c-Cbl Inhibition Improves Cardiac Function and Survival in Response to Myocardial Ischemia

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Background—The proto-oncogene Casitas b-lineage lymphoma (c-Cbl) is an adaptor protein with an intrinsic E3 ubiquitin ligase activity that targets receptor and nonreceptor tyrosine kinases, resulting in their ubiquitination and downregulation. However, the function of c-Cbl in the control of cardiac function is currently unknown. In this study, we examined the role of c-Cbl in myocardy death and cardiac function after myocardial ischemia.

Methods and Results—We show increased c-Cbl expression in human ischemic and dilated cardiomyopathy hearts and in response to pathological stress stimuli in mice. c-Cbl-deficient mice demonstrated a more robust functional recovery after myocardial ischemia/reperfusion injury and significantly reduced myocyte apoptosis and improved cardiac function. Ubiquitination and downregulation of key survival c-Cbl targets, epidermal growth factor receptors and focal adhesion kinase, were significantly reduced in c-Cbl knockout mice. Inhibition of c-Cbl expression or its ubiquitin ligase activity in cardiac myocytes offered protection against H2O2 stress. Interestingly, c-Cbl deletion reduced the risk of death and increased cardiac functional recovery after chronic myocardial ischemia. This beneficial effect of c-Cbl deletion was associated with enhanced neoangiogenesis and increased expression of vascular endothelial growth factor-a and vascular endothelial growth factor receptor type 2 in the infarcted region.

Conclusions—c-Cbl activation promotes myocyte apoptosis, inhibits angiogenesis, and causes adverse cardiac remodeling after myocardial infarction. These findings point to c-Cbl as a potential therapeutic target for the maintenance of cardiac function and remodeling after myocardial ischemia. (Circulation. 2014;129:2031-2043.)

Key Words: angiogenesis ■ apoptosis ■ myocardial ischemia ■ ubiquitin

Myocardial infarction (MI) due to coronary artery occlusion is a leading cause of death worldwide. The loss of blood flow after MI results in loss of cardiac myocytes in the ischemic zone, which diminishes cardiac contractility and impedes angiogenesis and repair. Although much is known about the pathways that promote the pathological remodeling responses, mechanisms that promote myocyte loss and impair cardiac function have not been as clearly defined. The ubiquitin proteasome system is largely responsible for the degradation of misfolded and damaged proteins as well as proteins involved in the control of components of the contractile apparatus and hypertrophic gene expression, thereby regulating cardiac hypertrophy and remodeling. Activation of the ubiquitin proteasome system in heart reduces muscle mass as occurs during muscle atrophy, promotes myocyte hypertrophy, and impairs cardiac remodeling.5

The formation of ubiquitin-protein conjugates involves 3 components that participate in a cascade of ubiquitin transfer reactions: a ubiquitin-activation enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) that acts at the last step of the cascade and presents the greatest tissue and substrate specificity. Once a protein has been marked with polyubiquitin conjugates, it is destined for degradation by the multicatalytic protease, the 26S proteasome complex. The proto-oncogene Casitas b-lineage lymphoma (c-Cbl) contains a highly conserved helical region and an adjacent RING finger domain that together bind an E2; as such, c-Cbl acts as a RING type ubiquitin ligase (E3) and negatively regulates receptor and nonreceptor tyrosine kinases by promoting their ubiquitination and lysosomal/proteasomal degradation. In addition to a tyrosine kinase–binding domain for binding to activated tyrosine kinases, c-Cbl also contains multiple protein interaction motifs; a tyrosine kinase binding domain that encompasses a 4-helical bundle,
an EF hand, a Src homology domain 2, and a proline-rich motif. These domains/motifs allow the interaction of c-Cbl with multiple signal transducers, including the p85 subunit of phosphoinositide-3 kinase, the Crk and Grb2 adaptor proteins, and Src family kinases, suggesting that c-Cbl acts as a docking protein to integrate signaling pathways. Although c-Cbl phosphorylation in response to a variety of cell surface receptors has been documented, the precise biological function of c-Cbl is unclear, and both negative and positive roles for c-Cbl have been proposed. In the heart, c-Cbl expression increases in response to right ventricular pressure overload stimuli, and 1 study linked c-Cbl to reduced insulin responsiveness in adult rat cardiomyocytes in vitro. However, specific functions of c-Cbl in myocyte growth and cardiac function are still not well understood, and no loss-of-function cardiac phenotypes of c-Cbl gene have yet been described.

We recently showed that activation of c-Cbl ubiquitin ligase by neutrophil-derived serine proteases leads to focal adhesion protein turnover and myofibril degeneration. In the present study, we show that c-Cbl expression increases in human cardiomyopathies and in response to myocardial stress in mice. Mice lacking c-Cbl display normal cardiac function but exhibit less myocyte loss and cardiac dysfunction in response to ischemia/reperfusion (IR) injury. c-Cbl–deficient mice are also protected against pathological remodeling of the heart after chronic MI. This protection appears to be mediated, at least in part, to neoangiogenesis in the infarcted region. We conclude that c-Cbl negatively regulates ventricular myocyte survival and angiogenesis after myocardial ischemia injury.

Methods

Subjects and Tissue Preparation

Human left ventricular (LV) myocardial tissue was obtained at the time of cardiac transplantation from patients with end-stage heart failure due to ischemic cardiomyopathy (ICM; n=5) and dilated cardiomyopathy (DCM; n=5). LV tissue was also obtained from nonfailing hearts (n=5) of brain-dead organ donors that could not be used for transplantation. Relevant clinical data were collected from all subjects providing heart tissue (Table I in the online-only Data Supplement).

Our protocol was approved by the Temple University institutional review board, according to the guidelines noted in our instructions to authors. All hearts were harvested in situ with cold, blood-containing, cardioplegia solution and promptly transported to the laboratory in Krebs-Henseleit buffer solution, as described previously. LV tissue slices were immediately snap-frozen in liquid nitrogen and stored at −80°C or fixed in formalin.

Myocardial Ischemia and IR Injury Procedure

All mice were anesthetized by a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) to perform a left anterior descending coronary artery ligation. Hearts were harvested after 2, 7, or 30 days (for animals subjected to myocardial ischemia) or after 24 hours of reperfusion (for animal subjected to IR injury). All mice were randomized to the aforementioned experimental protocol.

Data Analysis

Summary data are presented as means±SEM. For comparisons of ≥2 groups, 1-way ANOVA or, more generally, the generalized linear regression approach was used for normal distributions, and the Kruskal-Wallis test was used for nonnormal or small sample situations. Two-group comparisons were analyzed by the 2-sample t test or nonparametric Wilcoxon rank test, whenever appropriate (eg, when the sample size was small or the distribution was not normal). Bonferroni post hoc test adjustments were used for multiple pairwise group comparisons after the overall F or Kruskal Wallis test showed a statistical significance. The exact testing was used when the sample size was small (eg, when all group sizes were <10). The survival time was analyzed by the Kaplan-Meier product-limit approach and compared by the log-rank test. To make the plot and interpretation of the fold increase over WT sham easier to understand, we scaled the data value from each animal in each of the 4 groups, including individual sham values, using the mean of the WT sham group. All in vitro experiments were performed at least 3 times from 3 different cultures, and the data values were scaled to controls. A value of P<0.05 was considered statistically significant.

An expanded Methods section is included in the online-only Data Supplement.

Results

C-Cbl Expression Is Developmentally Downregulated

To determine the temporal pattern of c-Cbl expression during normal cardiac development in the mouse, we analyzed total protein extracts from series of fetal and postnatal time points. C-Cbl protein was highly expressed in fetal hearts at 12.5 days, and this expression decreased gradually throughout fetal life (−17±3% at E19.5 compared with E12.5; Figure 1A). After birth, c-Cbl expression decreased significantly at postnatal days 3 and 7 (−30±2% at 1 day, −37±2% at 3 days, and −46±3% at 7 days compared with E12.5) and reached low but detectable levels in adult hearts (−80±5% compared with E12.5). This decrease in c-Cbl expression in adult hearts was associated with a decrease in c-Cbl expression in adult isolated myocytes (Figure 1B).

C-Cbl Expression Is Upregulated in Failing Human Myocardium

Cardiac explants from human donors (clinical characteristics of heart donors are summarized in Table I in the online-only Data Supplement) were assessed for the expression of Western blot and immunohistochemistry. Figure 1C shows increased c-Cbl immunostaining in both ICM and DCM compared with nonfailing hearts. This c-Cbl accumulation was detected mainly in myocytes, and no appreciable
Figure 1. Proto-oncogene Casitas b-lineage lymphoma (c-Cbl) expression is upregulated in human cardiomyopathies. A and B, Representative immunoblots of cardiac lysates from indicated embryonic (E), postnatal day (P), or adult (Ad) Sprague-Dawley rats (A) or cardiomyocytes isolated from 1 day postnatal or adult rat hearts (B). GAPDH was shown as a loading control. Western blots are representative of 3 separate experiments. C, Representative immunostainings of paraffin-embedded nonfailing (NF), ischemic cardiomyopathy (ICM), and dilated cardiomyopathy (DCM) human heart sections stained for c-Cbl and counterstained with hematoxylin. Bar, 40 μm. *P<0.05 vs NF controls. D, Immunoblot analysis of whole lysates from NF (n=5), ICM (n=5), and DCM (n=5) human hearts. E, Lysates from neonatal rat cardiomyocytes untreated or treated with isoproterenol (Iso; 10 μmol/L), norepinephrine (NE; 10 μmol/L), thrombin (Thr; 1 U/mL), or tumor necrosis factor-α (TNF-α; 100 ng/mL) for 48 hours. Ctrl indicates control. Top, c-Cbl immunoblot with GAPDH taken as a loading control. Bottom, Quantification of experiments expressed as mean±SE from 3 separate cultures. *P<0.05 vs control.
labeling was observed at the level of fibroblasts in the fibrotic zone, smooth muscle cells of medial arteries, or endothelial cells. Some c-Cbl immunostaining was detected at the medial layer of small arterioles (data not shown). c-Cbl accumulation in failing human heart samples was 2.3-fold and 1.5-fold higher in the ICM and DCM human hearts, respectively, compared with signals in nonfailing hearts (Figure 1D).

To explore the mechanism of induction of c-Cbl, we examined the effect of stress stimuli on c-Cbl expression in neonatal rat cardiomyocytes. Myocytes challenged with hypertrophic agonists norepinephrine (primarily an α-adrenergic receptor agonist), isoproterenol (a nonselective β-adrenergic receptor agonist), thrombin, or tumor necrosis factor-α for 48 hours showed an increase in c-Cbl accumulation compared with controls (Figure 1E). These data together show the effect of hypertrophic agonists and inflammatory cytokines in modulating c-Cbl expression in cardiomyocytes.

c-Cbl Ablation Protects the Heart From IR Injury

To explore the functional effects associated with c-Cbl induction in failing hearts, c-Cbl KO mice and their WT controls were subjected to transient left anterior descending coronary artery ligation for 30 minutes followed by reperfusion for 24 hours. c-Cbl KO mice did not differ from WT mice with regard to their baseline ratio of heart weight to body weight, heart rate (WT, 457±10 bpm; c-Cbl KO, 471±24 bpm), LV end-systolic pressure (WT, 89±9 mm Hg; c-Cbl KO, 86±5 mm Hg), LV end-diastolic pressure (WT, 5.2±0.8 mm Hg; c-Cbl KO, 6.3±0.9 mm Hg), and maximal dP/dt (WT, 8517±780 mm Hg·min⁻¹; c-Cbl KO, 7517±395 mm Hg·min⁻¹); hemodynamic data were obtained in n=8 WT mice and n=8 c-Cbl KO mice. Interestingly, c-Cbl KO mice subjected to IR injury showed significantly less impairment of cardiac function as assessed by LV ejection fraction and fractional shortening percentage compared with WT mice (Figure 2A and 2B and Table). Ablation of c-Cbl also reduced the percentage of infarcted area normalized to area at risk (22±3%) compared with WT mice (34±5%; Figure 2C and 2D). Apoptotic cell death in the area at risk, as evaluated by terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining, was significantly lower in c-Cbl KO mice compared with WT mice (Figure 2E and 2F). Under baseline conditions, the proportion of TUNEL-positive cardiomyocytes was very low (<0.1%) in c-Cbl KO and WT mice. Consistent with TUNEL staining, caspase-3 activity was also lower in c-Cbl KO infarcted hearts compared with WT, thus confirming a lesser propensity for myocyte death by apoptosis in c-Cbl KO mice subjected to IR (Figure 2G). Circulating plasma levels of the cardiac-specific isoform of troponin-I were evaluated as an additional marker of myocardial injury (Figure 2H). Serum samples from c-Cbl KO mice subjected to IR injury were found to contain lower levels of cardiac-specific isoform of troponin-I compared with WT mice (43.2±2.2 in WT versus 28.1±3.5 ng/mL per milligram protein in c-Cbl KO), thus confirming the cytoprotective effect of c-Cbl deletion.

c-Cbl Deletion Exhibits Protective Signaling

Activation of c-Cbl enables it to act as a multivalent adaptor for a plethora of Src homology domain 2– or Src homology domain 3–containing signaling proteins to positively or negatively regulate signaling pathways and cell growth.⁹ Therefore, we next examined the molecular mechanisms responsible for c-Cbl–mediated cardioprotection. Relative to WT samples, c-Cbl KO heart samples showed increases in the levels of phospho-Akt(S⁴⁷³) (3.2±0.2-fold), phospho-Bad(S¹¹²) (3.3±0.4-fold), phospho-Erk1/2 (1.8±0.2-fold), and phospho-Erk1/2 (1.7±0.2-fold; Figure 2I). However, we observed no significant increase in the expression of total-Akt or c-IAP1 in c-Cbl KO compared with WT control heart samples. We also examined these signaling molecules 1 day after IR injury; relative to sham-operated controls, WT mice after IR injury showed significant increases in phospho-Akt(S⁴⁷³) (2.2±0.2-fold) and phospho-Erk1/2 (2.4±0.2-fold), along with a significant decrease in the expression of phospho-Bad(S¹¹²) (−0.78±0.07-fold), c-IAP-1 (−0.70±0.1-fold), and XIAP (−0.80±0.14-fold). In comparison, c-Cbl KO mice subjected to IR injury showed a stronger increase in phospho-Bad(S¹¹²), c-IAP, and XIAP expression compared with WT mice subjected to IR injury. No significant effect on total Akt expression or phosphorylation between WT and c-Cbl KO mice subjected to IR injury was detected. Thus, compared with WT hearts, c-Cbl KO hearts show significantly more elevation in the levels of antiapoptotic signaling molecules.

Cardiac-Specific Deletion of c-Cbl Is Cardioprotective

In light of the cardioprotective effect of global c-Cbl deletion, we generated mice homozygous for a loxp-P-flanked c-Cbl allele and positive for tamoxifen-inducible Cre recombinase driven by the cardiomyocyte-specific α-MHC promoter (α-MHC-MerCreMer/c-Cblflox/flox). Tamoxifen injections at the age of 8 weeks for 5 days induced Cre-mediated recombination after 4 weeks, and Western analysis of protein lysates confirmed that c-Cbl protein was reduced by 90% in cardiac tissue (Figure I in the online-only Data Supplement). To assess the potential consequences of cardiac c-Cbl loss of function on IR injury, we subjected CM-Cbl KO and c-Cblflox/flox mice to IR for 24 hours. Echocardiography before surgery showed no difference in cardiac function and geometry between the groups (Table II in the online-only Data Supplement). Twenty-four hours after surgery, CM-Cbl KO mice showed significantly improved ejection fraction and fractional shortening compared with c-Cblflox/flox mice (Figure II A and II B in the online-only Data Supplement). This was associated with reduced LV internal diameter (Figure II C and II D in the online-only Data Supplement). Cardiac myocyte–specific deletion of c-Cbl also reduced the percentage of infarcted area normalized to area at risk (25±4%; n=6) compared with c-Cblflox/flox hearts (38±3%; n=5; Figure III E in the online-only Data Supplement), reduced occurrence of TUNEL-positive cells in the infarcted hearts (Figure III A and III B in the online-only Data Supplement), and attenuated caspase-3 activity in infarcted hearts compared with c-Cblflox/flox mice (Figure III C in the online-only Data Supplement). These data collectively demonstrate that deletion of c-Cbl in cardiomyocytes offers cardioprotection in response to IR injury.
Figure 2. Proto-oncogene Casitas b-lineage lymphoma (c-Cbl) ablation protects against ischemia/reperfusion (IR) injury. The left anterior descending coronary artery was ligated for 30 minutes to induce ischemia, and it subsequently was reperfused for 24 hours. A and B, Echocardiography measurement of ejection fraction (A) and fractional shortening (B) in wild-type (WT) and c-Cbl knockout (KO) animals (n=8 for sham groups; n=9 for IR groups). C, Representative cross sections were stained with triphenyltetrazolium chloride and Evans blue to determine the extent of injury. D, Quantification of infarct area (IA) vs area at risk (AAR) after IR injury in the indicated groups (n=6 per group). E, Left ventricular (LV) tissue sections were assessed for apoptosis with the use of the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay (green), tropomyosin (red), and DAPI (4′,6-diamidino-2-phenylindole; blue) staining. Bars, 40 μm (a through d) or 20 μm (e and f). F, The number of TUNEL-positive myocytes in the ischemic area was expressed as a percentage of total nuclei detected by DAPI staining. G, Quantification of caspase-3 activity in LV with the use of caspase-3-specific fluorogenic substrate. RFU indicates relative fluorescence units. H, Serum levels of cardiac troponin I (cTnI) after IR injury in WT and c-Cbl KO mice (n=6 in each group). I, Representative immunoblots of LV lysates from WT or c-Cbl KO animals. GAPDH was included as a loading control. Left, Representative autoradiogram (with each lane from a single gel exposed for the same duration). Right, Fold induction (n=6 in each group). *P<0.05 vs WT shams; †P<0.05 vs WT IR.
IR mediates ubiquitination and degradation of signaling molecules involved in myocyte survival.

**c-Cbl Knockdown Induces Protection Against Oxidative Stress–Induced Myocyte Death**

To directly demonstrate that the cardioprotective effect of c-Cbl deletion after IR results from endogenous expression of c-Cbl in cardiomyocytes, we investigated the role of c-Cbl in oxidative stress–induced cardiac myocyte death. Treatment of neonatal rat cardiomyocytes with H$_2$O$_2$ induced an increase in c-Cbl ubiquitin ligase activity as assessed by in vitro auto-ubiquitination assay (Figure 4A). This increase was rapid, was sustained for >60 minutes after H$_2$O$_2$ treatment, and was potent compared with cells treated with EGF for 5 minutes. H$_2$O$_2$ also caused a transient increase in c-Cbl Y774 phosphorylation, with maximum phosphorylation observed at 30 minutes after H$_2$O$_2$ treatment. Concomitant with increased c-Cbl auto-ubiquitination, H$_2$O$_2$ treatment increased c-Cbl interaction with EGFR along with increased EGFR ubiquitination (Figure 4B), suggesting that c-Cbl mediates EGFR ubiquitination. Transduction of adenovirus carrying shRNA c-Cbl (Ad-shCbl), which reduced c-Cbl expression by \( \approx 75\% \) to 85% compared with cells infected with shRNA control (Ad-shCtrl), significantly decreased EGFR ubiquitination and p38 mitogen-activated protein kinase and AKT phosphorylation induced by H$_2$O$_2$ or EGF treatment for 10 minutes (Figure 4C and Figure IV in the online-only Data Supplement). Expression of Ad-shCbl enhanced the basal phosphorylation of prosurvival signaling molecules ERK$_{1/2}$ and AKT compared with Ad-shCtrl–infected cells. Furthermore, H$_2$O$_2$ treatment did not further increase or induce ERK$_{1/2}$ or JNK phosphorylation, respectively. Ad-shCbl–infected cells also showed a significantly attenuated phosphorylation of these 2 kinases in response to EGF treatment compared with Ad-shCtrl–infected cells. These data show that inhibition of c-Cbl expression enhances prosurvival signaling and suggest that c-Cbl–dependent and –independent pathways are involved in H$_2$O$_2$–induced downstream signaling.

To further demonstrate the role of c-Cbl ligase activity on EGFR ubiquitination and EGFR downstream signaling activation, we infected myocytes with recombinant adenoviruses expressing Lac.Z or 70Z-Cbl, a c-Cbl mutant in which a 17–amino acid deletion in the helix linker and RING finger domain abolishes the ubiquitin ligase activity, resulting in a dominant-negative mutant. Overexpression of 70Z-Cbl significantly attenuated EGFR ubiquitination and p38 mitogen-activated protein kinase phosphorylation induced by H$_2$O$_2$ or EGF treatment compared with Lac.Z–infected cells (Figure V in the online-only Data Supplement).

To assess whether c-Cbl activation is involved in H$_2$O$_2$–induced myocyte apoptosis, myocytes infected with recombinant adenovirus expressing shRNA c-Cbl, shRNA control, Lac.Z, or 70Z-Cbl were evaluated for apoptotic signaling. Overexpression of c-Cbl shRNA or 70Z-Cbl significantly attenuated myocyte apoptosis induced by H$_2$O$_2$ treatment for 16 hours, as demonstrated by a decrease in the percentage of TUNEL–positive myocytes and DNA fragmentation (Figure 4D and 4E and Figure V in the online-only Data Supplement). No effect of Ad-shCbl or 70Z-Cbl expression

### Table. Echocardiographic Measurements in WT and c-Cbl Knockout Mice Subjected to Ischemia/Reperfusion Injury

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<th>Sham</th>
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<tr>
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<td>LVESD, mm</td>
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<td>LVPW Ts, mm</td>
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<td>LVPWTs, mm</td>
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*P<0.05 vs WT sham group.
†P<0.05 vs WT ischemia/reperfusion group.

BW indicates body weight; c-Cbl, proto-oncogene Casitas b-lineage lymphoma; HR, heart rate; HW, heart weight; LVEDD, left ventricular end-diastolic dimension; LVEDD left, ventricular end-diastolic dimension; LVDPW Td, left ventricular posterior wall thickness diastole; LVDPWs, left ventricular posterior wall thickness systole; and WT, wild-type.
on apoptosis was observed in control myocytes. These results suggest that inhibition of endogenous c-Cbl during oxidative stress is protective and that the protective effect of c-Cbl is cardiomyocyte cell autonomous.

Mice Lacking c-Cbl Are Protected Against Pathological Remodeling Induced After Myocardial Ischemia

To explore the potential influence of c-Cbl on cardiac remodeling responses after myocardial ischemia, we examined c-Cbl localization and expression 2, 7, and 30 days after MI. Figure 5A shows increased c-Cbl immunostaining in border-zone cardiomyocytes and infiltrating inflammatory cells at 7 and 30 days after MI. c-Cbl was not immunodetected in fibroblasts or myofibroblasts of the infarcted area. Quantification of c-Cbl accumulation in shams and the border zone of the infarct showed a 2.7±0.6-, 3.5±0.2-, and 3.7±0.3-fold increase over sham control at 2, 7, and 30 days after MI, respectively (Figure 5B).

Survival up to 4 weeks after MI was comparable in WT and c-Cbl KO shams (97% versus 98%, respectively). However, c-Cbl–deficient mice displayed remarkably improved postinfarction survival compared with WT (85% in c-Cbl KO mice compared with 55% in WT mice; P<0.05), especially in the first week after the insult (Figure 5C). Echocardiography 1 and 4 weeks after MI surgery indicated that LV end-diastolic dimension, LV end-systolic dimension, and LV ejection fraction improved significantly in Cbl KO compared with WT mice, whereas there were no differences in the sham-operated animals (Figure 5D and 5E and Tables III and IV in the online-only Data Supplement). Morphometric analysis at 4 weeks after MI indicated the
presence of cardiac hypertrophy and concomitant signs of cardiac failure, such as an increase in lung weight, in WT mice, whereas this pathological remodeling response was lacking in the c-Cbl KO mice (Figure 5F and 5G). Myocyte cross-sectional area, an index of myocyte size, mirrored the changes in cardiac hypertrophy and was significantly attenuated in c-Cbl KO compared with WT infarcted hearts (Figure VI in the online-only Data Supplement). Infarct size measurements as percentage of LV circumference indicated that 45±5% of the LV was affected by the infarct in WT, whereas the infarct accounted for 29±4% of the LV in c-Cbl KO mice (Figure 5H). Examination of cell death in the border zone indicated fewer TUNEL-positive cells in the c-Cbl KO mice compared with WT animals subjected to MI (data not shown). Thus, the integrity of the infarcted area was better maintained after MI in c-Cbl KO mice and likely prevented the heart from dilating to the same extent as in the WT mice, which results in a better maintenance of cardiac function after MI.

**Upregulation of Vascular Endothelial Growth Factor a/b and Vascular Endothelial Growth Factor Receptor 2 in the Infarcted Region of c-Cbl KO Mice**

One of the beneficial effects of c-Cbl deficiency on the cardiovascular system may be through an improvement in myocardial perfusion by the formation of new capillaries and by the enlargement of preexisting collateral vessels. One key regulator of this proangiogenic response is vascular endothelial growth factor (VEGF)a, which encodes an extracellular ligand for its corresponding endothelial receptors VEGF receptor (VEGFr)1 and VEGFr2.22,23 VEGFa, VEGFb, and VEGFr2 levels were significantly upregulated in c-Cbl KO compared with WT hearts in both sham and MI groups (Figure 6A and
6B). This observation is consistent with the fact that VEGFa induces expression of its receptors to create a positive feedback loop, allowing endothelial cells to become responsive to and activated by VEGF. Immunohistochemistry 7 days after MI indicated that VEGFr2 expression was significantly induced in the infarct area and the border zone, which was even more pronounced in the c-Cbl KO mice (Figure 6C). Erk 1/2 and AKT phosphorylation was also upregulated in c-Cbl KO compared with WT hearts in shams (Figure VIIA and VIIB in the online-only Data Supplement). However, no change in Erk 1/2 and AKT phosphorylation levels was observed between c-Cbl KO and WT mice after MI. Collectively, these data indicate that removal of c-Cbl increases the expression of VEGFa and its downstream receptor, which likely promotes cardio-protection after ischemic damage.

We next visualized vasculature using anti-CD31 as an endothelial surface marker and smooth muscle α-actin as a marker for smooth muscle cells. There was a regular distribution of capillaries around cardiomyocytes in both sham-operated groups, with no detectable differences in vessel density (Figure 6D and 6E). Four weeks after MI, the border zone of the infarcted area contained regions of low vascularity in WT mice, whereas the loss of cardiac function was substantially attenuated in c-Cbl KO mice (n=6 for sham groups; n=8 for MI groups).

Figure 5. Proto-oncogene Casitas b-lineage lymphoma (c-Cbl) knockout (KO) mice are protected against post-myocardial infarction (MI) cardiac remodeling. A, Representative micrographs of paraffin-embedded sections from mouse hearts subjected to permanent left coronary artery ligation for 2, 7, and 30 days. Bar, 40 μm. B, top, Representative immunoblot of left ventricular heart lysates for c-Cbl. Sh indicates sham. B, bottom: Fold induction of c-Cbl accumulation (n=5 for sham groups; n=6 for MI groups). C, Comparison of post-MI mortality between wild-type (WT; n=18) and c-Cbl knockout (KO; n=16) mice. c-Cbl KO mice showed an increase in survival after MI compared with WT mice (P<0.05). D and E, Cardiac function was measured by echocardiography 4 weeks after MI. The data demonstrate cardiac dilation and loss of contractile function in WT mice, as indicated by left ventricular end-systolic dimension (LVESD; D) and ejection fraction (E), whereas the loss of cardiac function was substantially attenuated in c-Cbl KO mice (n=6 for sham groups; n=8 for MI groups). F and G, c-Cbl deletion attenuated MI-induced increase in the ratio of heart weight (HW) to tibia length (TL; F) and the ratio of lung weight (LW) to TL (G). H, Infarct size 4 weeks after MI expressed as a fraction of the total cross-sectional circumference of the left ventricle indicates that the infarct size in c-Cbl KO mice is significantly smaller than the infarct size in WT. *P<0.05 compared with WT sham group; †P<0.05 compared with WT MI group.
WT animals, with a more pronounced reduction in the infarct. However, both the border zone and the infarcted region of the c-Cbl KO appeared to be highly vascularized, with enlarged thin-walled vessels (Figure 6C and 6D). The number of capillaries counted in the remote region showed no significant difference between WT and c-Cbl KO mice after MI, whereas this number was significantly increased in the border zone of the c-Cbl KO mice (Figure 6E). These findings suggest that the cardioprotection observed in Cbl KO mice is attributable, at least in part, to an increase in vessel formation after MI, which might involve VEGFa and its receptor VEGFr2.23

**Discussion**

In this study, we identified a novel role for the E3 ubiquitin ligase and adaptor molecule c-Cbl in mediating early stress responses in the heart. c-Cbl expression was strongly induced...
in human failing hearts, and its inhibition provided protection against IR injury, evidenced by decreased cardiac apoptosis and improved LV function. c-Cbl directly ubiquitinates EGFR and FAK such that decreased EGFR and FAK ubiquitination in c-Cbl KO mice reduced their degradation and attenuated cardiomyocyte death induced after IR. More interestingly, c-Cbl deletion attenuated LV hypertrophy, enhanced angiogenesis, and improved LV contractility after chronic ischemic insult. This seems to be related, in part, to increased VEGFa and VEGF receptor 2 (VEGFR2) expression. These data highlight c-Cbl as one of the important signaling molecules that is specifically involved in pathological cardiac remodeling.

Using c-Cbl KO mice, we provide the first evidence, to our knowledge, that this multifaceted protein is involved in the early responses induced by IR injury. Adult c-Cbl-deficient mice have virtually normal hearts under nonstressed, baseline conditions. Moreover, baseline heart rate, blood pressure, and maximal dp/dt were comparable in c-Cbl-deficient and WT mice. However, c-Cbl-deficient mice had smaller infarct sizes, reduced cardiomyocyte apoptosis in the infarct border zone after IR injury, and preserved cardiac contractile function compared with WT controls, indicating that endogenous c-Cbl mediates myocardial tissue damage in vivo. The cardioprotective effects of c-Cbl deletion correlate with the induction of XIAP expression as well as the phosphorylation of Bad, 2 molecules known to prevent myocyte death.23 We also found high basal levels of Akt and Erk1/2 phosphorylation in c-Cbl-deficient mice compared with WT controls, suggesting cardioprotective effects of increased Akt and Erk1/2 phosphorylation in these mice. Interestingly, these beneficial effects of c-Cbl deletion on myocyte apoptosis and cardiac function induced after IR injury occurred independently of c-Cbl effects on the immune system, as shown by the lack of significant changes in leukocyte infiltration and activation between c-Cbl KO and WT mice (Figure VIII in the online-only Data Supplement).

Rather, deletion of c-Cbl in cardiac myocytes conferred this protection against IR injury. Using CM-Cbl KO mice, we demonstrated that endogenous deletion of c-Cbl in cardiac myocytes offered cardioprotection against IR injury that mirrored the effect of global c-Cbl deletion. CM-Cbl KO mice showed less myocyte apoptosis, reduced infarct size, and improved cardiac function in response to IR injury. Furthermore, inhibition of c-Cbl expression or of its E3 ubiquitin ligase activity markedly attenuated myocyte apoptosis induced in response to oxidative stress, suggesting that endogenous expression of c-Cbl in myocytes plays an important role in mediating the deleterious effect of IR injury.

c-Cbl activation occurs in response to a variety of stimuli, including activated EGFR, cytokines, and hormones.9 We describe in the present report that c-Cbl ubiquitin ligase activity and its tyrosine phosphorylation were increased in hearts of mice subjected to IR injury and in cultured myocytes after H2O2 stimulation. Furthermore, inhibition of c-Cbl expression by shRNA or inhibition of its ubiquitin ligase activity significantly reduced myocyte apoptosis in response to H2O2. Given that H2O2 is a potent inducer of myocyte death that is released as a result of IR injury, it may account for the c-Cbl activation and myocyte death in the early IR injury. However, stimulation from other factors released after IR injury could not be excluded. In this regard, we showed recently that c-Cbl ubiquitin ligase activity is involved in mediating focal adhesion, myofibril protein degradation, and myocyte death induced by neutrophil-derived serine proteases.13 Together, these findings show that activation of c-Cbl after IR increases myocyte apoptosis and that a similar mechanism may be critical in other settings of cardiac stress.

An important finding of the present study is the identification of the molecular mechanisms by which the c-Cbl exerts its cardioprotective effect. c-Cbl RING finger has intrinsic E3 ligase activity and can recruit E2s for the transfer of ubiquitin to substrates.9 Activation of c-Cbl ligase activity has been shown to target several activated receptor and non-receptor tyrosine kinases and mediates their downregulation, thus providing a means by which signaling processes can be negatively regulated.26-28 In this study, we found that c-Cbl activation after IR injury correlated with c-Cbl interaction with EGFR and FAK. 2 known targets of c-Cbl.9 Interestingly, EGFR and FAK ubiquitination and degradation were significantly decreased in c-Cbl KO mice, suggesting that c-Cbl activation presumably mediates EGFR and FAK ubiquitination and degradation induced by IR injury. To our knowledge, this is the first demonstration that EGFR and FAK are being ubiquitinated and downregulated in vivo. A similar decrease in EGFR and FAK expression was observed in human failing hearts, but the mechanisms of such downregulation have never been elucidated.27,28 Because selective downregulation of EGFR and focal adhesion proteins appears to be critical for reduction in myocyte survival,17,18 they might be directly linked to reduced myocyte loss and enhanced cardiac contractility observed after IR injury in c-Cbl KO mice. These data extend our previous findings showing the role of c-Cbl ubiquitin ligase activity in FAK and myofibril protein degradation and myocyte death in response to neutrophil-derived serine proteases.13 Although our data that deletion of c-Cbl or inhibition of its ubiquitin ligase activity results in reduced EGFR ubiquitination and attenuated myocyte apoptosis in response to H2O2 clearly support the established model that c-Cbl is a negative regulator of tyrosine kinase signaling, the implication of the phosphoinositide-3 kinase interacting domain of c-Cbl (located at Y731, Y770, Y774) remains to be elucidated. In this regard, c-Cbl has been suggested to function as a positive regulator of signaling pathways through its adaptor function to recruit phosphoinositide-3 kinase and other Src homology domain 2-containing molecules in some systems.9,28 It is noteworthy that hearts from c-Cbl KO mice showed high basal levels of Akt and Erk1/2 phosphorylation compared with WT control mice. However, this basal increase in Akt and Erk1/2 phosphorylation did not affect heart weight in c-Cbl KO mice. The explanation for this lack of hypertrophy in c-Cbl KO mice needs further investigation. However, it has been reported that c-Cbl KO mice exhibit elevated energy expenditure and improved insulin action,29 which could, in an organ with high energy demands like the heart, play a role in maintaining cardiac structure and function and prevent cardiac hypertrophy.

In addition to its role in protecting myocyte death, our results show that c-Cbl deficiency also affected the levels of VEGFa and its receptor VEGF receptor 2 (VEGFR2) in response to chronic myocardial ischemia. Because the angiogenic activity of VEGF...
is dependent on its expression levels, this increase in total VEGF levels would be expected to result in vascular development/angiogenesis. However, c-Cbl KO mice showed no obvious defects in vascular development in shams despite increased levels of VEGFa and VEGF-R2. Early studies have demonstrated that loss of c-Cbl alone was dispensable for normal embryonic development, whereas loss of both c-Cbl and Cbl-b was embryonically lethal. Therefore, it is tempting to speculate that c-Cbl activity is not stringently required during development, and the function of Cbl family proteins may be compensatory in their ability to regulate the activity of target proteins. These data are consistent with previous studies showing normal embryonic and postnatal angiogenesis but impaired ischemia-induced angiogenesis in several gene KO mouse models, suggesting that c-Cbl selectively plays a role in pathological angiogenesis. Moreover, c-Cbl inactivation has been shown to enhance endothelial cell proliferation and tube formation in response to VEGF in vitro and results in increased tumor angiogenesis and retinal neovascularization in vivo. This latter effect of c-Cbl is mediated by a direct regulation of VEGF-R2 expression through its phosphorylation on Y1052 and Y1057 residues, as well as indirectly through PLCγ1 activation. In addition, it is notable that the enhancement of overall vascularity may not come from neoangiogenesis exclusively but also from protecting preexisting vascular cells. In fact, c-Cbl deletion protects cells from undergoing apoptosis after MI. Irrespective of whether c-Cbl removal enhances cardiac protection through upregulation of angiogenesis, through an increase in protection of preexisting vascular cells, or a combination of both, our findings show that c-Cbl is a negative regulator for angiogenesis by functioning as a molecular switch to fine-tune angiogenic events during pathological conditions such as MI.

The role of muscle-specific protein ubiquitin ligases as molecules that affect the severity of cardiac diseases is complex and often ambiguous. Activation of E3 ligases CHIP and MDM2 protected myocardium from IR injury, whereas activation of Atrogin1 and MuRF1 attenuated cardiac hypertrophy. The present study adds c-Cbl to the list of E3 ubiquitin ligases that play a role in cardiac protection after myocardial ischemia. Expression of c-Cbl was increased in human hearts with ICM and DCM, and its activation provided a means by which signaling emanating from EGFR and FAK can be negatively regulated. Inactivation of ubiquitin ligase activity of c-Cbl or inhibition of its expression may lead to enhanced and prolonged signaling, a function that can explain the increased survival signaling in myocytes expressing a ligase-deficient mutant of c-Cbl or in c-Cbl KO hearts, respectively. These data, along with the recent findings linking mutation in c-Cbl RING finger domain to Noonan syndrome, one of the most common genetic syndromes associated with congenital heart disease in humans, suggest that c-Cbl inhibition may constitute a novel therapy offering cardioprotection.

In conclusion, the results of this study suggest that c-Cbl deletion protects the heart against IR injury by decreasing/preventing ubiquitination and subsequent degradation of EGFR and FAK and myocyte death in response to injury. These findings provide evidence for an important role of c-Cbl in direct modulation of EGFR and focal adhesion protein turnover in the heart. Given that EGFR and FAK signaling downregulation also underlie cardiac hypertrophy and other form of heart disease, it is likely that c-Cbl plays a role in a variety of stress settings. Thus, modulating c-Cbl activation to attenuate myocyte death and to enhance angiogenesis may have potential implications in the treatment of cardiac disease.

Sources of Funding
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Disclosures
None.

References
c-Cbl Deletion Is Cardioprotective

Myocardial infarction due to coronary artery occlusion is one of the leading causes of death worldwide. The loss of blood flow after myocardial infarction results in loss of cardiac myocytes in the ischemic zone, which diminishes cardiac contractility and impedes angiogenesis and repair. Although much is known about the pathways that promote the pathological remodeling responses, mechanisms that promote myocyte loss and impair cardiac function have not been as clearly defined. The ubiquitin proteasome system is largely responsible for the degradation of misfolded and damaged proteins. The ubiquitin proteasome system is largely responsible for the degradation of misfolded and damaged proteins, and nonreceptor tyrosine kinases, is increased in human failing hearts. Inhibition of c-Cbl in mice reduces myocyte death, thereby regulating cardiac hypertrophy and remodeling. In the present study, we show that expression of proto-oncogene Casitas b-lineage lymphoma (c-Cbl), an adaptor protein with an intrinsic E3 ubiquitin ligase activity that targets receptor and nonreceptor tyrosine kinases, is increased in human failing hearts. Inhibition of c-Cbl in mice reduces myocyte death, enhances angiogenesis, and improves cardiac function and survival in response to myocardial ischemia. Thus, modulating c-Cbl activation to attenuate myocyte death and to enhance angiogenesis may have potential implications in the treatment of cardiac disease.
c-Cbl Inhibition Improves Cardiac Function and Survival in Response to Myocardial Ischemia

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SUPPLEMENTAL MATERIAL

Supplemental MATERIALS & METHODS

Materials: Monoclonal antibodies to c-Cbl and ubiquitin were obtained from BD Biosciences, and polyclonal antibody for FAK was from Santa Cruz Biotechnology. Phospho-c-Cbl Y\textsuperscript{774}, EGFR receptor (EGFR), Bcl2, phospho-Bad-S\textsuperscript{112}, Bad, c-IAP1, and XIAP were from Cell Signaling. Monoclonal antibodies for phosphotyrosine and ubiquitin Lys\textsuperscript{48} were from Upstate Biotechnology and anti-troponin I was from Sigma. All other chemicals were from standard suppliers.

Generation of cardiac myocyte-specific c-Cbl knockout mice: Conditional mice bearing floxed c-Cbl alleles (c-Cbl\textsuperscript{floxfloxA}) have previously been described.\textsuperscript{1} Transgenic mice overexpressing Cre-recombinase protein fused to 2 mutant estrogen receptor ligand-binding domains under the control of the \(\alpha\)-myosin heavy chain (\(\alpha\)-MHC) promoter (\(\alpha\)-MHC-MerCreMer)\textsuperscript{2} were received from The Jackson Laboratory (JAX Mice and Services, Bar Harbor, Me). Homozygous mice with the floxed c-Cbl alleles were crossed with \(\alpha\)MHC-MerCreMer mice and bred back to c-Cbl\textsuperscript{floxfloxA} to produce \(\alpha\)MHC-MerCreMer\(\times\)c-Cbl\textsuperscript{floxfloxA} mice. c-Cbl\textsuperscript{floxfloxA} littermate were taken as controls. All animals were bred and maintained on a C57Bl/6 background.

To induce Cre-dependent recombination, tamoxifen (20 mg/kg body weight, Sigma-Aldrich) was injected into 8-weeks-old animals intraperitoneally (i.p.) for 5 days. Tissue-specific c-Cbl deficiency was achieved 4 weeks after injection as verified by western blot analysis.\textsuperscript{2}

Genotyping of mice: The c-Cbl\textsuperscript{floxfloxA} and c-Cbl\textsuperscript{wt/wt} alleles and the Cre transgene were detected in mouse tail DNA by PCR. Primers for c-Cbl\textsuperscript{wt/wt} and c-Cbl\textsuperscript{floxfloxA} result in a 600bp and a 700bp product, respectively. The primer sets used are: Cre (Forward 5'-
TCCAATTTACTGACCGTACACCAA -3'; Reverse, 5'- CCTGATCTGGCAATTTCGGCTA -3'); c-Cbl^{flox/flox} (Forward 5'-GTTTGAGATGTCTGGCTG TGTGTACACGCG-3'; Reverse 5'-GTTTGAGATGTCTGGCTG TGTGTACACGCG-3'), c-Cbl^{/-} (Forward 5'-TCCCCTCCCCTTTCCCATGTTTTTAATAGACTC-3'; Reverse 5'-TGGCTGGACGTAAACTCCTCTCAGACCTAATAAC-3'), and c-Cbl^{wt/wt} (Forward 5'-GACGATAGTCCCGTGGAAGAGCTTTCGACA-3', Reverse 5'-CCTAAGTGGTATGATTATAATTGCAAGCCACCAC-3').

Assessment of area at risk and infarct size: After 24 hours of reperfusion, the slipknot was retied and the right carotid artery was cannulated to allow injection of KCL (40 mEq/L) followed by 1% Evan's blue dye for identification of the area at risk (AR). The hearts were excised, rinsed briefly in PBS, and sliced transversely. Slices were then incubated with 1% triphenyl-tetrazolium chloride (TTC, pH 7.4 in phosphate buffer) at 37°C for 30 minutes, fixed in 10% formalin, photographed, and the images were used to quantify IR-induced myocardial infarction using Bioquant software. The Evan's blue stained area defined the perfused area, whereas the Evan's blue unstained area defined the area at risk (AAR). The area lacking the red TTC staining within the AAR was considered as the infarct area (IA). Both the surgeon and the evaluator of infarct size were blinded to mouse genotypes.

Heart function: Echocardiographic measurements were taken before surgery and at 24 hours after IR injury or 4 weeks after MI to determine the baseline heart function and ventricular dimensions in the experimental groups. Briefly, following light sedation with 1% Isoflurane, the mice were placed on a heated platform in the left lateral decubitus position for imaging. A Visualsonic Ultrasound System (Vevo770) containing a 40 Mhz variable frequency probe was used to capture the echocardiogram. Standard long and short axis M-Mode views were recorded
when the mouse possessed a target heart rate between 450 and 550 beats per minute. End-diastolic and end-systolic interventricular septum (IVSd, IVDs), posterior wall thickness (PWTd, PWTs) and left ventricular internal diameters (LVEDD, LVESD) were calculated and averaged from 4 consecutive contractions using manufacturer’s software. Percent fractional shortening was calculated using: % FS= [(LVEDD-LVESD)/LVEDD] x 100. LVEF was calculated by the cubed method as follows: LVEF = [(LVEDD)^3 – (LVESD)^3]/(LVEDD)^3.

Hemodynamic measurements were performed using a 1.0 F catheter tip micromanometer (Millar PVR-1035). Following calibration, the catheter (connected to a Powerlab 40/3 data acquisition box; ADInstruments) was inserted through the right carotid artery of anesthetized mice. The first derivative of left ventricular pressure (dP/dT) was assessed using PVAN 3.6 software (Millar).

Neonatal rat cardiomyocyte isolation: Myocytes were isolated from the ventricles of neonatal Sprague-Dawley rats by collagenase digestion as previously described. After 30 minutes of preplating (to eliminate non-myocyte cell contamination), myocytes were plated in collagen precoated dishes or in fibronectin (BD bioscience) precoated glass coverslips at a density of 160,000/cm² in 10% fetal bovine serum DMEM supplemented with 1 mmol/liter L-glutamine, antibiotic/antimycotic solution, and 100 µmol/L 5-bromo-2-deoxyuridine (BrdU). Under these high density conditions, the myocytes form cell-cell contacts and display spontaneous contractile activity within 24 hours of plating.

Cardiac troponin-I ELISA assay: Mice were exsanguinated after IR injury and the serum levels of the cardiac-specific isoform of troponin-I were assessed using ELISA assay (Life Diagnostics, PA).

Immunoprecipitation and immunoblot analysis: Extraction of proteins from heart tissue samples was performed as described previously. Briefly, lysates were cleared by centrifugation at
12,000 rpm and the supernatants (800 µg of protein/ml) were subjected to immunoprecipitation with corresponding antibodies. After overnight incubation at 4 °C, protein A- or G-agarose beads were added and left for an additional 3 hours. Immunocomplexes were then subjected to SDS-PAGE followed by Western blot analysis according to methods published previously or to the manufacturer's instructions. Each panel in each figure represents results from a single gel exposed for a uniform duration, with bands detected by enhanced chemiluminescence and quantified by laser scanning densitometry.

c-Cbl auto-ubiquitination assay: c-Cbl ubiquitin ligase activity was performed using auto-ubiquitination assay kit from Enzo Life Sciences with minor modification. Briefly, c-Cbl immunoprecipitates were washed twice with lysis buffer, once with ubiquitination buffer and then were incubated with 30 µL of ligase reaction buffer mixture containing ubiquitination buffer, 1 mmol/L DTT, 20 U/ml inorganic pyrophosphatase, 5 mmol/L Mg-ATP, 2.5 µM ubiquitin, 100 nmol/L E1, and 2.5 µM at 37°C for 1 h. The reaction was quenched by adding 30 µl of 2x non-reducing gel loading buffer and heating at 95°C for 5 minutes. Finally, the samples were assessed for immunoblotting with anti-ubiquitin for detecting ubiquitinated c-Cbl and for c-Cbl as a loading control.

Expression of adenoviral vectors: Production of recombinant adenovirus expressing Lac-Z, ligase deficient c-Cbl (70Z-Cbl), a mutant that lacks 17 amino acid in the RING finger domain necessary for ubiquitin ligase activity, was described elsewhere. The 70Z Cbl plasmids were a gift from Tsyganov A (Temple University, USA). Adenoviral vectors were purified using a kit from Virapur and titrated using BD Adeno-X rapid titer kit (BD Bioscience). After 24 hours of plating, NRCMs were infected with adenoviruses expressing Lac-Z (10 pfu/cell), or 70Z-Cbl-Cbl (5 pfu/cell) in DMEM for 2 h, then 5% fetal bovine serum DMEM was added, and cells were
incubated for an additional 48 h. Serum-free DMEM/F-12 medium was changed 1 hour before
the start of the experiments.

*Preparation of adenoviral shRNA:* The generation of adenovirus harboring shRNA constructs of
c-Cbl and control has been described previously. Briefly, DNA sequences that encoded short
hairpin RNA to c-Cbl (Ad-shCbl) 5′-
CACCGCAGATGGCTCAAGAGACACGAATGTCTCTTGAGCCATCTGC-3′ or an inactive
randomized control RNA (Ad-shCtrol) 5′-
CACCGCCTCCAGCACTGTGTTAACGAATTAACGACAGTGGAGG-3′ were cloned
into pENTRU6 vector, recombined into pAd/BLOCKit vector (Gateway system; Invitrogen), and
packaged into recombinant adenoviruses expressing Ad-shCbl or Ad-Ctrl by using U293 cells
(Invitrogen) following the manufacturer's instructions. Adenovirus was added to neonatal rat
cardiomyocytes after 1 day of culture on collagen at 10 plaque forming unit (PFU)/cell, which
resulted in a 75-85% down-regulation of c-Cbl relative to uninfected or control-shRNA-infected
myocytes.

*Immunostaining.* Paraffin heart sections were deparaffinized in xylene and re-hydrated. Antigen
retrieval was achieved by boiling the slides in citrate solution for 12-15 minutes and slides were
then washed with phosphate-buffered Saline (PBS). After quenching endogenous tissue
peroxidase activity with 3% H₂O₂ for 20 minutes, slides were then washed in PBS and samples
blocked in PBS containing 5% bovine serum albumin (BSA) at room temperature for 30
minutes. Primary antibodies to detect c-Cbl (BD Biosciences), CD31 (Santa-Cruz
Biotechnology), SM α-actin (Sigma), and VEGFr2 (Cell Signaling) were applied overnight at
4°C in PBS containing 2% BSA. The next day, samples were washed in PBS and then
sequentially incubated with Vectastain Elite ABC Kit (Avidin/Biotin/Horseradish Peroxidase-
System (Vector Laboratories) or with fluorophore-secondary antibody conjugates (Molecular Probes). The peroxidase reaction was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) and slides were counterstained with Hematoxylin.

Cathepsin G activity in LV tissue lysates. Snap frozen LV tissues were homogenized in ice-cold buffer containing 100 mmol/L Hepes, pH 7.5, 1 mol/L NaOH, 50 mmol/L CaCl₂, and 0.01% Igepal CA-630 in presence or absence of Cat.G inhibitor. After centrifugation, supernatants containing 100 µg proteins were used for Cat.G activity assay by measurement of the rate of cleavage of fluorogenic conjugated substrate Suc-Ala₂-Pro-Phe-Amc (R&D Systems).

Measurement of myeloperoxidase activity. MPO activity in LV homogenates was determined using MPO peroxidation assay kit from Cayman Chemicals. Briefly, LV samples were suspended in lysis buffer provided in the kit and homogenates were cleared by centrifuging at 10,000 rpm at 4°C. The samples were incubated with hydrogen peroxide and the substrate ADHP (10-acetyl-3,7-dihydroxyphenoxazine). Fluorescence was then analyzed with an excitation wavelength of 540 nm and emission wavelength of 595 nm. The specificity of the assay was confirmed by addition of a MPO inhibitor (4-aminobenzhydrazide) in the reaction mixture prior to the assay.

Terminal deoxynucleotidyl transferase (TdT) and tropomyosin immunolabeling. Three sections from each LV cut perpendicularly to the major axis of the heart were sampled. TdT assay was performed using kit from Promega. Positive cells were counted throughout the LV and were expressed as percentage of the total number of nuclei as determined by DAPI (Molecular probes) staining.
Apoptotic Cell Death ELISA: A cell death detection ELISA kit (Roche Applied Science) was used to quantitatively determine the apoptotic DNA fragmentation by measuring the cytosolic histone-associated mono- and oligo-nucleosomes fragments associated with apoptotic cell death.
**Supplemental Table 1.** Demographic and clinical data.

<table>
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<tr>
<th></th>
<th>Non-failing (n=5)</th>
<th>DCM (n=5)</th>
<th>ICM (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62 ± 9</td>
<td>49 ± 11</td>
<td>52 ± 9</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>1/4</td>
<td>2/3</td>
<td>5/0</td>
</tr>
<tr>
<td>Heart Weight (g)</td>
<td>418 ± 68</td>
<td>561 ± 218</td>
<td>514 ± 165</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>60 ± 5</td>
<td>14 ± 6 *</td>
<td>24 ± 22 *</td>
</tr>
<tr>
<td>Inotropes (yes/no)</td>
<td>1/4</td>
<td>2/3</td>
<td>2/3</td>
</tr>
<tr>
<td>ACE inhibitors (yes/no)</td>
<td>0/5</td>
<td>5/0</td>
<td>3/2</td>
</tr>
<tr>
<td>β-blockers (yes/no)</td>
<td>1/4</td>
<td>2/3</td>
<td>3/2</td>
</tr>
</tbody>
</table>

DCM: dilated cardiomyopathy; ICM: ischemic cardiomyopathy; ACE: angiotensin II converting enzyme. *p<0.001 compared to non-failing group.
Supplemental Table 2. Echocardiographic measurements in CM-Cbl KO and Cbl$^{flox/flox}$ littermates subjected or not to IR injury.

<table>
<thead>
<tr>
<th></th>
<th>Sham Cbl$^{flox/flox}$ (n=5)</th>
<th>Sham CM-Cbl KO (n=6)</th>
<th>IR Cbl$^{flox/flox}$ (n=5)</th>
<th>IR CM-Cbl KO (n=6)</th>
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<tbody>
<tr>
<td>HR (bpm)</td>
<td>465 ±18</td>
<td>453 ±14</td>
<td>473 ±24</td>
<td>485 ±32</td>
</tr>
<tr>
<td>HW (mg)</td>
<td>111 ±10</td>
<td>108 ±10</td>
<td>110 ±8</td>
<td>104 ±7</td>
</tr>
<tr>
<td>BW (g)</td>
<td>21 ±2</td>
<td>21 ±3</td>
<td>21 ±2</td>
<td>19 ±2</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>5.3 ±0.2</td>
<td>5.1 ±0.1</td>
<td>5.4 ±0.2</td>
<td>5.5 ±0.3</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.66 ±0.13</td>
<td>3.43 ±0.12</td>
<td>3.97 ±0.11*</td>
<td>3.63 ±0.12†</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.34 ±0.12</td>
<td>2.18 ±0.14</td>
<td>3.12 ±0.13*</td>
<td>2.60 ±0.15*†</td>
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<tr>
<td>LVPWTd (mm)</td>
<td>0.78 ±0.01</td>
<td>0.83 ±0.02</td>
<td>0.76 ±0.04</td>
<td>0.86 ±0.06</td>
</tr>
<tr>
<td>LVPWTs (mm)</td>
<td>1.12 ±0.05</td>
<td>1.17 ±0.08</td>
<td>0.94 ±0.06</td>
<td>1.07 ±0.10</td>
</tr>
</tbody>
</table>

HR indicates heart rate; HW, heart weight; BW, body weight; LVEDD, LV end diastolic dimension; LVESD, LV end systolic dimension; LVPWTd, LV posterior wall thickness diastole; and LVPWTs, LV posterior wall thickness systole. *$P<0.05$ vs. WT shams, †$P<0.05$ vs. WT IR
**Supplemental Table 3.** Echocardiographic measurements in WT and c-Cbl KO mice subjected or not to 7d MI injury.

<table>
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<tr>
<th></th>
<th>Sham</th>
<th>7d MI</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>c-Cbl KO</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td>(n=5)</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>428 ±13</td>
<td>471 ±24</td>
</tr>
<tr>
<td>HW (mg)</td>
<td>137 ±5</td>
<td>117 ±10</td>
</tr>
<tr>
<td>BW (g)</td>
<td>24 ±2</td>
<td>25 ±2</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>5.7 ±0.3</td>
<td>5.5 ±0.3</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>65 ±2</td>
<td>64 ±2</td>
</tr>
<tr>
<td>LVFS (FS)</td>
<td>36 ±2</td>
<td>34 ±3</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.39 ±0.10</td>
<td>3.15 ±0.15</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.19 ±0.1</td>
<td>2.00 ±0.12</td>
</tr>
<tr>
<td>LVPWTd (mm)</td>
<td>0.98 ±0.01</td>
<td>0.94 ±0.01</td>
</tr>
<tr>
<td>LVPWTs (mm)</td>
<td>1.45 ±0.04</td>
<td>1.49 ±0.03</td>
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</tbody>
</table>

HR indicates heart rate; HW, heart weight; BW, body weight; LVEF, LV ejection fraction; LVFS, LV fractional shortening; LVEDD, LV end diastolic dimension; LVESD, LV end systolic dimension; LVPWTd, LV posterior wall thickness diastole; and LVPWTs, LV posterior wall thickness systole. *P<0.05 vs. WT shams, †P<0.05 vs. WT MI
**Supplemental Table 4.** Echocardiographic measurements in WT and c-Cbl KO mice subjected or not to 30d MI injury.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>30d MI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n=6)</td>
<td>c-Cbl KO (n=6)</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>439 ±13</td>
<td>471 ±24</td>
</tr>
<tr>
<td>HW (mg)</td>
<td>135 ±5</td>
<td>117 ±10</td>
</tr>
<tr>
<td>BW (g)</td>
<td>25 ±2</td>
<td>25 ±2</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>5.2 ±0.2</td>
<td>5.6 ±0.4</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>63 ±2</td>
<td>64 ±2</td>
</tr>
<tr>
<td>LVFS (FS)</td>
<td>34 ±2</td>
<td>35 ±2</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.41 ±0.10</td>
<td>3.23 ±0.16</td>
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<tr>
<td>LVESD (mm)</td>
<td>2.10 ±0.1</td>
<td>2.04 ±0.20</td>
</tr>
<tr>
<td>LVPWTd (mm)</td>
<td>0.99 ±0.01</td>
<td>0.93 ±0.02</td>
</tr>
<tr>
<td>LVPWTs (mm)</td>
<td>1.58 ±0.04</td>
<td>1.38 ±0.04</td>
</tr>
</tbody>
</table>

HR indicates heart rate; HW, heart weight; BW, body weight; LVEF, LV ejection fraction; LVFS, LV fractional shortening; LVEDD, LV end diastolic dimension; LVESD, LV end systolic dimension; LVPWTd, LV posterior wall thickness diastole; and LVPWTs, LV posterior wall thickness systole. *P<0.05 vs. WT shams, †P<0.05 vs. WT MI
SUPPLEMENTARY FIGURE LEGEND

Supplemental Figure S1. Targeting strategy for specific disruption of c-Cbl in cardiac myocytes. (A) Generation of c-Cbl floxed mutant mice has been previously described. In brief, the floxed Cbl allele was generated by introducing two loxP sequences (triangles) into the introns flanking one of the exons of c-Cbl (corresponding to nucleotides 681–837 of the mouse c-Cbl cDNA) by gene targeting. Removal of the floxed exon by Cre-loxP-mediated DNA recombination generated transcripts out-of-frame for translation. PTB, phosphotyrosine binding domain; RF, RING-finger domain; LZ, leucine-zipper domain; S, SphI site. (B) Cardiac myocyte-specific deletion of c-Cbl mediated by αMHC-MerCreMer transgene expression. Genomic DNA samples from heart tissues obtained from Cbl<sup>flox/flox</sup> and CM-Cbl KO mice (α-MHC-MerCreMer<sup>×</sup>c-Cbl<sup>flox/flox</sup>) were digested with SphI and hybridized with the probe in (A). (C) Immunoblot analysis of c-Cbl deletion in the heart. Heart lysates were immunoblotted with c-Cbl antibodies. The same membrane was reprobed for GAPDH as a loading control.

Supplemental Figure S2: Cardiac myocyte-specific deletion of c-Cbl improves cardiac function. The left anterior descending artery was ligated for 30 minutes to induce ischemia and it subsequently was reperfused for 24 hours. Cardiac function was measured by echocardiography after IR injury. The data demonstrate improved left ventricular ejection fraction (A) and fractional shortening (B) in CM-Cbl KO compared to c-Cbl<sup>flox/flox</sup> mice (n=7 in sham groups; n=8 in MI groups). (C and D) Cardiac-myocyte deletion of c-Cbl attenuated IR-induced increase in LV end-systolic (C) and end-diastolic dimension (D). (E) Quantification of infarct area (IA) vs. area at risk (AAR) after IR injury shows significantly reduced infarct in CM-Cbl KO compared to c-Cbl<sup>flox/flox</sup> mice (n=6 per group). *P<0.05 vs. c-Cbl<sup>flox/flox</sup> shams. †P<0.05 vs. c-Cbl<sup>flox/flox</sup> IR.
Supplemental Figure S3: Cardiac myocyte-specific deletion of c-Cbl reduces apoptotic cell death. The left anterior descending artery was ligated for 30 minutes to induce ischemia and it subsequently was reperfused for 24 hours. (A) LV tissue sections from Cbl^{flox/flox} (a, c) or CM-Cbl KO (b, d) were assessed for apoptosis using TUNEL assay (green), tropomyosin (red), and DAPI (4',6-diamidino-2-phenylindole) (blue) staining. Arrows indicate apoptotic myocytes. Bar=40 µm (a, b) or 20 µm (c, d). (B) The number of TUNEL-positive myocytes in the ischemic area was expressed as a percentage of total nuclei detected by DAPI staining. (C) Quantification of caspase-3 activity in the LV using caspase-3 specific fluorogenic substrate. *P<0.05 vs. Cbl^{flox/flox} shams. †P<0.05 vs. Cbl^{flox/flox} IR.

Supplemental Figure S4. c-Cbl deletion attenuates H2O2-induced AKT, but not JNK, phosphorylation. Neonatal rat cardiac myocytes were transduced with adenoviruses expressing shRNA c-Cbl or shRNA control for 48 hours and then untreated or treated with 100 ng/ml EGF or 100 µM H2O2 for 10 minutes. Cell lysates were assayed for immunoblot analysis. Experiments were performed at least three times from three different cultures and the data values were scaled to untreated Ad-shCtrl. *P<0.05 vs. control; †P<0.05 vs. treated myocytes.

Supplemental Figure S5. c-Cbl ubiquitin ligase activity is involved in H2O2-induced myocyte apoptosis. (A) Neonatal rat cardiac myocytes were transduced with adenoviruses expressing Lac.Z or ligase deficient c-Cbl mutant 70Z-Cbl for 48 hours and then untreated or treated with 100 ng/ml EGF or 100 µM H2O2 for 10 minutes. Cell lysates were assayed for EGFR immunoprecipitation assays (A) or immunoblot analysis (B). Experiments were
performed at least three times from three different cultures and the data values were scaled to untreated Ad-shCtrl. (C-D) Myocyte apoptosis induced by H2O2 as assessed by percentage of TUNEL-positive myocytes in culture (C) or DNA fragmentation assay (D). Results are expressed as (OD410-OD500)/mg DNA (D) for triplicate determinations from a single experiment (mean ± SE). *P<0.05 vs. control; †P<0.05 vs. treated myocytes.

Supplemental Figure S6. Deletion of c-Cbl reduces myocyte hypertrophy 4 weeks after MI. c-Cbl or WT mice were subjected to MI for 4 weeks. (A) Histological assessment of cellular pathology of c-Cbl KO or WT mice by Masson’s trichrome staining. Scale Bar: 40 µm. (B) Histological analysis of myocyte cross-sectional area from sham and infarcted LV. Values are mean ±SE. *p<0.05 vs. WT sham; †P<0.05 vs. WT MI.

Supplemental Figure S7. Effect of c-Cbl deletion on Erk1/2 and AKT phosphorylation in infarcted LV. Top: Immunoblot analysis indicates a similar increase in Erk1/2 and AKT phosphorylation in the infarcted region of the c-Cbl KO mice and the WT 7 days post-MI. Bottom: Data are represented as fold change compared to WT animals (n=6 per group; means±SEM). *P<0.05 compared with the WT sham group, †P<0.05 compared with the WT MI group.

Supplemental Figure S8. Leukocyte infiltration and activation induced after IR injury is minimally affected by c-Cbl deletion. The left anterior descending artery was ligated for 30 minutes to induce ischemia and it subsequently was reperfused for 24 hours. (A) Representative immunolabeling of paraffin-embedded heart sections stained for MPO and counterstained with
hematoxylin. Scale Bar: 40 µm. (B) Quantification of MPO positive cells in the ischemia reperfused area. (C-D) LV homogenates from shams or mice subjected to IR for 24 hours were assessed for myeloperoxidase (MPO) (C) or cathepsin G (Cat.G)(D) activity assay using specific fluorogenic substrates. Results are expressed as relative fluorescence unit (RFU)/min/g protein. Values are mean ±SEM. *p<0.05; **p<0.01 vs. WT sham.
Supplemental Figure S1
Supplemental Figure S2
Supplemental Figure S3

A

Cbl<sup>flox/flox</sup>

CM-Cbl KO

B

![](image)

C

![](image)
Supplemental Figure S4
Supplemental Figure S6
Supplemental Figure S7
Supplemental Figure S8
SUPPLEMENTAL REFERENCES


