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The ubiquitin-proteasome system (UPS) degrades a protein molecule via 2 main steps: ubiquitination and proteasomal degradation. Extraproteasomal ubiquitin receptors are thought to couple the 2 steps, but this proposition has not been tested in vivo with vertebrates. More importantly, impaired UPS performance plays a major role in cardiac pathogenesis, including myocardial ischemia-reperfusion injury (IRI), but the molecular basis of UPS impairment remains poorly understood. Ubiquilin1 is a bona fide extraproteasomal ubiquitin receptor. Here, we report that mice with a cardiomyocyte-restricted knockout of Ubiquilin1 (Ubqln1-CKO mice) accumulated a surrogate UPS substrate (GFPdgn) and increased myocardial ubiquitinated proteins without altering proteasome activities, resulting in late-onset cardiomyopathy and a markedly shortened life span. When subject to regional myocardial ischemia-reperfusion, young Ubqln1-CKO mice showed substantially exacerbated cardiac malfunction and enlarged infarct size, and conversely, mice with transgenic Ubqln1 overexpression displayed attenuated IRI. Furthermore, Ubqln1 overexpression facilitated proteasomal degradation of oxidized proteins and the degradation of a UPS surrogate substrate in cultured cardiomyocytes without increasing autophagic flux. These findings demonstrate that Ubiquilin1 is essential to cardiac ubiquitination-proteasome coupling and that an inadequacy in the coupling represents a major pathogenic factor for myocardial IRI; therefore, strategies to strengthen coupling have the potential to reduce IRI.
Inadequate ubiquitination-proteasome coupling contributes to myocardial ischemia-reperfusion injury

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The ubiquitin-proteasome system (UPS) degrades a protein molecule via 2 main steps: ubiquitination and proteasomal degradation. Extraproteasomal ubiquitin receptors are thought to couple the 2 steps, but this proposition has not been tested in vivo with vertebrates. More importantly, impaired UPS performance plays a major role in cardiac pathogenesis, including myocardial ischemia-reperfusion injury (IRI), but the molecular basis of UPS impairment remains poorly understood. Ubiquilin1 is a bona fide extraproteasomal ubiquitin receptor. Here, we report that mice with a cardiomyocyte-restricted knockout of Ubiquilin1 (Ubqln1-CKO mice) accumulated a surrogate UPS substrate (GFPdgn) and increased myocardial ubiquitinated proteins without altering proteasome activities, resulting in late-onset cardiomyopathy and a markedly shortened life span. When subject to regional myocardial ischemia-reperfusion, young Ubqln1-CKO mice showed substantially exacerbated cardiac malfunction and enlarged infarct size, and conversely, mice with transgenic Ubqln1 overexpression displayed attenuated IRI. Furthermore, Ubqln1 overexpression facilitated proteasomal degradation of oxidized proteins and the degradation of a UPS surrogate substrate in cultured cardiomyocytes without increasing autophagic flux. These findings demonstrate that Ubiquilin1 is essential to cardiac ubiquitination-proteasome coupling and that an inadequacy in the coupling represents a major pathogenic factor for myocardial IRI; therefore, strategies to strengthen coupling have the potential to reduce IRI.

Introduction

Targeted removal of individual abnormal protein molecules in the cell is primarily performed by the ubiquitin-proteasome system (UPS). Hence, the UPS is vital to cellular protein quality control (PQC), which functions to minimize the level and toxicity of misfolded proteins. By regulatory degradation of most cellular proteins that are normal but no longer needed, the UPS also participates in the regulation and execution of virtually all cellular processes (1, 2). Thus, the proper functioning of the UPS is pivotal to both the functioning and survival of the cell. UPS-mediated degradation of a protein molecule is generally known to take 2 main steps: step 1 is the covalent attachment of a ubiquitin (Ub) chain to a target protein molecule via a process known as ubiquitination, in which Ub ligases (E3) are rate limiting and determine substrate specificity; step 2 is proteasome-mediated degradation of the ubiquitinated protein molecule (1, 2). The pathophysiological significance of defects in either step is being rapidly elucidated, as exemplified by identification of genetic mutations of Ub and Ub ligases in human diseases (3–7), by revelation of the essential roles of Ub ligases in organ/system (patho)physiology (6–14), and by demonstration of the therapeutic effectiveness of measures priming the proteasome in some of the most common and debilitating diseases or pathological processes in animal models (15–18). These exciting recent advances in UPS research have illustrated that dysfunction in either the ubiquitination or the proteasomal steps can impair UPS functioning and play important pathogenic roles. Notably, studies using cell culture and lower species have suggested that the coupling between ubiquitination and proteasomal degradation also represents an important step in UPS-mediated proteolysis (19); however, the coupling factors have rarely been investigated using vertebrates. More importantly, the pathophysiological significance of impaired or inadequate coupling has, for the most part, not been explored. The present study was conducted to fill these critical gaps.

Polyubiquitination and proteasomal degradation are coupled by a family of proteins known as Ub receptors, which recruit polyubiquitinated proteins and dock them to the proteasome for degradation (20). The 19S proteasome subunits Rpn10/S5a and Rpn13 harbor Ub interacting motif (UIM) domains and a pleckstrin-like receptor for Ub (Pru) domain, respectively; hence, they can serve as intraproteasomal Ub receptors to directly bind polyubiquitinated proteins for degradation (21). However, the recruitment and docking of polyubiquitinated proteins to the 26S proteasome seem to also employ shuttling factors or extraproteasomal Ub receptors. The latter must be capable of both binding ubiquitinated substrates and interacting with the 19S proteasome (21). The UBL-UBA family proteins, including Ubiquilin1 (Ubqln1) and the mammalian homologs of Rad23 and Ddi1, are ideally suited to do so and are regarded as the shuttling Ub receptors. These...
include the Ub-like (UBL) domain at the N terminus and the Ub-associated (UBA) domain or domains at the C terminus. The UBA binds polyubiquitinated proteins, while the UBL can interact with the 19S proteasome via Rpn10 or Rpn1 (20).

Among UBL-UBA proteins, Rad23 is extensively studied for its role in DNA repair (22); however, very few reported studies have examined the physiological and pathophysiological significance of the remote recruitment of ubiquitinated proteins to the proteasome in a mammalian organ (23, 24). Defective or inadequate ubiquitination-proteasome coupling has been implicated by the frequent coexistence of increased ubiquitinated proteins with normal or even increased proteasome peptidase activities in the heart under many pathological conditions, such as proteinopathy (25, 26), pressure overload (27, 28), myocardial ischemia (29). Therefore, unraveling the pathophysiological significance of this inadequate coupling in the heart is expected to improve understanding of cardiac UPS dysfunction and its role in cardiac pathogenesis.

Members of the Ubiquilin (Ubqln) family of Ub receptors are mammalian orthologues of yeast Dsk2, which is the primary Ub receptor for shuttling ubiquitinated misfolded proteins to the proteasome for degradation in yeast (19). Ubqln genes are highly conserved among mammals. Ubqln1 is ubiquitously expressed and Ubqln2 and Ubqln4 are expressed in most tissues, including the heart, but Ubqln3 is only expressed in testis (30). Ubqln proteins share a high degree of sequence and domain structural homology. Like other UBL-UBA proteins, Ubqln1 harbors a UBL domain at the N terminus and a UBA domain at the C terminus. Compared with 7 other Ub-binding domains, the UBA domain of Ubqln1 was found to bind the broadest range of Ub moieties, capturing most types of Ub moieties (31), with binding affinity similar to that of K48- and K63-linked poly-Ub chains (32). Located between UBL and UBA domains is a central region containing multiple STI1 motifs that may confer Ubqln1 with a chaperone-like function. Hence, Ubqln1 is purported to deliver polyubiquitinated proteins to the proteasome for degradation (33). Endoplasmic reticulum–associated (ER-associated) PQC is responsible for the quality control of proteins targeted to the secretory pathway, which is pivotal to cell functioning and survival (34). Terminally misfolded ER proteins are retrotranslocated to the cytosolic side of the ER membrane, where they are degraded via the ER-associated degradation (ERAD) pathway, which is activated by the UPS. ER stress is induced by an accumulation of misfolded proteins in the ER and triggers the classical unfolded protein response (UPR), which attempts to resolve the ER stress through suppressing nonessential protein synthesis and increasing ER chaperoning and ERAD capacity via various transcriptional and posttranscriptional mechanisms (34, 35). Work using mammalian model cell lines (e.g., HEK293 cells) has shown that Ubqln1 is upregulated by ER stress and is recruited to the ERAD complex, where it interacts with UBXD2 (also known as erasin) along with p97/VCP (36), indicating that the shuttling role of Ubqln1 is likely important for ERAD, in addition to ER-independent PQC (37). To date, the role of Ubqln in cardiomyocytes and in the heart remains undocumented.

In the present study, we demonstrate that Ubqln1 plays an important role in cardiac ubiquitination-proteasome coupling and that mice with perinatal cardiomyocyte-restricted knockout of the Ubqln1 gene (Ubqln1-CKO) develop late-onset cardiomyopathy. Moreover, we provide proof-of-principle evidence that inadequacy in ubiquitination-proteasome coupling represents a major pathogenic factor for myocardial ischemia-reperfusion injury (IRI), suggesting that facilitating the coupling may be a novel therapeutic strategy to reduce IRI.

**Results**  
Ubqln1 colocalizes with the proteasome and is recruited to the ERAD upon ER stress in cardiomyocytes. To investigate the distribution of Ubqln1 proteins in cardiomyocytes, we performed immunofluorescence confocal microscopy of cultured neonatal rat ventricular myocytes (NRVMs) overexpressing a FLAG-tagged full-length Ubqln1. Double-immunostaining revealed that Ubqln1 and Psmb5 (a stoichiometric subunit of the 20S proteasome) are colocalized, showing a striated pattern of enrichment (Figure 1A). The Psmb5-marked proteasome distribution in the cultured cardiomyocytes was in agreement with prior reports showing the enrichment of cardiac proteasomes in the intermyofibrillar space at the z-line level (38, 39). The colocalization between Ubqln1 and proteasomes is consistent with the purported role of Ubqln1 in coupling ubiquitination to proteasomal degradation. The physical interaction of Ubqln1 with the proteasome is further confirmed by our coimmunoprecipitation (co-IP) studies, which revealed that Rpt2, a stoichiometric subunit of the 19S proteasome, was detected in the Ubqln1 IP (Figure 1B). In HEK293 cells, Ubqln1 was shown to bind UBXD2 (also known as Erasin) (36), a known component of the ERAD machinery (40); our co-IP experiments confirmed that this also occurs in cardiomyocytes when ER stress is induced with tunicamycin, a prototype inducer of ER stress that achieves that induction through inhibition of protein glycosylation (Figure 1B), suggesting that Ubqln1 is likely involved in ERAD in cardiomyocytes during ER stress.

We performed Western blot analysis for myocardial Ubqln1 levels of WT mice subject to sham surgery or myocardial IRI induced via left anterior descending coronary artery (LAD) ligation for 30 minutes, followed by release for 24 hours. The result revealed that Ubqln1 was only detected in the soluble fraction of myocardial proteins in both IRI and sham surgery control groups and that Ubqln1 protein levels in the region remote to the IRI were considerably higher in the IRI group than in the sham group (Figure 1, C and D).

**Mice with perinatal Ubqln1-CKO develop late-onset cardiomyopathy.** By coupling a Ubqln1 floxed allele (Ubqln1lox/lox) with the cre transgene driven by the Myh6 promoter (Myh6-cre) (41), we successfully generated perinatal Ubqln1-CKO mice. In homozygous Ubqln1-CKO (Ubqln1lox/lox::Myh6-cre) mice, myocardial Ubqln1 protein levels were markedly reduced at postnatal day 1 and further reduced at day 3 and day 7. By day 7, the remaining myocardial Ubqln1 proteins were less than 10% of those of the Ubqln1lox/lox and Myh6-cre control mice (Figure 2A). The residual Ubqln1 proteins were likely from the noncardiomyocyte compartment. Offspring from breeding pairs between Ubqln1lox/lox and Ubqln1lox/lox::Myh6-cre or between Ubqln1lox/lox and Ubqln1lox/lox::Myh6-cre displayed the expected genotypes of the Mendelian ratio at birth, suggesting no embryonic lethality. No gross abnormality was discernible during the first 7 months of age in any of these genotypes, including homozygous...
Ubqln1-CKO mice. However, by 5 months of age, echocardiography revealed cardiac malfunction, as reflected by moderate but statistically significant increases in left ventricle (LV) end-systolic internal diameter (LVIDs) and decreases in ejection fraction (EF), fractional shortening (FS), stroke volume (SV), and cardiac output (CO) in the homozygous Ubqln1-CKO mice compared with the control group (Figure 2, B and C). Strikingly, homozygous Ubqln1-CKO mice died prematurely, with a median life span of 322 days, which is significantly shorter than that of the heterozygous Ubqln1-CKO (Ubqln1fl/fl::Myh6-cre) mice (Figure 2D). These observations show that perinatal Ubqln1-CKO causes a late-onset cardiomyopathy in mice, demonstrating that Ubqln1 is dispensable for perinatal and postnatal cardiac development, but that it inevitably plays an important role in maintaining normal cardiac function. Notably, the heterozygous Ubqln1-CKO mice lived longer than the homozygotes, but still showed a significantly shortened life span compared with the littermate Ubqln1fl/fl mice (Figure 2D), which suggests a gene-dosage effect. However, a gene-dosage effect on life span is uncertain, as the potential contribution of Myh6-cre to this phenotype observed in the heterozygous Ubqln1-CKO could not be ruled out.

Ubqln1-CKO compromises ubiquitination-proteasome coupling and impairs UPS performance in the heart. As a mammalian orthologue of yeast Dsk2, Ubqln1 is purported to function as a Ub receptor that shuttles ubiquitinated proteins to the proteasome for degradation, which is critical to UPS-mediated protein degradation (19); however, this proposition has not been formally tested in animals. Hence, we assessed cardiac UPS performance and the coupling between ubiquitination and proteasomal degradation in Ubqln1-CKO mice. First, we examined the myocardial levels of steady-state Ub conjugates and found that total ubiquitinated proteins and K48-linked poly-Ub chains (Figure 3) as well as K63-linked poly-Ub chains (Supplemental Figure 3; supplemental material available online with this article; https://doi.org/10.1172/JCI98287DS1) were moderately but statistically significantly increased in the homozygous Ubqln1-CKO mice. This suggests that the degradation of ubiquitinated proteins in cardiomyocytes is impaired by Ubqln1 deficiency. To further probe myocardial UPS proteolytic performance, we introduced transgenic (Tg) GFPdgn, a well-proven surrogate substrate of the UPS created via carboxyl fusion of degron CL1 to an enhanced green fluorescence protein (42), into the Ubqln1-CKO and litter-
Ubqln1 plays an important role in UPS-mediated protein degradation through coupling ubiquitination with proteasomal degradation and that the Ubqln1-CKO mice represent a mouse model of cardiomyocyte-restricted impaired coupling between ubiquitination and the proteasome.

Ubqln1-CKO exacerbates myocardial IRI. Perinatal Ubqln1-CKO mice do not show apparent abnormality until 5 months of age, and we observed a significant increase in myocardial Ubqln1 protein levels in WT mice subjected to myocardial IRI (Figure 1, C and D); hence, we hypothesized that Ubqln1 might play a more critical role in UPS-mediated protein degradation under a stress condition than at baseline. To test this hypothesis, we subjected 10-week-old Ubqln1-CKO and control mice to myocardial IRI, which was induced as described in Figure 5. Hemodynamic measurements revealed that the comparable IRI procedure induced more severe impairment of LV systolic and diastolic function in Ubqln1-CKO mice than in control mice, as reflected by significantly lower LV peak pressure (LVSP), diminished maximum rate of rise of left ventricular pressure (dP/dt), and elevated minimum dP/dt (Figure 5). Notably, compared with the control sham group, the Ubqln1-CKO sham group displayed a significantly elevated minimum dP/dt (P < 0.01), comparable to that induced by IRI in the control mice, although the parameters of systolic function (LVSP, maximum dP/dt) were not discernibly different between the 2 sham groups. This suggests 2 possibilities: (a) mild diastolic malfunction of Ubqln1-CKO mice at baseline that was not detected with echocardiography at this age became detectable with the more sensitive catheterization-based LV mechanical assessment; (b) the stress from the sham operation, which involves a major open chest surgery, unmasked the compromised LV diastolic function in Ubqln1-CKO mice. Furthermore, comparable

mate control mice via mouse crossbreeding. At 3 weeks of age, when gross and cardiac abnormalities had not become discernible yet, myocardial GFPdgn protein levels were significantly higher in the Ubqln1-CKO mice than in littermate controls (Figure 4, A and B), indicative of impairment of cardiac UPS performance by Ubqln1-CKO. The increased ubiquitinated proteins and impaired UPS performance in Ubqln1-CKO hearts are not caused by a decrease in the intrinsic function of the proteasome because all 3 types of myocardial proteasomal peptidase activities from both the 20S and the 26S proteasomes were comparable between Ubqln1-CKO and control mice (Figure 4, C–H). Taken together, these findings demonstrate that Ubqln1 plays an important role in UPS-mediated protein degradation through coupling ubiquitination with proteasomal degradation and that the Ubqln1-CKO mice represent a mouse model of cardiomyocyte-restricted impaired coupling between ubiquitination and the proteasome.

Figure 2. Perinatal ablation of Ubqln1 in cardiomyocytes causes late-onset cardiomyopathy in mice. Equal numbers of male and female mice were analyzed for each group. (A) Western blot analyses of myocardial Ubqln1 in Ubqln1-CKO (CKO) and littermate control (CTL) mice. (B and C) Echocardiographic data of mice at 5 months of age. Scatter dot plots are superimposed by mean ± SD. Two-tailed Student’s t test. LVPW, left ventricular posterior wall thickness; LVPWd, LVPW at end of diastole; LVPWs, LVPW at end of systole; LVIP, LV internal dimension; LVIDd, end-diastolic LVID; LVIDs, end-systolic LVID. Each lane (A) or dot (B and C) represents a mouse. (D) Kaplan–Meier survival curves, log-rank test.
IRI-induced LV functional impairment was remarkably less severe in the Tg mice than in the nTg mice (Figure 7). Infarct size assessment using TTC staining also revealed that the same initial ischemia caused significantly smaller infarct size in Ubqln1 Tg mice than in nTg mice (Figure 8). These experiments demonstrate that Ubqln1 gain of function protects myocardium against acute IRI, which indicates that inadequate coupling between ubiquitination and proteasomal degradation is a major pathogenic factor for IRI.

Ubqln1 overexpression promotes proteasomal degradation of oxidized proteins and a surrogate misfolded protein without altering autophagic flux in cardiomyocytes. In Ubqln1-CKO mice, the loss of Ubqln1 is restricted to cardiomyocytes, but Tg Ubqln1 overexpression is not; thus, it remains an important question whether the protective effects of Ubqln1 gain of function on IRI observed in the Ubqln1 Tg mice were necessarily derived from the Ubqln1 increase in the cardiomyocyte compartment. To answer this question, we performed cardiomyocyte culture studies.

Figure 3. Western blot analyses for myocardial total and K48-linked Ub conjugates in Ubqln1-CKO and littermate control mice at 3 weeks of age. The 1% Triton X-100 soluble and insoluble fractions of protein extracts from ventricular tissues were subject to SDS-PAGE and immunoblotting. GAPDH and sarcomeric α-actinin (Actn) were reprobed as the loading controls for the soluble and insoluble fractions, respectively. Representative images (A, C, E, G) and pooled densitometry data (B, D, F, H) are presented. A–D, Changes in total ubiquitinated proteins in the soluble (A and B) and insoluble (C and D) fractions. E–H, Changes in K48-linked ubiquitinated proteins in the soluble (E and F) and insoluble (G and H) fractions. P values were derived from the 2-tailed t test with Welch's correction. Each dot and each lane in Western blot image represents an independent animal.
that the primary mechanism underlying protection by Ubqln1 against myocardial IRI works to facilitate UPS-mediated degradation of oxidized proteins in cardiomyocytes. To examine this hypothesis, we subjected cultured NRVMs to increased oxidative stress produced by hydrogen peroxide and examined the impact of forced Ubqln1 overexpression on the dynamic changes in the levels of steady-state protein carbonyls. The latter is the signature change of oxidative modifications on proteins and can be derivatized to 2,4-dinitrophenyl–hydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH) and then detected with anti-DNP Abs (45). We found that hydrogen peroxide treatment induced dose-dependent increases in DNP-derivatized protein carbonyls and that the increases were substantially less in NRVMs overexpressing Ubqln1 compared with cells without Ubqln1 overexpression. Moreover, proteasome inhibition significantly increased protein carbonyls in H2O2-treated NRVMs, and this increase could not be attenuated by Ubqln1 overexpression. In other words, the decreasing protein carbonyl levels by Ubqln1 overexpression were nearly completely prevented by proteasome inhibition with bortezomib (Figure 9). These findings demonstrate that, under acute oxidative stress, there is an inadequate coupling between ubiquitination and proteasomal degradation and that overexpression of Ubqln1 is sufficient to promote UPS-mediated removal of oxidized proteins.

To determine whether Ubqln1 overexpression enhances UPS performance in cardiomyocytes, we tested the effect of Ubqln1 overexpression on the protein level of a known UPS substrate, GFPu, in cultured NRVMs (46), a slightly different version of GFPdgn, as expressed in the GFPdgn Tg mice used in the Ubqln1-CKO in vivo study (Figure 4, A and B). It should be pointed out that GFPu/GFPdgn is also a surrogate for misfolded proteins (15, 16, 47). Consistently with what was observed in Ubqln1-CKO hearts, overexpression of full-length Ubqln1 significantly decreased, while overexpression of a UBL-deleted form of Ubqln1 (Ubqln1ΔUBL) markedly increased, the steady-state GFPu protein level (Figure 10, A–D), indicating that Ubqln1 gain of function promotes UPS-mediated degradation of misfolded proteins and that the Ubqln1ΔUBL, which is able to bind ubiquitinated proteins but unable to interact with the proteasome, acts as a decoy Ub receptor and thereby prevents ubiquitinated substrates from proteasomal degradation in cardiomyocytes. Furthermore, we tested to determine whether autophagy is involved in the Ubqln1 promotion of oxidized protein removal and cardioprotection, and we found that Ubqln1 overexpression did not alter LC3-II flux, a commonly used indicator of autophagic activity (48), in cardiomyocytes (Figure 10, E and F).

**Discussion**

Failure of cardiac UPS to meet the increased demand for timely removal of unwanted and no longer needed proteins in cardiomyocytes contributes to acute myocardial IRI (16, 49). Prior to the present study, proteasomal functional insufficiency, or proteasomal impairment, was the only factor established as the cause for UPS inadequacy in IRI myocardium (16, 38, 49). Here, we demon-
strate for the first time, to our knowledge, that Ubqln1 exerts an essential role in coupling ubiquitination to proteasomal degradation in heart muscle cells, and moreover, we have identified inadequate coupling between ubiquitination and proteasomal degradation as another major cause for the UPS functional deficit that thereby contributes to myocardial IRI. This provides compelling evidence that, not only priming the proteasome, but also enhancing the coupling between ubiquitination and the proteasome, should be explored as therapeutic strategies to reduce myocardial IRI, a major and common pathological process that occurs in both the natural progression and therapeutic intervention of ischemic heart disease as well as during heart transplantation.

Ubqln1 contributes to cardiac ubiquitination-proteasome coupling. Since Dsk2 plays an essential role in shuttling K48-linked ubiquitinated proteins to the proteasome for degradation in yeast (50), Ubqln1 as an orthologue of yeast Dsk2 was naturally purported to serve a similar role in mammals. However, this supposed role of Ubqln1 has not been formally tested in vivo. Our creation and characterization of the Ubqln1-CKO mice allowed this critical gap to be filled. Taking advantage of a well-established reverse reporter (GFPdgn) of UPS performance, we detected impairment of cardiac UPS performance in Ubqln1-CKO mice, as reflected by significantly higher myocardial GFPdgn protein levels in Ubqln1-CKO::GFPdgn mice compared with control::GFPdgn (CTL::GFPdgn) mice (Figure 4, A and B). This myocardial UPS impairment by Ubqln1-CKO was detected at 3 weeks of age when no abnormal phenotypes were discerned, indicating that this is a primary effect of Ubqln1 deficiency. Similarly to what we had found in mouse brains with neuronal ablation of Ubqln1 (43), here we found Ubqln1-CKO caused myocardial accumulation of total ubiquitinated proteins (Figure 3, A–D). Moreover, we unveiled for what we believe is the first time that in both the soluble and insoluble fractions of Ubqln1-CKO hearts, the level of proteins conjugated with K48-linked polyUb chains and the canonical signal targeting proteins for proteasomal degradation (51) were significantly increased (Figure 3, E–H), indicating that Ubqln1 deficiency impairs the degradation of K48-linked polyubiquitinated proteins by the proteasome. This impairment conceivably can result from either impaired intrinsic activity of the proteasome or reduced delivery of the polyubiquitinated proteins to the proteasome. Our data support the latter. This is because proteasomal activities were examined for the first time, to our knowledge, in a mammalian organ deficient of Ubqln1 by the present study, and it was revealed that all 3 types of proteasomal peptidase activities in either the 20S or the 26S proteasomes were not discernibly altered by Ubqln1-CKO (Figure 4, C–H). In general, proteins conjugated with polyubiquitin chains, especially those with K48-linked Ub chains, are efficiently degraded by the proteasome (51); hence, this coexistence of increased K48-linked Ub conjugates with unaltered proteasome proteolytic activities in Ubqln1-CKO mouse hearts indicates an apparent uncoupling between ubiquitination and proteasomal degradation by Ubqln1 deficiency. The uncoupling and the resultant UPS impairment are not without consequence, as Ubqln1-CKO mice developed late-onset cardiomyopathy. The latter is evidenced by echocardiography that detected in 5-month-old (Figure 2), but not 10-week-old (Supplemental Table 1), mice with perinatal Ubqln1-CKO significant mor-
A coupling factor to shuttle ubiquitinated proteins to the proteasome in vivo provides compelling evidence that Ubqln1 acts as an important contributor to UPS impairment in IRI hearts, which thereby plays a major pathogenic role in IRI. In the present study, we were able to demonstrate that improving the coupling between ubiquitination and proteasomal degradation protects against IRI despite a significant increase in Ubqln1 proteins in IRI myocardium (Figure 1A). This is because the same myocardial IRI insult causes significantly less cardiac malfunction in Ubqln1-CKO versus WT mice. Complemented by the Ubqln1 loss-of-function study described earlier, the protection of Ubqln1 overexpression against myocardial IRI provides compelling evidence that inadequate coupling between ubiquitination and proteasomal degradation represents a major cause for UPS functional insufficiency observed in IRI hearts, identifying enhancement of ubiquitination-proteasome coupling as a strategy for reducing myocardial IRI.

It should be pointed out that diastolic dysfunction undetected by echocardiography at baseline was detected in the Ubqln1-CKO sham control group (Figure 5D); hence, it is possible that the diastolic malfunction induced by Ubqln1-CKO might have contributed to the IRI exacerbation observed in the Ubqln1-CKO mice. Nevertheless, this inevitable ambiguity in the interpretation of the in vivo studies of this nature is compellingly compensated by the findings from our in vitro and in vivo gain-of-function studies.

Mechanisms underlying Ubqln1 protection against IRI. Ubqln1 overexpression had been found to facilitate the degradation of overexpressed neural degenerative diseases causing polyglutamine-expanded — or polyalanine-expanded — proteins and protect against their cytotoxicity in cultured noncardiac cells (57–59). More recently, it was shown that the protection of Ubqln1 overexpression against brain damage in an ischemic stroke model was associated with marked attenuation of the accumulation of insoluble ubiquitinated proteins in the affected brain tissues (60). However, the exact pathway taken by Ubqln1 to enhance degradation of misfolded proteins remains unclear, although promoting the UPS-mediated proteolysis has been implicated (33, 43, 60). In the present study, the results from our cell-culture

Figure 6. Cardiac Ubqln1 ablation increases infarct size induced by acute myocardial IRI in mice. Myocardial IRI was produced as described in Figure 5. At 24 hours of reperfusion, the animal was sacrificed, the original coronary ligature was retied, and the heart was subject to retrograde perfusion with 5% phthalocyanine blue to demarcate the original ischemic area or AAR before undergoing TTC staining to reveal the infarct area. (A) Representative pair of TTC staining images. (B) AAR presented is the percentage of LV that was subject to ischemia during the initial LAD ligation phase. (C) Changes in infarct size. Two-tailed Student’s t test. Each dot represents an individual animal.
experiments demonstrate that Ubqln1 overexpression promotes the degradation of oxidized proteins in cardiomyocytes and, more importantly, that this promotion can be completely abolished by proteasome inhibition (Figure 9), thereby establishing the UPS as the primary pathway by Ubqln1 to facilitate the degradation of oxidized/damaged proteins.

The UPR triggered by ER stress attempts to resolve ER stress, but the UPR can also, by sustained ER stress, cause cell death. ER stress/UPR can occur in myocardial IRI and has proven to be a major factor affecting IRI. For example, the activating transcription factor 6α (ATF6) branch of the UPR mediates the transcription of ER stress-response genes (34); overexpression of an activated form of ATF6 protects, and conversely, ATF6 knockout exacerbates IRI in mouse hearts and cultured cardiomyocytes (61, 62). Since our data confirm that Ubqln1 is recruited to the ERAD machinery by ER stress in cardiomyocytes (Figure 1), it is very likely that promoting ERAD and thereby alleviating ER stress is an underlying mechanism for Ubqln1 to protect against myocardial IRI.

By removal of protein aggregates and damaged organelles, macroautophagy (commonly known as autophagy) is known to play a role in quality control in the cell (63, 64). Although opposing reports do exist (65), some recently reported studies support a protective role of increasing macroautophagy against IRI (66). This raises a question of whether enhancing macroautophagy plays a role in the protection of Ubqln1 against IRI. Addressing this question is important because a prior report showed that Ubqln1 could be found in autophagosomes and LC3 complexes and that siRNA-mediated Ubqln1/Ubqln2 knockdown reduced LC3-II levels in cultured HeLa cells, leading to a conclusion that Ubqln1 may function in macroautophagy (67). However, this conclusion was made without a rigorous assessment of autophagic flux, and more importantly, the effect of Ubqln1 gain of function on macroautophagy was not examined in the study (67). In the present study, we have tested the impact of Ubqln1 overexpression on autophagic flux in cultured NRVMs and the results do not support the notion that Ubqln1 overexpression significantly increases autophagic activity (Figure 10). Thus, it is very unlikely that modulating autophagy plays a significant role in the protection of Ubqln1 overexpression against myocardial IRI. Nevertheless, it is possible that Ubqln1 may exert cardiac protection through additional mechanisms, beyond enhancing removal of damaged and toxic proteins. For example, it may inhibit cell death by acting directly on specific proteins that regulate cell-death pathways. Beverly et al. reported that BCL2L10/BCLb, an antiapoptotic BCL2-like protein, was stabilized by Ubqln1 in its monoubiquitinated form (68). It remains unclear how Ubqln1 stabilizes monoubiquitinated BCLb while it promotes the degradation of polyubiquitinated proteins.

In summary, this study provides compelling evidence that the coupling of ubiquitination to proteasomal degradation becomes inadequate during IRI and that such inadequacy contributes to myocardial IRI, identifying improving the coupling via, for example, upregulation of Ubqln1 as a therapeutic strategy for preventing or better treating acute myocardial IRI.

Methods

Genetically modified mouse models. The creation of mice harboring a floxed Ubqln1 allele in a C57BL/6 inbred background and the cre-
Echocardiography. 2D echocardiogram-guided M-mode echocardiography was performed on mice as previously described (69). In brief, mice were anesthetized by inhalation of isoflurane (4.5% for induction and 1.5% for maintenance) via a nose cone. Transthoracic echocardiograph was recorded using the Vevo2100 echocardiography system with a 40 MHz transducer. The LV morphometric and functional parameters were analyzed offline (69).

Myocardial IRI. Under anesthesia with isoflurane, mice of both sexes (male/female = roughly 1:1) at approximately 10 weeks of age were subject to surgical ligation (30 minutes) and subsequent release (24 hours) of the LAD.

Cardiac function assessment. At 23.5 hours of reperfusion, a micro-tipped pressure transducer catheter (1.4F, model SPR-835, Millar Instruments Inc.) was placed into the LV via the apex, and the LV pressure and dP/dt were recorded and analyzed as we previously described (16).

Ischemic area and infarct size determination. At the end of 24 hours of reperfusion, the suture was retied and 250 μl of 5% phtha-locyanine blue was injected into the LV chamber. The heart was quickly excised, immediately frozen in a–20°C freezer for 30 minutes, and sliced into 5 short-axis sections, which were incubated in 1% TTC solution for 15 minutes. Each section was weighed and digitally photographed. The area not at risk (phthalocyanine blue stain), the AAR (including viable myocardium and infarcted area), and the total LV areas from both sides of each section were measured using Image-Pro Plus software (Media Cybernetics). The AAR was expressed as percentage of total LV, and infarct size was expressed as percentage of the AAR and LV (16).

Western blot analysis. Frozen ventricular myocardial tissues were homogenized in 1× SDS sampling buffer (50 mM Tris-HCl at pH 6.8, 2% SDS, and 10% glycerol). The supernatant was obtained following a 12,000 g centrifugation for 10 minutes at 4°C. The protein concentra-
tion was measured using the bicinchoninic acid (BCA) method. Equal amounts of protein sample were resolved by SDS-PAGE, transferred to PVDF membrane, and probed with appropriate primary Ab (see Supplemental Table 2 for sources of primary Abs) and horseradish peroxidase–conjugated secondary Ab, followed by detection with enhanced chemiluminescence (ECL-Plus) reagents (GE Healthcare) and a VersaDoc 4000 imaging system (Bio-Rad). The signal was quantified with Quantity One software (Bio-Rad) (16).

**NRVM culture and adenoviral gene delivery.** The isolation and culture of NRVMs as well as the infection of replication-deficient recombinant adenoviruses expressing Flag-tagged murine full-length Ubqln1 (Ad-Ubqln1), UBL domain–deleted Ubqln1 (Ad-Ubqln1-ΔUBL), GFPu (Ad-GFPu), or β-galactosidase (Ad–β-Gal) as control were performed as we previously reported (47).

**Immunofluorescence confocal microscopy.** This was conducted as we previously described (70). In brief, NRVMs cultured in fibronectin-coated chamber slides were fixed with 4% paraformaldehyde at 48 hours after infection of Ad-Ubqln1. The fixed cells were then subject to double-immunostaining with the mouse mAb against the FLAG tag (catalog v8146, Cell Signaling Technology) and the custom-made and previously validated rabbit anti-rodent Psmb5 (i.e., the β5 subunit of the 20S proteasome) primary Ab (38). The bound primary Abs were visualized with DAPI. The triple fluorescence staining was imaged with a triple-laser confocal microscope (Olympus Fluoview 500, Olympus).

**Co-IP.** Co-IP was done with the Pierce Co-Immunoprecipitation Kit (catalog 26149, Thermo Fisher Scientific), which provides covalent Ab immobilization so that potential interference of the IP Abs is completely avoided. All the components required for a co-IP experiment, including cell-lysis buffer, were from the kit. IP flag-Ubqln1 used the mouse mAb against FLAG tag (catalog v8146, Cell Signaling Technology). Immunoblot for Ubqln1 and UBXD2 (also known as Erasin) used, respectively, the rabbit polyclonal anti-Ubqln1 Ab (ab3341, Abcam) and a rabbit polyclonal Ab against UBXD2 (catalog 21052-1-AP, Proteintech Group).

**Detection of oxidized proteins.** The Oxidized Protein Western Blot Detection Kit (ab178020, Abcam) was used for detection and quantification of carbonyl groups, a hallmark of proteins with oxidative modifications. In brief, the protein carbonyl groups were derivatized to DNP-hydrazone by reaction with DNPH. The DNP-derived protein samples were then fractionated with SDS-PAGE, followed by transfer to PVDF membrane and immunoblotting with a rabbit anti-DNP polyclonal Ab (ab3341, Abcam) and a rabbit polyclonal Ab against UBXD2 (catalog 21052-1-AP, Proteintech Group).

**Statistics.** Unless indicated otherwise, data of continuous variables are presented as scatter dot plots with mean ± SD superimposed, where differences between 2 groups or among multiple groups were evaluated in SPSS for statistical significance using, respectively, unequal variance 2-tailed t test with Welch’s correction. Statistical tests for other results are specified in figure legends. P < 0.05 was considered statistically significant.

**Study approval.** The animal care and use protocol of this study was approved by the IACUC of the University of South Dakota. The protocol for animal use and care in this study conforms to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996).
Author contributions

XW, YT, CH, and JL designed research studies. CH, YT, BP, and PW conducted experiments. CH, YT, HX, EMT, and PW acquired data. XW, CH, YT, HX, FL, and JL analyzed data. HW and FL provided reagents. XW, YT, and CH wrote the manuscript.

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