an influential role during myocardial ischemia to promote AMPK activation. Sestrin2 protein was found to be expressed in adult cardiomyocytes and accumulated in the heart during ischemic conditions. Sestrin2 knockout (KO) mice were used to determine the importance of Sestrin2 during ischemia and reperfusion (I/R) injury. When wild-type (WT) and Sestrin2 KO mice were subjected to *in vivo* I/R, myocardial infarct size was significantly greater in Sestrin2 KO compared with WT hearts. Similarly, Langendorff perfused hearts indicated exacerbated postischemic contractile function in Sestrin2 KO hearts compared with WT. Ischemic AMPK activation was found to be impaired in the Sestrin2 KO hearts. Immunoprecipitation of Sestrin2 demonstrated an association with AMPK. Moreover, liver kinase B1 (LKB1), a major AMPK upstream kinase, was associated with the Sestrin2-AMPK complex in a time-dependent manner during ischemia, whereas this interaction was nearly abolished in Sestrin2 KO hearts. Thus, Sestrin2 plays an important role in cardioprotection against I/R injury, serving as an LKB1-AMPK scaffold to initiate AMPK activation during ischemic insults.—Morrison, A., Chen, L. Wang, J., Zhang, M., Yang, H., Ma, Y., Budanov, A., Lee, J. H., Karin, M., Li, J. Sestrin2 promotes LKB1-mediated AMPK activation in the ischemic heart. *FASEB J.* 29, 408–417 (2015). www.fasebj.org**

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AMPK HAS EMERGED as a pertinent stress-activated protein kinase shown to have substantial cardioprotective capabilities against myocardial ischemia/reperfusion (I/R) injury (1–4). When activated during ischemia, AMPK produces effects that have been demonstrated to be beneficial to the myocardium by means of increasing glucose transporter type 4 (GLUT4) translocation and glucose uptake (5–7), activating eNOS (8), decreasing apoptosis (2, 9), and increasing autophagic flux (10). However, the molecular basis behind the regulation of AMPK activity in the ischemic and reperfused heart remains incompletely understood.

AMPK is a heterotrimeric enzyme composed of a catalytic α subunit and regulatory β and γ subunits. Upon an intracellular rise in AMP, AMP binds to the γ subunit and allosterically induces a conformational change in AMPK, rendering it a more suitable substrate for phosphorylation on Thr^{172} on the α subunit by an upstream AMPK kinase (AMPKK) (11, 12). The phosphorylation on Thr^{172} by an AMPKK is suggested to be essential for increasing AMPK's kinase activity (13). Of the 3 known AMPKKs, liver kinase B1 (LKB1) has recently been characterized as a major upstream kinase of AMPK in the heart, as ischemic AMPK activation is nearly abolished in LKB1-deficient mice (14). Paradoxically, studies have shown that myocardial ischemia does not increase the kinase activity of LKB1 to any degree (14–16), which suggests a constitutively active enzyme and the presence of other mechanisms governing LKB1-induced AMPK phosphorylation in the ischemic heart.

A group of proteins known as the Sestrins, particularly Sestrin2 (Sesn2), was recently shown to interact with and activate AMPK *in vitro* and *in vivo*, although they are not

Abbreviations: AAR, area at risk; ACC, acetyl-CoA carboxylase; AICAR, 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide; AMPKK, AMPK kinase; CaMKKβ, Ca^{2+}/calmodulin-dependent protein kinase kinase β; ECG, electrocardiogram; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT4, glucose transporter type 4; I/R, ischemia/reperfusion; KHB, Krebs-Henseleit buffer; KO, knockout; LAD, left anterior descending; LKB1, liver kinase B1; LV, left ventricle; LVDP, left ventricular developed pressure; PV, pressure-volume; RPP, heart rate-LV pressure products; Sesn2, Sestrin2; TTC, 2,3,5-triphenyltetrazolium chloride; WT, wild-type

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protein kinases (17). Studies have also shown that Sesn2-null mouse embryonic fibroblasts and liver from Sesn2 knockout (KO) mice exhibit impaired AMPK activity when subjected to stress (17). However, the mechanism by which Sesn2 stimulates AMPK activation remains elusive. Intriguingly, Sesn2 is induced in response to hypoxic stimuli (18–20) and Sestrin gene deletion in *Drosophila* results in cardiac arrhythmia and impaired AMPK activation (21). The role of Sesn2 in the mammalian heart has not yet been investigated. In this study, we aimed to determine the role of Sesn2 during ischemic insults and investigate how Sesn2 modulates cardiac AMPK activation.

MATERIALS AND METHODS

Animals

Male wild-type (WT) and Sesn2 KO mice (all C57BL/6 background), 4–6 mo of age, were used. Sesn2 KO mice were generated as previously described (17). All animal procedures carried out in this study were approved by the University at Buffalo-State University of New York Institutional Animal Care and Use Committee.

Isolation of cardiomyocytes

Adult cardiomyocytes were harvested as previously described (16, 22). Briefly, mice were anesthetized with sodium pentobarbital (100 mg/kg i.p.), and hearts were rapidly excised and perfused in a Langendorff fashion. Hearts were perfused at 37°C with a Ca^{2+}-free Krebs-Henseleit–based buffer (KHB; pH 7.3) containing 0.6 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid , 4.7 mM KCl, 1.7 mM $MgSO_4$, 120.3 mM NaCl, 4.6 mM $NaHCO_3$, 30 mM taurine, 10 mM glucose, and 10 mM 2,3-butanedione monoxime that was bubbled with 95% O_2/5% CO_2. Perfusion was then initiated with buffer containing 0.067 mg/ml Liberase Blendzyme 4 (Roche, Indianapolis, IN, USA) until the heart became flaccid. After perfusion, the atria were removed, and the left ventricle (LV) was isolated and minced to disperse cardiomyocytes into the Ca^{2+}-free buffer without butanedione monoxime. Extracellular Ca^{2+} was then added slowly and incrementally back to 1 mM.

Cardiac phenotype

Heart weight and body weight were measured and expressed as a percentage of body weight. Hearts were fixed in phosphate-buffered formalin and stained with hematoxylin and eosin to assess differences in physical characteristics (2). For the pressure-volume (PV) studies, mice were anesthetized with inhaled 0.5–1% isoflurane, and body temperature was maintained at 37°C with a heating pad. The heart was then subjected to apical LV cardiac catheterization with a catheter tipped with a 1.2 F micro-transducer (Scisense, London, ON, Canada), which was inserted into the lumen of the LV to assess real-time LV function. Hemodynamic parameters such as heart rate, cardiac output, and ejection fraction were measured and calculated with the iWorx software (Dover, NH, USA).

Assessment of myocardial Infarction

Mice were anesthetized with sodium pentobarbital (100 mg/kg i.p.) and placed on a ventilator (Harvard Rodent Ventilator; Harvard Apparatus, Holliston, MA, USA), and core temperature was maintained at 37°C with a heating pad. After left lateral thoracotomy, the left anterior descending coronary artery was occluded for 20 min with an 8-0 nylon suture and polyethylene tubing to prevent arterial injury and then reperfused for 4 h. An electrocardiogram (ECG) and blanching of the LV confirmed ischemic repolarization changes (ST-segment elevation) during coronary occlusion (ADInstruments, Colorado Springs, CO, USA). The hearts were then excised and stained with 2,3,5-triphenyltetrazolium (TTC) and Evans blue dye to delineate the extent of myocardial necrosis as a percent of the ischemic area at risk (AAR). Hearts were then fixed, sectioned, and photographed with a Leica microscope and analyzed with the ImageJ Software (U.S. National Institutes of Health, Bethesda, MD, USA) (23, 24).

Isolated heart perfusions

Mice were anesthetized with sodium pentobarbital (100 mg/kg i.p.), and hearts were excised and perfused in a Langendorff fashion with a KHB buffer containing 7 mM glucose, 0.4 mM oleate, 1% bovine serum albumin, and 10 μU/ml insulin. Hearts were perfused for 20 min at a flow of 4 ml/min, followed by 20 min of global, no-flow ischemia and 30 min of reperfusion. A fluid-inflated balloon connected to the Chart5 system from ADInstruments was inserted into the LV to measure heart rate and left ventricular developed pressure (LVDP). The balloon was filled to achieve a baseline LV end-diastolic pressure of 5 mm Hg that was kept constant during ischemia and reperfusion (6, 9, 25).

Immunoblotting

Immunoblots and immunoprecipitation were performed as previously described (6, 25, 26). Rabbit antibodies p-AMPKα (Thr^{172}), AMPKα, p-acetyl-CoA carboxylase (ACC) (Ser^{79}), ACC, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and LKB1 were obtained from Cell Signaling (Danvers, MA, USA). Rabbit Sesn2 antibody was obtained from ProteinTech (Chicago, IL, USA). Goat LKB1 (M-18) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse LKB1 and mouse Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) was obtained from Novus Biologicals (Littleton, CO, USA). Mouse FLAG antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

HL-1 cardiomyocytes were given to us as a kind gift from Dr. William Claycomb. Cells were cultured in Claycomb medium supplemented with 10% fetal bovine serum (Sigma-Aldrich and Atlanta Biologicals, Lawrenceville, GA, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM norepinephrine, and 2 mM L-glutamine (Sigma-Aldrich) and were incubated at 37°C with 5% CO_2 (27). HeLa cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated at 37°C with 5% CO_2. Pharmacological treatment with either vehicle (PBS) or 1 mM 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR) (Enzo Life Sciences, Farmingdale, NY, USA) was administered to these cells when 80% confluency was reached. Cells were treated for 30 min at 37°C and then immediately washed with PBS, lysed, and prepared for biochemical analysis. Hypoxia treatment was initiated when 80% cell confluency was reached. Cells dishes were placed into a hypoxia chamber (Billups-Rothenberg, Del Mar, CA, USA), and a tube containing 95% N_2/5% CO_2 hypoxic gas was turned on to flush gas through the chamber for 10 min to completely deoxygenate the chamber. The chamber's gas input/output clamps were then immediately

clamped, and the chamber was put into a 37°C incubator for the desired period of time. At the end of hypoxic incubation, the cells were immediately washed, lysed, and prepared for biochemical analysis.

Transfection

A bacterial stab of the plasmid pcDNA3-FLAG-LKB1 (28) was purchased from Addgene (plasmid 8590; Cambridge, MA, USA). Agar plates containing ampicillin were streaked with the bacteria (*Escherichia coli*) from the stab and incubated overnight at 37°C to obtain single bacterial colonies. A single colony was chosen to inoculate 3 ml of Luria broth containing 100 mg/ml of ampicillin and was shaken at 37°C for 8 h. A starter culture was then prepared for plasmid DNA purification using the HiSpeed Plasmid Maxi Kit (Qiagen, Valencia, CA, USA) per the manufacturer's instructions. After measuring the DNA concentration using a NanoDrop (Thermo Scientific, Rockford, IL, USA), purified plasmid DNA was then transfected to HeLa cells at 70% confluency using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA), and the procedure was carried out according to the manufacturer's instructions.

Cardiac troponin-I assay

Isolated hearts were subjected to I/R in the Langendorff mode. On reperfusion, the perfusate was collected into a beaker that was kept on ice. A 1 ml aliquot of the total perfusate was used for detection of troponin-I using the High Sensitivity Mouse Cardiac Troponin-I ELISA Kit (Life Diagnostics, West Chester, PA, USA) and was assayed according to the manufacturer's instructions as described previously (29).

Statistical analysis

Values are means ± SEM. Data analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Significance was determined by either a 2-tailed, unpaired Student's *t* test or ANOVA using Tukey's posttest. Data from the isolated heart perfusion experiments comparing cardiac function over time were analyzed by 2-way repeated-measures ANOVA. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Sesn2 is expressed in the heart and cardiomyocytes

Previous studies have demonstrated that Sesn2 mRNA is expressed in human and mouse heart tissue (30). To verify that Sesn2 mRNA found in heart tissue translates to protein in the parenchymal cells of the heart, cardiomyocytes and heart homogenates were prepared for immunoblotting. The results shown in **Fig. 1*A, B*** establish that Sesn2 protein is expressed in the adult mammalian heart and cardiomyocytes. The band was confirmed to be Sesn2 with the use of lysates from Sesn2 KO hearts. Because Sesn2 has been demonstrated to be responsive to hypoxic and ischemic conditions (18), Sesn2 was examined for its expression in cardiac tissue at basal and ischemic conditions at different time points in mouse hearts. Interestingly, Sesn2 expression was significantly increased at just 5 min of myocardial ischemia (Fig. 1*C*).

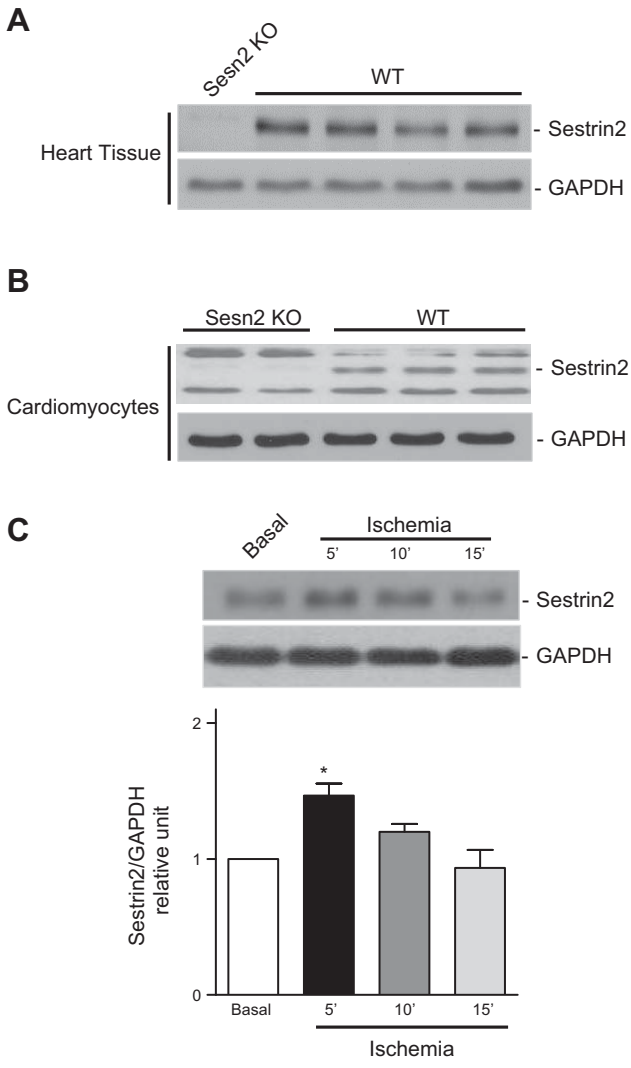

Figure 1. Expression of Sesn2 in cardiac tissue. *A*) Immunoblot of Sesn2 in WT mouse heart tissue collected from the LV. *B*) Immunoblot of Sesn2 in WT primary cardiomyocytes isolated from adult mouse hearts. *C*) Immunoblot of Sesn2 in WT hearts during basal conditions and different time points of myocardial ischemia induced by *in vivo* regional ischemia. Representative blots and the respective quantifications are expressed as a ratio of Sesn2 to GAPDH. Values are means ± SEM for 3–4 independent experiments. $^*P < 0.05$ *vs.* basal.

Effects of Sesn2 on cardiac function during myocardial I/R

Since the generation of Sesn2 KO mice (17), no cardiac phenotype analysis has been conducted to determine any possible morphologic or hemodynamic alterations in these hearts. Histologically, hematoxylin and eosin staining revealed that, structurally, the hearts of WT and Sesn2 KO mice were essentially identical (Supplemental Fig. 1*A*). To determine possible hemodynamic differences at baseline, WT and Sesn2 KO mice were subjected to LV catheterization with a 1.2 F microtransducer (Scisense) to acquire real-time PV relationships. Exemplary PV loops of WT and Sesn2 KO mice demonstrate no significant differences (Supplemental Fig. 1*B*). Moreover, measures

of pump function such as heart rate, cardiac output, and ejection fraction were not significantly different between WT and Sesn2 KO mice (Supplemental Fig. 1*B*).

To test the hypothesis that Sesn2 is an important stress-responsive protein with cardioprotective capabilities against ischemic injury, WT and Sesn2 KO mice were subjected to *in vivo* regional I/R to assess the extent of myocardial necrosis. After 20 min of left anterior descending artery (LAD) ligation followed by 4 h of reperfusion, Sesn2 KO hearts demonstrated a significant increase in myocardial infarct size compared with WT hearts (**Fig. 2*A***). Isolated hearts from WT and Sesn2 KO mice were also subjected to Langendorff heart perfusion to further investigate the ability of Sesn2 to protect the heart against ischemic injury and improve postischemic cardiac function. Hearts were perfused for 20 min at basal conditions followed by 20 min of global, no-flow ischemia, and were then reperfused for 30 min (Fig. 2*B*). Compared with WT hearts, postischemic cardiac function in Sesn2 KO hearts was significantly impaired during reperfusion as demonstrated by decreased LVDP, dP/dt, and heart rate-LV pressure products (RPPs) (Fig. 2*B*). There were no significant changes in heart rate between WT and Sesn2 KO hearts (Fig. 2*B*), suggesting that the impairment in cardiac function after ischemic insults could not be ascribed to changes in chronotropic effects. Because I/R has been demonstrated to cause myocardial stunning where salvaged myocytes display a prolonged period of contractile dysfunction despite the absence of irreversible damage (31), the question was then asked as to whether the impaired recovery of function in the Sesn2 KO hearts was due to either myocardial stunning or exacerbated cell death. Both WT and Sesn2 KO hearts were stained with TTC to determine the extent of necrosis, and myocardial infarction size was markedly increased in the Sesn2 KO hearts (Fig. 2*C*). As the amount of myocardial infarction correlates to the amount of cardiac-specific troponin-I released from cardiomyocytes (32), the amount of troponin-I release into the perfusate was measured and was found to be significantly elevated to the same degree as the extent of myocardial infarct size in Sesn2 KO hearts (Fig. 2*D*). We also observed in the isolated Langendorff hearts that as the ischemic time was prolonged, the end-diastolic pressure in the Sesn2 KO hearts began to increase at a significantly higher rate than the WT hearts, suggesting impaired ischemic myocardial energetics in the absence of Sesn2 (Fig. 2*E*).

Sesn2 modulates AMPK activation in the ischemic heart

In line with the hypothesis of impaired ischemic myocardial energetics in the Sesn2 KO hearts, the question was asked as to whether Sesn2 could be modulating AMPK activation to promote cardioprotection because AMPK can improve myocardial energetics by increasing glucose uptake (2, 6) and activating phosphofructokinase-2 to increase rates of glycolysis and ATP generation during ischemia (33). Isolated WT and Sesn2 KO hearts perfused in the Langendorff mode were subjected to either basal or ischemic conditions and prepared for immunoblotting. Interestingly, ischemic AMPK phosphorylation was significantly impaired in Sesn2 KO hearts (**Fig. 3*A***). The ability of AMPK to phosphorylate its immediate downstream target ACC was also significantly impaired in Sesn2 KO hearts during ischemia (Fig. 3*A*). Specifically, the effects of both AMPKα1 and AMPKα2 to phosphorylate ACC were determined by blotting for p-ACC (Ser^{79}) in the respective immune complexes from the basal and ischemic heart samples. ACC phosphorylation by both AMPKα1 and AMPKα2 catalytic subunits was markedly reduced in Sesn2 KO hearts (Fig. 3*B*).

Sesn2 is a scaffold for LKB1 and AMPK during ischemia

To better understand the activation of AMPK during the time course of ischemia and reperfusion, phosphorylation on Thr^{172} of the α-catalytic subunit was detected at progressive time points during ischemia and I/R. Compared with baseline, there is a dramatic and robust increase in the phosphorylation status of AMPK during ischemia, and during reperfusion, AMPK phosphorylation returns rapidly back to baseline (**Fig. 4*A***). As other reports have demonstrated evidence of a Sesn2-AMPK complex *in vitro* and *in vivo* (17, 34), it was sought to determine whether this same interaction exists in the heart. To examine this, Sesn2 was immunoprecipitated from heart tissue lysates that underwent the time course of ischemia and I/R. The immunoblots of Sesn2 immunoprecipitates demonstrated that Sesn2 does indeed form a complex with AMPKα in the heart (Fig. 4*A*). Intriguingly, when detecting AMPK phosphorylation in the Sesn2 immune complexes, there is minimal interaction during basal conditions, although with progressive ischemic time, the interaction increases and approaches basal interactions during reperfusion (Fig. 4*A*). It was then asked how Sesn2 could be influencing AMPK activation, as it does not possess kinase characteristics. Sesn2 immune complexes revealed a striking interaction between Sesn2 and LKB1 that occurs at the onset of ischemia and becomes weakened on reperfusion (Fig. 4*A*). Moreover, these events mirror the kinetics of AMPK phosphorylation observed in the total lysates, suggesting that Sesn2 is an ischemic-induced scaffold that initiates AMPK phosphorylation via the recruitment of LKB1.

To confirm that Sesn2 was indeed promoting AMPK activation via a complex with LKB1 and AMPKα during ischemia, WT and Sesn2 KO hearts were subjected to myocardial ischemia, and LKB1 was then immunoprecipitated from these heart lysates. The LKB1 immune complexes revealed an increased LKB1-AMPKα interaction during ischemia compared with basal conditions in WT hearts. However, in the Sesn2 KO hearts, there was little association between LKB1 and AMPKα during ischemia, demonstrating that this mechanism is mitigated in the absence of Sesn2 (Fig. 4*B*). The depleted interaction between LKB1 and AMPKα during ischemia could have been potentially due to decreased LKB1 expression in the Sesn2 KO hearts. Therefore, LKB1 expression and the expression of LKB1 partners, mouse protein 25α and STE20-related adaptor protein α, were examined in WT and Sesn2 KO hearts, and no differences were found (Supplemental Fig. 2*A*). To rule out the

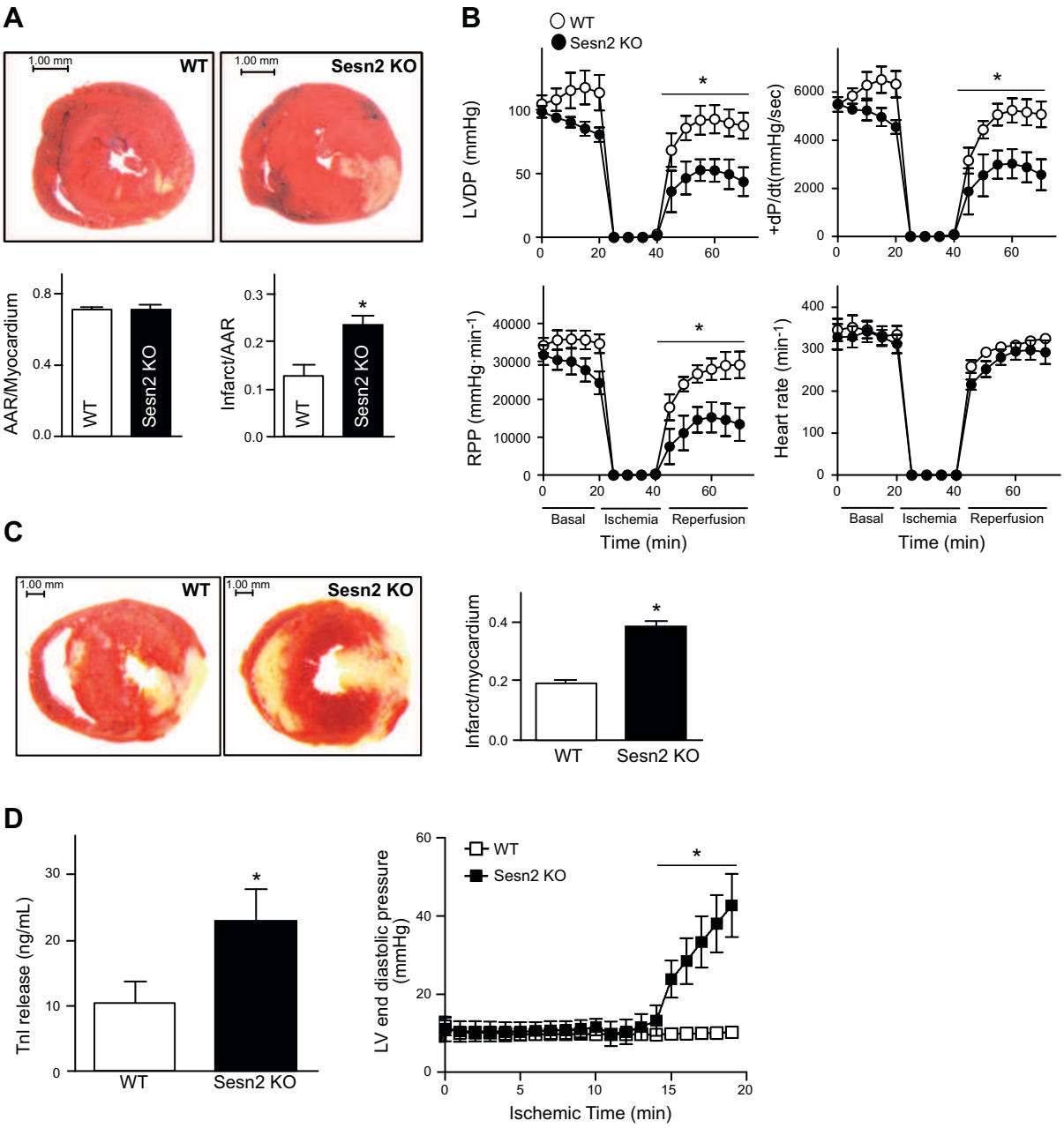

Figure 2. Effects Sesn2 on cardiac function in response to I/R. *A*) WT and Sesn2 KO mouse hearts were subjected to 20 min of ischemia followed by 4 h of reperfusion via LAD ligation *in vivo*. Myocardial infarct size is presented as a percent of the AAR. Representative sections of the extent of myocardial infarction (upper); ratio of the AAR to total myocardium (left) and ratio of the infarcted area to the AAR (right). Values are means ± SEM for 4–5 independent experiments. $*P < 0.05$ *vs.* WT. *B*) Isolated WT and Sesn2 KO hearts were perfused using the Langendorff method *ex vivo* at basal conditions for 20 min, subjected to 20 min of global, no-flow ischemia, and then reperfused for 30 min. Cardiac function during experiments is shown by the following: LVDP (upper left), dP/dt, RPP (lower left), and heart rate (lower right). Values are means ± SEM; $*P < 0.05$, $n = 5$–6 per group. *C*) Isolated WT and Sesn2 KO hearts were perfused *ex vivo* for 20 min, subjected to 20 min of global, no-flow ischemia, and then reperfused for 30 min. Representative TTC-stained sections of WT and Sesn2 KO hearts show the extent of myocardial necrosis (left) and ratio of the myocardial infarct area to the total myocardium (right). Values are means ± SEM; $*P < 0.05$ *vs.* WT, $n = 5$–6 per group. *D*) Measurement of total cardiac troponin-I released into the coronary effluent at the end of reperfusion. Values are means ± SEM; $*P < 0.01$, $n = 4$–6 per group. *E*) LV end-diastolic pressure in WT and Sesn2 KO hearts during the time course of myocardial ischemia *ex vivo*. Values are means ± SEM; $*P < 0.05$, $n = 5$–6 per group.

possibility of another known upstream kinase being present in the complex with Sesn2 that could trigger AMPK phosphorylation, Sesn2 immune complexes were probed for CaMKKβ (Supplemental Fig. 2*B*), and the absence of CaMKKβ was suggestive of an LKB1-dependent mechanism.

LKB1 phosphorylates AMPK during hypoxia when complexed with Sesn2

To directly determine whether LKB1 was actually phosphorylating AMPKα (Thr^{172}) when associated

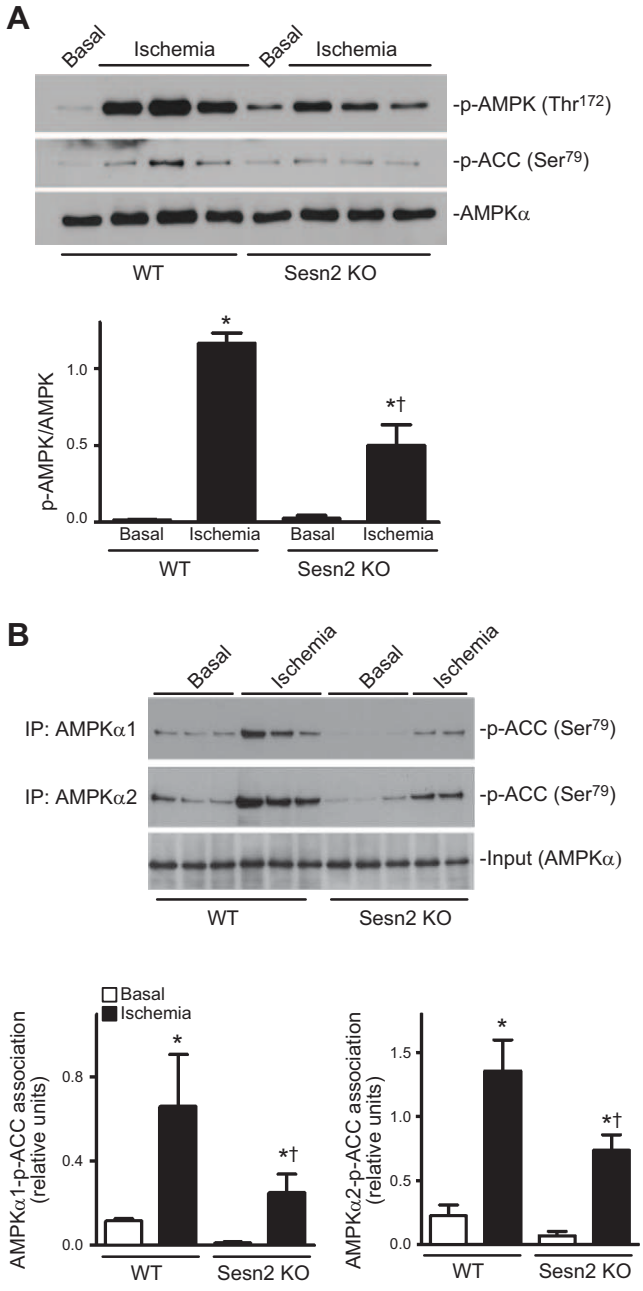

Figure 3. Effect of Sesn2 on AMPK activation in the ischemic heart. *A*) Immunoblots of p-AMPKα (Thr^{172}), p-ACC (Ser^{79}), and AMPKα from WT and Sesn2 KO heart tissue during basal conditions or myocardial ischemia (upper), and bar graphs show the ratio of phosphorylated to total AMPK (lower). Values are means ± SEM for 3 independent experiments, *$P < 0.05$ *vs.* basal; †$P < 0.05$ *vs.* WT ischemia. *B*) Immunoblots of p-ACC (Ser^{79}) from WT and Sesn2 KO heart tissue subjected to myocardial ischemia. AMPKα1 or AMPKα2 was immunoprecipitated from the heart tissue homogenates to show the association of p-ACC during basal or ischemic conditions (upper), and bar graphs show the quantification of the amount of p-ACC associated with AMPKα1 and AMPKα2, respectively (lower). Values are means ± SEM, *$P < 0.05$ *vs.* basal; †$P < 0.05$ *vs.* WT ischemia; $n = 3$ per group.

with this complex, HeLa cells were used as the model system for these experiments as HeLa cells do not express LKB1 (35), although they do express AMPK and Sesn2 (Supplemental Fig. 3*A*). To mimic ischemic stress, HeLa cells were subjected to hypoxic conditions and analyzed for AMPK phosphorylation. As expected, there was no evidence of AMPK activation during hypoxia (Supplemental Fig. 3*A*). Alternatively, when cells such as the HL-1 cardiomyocytes (27), which express LKB1, AMPK, and Sesn2, were subjected to hypoxia, a robust phosphorylation of AMPK was seen (Supplemental Fig. 3*B*). To address the question of whether or not hypoxia-induced AMPK phosphorylation could be restored in HeLa cells when LKB1 expression is reinstated, HeLa cells were transfected with a FLAG-tagged LKB1 plasmid (Fig. 4*C*). HeLa cells with or without LKB1 expression were then subjected to a time course of normoxic or hypoxic conditions of 6 or 9 h. Interestingly, in the HeLa cells that were transfected with LKB1, AMPK phosphorylation was increased time dependently during the hypoxic treatment (Fig. 4*C*). To determine whether LKB1 was responsible for phosphorylating AMPK when complexed with Sesn2, Sesn2 was immunoprecipitated from the HeLa cell lysates with or without LKB1 that were subjected to normoxia or hypoxia for 6 or 9 h. Both FLAG and LKB1 were found to coprecipitate with Sesn2 only in the HeLa cells that expressed LKB1 (Fig. 4*D*). Interestingly, p-AMPKα (Thr^{172}) associated with Sesn2 and increased time dependently with hypoxic treatment. Although LKB1 was associated with Sesn2 during both normoxic and hypoxic conditions, AMPK phosphorylation was only triggered on hypoxic stimulus (Fig. 4*D*).

DISCUSSION

The results of this study reveal the importance of Sesn2 in the heart during myocardial I/R injury. First, it is now established that Sesn2 is expressed in adult mammalian cardiomyocytes and is responsive to ischemic conditions in cardiac tissue. The results also elucidate the fact that Sesn2 is an essential part of the adaptive response to I/R as Sesn2 KO hearts display exacerbated myocardial infarction and impaired post-ischemic cardiac function as indicated by the *in vivo* and *ex vivo* heart perfusion experiments. Furthermore, we identified a unique mechanism by which Sesn2 promotes AMPK activation during myocardial ischemia where Sesn2 acts as an ischemia-induced scaffold protein to initiate AMPK phosphorylation via a time-dependent interaction with LKB1.

Sesn2 is a member of a highly conserved group of individual proteins collectively known as the Sestrins (36) and is expressed in various tissues including the heart. The induction and regulation of Sesn2 expression has previously been demonstrated to be stress inducible and initiated as a result of DNA damage and oxidative stress via a p53-dependent manner (17). Previous studies have attributed Sesn2 to many important cellular processes and functions such as autophagy (37), metabolism (38), and reactive oxygen species quenching (39). Moreover, Sesn2 is also induced in response to hypoxia, simulated ischemic

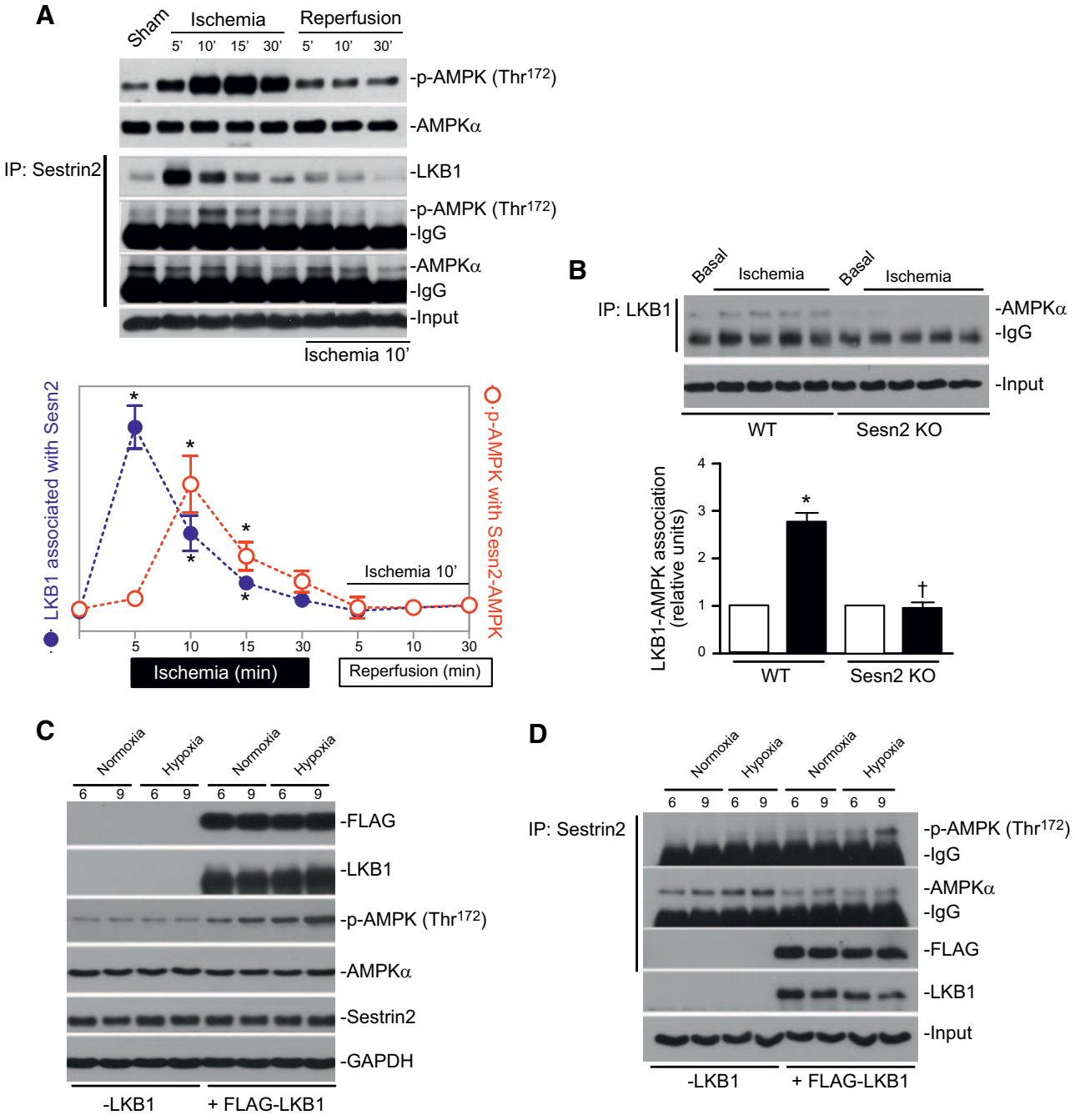

Figure 4. Sesn2 is a scaffold for LKB1 and AMPK in response to ischemic stress. *A*) Immunoblots of p-AMPKα (Thr^{172}) and AMPKα from WT heart tissue baseline, ischemia, or I/R (top 2 panels); Sesn2 was immunoprecipitated from the heart tissue homogenates to show the association of Sesn2 with LKB1, p-AMPKα, and AMPK*α* where indicated. The curve shows the quantification of the amount of LKB1 associated with Sesn2 is expressed relative to the amount protein input used for the IP, and the amount of p-AMPK associated with Sesn2 is expressed relative to the amount protein input used for the IP. Values are means ± SEM; $*P < 0.05$ *vs.* basal; $n = 3$ per group. *B*) Immunoblot of AMPKα from LKB1 immune-complexes during basal or ischemic conditions in WT and Sesn2 KO hearts. *C*) Immunoblots of FLAG, LKB1, p-AMPK*α* (Thr^{172}), AMPKα, Sesn2, and GAPDH in HeLa cells transfected with a FLAG-tagged LKB1 plasmid that were exposed to 6 or 9 h of normoxia or hypoxia. *D*) Immunoblots of p-AMPKα (Thr^{172}), AMPKα, FLAG, and LKB1 from Sesn2 immune-complexes in HeLa cells transfected with LKB1 plasmid that were exposed to 6 or 9 h of normoxia or hypoxia.

conditions such as hypoxia/glucose deprivation, and cerebral ischemia (18–20). Consistent with these results, there was a slight but significant increase in Sesn2 accumulation during myocardial ischemia. Studies suggest that Sesn2 is a target of hypoxia-inductible factor-1, which is rapidly activated during myocardial ischemia (16), as experiments conducted in hypoxia-inductible factor-1*α*–depleted cells demonstrate an absence of or diminished Sesn2 expression (19, 20). Alternatively, it is also likely that the rapid

accumulation of Sesn2 is due to a posttranslational mechanism. Sesn2 does contain several phosphorylation sites that may be of importance in terms of promoting its stabilization or preventing degradation (18).

Elegant studies creating a cardiac-specific Sestrin KO in *Drosophila* (because *Drosophila* express only one Sestrin gene) have demonstrated that Sestrin plays a significant role in maintaining basal cardiac integrity as Sestrin-deficient hearts display increased arrhythmias, bradycardia, disrupted myofibrils, and defective autophagy (21). In the current study, we did not detect any significant changes in the basal cardiac phenotype between WT and Sesn2 KO mice that may have influenced any sensitivity when the ischemic insults were conducted. Because Sesn2 is a stress-inducible protein, these results were not surprising, as we did not expect any significant changes when examining basal cardiac hemodynamics. Furthermore, as there are 2 other Sestrin genes (1, 3), there is likely to be compensation maintaining basal cardiac integrity, warranting future investigations with other Sestrin KOs or combinations thereof. Despite the absence of any basal cardiac phenotype abnormalities, subjecting Sesn2 KO hearts to myocardial ischemia and subsequent reperfusion demonstrated exacerbated myocardial infarction and impaired contractile function compared with WT hearts. Therefore, Sesn2 is cardioprotective in nature, as the reason for impaired cardiac function after I/R was due to increased cell death and not aggravated myocardial stunning as demonstrated by TTC staining and increased troponin-I release. Sesn2 is thus a significant Sestrin in the heart during ischemic stress and, to the best of our knowledge, the only Sestrin to date implicated in the hypoxic response.

The results presented here indicate that the mechanism of increased sensitivity to I/R injury in the Sesn2 KO hearts is due to the significant impairment of ischemic AMPK activation as evidenced by depleted AMPK and ACC phosphorylation. Moreover, we have identified that the mechanism of Sesn2-induced AMPK activation occurs through the cooperative interaction with LKB1 during ischemia. It is important to note, however, that moderate and significant AMPK activation still exists in the absence of Sesn2 in the heart, suggesting that this is not the sole mechanism for how LKB1 might stimulate AMPK. Similarly, as LKB1 primarily affects AMPKα2 activity (14), in our hands, both α1 and α2 were affected with Sesn2 deficiency. Our findings are in agreement with studies that have also detected an apparent Sesn2-AMPK complex (17, 34). It was initially postulated that because Sesn2 and AMPK are associated in a complex and kinase activity is not possessed by Sesn2, Sesn2 could enhance AMPK activity simply by induced autophosphorylation, as LKB1 was not found to be in Sesn2 immune complexes (17). We provide evidence that LKB1 is recruited to this complex during myocardial ischemia in a robust time-dependent manner to phosphorylate AMPKα, suggesting that this induced protein-protein interaction is very sensitive to ischemic conditions, which may serve to enhance the stability of Sesn2. Interestingly, the ischemia-induced LKB1-AMPKα interaction was significantly blunted in the Sesn2 KO hearts, suggesting the pivotal role of Sesn2 as a scaffold protein to modulate this reaction in the heart. The results of the LKB1 transfection experiments conducted in HeLa cells were able to confirm the molecular mechanism of Sesn2 as a scaffold to induce the phosphorylation of AMPK by LKB1. Due to the overexpression of LKB1 in these cells, there was an apparent competition between LKB1 and AMPKα to associate with Sesn2, demonstrating the robustness of the apparent Sesn2-LKB1 interaction and cooperation in stimulating AMPK activation. Despite reduced Sesn2-AMPK association in the presence of increased LKB1, phosphorylation of AMPK was still triggered by LKB1 in this complex, however, only when the cells were exposed to the hypoxic stimulus.

A recent paper by Sanli and colleagues (34) demonstrated results complimentary to ours that further indicates the role of Sesn2 as an LKB1 scaffold to trigger AMPK activation in cells exposed to ionizing radiation. Other studies also demonstrated the capacity of AMPK to cooperate with scaffold proteins, such as transforming growth factor-β–activated protein kinase 1-binding protein 1, in the ischemic heart, which in this complex, recruits and activates p38 MAPK to cause increased glucose uptake during myocardial ischemia (26). Moreover, scaffold proteins are thought to be key mechanisms for insulating the particular target kinase and preventing "signal drifting" from adjacent signaling pathways (40, 41). Therefore, in the case of an upstream kinase such as LKB1, Sesn2 provides a structural platform whereby LKB1 is able to phosphorylate AMPK on either hypoxic or ischemic stimuli.

Previous studies have found that AMP is not required for Sesn2-induced AMPK activation, as the degree of AMPK phosphorylation in control compared with Sesn2-deficient cells when treated with AICAR remains the same (17). The results obtained in this study are consistent with these results in that AMP is not required for Sesn2-induced AMPK phosphorylation because Sesn2 KO cardiomyocytes treated with AICAR demonstrated significant AMPK phosphorylation (Supplemental Fig. 4*A*) to the same degree as WT cardiomyocytes. Complementary to these results, in HeLa cells lacking the expression of LKB1, we observed no increase in AMPK activation when exposed to hypoxia (Supplemental Fig. 3*A*), whereas HeLa cells treated with AICAR still demonstrated an increase in AMPK phosphorylation (Supplemental Fig. 4*B*). Therefore, LKB1-mediated AMPK activation through the scaffolding mechanism of Sesn2 suggests a dependence on hypoxic or ischemic stimuli rather than increased concentrations of AMP. Nevertheless, LKB1-mediated AMPK phosphorylation can also be potentiated in an AMP-dependent manner (12, 14), which may be independent of Sesn2.

In summary, we demonstrated that Sesn2 is a significant player in the adaptive response to ischemic injury by influencing AMPK activation. Further understanding of AMPK signal transduction remains an important feat for the development of novel therapeutic strategies against I/R injury. Future studies are warranted to

determine how Sesn2-mediated AMPK activation directs AMPK signaling to achieve a cardioprotective response. FJ

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REFERENCES

1. Young, L. H. (2008) AMP-activated protein kinase conducts the ischemic stress response orchestra. *Circulation* **117**, 832–840
2. Russell III, R. R., Li, J., Coven, D. L., Pypaert, M., Zechner, C., Palmeri, M., Giordano, F. J., Mu, J., Birnbaum, M. J., and Young, L. H. (2004) AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. *J. Clin. Invest.* **114**, 495–503
3. Morrison, A., and Li, J. (2011) PPAR-γ and AMPK—advantageous targets for myocardial ischemia/reperfusion therapy. *Biochem. Pharmacol.* **82**, 195–200
4. Moussa, A., and Li, J. (2012) AMPK in myocardial infarction and diabetes: the yin/yang effect. *Acta Pharmaceutica Sinica B* **2**, 368–378
5. Miller, E. J., Li, J., Leng, L., McDonald, C., Atsumi, T., Bucala, R., and Young, L. H. (2008) Macrophage migration inhibitory factor stimulates AMP-activated protein kinase in the ischaemic heart. *Nature* **451**, 578–582
6. Wang, J., Tong, C., Yan, X., Yeung, E., Gandavadi, S., Hare, A. A., Du, X., Chen, Y., Xiong, H., Ma, C., Leng, L., Young, L. H., Jorgensen, W. L., Li, J., and Bucala, R. (2013) Limiting cardiac ischemic injury by pharmacological augmentation of macrophage migration inhibitory factor-AMP-activated protein kinase signal transduction. *Circulation* **128**, 225–236
7. Costa, R., Morrison, A., Wang, J., Manithody, C., Li, J., and Rezaie, A. R. (2012) Activated protein C modulates cardiac metabolism and augments autophagy in the ischemic heart. *J. Thromb. Haemost.* **10**, 1736–1744
8. Li, J., Hu, X., Selvakumar, P., Russell III, R. R., Cushman, S. W., Holman, G. D., and Young, L. H. (2004) Role of the nitric oxide pathway in AMPK-mediated glucose uptake and GLUT4 translocation in heart muscle. *Am. J. Physiol. Endocrinol. Metab.* **287**, E834–E841
9. Kim, A. S., Miller, E. J., Wright, T. M., Li, J., Qi, D., Atsina, K., Zaha, V., Sakamoto, K., and Young, L. H. (2011) A small molecule AMPK activator protects the heart against ischemia-reperfusion injury. *J. Mol. Cell. Cardiol.* **51**, 24–32
10. Matsui, Y., Takagi, H., Qu, X., Abdellatif, M., Sakoda, H., Asano, T., Levine, B., and Sadoshima, J. (2007) Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. *Circ. Res.* **100**, 914–922
11. Hawley, S. A., Davison, M., Woods, A., Davies, S. P., Beri, R. K., Carling, D., and Hardie, D. G. (1996) Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. *J. Biol. Chem.* **271**, 27879–27887
12. Baron, S. J., Li, J., Russell III, R. R., Neumann, D., Miller, E. J., Tuerk, R., Wallimann, T., Hurley, R. L., Witters, L. A., and Young, L. H. (2005) Dual mechanisms regulating AMPK kinase action in the ischemic heart. *Circ. Res.* **96**, 337–345
13. Stein, S. C., Woods, A., Jones, N. A., Davison, M. D., and Carling, D. (2000) The regulation of AMP-activated protein kinase by phosphorylation. *Biochem. J.* **345**, 437–443
14. Sakamoto, K., Zarrinpashneh, E., Budas, G. R., Pouleur, A. C., Dutta, A., Prescott, A. R., Vanoverschelde, J. L., Ashworth, A., Jovanović, A., Alessi, D. R., and Bertrand, L. (2006) Deficiency of LKB1 in heart prevents ischemia-mediated activation of AMPKalpha2 but not AMPKalpha1. *Am. J. Physiol. Endocrinol. Metab.* **290**, E780–E788
15. Altarejos, J. Y., Taniguchi, M., Clanachan, A. S., and Lopaschuk, G. D. (2005) Myocardial ischemia differentially regulates LKB1 and an alternate 5′-AMP-activated protein kinase kinase. *J. Biol. Chem.* **280**, 183–190
16. Ma, H., Wang, J., Thomas, D. P., Tong, C., Leng, L., Wang, W., Merk, M., Zierow, S., Bernhagen, J., Ren, J., Bucala, R., and Li, J. (2010) Impaired macrophage migration inhibitory factor-AMP-activated protein kinase activation and ischemic recovery in the senescent heart. *Circulation* **122**, 282–292
17. Budanov, A. V., and Karin, M. (2008) p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. *Cell* **134**, 451–460
18. Budanov, A. V., Shoshani, T., Faerman, A., Zelin, E., Kamer, I., Kalinski, H., Gorodin, S., Fishman, A., Chajut, A., Einat, P., Skaliter, R., Gudkov, A. V., Chumakov, P. M., and Feinstein, E. (2002) Identification of a novel stress-responsive gene Hi95 involved in regulation of cell viability. *Oncogene* **21**, 6017–6031
19. Essler, S., Dehne, N., and Brüne, B. (2009) Role of sestrin2 in peroxide signaling in macrophages. *FEBS Lett.* **583**, 3531–3535
20. Olson, N., Hristova, M., Heintz, N. H., Lounsbury, K. M., and van der Vliet, A. (2011) Activation of hypoxia-inducible factor-1 protects airway epithelium against oxidant-induced barrier dysfunction. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **301**, L993–L1002
21. Lee, J. H., Budanov, A. V., Park, E. J., Birse, R., Kim, T. E., Perkins, G. A., Ocorr, K., Ellisman, M. H., Bodmer, R., Bier, E., and Karin, M. (2010) Sestrin as a feedback inhibitor of TOR that prevents age-related pathologies. *Science* **327**, 1223–1228
22. Chen, S., Zhu, P., Guo, H. M., Solis, R. S., Wang, Y., Ma, Y., Wang, J., Gao, J., Chen, J. M., Ge, Y., Zhuang, J., and Li, J. (2014) Alpha1 catalytic subunit of AMPK modulates contractile function of cardiomyocytes through phosphorylation of troponin I. *Life Sci.* **98**, 75–82
23. Morrison, A., Yan, X., Tong, C., and Li, J. (2011) Acute rosiglitazone treatment is cardioprotective against ischemia-reperfusion injury by modulating AMPK, Akt, and JNK signaling in nondiabetic mice. *Am. J. Physiol. Heart Circ. Physiol.* **301**, H895–H902
24. Wang, J., Yang, L., Rezaie, A. R., and Li, J. (2011) Activated protein C protects against myocardial ischemic/reperfusion injury through AMP-activated protein kinase signaling. *J. Thromb. Haemost.* **9**, 1308–1317
25. Tong, C., Morrison, A., Mattison, S., Qian, S., Bryniarski, M., Rankin, B., Wang, J., Thomas, D. P., and Li, J. (2013) Impaired SIRT1 nucleocytoplasmic shuttling in the senescent heart during ischemic stress. *FASEB J.* **27**, 4332–4342
26. Li, J., Miller, E. J., Ninomiya-Tsuji, J., Russell III, R. R., and Young, L. H. (2005) AMP-activated protein kinase activates p38 mitogen-activated protein kinase by increasing recruitment of p38 MAPK to TAB1 in the ischemic heart. *Circ. Res.* **97**, 872–879
27. Claycomb, W. C., Lanson, N. A., Jr., Stallworth, B. S., Egeland, D. B., Delcarpio, J. B., Bahinski, A., and Izzo, N. J., Jr. (1998) HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc. Natl. Acad. Sci. USA* **95**, 2979–2984
28. Shaw, R. J., Kosmatka, M., Bardeesy, N., Hurley, R. L., Witters, L. A., DePinho, R. A., and Cantley, L. C. (2004) The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc. Natl. Acad. Sci. USA* **101**, 3329–3335
29. Wang, J., Wang, Y., Wang, J., Gao, J., Tong, C., Manithody, C., Li, J., and Rezaie, A. R. (2013) Antithrombin is protective against myocardial ischemia and reperfusion injury. *J. Thromb. Haemost.* **11**, 1020–1028

30. Peeters, H., Debeer, P., Bairoch, A., Wilquet, V., Huysmans, C., Parthoens, E., Fryns, J. P., Gewillig, M., Nakamura, Y., Niikawa, N., Van de Ven, W., and Devriendt, K. (2003) PA26 is a candidate gene for heterotaxia in humans: identification of a novel PA26-related gene family in human and mouse. *Hum. Genet.* **112**, 573–580
31. Braunwald, E., and Kloner, R. A. (1982) The stunned myocardium: prolonged, postischemic ventricular dysfunction. *Circulation* **66**, 1146–1149
32. Antman, E. M., Tanasijevic, M. J., Thompson, B., Schactman, M., McCabe, C. H., Cannon, C. P., Fischer, G. A., Fung, A. Y., Thompson, C., Wybenga, D., and Braunwald, E. (1996) Cardiac-specific troponin I levels to predict the risk of mortality in patients with acute coronary syndromes. *N. Engl. J. Med.* **335**, 1342–1349
33. Marsin, A. S., Bertrand, L., Rider, M. H., Deprez, J., Beauloye, C., Vincent, M. F., Van den Berghe, G., Carling, D., and Hue, L. (2000) Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. *Curr. Biol.* **10**, 1247–1255
34. Sanli, T., Linher-Melville, K., Tsakiridis, T., and Singh, G. (2012) Sestrin2 modulates AMPK subunit expression and its response to ionizing radiation in breast cancer cells. *PLoS ONE* **7**, e32035
35. Tiainen, M., Ylikorkala, A., and Mäkelä, T. P. (1999) Growth suppression by Lkb1 is mediated by a G(1) cell cycle arrest. *Proc. Natl. Acad. Sci. USA* **96**, 9248–9251
36. Budanov, A. V., Lee, J. H., and Karin, M. (2010) Stressin' Sestrins take an aging fight. *EMBO Mol. Med.* **2**, 388–400
37. Maiuri, M. C., Malik, S. A., Morselli, E., Kepp, O., Criollo, A., Mouchel, P. L., Carnuccio, R., and Kroemer, G. (2009) Stimulation of autophagy by the p53 target gene Sestrin2. *Cell Cycle* **8**, 1571–1576
38. Lee, J. H., Budanov, A. V., Talukdar, S., Park, E. J., Park, H. L., Park, H. W., Bandyopadhyay, G., Li, N., Aghajan, M., Jang, I., Wolfe, A. M., Perkins, G. A., Ellisman, M. H., Bier, E., Scadeng, M., Foretz, M., Viollet, B., Olefsky, J., and Karin, M. (2012) Maintenance of metabolic homeostasis by Sestrin2 and Sestrin3. *Cell Metab.* **16**, 311–321
39. Budanov, A. V., Sablina, A. A., Feinstein, E., Koonin, E. V., and Chumakov, P. M. (2004) Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. *Science* **304**, 596–600
40. Dhanasekaran, D. N., Kashef, K., Lee, C. M., Xu, H., and Reddy, E. P. (2007) Scaffold proteins of MAP-kinase modules. *Oncogene* **26**, 3185–3202
41. Kim, A. S., Miller, E. J., and Young, L. H. (2009) AMP-activated protein kinase: a core signalling pathway in the heart. *Acta Physiol. (Oxf.)* **196**, 37–53

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