ORIGINAL RESEARCH

Loss of Rbl2 (Retinoblastoma-Like 2) Exacerbates Myocardial Ischemia/Reperfusion Injury

Jingrui Chen, PhD; Peng Xia, PhD; Yuening Liu, PhD; Clark Kogan, PhD; Zhaokang Cheng, PhD

BACKGROUND: The postmitotic state of adult cardiomyocytes, maintained by the cell cycle repressor Rbl2 (retinoblastoma-like 2), is associated with considerable resistance to apoptosis. However, whether Rbl2 regulates cardiomyocyte apoptosis remains unknown.

METHODS AND RESULTS: Here, we show that ablation of Rbl2 increased cardiomyocyte apoptosis following acute myocardial ischemia/reperfusion injury, leading to diminished cardiac function and exaggerated ventricular remodeling in the long term. Mechanistically, ischemia/reperfusion induced expression of the proapoptotic protein BCL2 interacting protein 3 (Bnip3), which was augmented by deletion of Rbl2. Because the Bnip3 promoter contains an adenoviral early region 2 binding factor (E2F)-binding site, we further showed that loss of Rbl2 upregulated the transcriptional activator E2F1 but downregulated the transcriptional repressor E2F4. In cultured cardiomyocytes, treatment with H2O2 markedly increased the levels of E2F1 and Bnip3, resulting in mitochondrial depolarization and apoptosis. Depletion of Rbl2 significantly augmented H2O2-induced mitochondrial damage and apoptosis in vitro.

CONCLUSIONS: Rbl2 deficiency enhanced E2F1-mediated Bnip3 expression, resulting in aggravated cardiomyocyte apoptosis and ischemia/reperfusion injury. Our results uncover a novel antiapoptotic role for Rbl2 in cardiomyocytes, suggesting that the cell cycle machinery may directly regulate apoptosis in postmitotic cardiomyocytes. These findings may be exploited to develop new strategies to limit ischemia/reperfusion injury in the treatment of acute myocardial infarction.

Key Words: apoptosis ■ cardiac myocytes ■ cell cycle ■ cell death ■ cyclin-dependent kinase ■ myocardial infarction
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the human RBL2 gene is subject to loss-of-function mutations and deletions.13–15 To date, the role of Rbl2 in cardiomyocyte apoptosis and its pathophysiological significance have not been established yet.

In the present study, we show that loss of Rbl2 significantly exacerbated cardiomyocyte apoptosis and cardiac dysfunction following I/R injury. Mechanistically, Rbl2 deficiency enhanced I/R-induced adenoviral early region 2 binding factor 1 (E2F1) activation, leading to increased expression of the proapoptotic protein BCL2 interacting protein 3 (Bnip3). Our findings uncover a novel antiapoptotic role for the cell cycle inhibitory protein Rbl2 in cardiomyocytes.

METHODS

The authors declare that all supporting data are available within the article and its supplemental files.

Animals

Rbl2 homozygous knockout mice (Rbl2−/−). The Jackson Laboratory, stock number 00817616 were crossed with C57BL/6 mice (Envigo) to generate heterozygous Rbl2+/− mice. Subsequent intercross of the Rbl2+/− mice led to the generation of the Rbl2−/− mice and wild-type Rbl2+/+ littersmates. Eight- to 12-week-old sex-matched Rbl2−/− and Rbl2+/+ mice were used in this study. Sprague-Dawley rats were purchased from Envigo. All animal studies were approved by the Institutional Animal Care and Use Committee at Washington State University.

Cell Culture and Transfection

Neonatal rat cardiomyocytes (NRCMs) were isolated from 2- to 4-day-old Sprague-Dawley rats as described.17 NRCMs were cultured in serum-free medium 199 in 0.2% gelatin-coated plates and transfected with small interfering RNAs (siRNAs) using HiPerfect transfection reagent (Qiagen). The siRNA sequences used were as follows: Rbl2 siRNA, CCGGAAAGAACUUGUGAAC[dT][dT]; and scrambled control siRNA, UAAGGCUAUGAAGAGAUAC[dT][dT].

Adult mouse cardiomyocytes (AMCMs) were isolated from C57BL/6 mice, Rbl2−/− or Rbl2+/+ littersmates with collagenase II (0.5 mg/mL), collagenase IV (0.5 mg/mL), and protease XIV (0.05 mg/mL) as described.18 AMCMs were cultured in serum-free medium 199 with 0.1% bovine serum albumin, 1% insulin-transferrin-sodium selenite (I3146, Sigma-Aldrich), 10 mM 2,3-butanediol monoxime (B0753, Sigma-Aldrich), and 1% chemically defined lipid (11905–031, ThermoFisher Scientific) in laminin-coated dishes.

Cell Viability Assay

NRCMs plated in a 96-well plates were transfected with specific siRNAs before treatment with H2O2 as indicated. Cell viability was assessed using Cell Proliferation Kit I (3,4,5-dimethylthiazol-2-y]-2,5-diphenyl-tetrazolium bromide; Roche) as described previously.18

Western Blotting

Cells and heart tissue were homogenized in radioimmune precipitation assay buffer supplemented with protease and phosphatase inhibitors (ThermoFisher Scientific). Western blotting was performed with the following antibodies: rabbit anti-phospho-Rbl2 (S952, ab68136, Abcam, 1:1000), mouse anti-Rbl2 (610 262, BD Biosciences, 1:1000), rabbit anti-phospho-CDK2 (cyclin-dependent kinase 2) (Thr160, 2561, Cell Signaling Technology, 1:1000), rabbit anti-CDK2 (sc-163, Santa Cruz Biotechnology, 1:1000), rabbit anti-phospho-CDK2 (cyclin-dependent kinase 2) (Thr160, 2561, Cell Signaling Technology, 1:1000), rabbit anti-CDK2 (sc-163, Santa Cruz Biotechnology, 1:1000), rabbit anti-CDK2 (sc-163, Santa Cruz Biotechnology, 1:1000), rabbit anti-caspase 9 (9662, Cell Signaling Technology, 1:1000), rabbit anti-cleaved caspase 3 (9664s, Cell Signaling Technology, 1:500), rabbit anti-Bnip3 (3769, Cell Signaling Technology, 1:1000), mouse anti-E2F1 (sc-251, Santa Cruz Biotechnology, 1:1000), mouse anti-E2F4 (sc-511, Santa Cruz Biotechnology, 1:1000), and rabbit anti-GAPDH

CLINICAL PERSPECTIVE

What Is New?

• Rbl2 (retinoblastoma-like 2) confers resistance to apoptosis in cardiomyocytes.
• Cardiomyocytes lacking Rbl2 are more sensitive to apoptosis under oxidative stress conditions.
• Loss of Rbl2 downregulates adenoviral early region 2 binding factor 4 (E2F4) but upregulates the proapoptotic proteins E2F1 and BCL2 interacting protein 3 (Bnip3).

What Are the Clinical Implications?

• E2F1-mediated apoptosis is a potential drug target in the treatment of heart attack.
• Loss-of-function mutations or deletions of the human RBL2 gene might be associated with worse outcomes after a heart attack.

Nonstandard Abbreviations and Acronyms

| AMCM | adult mouse cardiomyocyte |
| I/R | ischemia/reperfusion |
| NRCM | neonatal rat cardiomyocyte |
| Rbl2 | retinoblastoma-like 2 |

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infarct area to area at risk as described previously. Chloride staining and defined as the percentage of reperfusion. Evans blue/2,3,5-triphenyltetrazolium chloride staining revealed that loss of Rbl2 significantly increased infarct size as measured by the percentage of infarct area to area at risk (Figure 2A). Areas at risk were comparable between both groups (Figure 2A). Importantly, ablation of Rbl2 also significantly increased the serum lactate dehydrogenase level at 4 and 24 hours after I/R (Figure 2B). These results suggested that loss of Rbl2 exacerbated I/R-induced acute myocardial injury.

RESULTS

Oxidative Stress Induced Rbl2 Phosphorylation at S952 in Cardiomyocytes

The biological function of Rbl2 is repressed by CDK (cyclin-dependent kinase)-mediated phosphorylation at multiple sites including Ser 952. Because myocardial I/R induces CDK2 activation, we first measured Rbl2 phosphorylation in heart lysates following I/R. As expected, the level of phospho-Rbl2 (Ser 952) was rapidly increased within 45 minutes of I/R followed by a gradual decline afterward (Figure 1A), indicating acute Rbl2 inactivation. I/R is associated with massive ROS production in the ischemic myocardium. To determine whether ROS overload provokes Rbl2 phosphorylation, AMCMs were incubated with \( \mathrm{H}_2\mathrm{O}_2 \). Immunofluorescent staining revealed that \( \mathrm{H}_2\mathrm{O}_2 \) dramatically increased phospho-Rbl2 (Ser 952) signal intensity in the nuclei of cardiomyocytes (Figure 1B). Moreover, \( \mathrm{H}_2\mathrm{O}_2 \) treatment also significantly increased the protein levels of phospho-Rbl2 (Ser 952) in NRCMs (Figure 1C). Interestingly, \( \mathrm{H}_2\mathrm{O}_2 \)-induced Rbl2 phosphorylation is associated with increased protein levels of phospho-CDK2 (T160, a marker of CDK2 activation) in both NRCMs (Figure S1A) and AMCMs (Figure S1B). \( \mathrm{H}_2\mathrm{O}_2 \) treatment also upregulated both the p39 and p33 isoforms of CDK2 in NRCMs (Figure S1A). Together, these results suggested that I/R-related oxidative stress likely induced CDK2-mediated Rbl2 phosphorylation, leading to inhibition of Rbl2 function.

Measurement of Mitochondrial Membrane Potential

NRCMs were stained with JC-1 (Cayman Chemical) to evaluate the mitochondrial membrane potential (\( \Delta \Psi_m \)). Loss of \( \Delta \Psi_m \), an indicator of mitochondrial damage, is determined as an increase in the ratio of JC-1 monomers (green)/J-aggregates (red) fluorescent intensity.

In Vivo Studies

Myocardial I/R injury was induced as previously described. Briefly, mice were subjected to 30 minutes of ischemia via ligation of the left anterior descending coronary artery, followed by reperfusion. Serum lactate dehydrogenase level was measured using the Cytotoxicity Detection Kit (11 644793001, Roche) according to the manufacturer’s instructions. Infarct size was evaluated by Evans blue/2,3,5-triphenyltetrazolium chloride staining and defined as the percentage of infarct area to area at risk as described previously. Cardiac function was monitored by echocardiography using Vevo 2100 (VisualSonics). Myocardial fibrosis was examined by Masson’s trichrome staining.

Statistical Analysis

GraphPad Prism 7 (GraphPad Software) was used for statistical analysis. Results are expressed as mean±SEM. Sample size was estimated based on our previously published data. For experiments with a small sample size (n<6), normality was not assessed, and statistical analyses were performed using nonparametric tests. Differences between 2 groups were compared using the Mann-Whitney test. Differences among multiple groups were analyzed using 2-way ANOVA followed by post hoc Tukey test. A P value of <0.05 was considered statistically significant.
Rbl2 Deficiency Exaggerated Chronic Cardiac Dysfunction and Fibrosis Following I/R

We next evaluated the long-term effect of Rbl2 deletion following I/R for up to 4 weeks. Although basal heart function was comparable between both groups of mice, left ventricular ejection fraction (Figure 3A and 3B) and fractional shortening (Figure 3A and 3C) were significantly lower in Rbl2−/− mice than in wild-type mice following I/R. Left ventricular anterior wall thickness at end systole was significantly decreased by Rbl2 ablation at 2 weeks, with a similar trend at 4 weeks after I/R (Figure 3A and 3D). Myocardial fibrosis was minimal and comparable between Rbl2−/− and wild-type mice at baseline (data not shown). However, the fibrotic area was significantly larger in Rbl2−/− than in Rbl2+/+ hearts at 4 weeks after I/R (Figure 3E). Collectively, Rbl2 deficiency exacerbated I/R-induced cardiac dysfunction and fibrotic remodeling in the long term.

Depletion of Rbl2 Augmented Oxidative Stress-Induced Cardiomyocyte Apoptosis

To determine the role of Rbl2 in apoptosis, a separate cohort of Rbl2−/− or wild-type mice were subjected to 30 minutes of ischemia followed by 24 hours of reperfusion. As shown in Figure 4A, ablation of Rbl2 increased TUNEL-positive nuclei in the infarcted myocardium, indicating increased apoptosis. We next isolated AMCMs from healthy Rbl2−/− and wild-type hearts and incubated these cells with H2O2 in vitro. Deletion of Rbl2 again increased TUNEL-positive nuclei in cultured AMCMs (Figure 4B). To further confirm this finding, NRCMs were transfected with Rbl2 siRNAs before treatment with H2O2 for 24 hours. Silencing
of Rbl2 significantly increased H$_2$O$_2$-induced cleavage of poly (ADP-ribose) polymerase and caspase-3, 2 widely used markers of apoptosis (Figure 4C). Knockdown of Rbl2 also increased the percentage of TUNEL-positive NRCMs (Figure 4D). Moreover, 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide assay revealed that Rbl2 depletion significantly reduced cell viability after incubation with H$_2$O$_2$.
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for 24 hours (Figure 4E). These data suggested that depletion of Rbl2 augmented oxidative stress-induced cardiomyocyte apoptosis.

Rbl2 Depletion Exaggerated Oxidative Stress-Induced Mitochondrial Damage

Apoptosis can be accelerated by loss of $\Delta \Psi_m$. To determine whether Rbl2 regulates mitochondrial depolarization under oxidative stress, NRCMs were transfected with Rbl2 siRNAs before treatment with $H_2O_2$ for 24 hours. JC-1 staining revealed that $H_2O_2$ treatment significantly increased the ratio of JC-1 monomer/J-aggregates, indicating loss of $\Delta \Psi_m$ (Figure 5). Importantly, $H_2O_2$-induced loss of $\Delta \Psi_m$ was significantly enhanced by knockdown of Rbl2, suggesting that Rbl2 depletion exaggerated mitochondrial damage under oxidative stress.

Oxidative Stress-Induced Bnip3 Expression Was Augmented by Rbl2 Depletion

It is well documented that the BCL2 homology domain 3 (BH3)-only protein Bnip3 localizes at mitochondria...
to mediate mitochondrial depolarization and cardiomyocyte apoptosis during I/R injury. Intriguingly, simulated I/R for up to 2 hours fails to induce Bnip3 expression in the ex vivo heart. Therefore, we investigated myocardial Bnip3 expression following in vivo I/R for up to 24 hours. Western blotting revealed that the cardiac Bnip3 protein level was significantly increased as early as 4 hours after I/R (Figure 6A). Ablation of Rbl2 further augmented I/R-induced upregulation of Bnip3 (Figure 6B). To determine whether I/R-related ROS production induces Bnip3 expression, NRCMs were incubated with H2O2 for various periods of time. As expected, H2O2 treatment significantly increased Bnip3 protein level (Figure 6C), which was again augmented by knockdown of Rbl2 (Figure 6D). These findings suggested that depletion of Rbl2 enhanced oxidative stress-induced expression of the proapoptotic protein Bnip3.

Oxidative Stress Upregulated E2F1 but Downregulated E2F4 in Cardiomyocytes

The Bnip3 promoter contains an E2F-binding site, which can be recognized by both E2F1 and E2F4, resulting in activation and repression of transcription, respectively. Consistent with the upregulation of Bnip3 (Figure 6A), I/R also increased E2F1 but reduced E2F4 levels in the mouse heart (Figure 7A). In response to H2O2 treatment, AMCMs exhibited a dramatic increase in E2F1 signal intensity (Figure 7B) and a decrease in E2F4 intensity (Figure 7C). Notably, both E2F1 and E2F4 were localized in the nuclei of AMCMs. Because nuclear localization is necessary for activation of E2Fs, our data suggested that oxidative stress activated E2F1 but repressed E2F4 in cardiomyocytes.

Hearts Lacking Rbl2 Displayed Higher E2F1 but Lower E2F4 Levels at Baseline and After I/R

Because Rbl2 depletion increased expression of the E2F target protein Bnip3 (Figure 6B and 6D), we next evaluated the levels of E2F1 and E2F4 in the Rbl2−/− mouse heart. Western blotting revealed that ablation of Rbl2 increased the protein level of the transcription activator E2F1 at basal conditions (Figure 8A). In contrast, loss of Rbl2 reduced the level of the transcription activator E2F4 at basal conditions (Figure 8B), resulting in a decrease in E2F1/E2F4 ratio (Figure 8C). This finding indicated that Rbl2 negatively regulates E2F4 expression during I/R injury, thereby promoting cardiomyocyte survival.

Figure 5. Rbl2 (retinoblastoma-like 2) depletion exaggerated oxidative stress-induced mitochondrial damage.

Neonatal rat cardiomyocytes were transfected with siControl or siRbl2 before incubation with H2O2 (100 μmol/L) for 24 hours (n=6). Cells were then incubated with JC-1 to evaluate mitochondrial membrane potential. JC-1 monomers (green) and J-aggregates (red) indicate damaged and healthy mitochondria, respectively. Scale bar=20 μm. Depletion of Rbl2 exacerbated H2O2-induced mitochondrial depolarization. Two-way ANOVA with Tukey test, **P<0.01. Interaction between 2 factors: P<0.0001.
repressor E2F4 in the normal heart (Figure 8A). Compared with wild-type myocytes, AMCMs lacking Rbl2 exhibited higher E2F1 but lower E2F4 levels (Figure 8B and 8C). Moreover, ablation of Rbl2 also upregulated E2F1 but downregulated E2F4 following I/R (Figure 8D). These data suggested that loss of Rbl2 enhanced activation of cardiac E2F1, a key transcription factor for genes involved in apoptosis including Bnip3 and others.24,25

**DISCUSSION**

Adult cardiomyocytes are more resistant to apoptosis when compared with pediatric cardiomyocytes.3,4 Mature cardiomyocytes in the adult heart are maintained in a postmitotic state by the RB family of proteins including Rbl2.8,9 In the present study, we demonstrated that loss of Rbl2 exacerbated cardiomyocyte apoptosis and myocardial I/R injury. Mechanistically,
ablation of Rbl2 upregulated the transcriptional activator E2F1, resulting in increased expression of the proapoptotic E2F target protein Bnip3 (Figure 8E). This study provides the first direct evidence that Rbl2 plays an antiapoptotic role in cardiomyocytes.

Rbl2 is primarily known as a cell cycle regulator, but its role in apoptosis has been emerging.7 In postmitotic cells such as neurons, knockdown of Rbl2 provoked apoptosis.27 Our results also revealed that loss of Rbl2 augmented oxidative stress-induced apoptosis in cardiomyocytes. Intriguingly, Rbl2 is necessary for Akt inhibitor-induced apoptosis in actively cycling cells including human embryonic kidney 293 (HEK293), lung cancer, and mesothelioma cells.28 Overexpression of Rbl2 increased apoptosis because of downregulation of the antiapoptotic proteins (B-cell lymphoma-xl [Bcl-xl], B-cell lymphoma-2 [Bcl-2]) and upregulation of the proapoptotic proteins (BCL2 associated X [Bax], p73) in glioblastoma cells and marrow stromal stem cells.29,30 These studies suggested that Rbl2 may be antiapoptotic in postmitotic, terminally differentiated cells, but proapoptotic in rapidly dividing cells. In addition, the role of Rbl2 in apoptosis may also depend on the apoptotic stimuli.31

Rbl2 is thought to mediate nuclear translocation of E2F4, which then binds the E2F-responsive sites in gene promoters at G0/G1 to repress transcription. At the G1/S transition, CDK-mediated Rbl2 hyperphosphorylation induces the dissociation of E2F4 from Rbl2, resulting in displacement of E2F4 by E2F1 at the promoter and subsequent transcription of E2F target genes.25,26 In this study, we showed that I/R induced Rbl2 phosphorylation at the CDK target site S952. Phosphorylation of Rbl2 at S952 may be mediated by CDK2, which is known to be activated by various pathological challenges including I/R or doxorubicin exposure.12,17,32 We further showed that ablation of Rbl2 reduced E2F4 level but increased E2F1 level in the heart. Intriguingly, hearts lacking E2F4 exhibit spontaneous apoptosis and deteriorated function at 6 weeks of age.33 Overexpression of E2F1 is sufficient to cause apoptosis in cardiomyocytes.34–36 Conversely, ablation
of E2F1 protects against I/R-induced apoptosis.\textsuperscript{37} Therefore, E2F4 downregulation and E2F1 upregulation are likely important mechanisms of the increased apoptosis in Rbl2-deficient heart after I/R. Interestingly, genotoxic stress-induced apoptosis in postmitotic neurons is also augmented by deletion of E2F4 and attenuated by ablation of E2F1.\textsuperscript{38}

In the current study, we demonstrated that loss of Rbl2 augmented I/R-induced E2F1 upregulation, which was accompanied by increased expression of the E2F target protein Bnip3. As a redox sensor, Bnip3 undergoes ROS-mediated oxidation and homodimerization to activate the mitochondrial apoptosis pathway.\textsuperscript{39} Bnip3 may also contribute to cardiac damage by provoking mitochondrial permeability transition pore opening and cardiomyocyte necrosis.\textsuperscript{40} Ablation of Bnip3 attenuates cardiomyocyte death and myocardial I/R injury.\textsuperscript{22} In addition to Bnip3, E2F1 is known to mediate transcription of additional proapoptotic genes including Apaf1, p73, Bad, and Bak1,\textsuperscript{25} which may also account for the increased apoptosis in hearts lacking Rbl2.

It is noteworthy that E2F1-induced apoptosis of cardiomyocytes is often accompanied by cell cycle re-entry.\textsuperscript{34,35} Mechanistically, E2F1 is known to mediate transcription of genes involved in either apoptosis or cell cycle progression.\textsuperscript{25} Whether E2F-mediated apoptosis can be uncoupled from cell cycle reentry has been an interesting area of investigation. The protein level of E2F1 appears to be a key determinant of cell fate, with low, moderate, and high levels of E2F1 inducing proliferation, cell cycle arrest, and apoptosis, respectively.\textsuperscript{51} The presence of DNA damage has been shown to direct E2F1 from E2F target genes involved...
in cell cycle progression to those involved in apoptosis. Moreover, EPC1 (epigenetic modifier enhancer of polycomb homolog 1) is able to induce a switch in transcription from apoptotic to metastatic E2F target genes. In addition, E2F2 or E2F4 appears to induce transcription from apoptotic to metastatic E2F target genes. In conclusion, we showed that Rbl2 deficiency exacerbated I/R-induced cardiomyocyte apoptosis and cardiac dysfunction. Mechanistically, ablation of Rbl2 augmented I/R-induced, E2F1-mediated expression of the proapoptotic protein Bnip3. These results uncover a critical role of the cell cycle protein Rbl2 in the regulation of cardiomyocyte apoptosis.

ARTICLE INFORMATION
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Disclosures
None.

Supplemental Material
Figure S1

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Figure S1. Oxidative stress induced activation of cyclin-dependent kinase 2 (CDK2) in cardiomyocytes.

(A) Neonatal rat cardiomyocytes (NRCMs) were treated with H$_2$O$_2$ (100 µM) for various periods of time. Western blot analysis revealed that H$_2$O$_2$ treatment significantly increased protein level of phospho-CDK2 (T160), a reliable marker of CDK2 activation. H$_2$O$_2$ treatment also increased both the p39 and p33 isoforms of CDK2. Kruskal-Wallis with Dunn's test. * P<0.05 vs. Time 0.

(B) Adult mouse cardiomyocytes (AMCMs) were treated with H$_2$O$_2$ (100 µM) or vehicle for 4 h, followed by immunofluorescent staining for phospho-CDK2 (T160, green), cardiac troponin T (cTnT, red) and nuclei (4',6-diamidino-2-phenylindole (DAPI), blue). Scale bar = 50 µm.