Sumo E2 Ligase UBC9 is Required for Efficient Protein Quality Control in Cardiomyocytes

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ABSTRACT

Rationale: Impairment of proteasomal function is pathogenic in a number of cardiac proteinopathies and can eventually lead to heart failure. Loss of proteasomal activity often results in the accumulation of large protein aggregates. The ubiquitin proteasome system (UPS) is primarily responsible for cellular protein degradation and, while the role of ubiquitination in this process is well studied, the function of an ancillary post-translational modification, SUMOylation, in protein quality control (PQC) is not fully understood.

Objective: To determine the role of UBC9, a SUMO-conjugating enzyme, in cardiomyocyte PQC.

Methods and Results: Gain- and loss-of-function approaches were used to determine the importance of UBC9. Overexpression of UBC9 enhanced UPS function in cardiomyocytes while knockdown of UBC9 by siRNA caused significant accumulations of aggregated protein. UPS function and relative activity was analyzed using a UPS reporter protein, GFPu. Subsequently, UBC9’s effects on UPS function were tested in a proteotoxic model of desmin-related cardiomyopathy, caused by cardiomyocyte specific expression of a mutated alpha B crystallin, CryAB R120G. CryAB R120G expression leads to aggregate formation and decreased proteasomal function. Co-infection of UBC9 adenovirus with CryAB R120G virus reduced the proteotoxic sequelae, decreasing overall aggregate concentrations. Conversely, knockdown of UBC9 significantly decreased UPS function in the model and resulted in increased aggregate levels.

Conclusions: UBC9 plays a significant role in cardiomyocyte PQC and its activity can be exploited to reduce toxic levels of misfolded or aggregated proteins in cardiomyopathy.

Keywords: Quality control, SUMO ligase, proteasome, cardiomyopathy, autophagy, cardiac dysfunction, protein

Nonstandard Abbreviations and Acronyms:
Ad adenovirus
CMV cytomegalovirus promoter
CryAB alpha B crystallin
E1 ubiquitin-activating enzyme
E2 ubiquitin-conjugating enzyme
E3 ubiquitin ligase
ERSE ER (endoplasmic reticulum) stress response element
MG-132 Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal, Z-LLL-CHO
MuRF1 muscle-specific RING finger 1
NRVCs neonatal rat ventricular cardiomyocytes
PAO pre amyloid oligomer
PQC protein quality control
SAE1 SUMO1 activating enzyme subunit 1
SAE2 SUMO1 activating enzyme subunit 2
SUMO small ubiquitin-like modifier
SUMO E1 SUMO activating enzyme
SUMO E2 SUMO conjugating enzyme 2
TAC transverse aortic constriction
UBC9 ubiquitin-conjugating enzyme 9/SUMO E2 ligase
UPS ubiquitin proteasome system
UPR unfolded protein response
INTRODUCTION

Cardiovascular disease remains the number one killer in developed countries. During disease onset and the ensuing pathological processes, protein homeostasis in the stressed cells can be perturbed, and misfolded or damaged proteins may gradually accumulate and form large aggregates or inclusion bodies in the cells. In addition to more subtle pathogenic consequences, these aggregates can interfere with normal contractile function of the cells, leading to increased mechanical and metabolic stress.1-3 The pathogenic consequences of accumulating misfolded and damaged proteins is generally referred to as proteotoxicity.4 Multiple mechanisms of proteotoxicity have been proposed including: alteration of transcriptional programs, oxidative stress, mitochondrial dysfunction and inhibition of protein quality control (PQC) in the cell.5,5 Altered or compromised PQC has been noted in the stressed heart as a result of diverse etiologies including ischemia reperfusion,6,7 congenital hypertrophy8 and desmin related cardiomyopathy.9,10

Under normal circumstances, the majority of cardiac proteins are subject to degradation via the ubiquitin proteasome system (UPS).11 A damaged or misfolded protein is targeted for degradation via a specific process in which it is recognized as such, and is post-translationally modified by the attachment of a small signaling protein, ubiquitin, with the help of sequential enzymatic reactions. The modified, poly-ubiquitinated protein is then shuttled to the proteasome where it is subsequently degraded. This process has been reviewed in detail.12 However, cardiac disease is often accompanied by proteasome functional insufficiency,10,13,14 and misfolded or damaged proteins can accumulate and form aggregates that are easily discerned by standard histological analyses.15 Compromised proteasomal degradation has been hypothesized as potentially playing a pathogenic role during the development of cardiac disease, further compromising the stressed cells and enhancing the downward spiral into heart failure.2

In addition to ubiquitination, other post-translational modifications may play a signaling role during the degradation of misfolded proteins. However these are not well understood and the underlying physiological stimuli and consequences remain obscure. A number of studies have suggested that the SUMOylation system can be upregulated during cellular stress and is involved in the degradation of misfolded proteins and reduction of cellular aggregates.16-19 Modification via SUMOylation also occurs through a series of conjugation steps that are quite similar to those that underlie ubiquitination. SUMO protein is first activated with SUMO E1 (heterodimeric enzyme, SAE1/SAE2), and the activated SUMO is then ligated to substrate with the help of a SUMO E2 ligase, UBC9. UBC9 ligates SUMO to the substrate by forming isopeptide bonds between the C-terminal Gly-Gly sequence of the SUMO protein and the amino group of the target lysine. In vitro, UBC9 directly ligates SUMO protein to the substrate but in vivo, SUMO E3 ligase determines the substrate specificity of UBC9.20

There are several isoforms of SUMO (SUMO 1-4) that are encoded by the human genome. Previously, the role of SUMOylation in PQC has been studied using individual components and it appears that SUMO2/3 is involved in protein degradation.17,21 Initially it was postulated that SUMO and ubiquitin were competitors, conjugating to the same lysine site(s). Thus, SUMO might interfere with ubiquitin-mediated degradation. However, current hypotheses now focus on the tight integration of SUMO with the UPS. SUMO acts as a signal for the degradation of several proteins and SUMO modification can be recognized by the E3 ubiquitin ligase, promoting poly ubiquitination and degradation through the UPS.22 SUMO modification can, in some cases, even serve as an adaptor for the poly ubiquitination and degradation of protein through the UPS.23

In yeast and mammalian cell lines, UBC9 activity inhibited aggregate formation through both SUMO dependent and independent mechanisms.18,24,25 UBC9 also interacts with several ubiquitin E3 ligases, such as MuRF1 and may participate in the removal of protein aggregates.18,26 UBC9 expression increases overall patterns of cellular ubiquitination of cellular proteins and degradation through the UPS pathway.27,28 However, the underlying mechanism of UBC9-mediated aggregate reduction is not fully
understood. UBC9 has enzymatic functions but may also partially regulate several other pathways involved in post-translational protein modification and homeostasis, and can inhibit free radical generation during environmental stress.29

Herein, we study the role of UBC9 in a cardiomyocyte model of the proteotoxic disease, Desmin Related Cardiomyopathy, which we created by expressing a mutant alpha B (CryABR120G).15,30 Using both loss of function and gain of function approaches, we explored the functional consequences of up- and down-regulating UBC9 levels. Increased UBC9 effectively decreased protein aggregates and maintained normal proteasomal function, which is otherwise compromised in this disease model.

METHODS

Mice.
Twelve week-old FVBN mice were subjected to transverse aortic constriction (TAC) to produce pressure overload.31 CryABR120G mice were used for the expression analysis.2 Animals were handled in accordance with the principles and procedures of the Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital approved all experimental procedures.

Neonatal Rat Ventricular Cardiomyocyte (NRVC) Cultures and Adenovirus (Ad) Infection.
Primary NRVCs were isolated from the ventricles of 1-2 day old Harlan Sprague-Dawley rats and plated at a density of 1.5x10^6 cells in 10 cm plates or 1x10^5 in a two-well chamber slide. Initially the cells were grown in DMEM with 10% FBS for 24 hours. Twenty-four hours after plating, the cells were infected with adenovirus for 2 hours in DMEM without serum medium, which was then replaced with DMEM with 2% serum and 1X penicillin/streptomycin (Gibco).

Preparation of recombinant adenovirus.
We generated a recombinant adenovirus that encodes mouse wild type UBC9. The UBC9 gene was amplified from mouse cDNA and a HA-tag epitope (MYPYDVPDYA) incorporated at the N-terminus to distinguish the recombinant UBC9 from endogenous protein. The cDNA was cloned in the pShuttle-CMV vector to generate adenovirus using an adenovirus-packaging kit (Agilent Technologies). For controls, we used an empty β-gal reporter adenovirus. The adenoviral vector expressing an inverse reporter of the UPS (Ad-GFPu) was previously described.32

Knockdown of UBC9 in NRVC.
UBC9 protein was knocked down using siRNA1 (s120806) and siRNA2 (s75672) (Life Technologies). A non-specific Stealth RNAi™ was used as negative control in all silencing experiments (Cat. #:12935200, Invitrogen). Twenty-four hours after plating, NRVCs were transfected with 10 nM siRNA, using Lipofectamine 2000 as a transfection reagent (Invitrogen) in a reduced serum medium OptiMem (Invitrogen). Cells were incubated overnight and the media replaced with DMEM with 2% serum.

Protein extraction and Western Blot analysis.
Cultured cardiomyocytes were washed twice with 1X PBS and then lysed in RIPA buffer (Sigma) containing complete protease inhibitor cocktail (Roche). Unbroken cells and cell debris were removed by centrifugation at 10,000 x g and the supernatant used for further analysis. Ventricular tissue was homogenized with a Bead Beater (Bertin Technologies) in RIPA buffer (Sigma) and centrifuged at 10,000 x g to sediment unbroken cells and large debris. Preparation and analyses of the insoluble proteins have been described.1 Protein concentration was determined using a protein assay kit (BioRad). For Western blot
analyses, proteins were dissolved in 1X Laemmli buffer and heated at 100 °C in a water bath for 5 minutes. Proteins were electrophoresed on SDS-PAGE and transferred to a PVDF membrane (BioRad) in a wet transfer apparatus. The membrane was blocked in 1X blocking buffer (Roche) in 1X TBS for 1 hour at room temperature. Subsequently, the blot was incubated with primary antibody at 4 °C overnight, washed 3 times with 0.1% Tween 20 for 5 minutes and incubated with alkaline phosphatase conjugated secondary antibody for 2 hours. The membrane was then washed 3 times with 0.1% Tween 20 and 1 time with 1X TBS. The immune complex was detected with ECF detection reagents (GE Healthcare). Images were captured in a STORM 820 fluorescent scanner (GE Healthcare). The following antibodies were used for immunoblotting: UBC9, GFP, secondary antibody conjugated with alkaline phosphatase from (Santa Cruz Biotechnology), SUMO1, SUMO2/3, GAPDH and ubiquitin (Millipore), CryAB (Enzo Life Sciences), Bip (Sigma), PDI, HA tag and β-actin (Cell Signaling), ATF4, XB1 and VCP (Abcam).

**Proteasomal inhibitor study.**
NRVCs were treated with MG132 or lactacystin at dosages indicated for 12 hours in order to inhibit proteasomal activity. Aggresome clearance was measured essentially as described. In brief, NRVCs were infected with either Ad-β-gal or Ad-UBC9 and cells incubated in the 2% DMEM for 24 hours. For inhibition of proteasomal activity, NRVCs were treated with the proteasomal inhibitor MG132 for 12 hours and cells were washed twice with DMEM plus 2% serum. The cells were grown in DMEM with 2% serum for an additional 24 hours and harvested at 0, 12, and 24 hours. They were subsequently fixed and permeabilized as described below. Aggregates were stained with poly ubiquitin antibody and aggregates were quantitated using MetaMorph software.

**Proteasome activity assay.**
Proteasomal activity was measured using the 20S Proteasome Activity assay kit (Millipore). NRVCs were infected with either Ad-β-gal or Ad-UBC9 for 3 days. Crude proteasomes were isolated as per the manufacturer’s protocol. Unbroken cells and cellular debris were removed by centrifugation at 12,000 x g. The assay was done using proteasomal substrate (Suc-LLVY-AMC) and activity measured in a spectrophotometer (Synergy 2, BioTek) using a 360/460 nm filter. Activity was recorded as changes in fluorescence at different time intervals.

**Immunofluorescence microscopy.**
NRVCs were first washed with 1X PBS and then treated with cell fixative and permeabilization buffer (4% paraformaldehyde, 0.5% Triton-X in 1X PBS) for 10 minutes. Fixed cells were washed twice with 1X PBS and then incubated with 0.1mol/L glycine (pH 3.5) for 30 minutes. Excess glycine buffer was washed with 1X PBS and the fixed cells blocked using blocking buffer (1% BSA, 0.1% Tween-20 in 1X PBS) for 1 hour at room temperature. Cells were incubated with primary antibody diluted in blocking buffer at 4 °C. Cells were washed and then incubated with secondary antibody conjugated to Alexa Fluor (Molecular Probes) for 1 hour at room temperature. In some experiments, cardiomyocytes were counterstained with sarcomere specific antibodies such as TnI. In such cases the cells were again blocked with blocking buffer for 30 minutes followed with the primary antibody and secondary antibody incubations for 2 hours each. For detection of the nucleus, a nucleic acid specific stain, DAPI (Molecular Probes), was used in 1X PBS at a 1:5000 dilution for 5 minutes. Cells were washed twice with 1X PBS and then mounted using Vectashield Hard Set mounting medium (Vector labs), and allowed to dry at room temperature for 30 minutes. Slides were kept at 4 °C until microscopy was performed. Cells were analyzed using an A1 confocal microscope (Nikon) using the following antibodies: TnI, ubiquitin (Millipore), cMyBP-C, CryAB (Enzo Life Sciences), anti-oligomer antibody A11 and UBC9 (Santa Cruz Biotechnology).

**Cellular toxicity and death assays.**
Cellular toxicity was analyzed by measuring the release of adenylate kinase from damaged cells using the ToxiLight non-destructive cytotoxicity bioassay kit (Lonza). Chemiluminescence was measured in a Luminometer (GLOMAX, Promega). Cell death was detected by TUNEL staining using the In Situ Cell
Death Detection kit (Roche). Cells were observed under an A1 confocal microscope (Nikon) using the green channel (TUNEL positive).

**UBC9’s role in the unfolded protein response using an ER Stress Response Element (ERSE) reporter.**
ERSE reporter activity was determined in HEK239T cells using the Cignal ERSE Reporter (Luc) assay kit. Cells were transfected with reporter plasmid in the presence of 100 ng of pShuttle CMV vector or 100 ng of pShuttle CMV-UBC9 using lipofectamine (Invitrogen). Activity was measured after 48 hours of transfection using the Dual-Glo Luciferase assay system (Promega) and recorded in a Luminometer (GLOMAX, Promega).

**Statistical analyses.**
All biochemical and functional assays were performed in mice with mixed genders. Experiments were repeated multiple times. Results are shown as means ±SD. Paired data were evaluated by Student’s t test. For multiple comparisons, ANOVA with post hoc Tukey’s test was used. Statistical analysis was done using SPSS software. A value of $P<0.05$ was considered significant. The authors had full access to and take full responsibility for the integrity of the data. The authors have read and agree to the manuscript as written.

**RESULTS**
SUMOylation can be upregulated during cellular stress. As UBC9 is thought to be associated with increased SUMO activity, we first tested adult (14 weeks) CryAB$^{R120G}$ hearts. UBC9 levels were increased (Figure 1A and B), SUMO 1 expression decreased while SUMO2/3 was unchanged. Increases in UBC9 are not restricted to this model of cardiomyopathic stress, as we confirmed that the UBC9 protein is also increased in a pressure overload model of hypertrophy induced by transverse aortic constriction (TAC). At 2 weeks post surgery UBC9 levels are elevated and SUMO1 expression is also significantly decreased (Figure 1C and D). Changes in SUMO2/3 did not reach statistical significance. Although UBC9 was elevated in the CryAB$^{R120G}$ hearts, SUMOylated proteins were significantly decreased (Figure 1E and F). In healthy neonatal rat cardiomyocytes (NRVC), forced UBC9 expression increased overall levels of SUMOylation while UBC9 knockdown resulted in hypo-SUMOylation (Online Figure I) and we believe that, in the CryAB$^{R120G}$ cardiomyocytes, increased levels of UBC9 constitute a compensatory attempt to maintain normal SUMOylation.

We then determined if enhanced expression of UBC9 could directly affect aggregate accumulation in cardiomyocytes expressing CryAB$^{R120G}$. We generated adenovirus in which the CMV promoter was used to drive UBC9 expression in NRVCs. Western blot analyses showed that UBC9 expression could be modulated in the cells and a dose response curve generated (Figure 2A and B), with the protein being present in both the cytosol and nucleus (Figure 2C). Up- or down-regulation of UBC9 expression showed no discernible effect on endogenous CryAB levels or on endogenous cellular proteins such as -actin. Overexpression of UBC9 in NRVCs had no effect on cell viability but, in contrast, knockdown of UBC9 significantly increased cell death (Online Figure III).

To test the hypothesis that UBC9 expression could affect aggregate levels under proteotoxic conditions, UBC9 protein was co-expressed with CryAB$^{R120G}$ in NRVCs. Aggregated protein was detected by staining for CryAB and quantitation showed that overexpression of UBC9 reduced CryAB$^{R120G}$ aggregate content in a dose dependent manner (Figure 3A and B). As UBC9 overexpression does not alter endogenous CryAB expression in cardiomyocytes (Online Figure II), the data suggest that UBC9 may specifically enhance the removal of misfolded proteins in the absence of increased chaperone. We also noted that UBC9 expression reduced ubiquitinated protein load in the CryAB$^{R120G}$ cells (Figure IIIC and D).
but had no effect on overall ubiquitinated protein levels in normal NRVCs (Online Figure IVA, B and C). However, knockdown of UBC9 did increase ubiquitination in the insoluble fraction (Online Figure IVD, E and F). This suggests that UBC9 in general can reduce aggregate content in cells undergoing a proteotoxic response and that endogenous UBC9 levels can impact on protein aggregation.

As noted above (Online Figure III), knockdown of UBC9 in healthy NRVCs had a detrimental effect on cell viability, suggesting that UBC9 is a crucial component of cardiomyocytes for cellular function and survival. To further explore its role in the proteotoxic environment induced by CryABR120G expression, we modulated UBC9 levels in NRVC infected with adenovirus carrying the CryABR120G cDNA. We decreased UBC9 expression in these cells with siRNA. Western blot analysis showed that UBC9 siRNA1 and UBC9 siRNA2 knocked down expression, with siRNA2 being more effective (Figure 4A and B). Knockdown in these cells led to significantly increased aggregation (Figure 4C and D), suggesting that UBC9 levels can affect the stable accumulation of misfolded and aggregated proteins.

We have previously shown that CryABR120G expression results in compromised proteasomal function.9 To further define a potential direct role of UBC9 in the UPS we utilized the inverse reporter protein (GFPu) of the UPS. GFPu is an unstructured protein that is efficiently degraded through the UPS and its metabolism can be easily monitored by measuring GFPu levels.32 Overexpression of UBC9 led to significantly decreased GFP levels compared to control cells (Figure 5A and B), indicating improved UPS performance, while knockdown of UBC9 via siRNA resulted in increased GFPu (Figure 5C and D), suggesting UPS impairment. These data are consistent with the hypothesis that, in a cellular context, UBC9 levels can help regulate UPS function. To confirm that this effect was not due to a direct alteration of the proteasomal core on the part of UBC9, we looked at UBC9’s effects in an isolated system, in which a direct measure of 20S proteasomal activity could be determined (Methods). The data showed that UBC9 had no direct effect on the activity of the 20S proteasome (Online Figure V).

To determine if proteasomal activity was essential in helping to mediate the effect of UBC9 on decreasing aggregate content, we used the proteasomal inhibitors MG-132 and lactacystin.11,35 NRVCs were co-infected with UBC9 and CryABR120G and then treated with proteasomal inhibitor for 12 hours before fixation. Quantification of aggregate levels showed that inhibition of the UPS abolished any effect that enhanced UBC9 expression had on aggregate accumulation, implying that its actions are mediated at least in part through the proteasome based UPS (Figure 6). As expected on the basis of these data, knockdown of UBC9 increased the insoluble ubiquitinated protein levels in CryABR120G expressing NRVCs (Online Figure VI). Recent studies suggest that UBC9 may also impact on ER homeostasis; for example UBC9 protein regulates the expression of XBP-1 and the transcript’s stability.36 We confirmed that components of the UPR, including XBP-1, are upregulated by elevated levels of UBC9 (Online Figure VII) and UBC9 can directly target promoter elements involved in UPR activation (Online Figure VIIC and D).

We wished to determine if enhanced UBC9 activity could effectively remove preexisting aggregates from the NRVCs. Inhibition of proteasomal activity led to the accumulation of ubiquitinated aggregate proteins as detected by staining with polyubiquitin antibody (Figure 7). We used the reversible proteasomal inhibitor MG132 to block proteasomal activity for 12 hours33 and subsequently removed it from the cells, following the temporal disappearance of the aggregate content in the absence and presence of UBC9 expression. UBC9 effectively accelerated aggregate clearance in the NRVCs over the subsequent 24 hour period, suggesting that UBC9 can play a vital role in the clearance of aggregated protein.

In the CryABR120G hearts, accumulation of protein aggregates and impairment of systems involved in protein quality control is accompanied by the presence of toxic pre-amyloid oligomers (PAO).15 PAOs are soluble, highly proteotoxic entities and expression of peptide fragments that generate PAO in cardiomyocytes is sufficient to cause heart failure and death in transgenic mice.37,38 We therefore determined if enhanced UBC9 expression could reduce the PAO generated as a result of CryABR120G
expression in NRVCs. PAO was detected using the conformer specific antibody A11. Enhanced UBC9 expression had a dramatic effect on PAO levels, rendering them almost undetectable (Figure 8A and B). As we have shown that PAO is also toxic to the cardiomyocyte, we examined the effect of UBC9 on cell viability (Figure 8C) and confirmed that cell death, as measured by the TUNEL assay, was dramatically reduced as well (Figure 8D and E). As PAO generation appears to be quite common in diseased hearts suffering from different primary etiologies these data further suggest that UBC9 expression is beneficial for cardiomyocytes subjected to proteotoxic stress.

**DISCUSSION**

Recent data point to the potential contribution of compromised PQC in developing cardiac disease and heart failure. Proteasomal degradation plays a major role in PQC and if impaired, affects the cell’s ability to remove damaged or misfolded proteins. If the UPS remains compromised, the proteotoxic load on the cell can increase, which often leads to the development of large aggregates, aggresomes or inclusion bodies. These aggregates often display sequestration of chaperones, which further compromises the cell’s PQC, causing a general inhibition of normal protein synthesis and function. We have found that in cardiomyocytes, these proteotoxic processes are often accompanied by the generation of extremely toxic PAOs and have confirmed cardiomyocyte-specific PAO accumulation can lead to cell pathology and death in the CryABR120G animals.

Post-translational modification of damaged proteins, such as ubiquitination, can target them for degradation. Although the role that ubiquitination plays in the normal and diseased heart has been intensively studied, the possibility of therapeutically targeting a specific degradative process is only just beginning to be explored. Herein, we extend the concept of PQC impacting on cardiac pathology to another post-translational process, SUMOylation. SUMOylation enzymes belong to the ubiquitin class of proteins and recent studies have suggested that overexpression of SUMO protein can be cardioprotective. The full impact of SUMOylation is still being defined but it can clearly act on multiple cellular functions. SUMO modification can activate the transcriptional activity of GATA4, TBXs, Nkx2.5 but can also repress transcriptional activity by destabilizing other transcriptional factors. SUMO modification is crucial for cardiomyocyte proliferation and normal cardiac development as well, and enhanced deSUMOylation caused cardiac abnormalities and perinatal death. SUMO modification can stabilize and enhance the ion channel activity of SERCA and inhibit Kv2.1 activity.

SUMOylation appears to be associated with degradation of proteins through the UPS although the mechanistic linkages remain obscure. UPS inhibition resulted in accumulation of SUMO2/3 conjugated proteins. Mass spectroscopy studies suggest that CryAB can be a substrate for ubiquitin and SUMO modification, but the functional significance is unclear. After determining that free SUMO protein is down-regulated in the CryABR120G hearts and in a TAC model, we chose to explore the role of SUMOylation in cardiomyocytes by manipulating levels of the critical SUMOylation enzyme UBC9. SUMOylation components can be associated with protein aggregates in different proteinopathy models. However, the role of UBC9 in cardiomyocytes has not been determined. UBC9 is a SUMO E2 ligase and previous studies suggested that it plays a role in degrading short-lived proteins as well as in the removal of misfolded proteins. Immunohistochemical analyses of UBC9 in NRVCs both with and without CMV promoter-driven UBC9 overexpression confirmed the ubiquitous distribution of the protein in both the cytoplasmic and nuclear compartments. Previous data derived from a variety of cell types are consistent with these results as UBC9 protein has been found in the cytosol, nucleus, nuclear membrane, cell membrane and endoplasmic reticulum. Overexpression of UBC9 in the CryABR120G-expressing NRVC had a striking effect on aggregate accumulation, decreasing the volume occupied by the aggregates by almost 90% (Figure
3), demonstrating that UBC9 levels can significantly affect the cardiomyocyte’s efficiency in removing protein aggregates. UBC9 expression also reduced ubiquitinated protein levels, suggesting that UBC9 may function in controlling the overall proteasomal activity in the cell. UBC9 expression not only reduced protein aggregate accumulations but also improved cell viability, particularly when the NRVCs were challenged with a proteotoxic environment.

Confirming the protein’s importance in aggregate removal, UBC9 knockdown via siRNA treatment dramatically increased aggregate volume in the NRVCs. It is presently unclear how SUMOylation actually mediates aggregate reduction. It has been proposed that SUMOylation inhibits aggregation by reducing the β-sheet structure formation of mutant or unfolded proteins, and this is entirely consistent with the dramatic reduction in PAO levels that we observed upon upregulating UBC9 levels as the conformer is dependent, among other things, upon a β-sheet conformation.

By measuring UPS performance directly in the intact cell using a degron-based reporter, we were able to confirm an effect of UBC9 levels on the UPS. Elevated UBC9 levels enhanced UPS function while decreased UBC9 inhibited UPS mediated protein degradation. These experiments suggest that UBC9 plays an important role in modulating proteasomal activity and an essential role of the proteasome in mediating UBC9’s effects was confirmed using the proteasomal inhibitors MG-132 and lactacystin. Available data on the removal of the aggregates in the CryAB model points to both autophagy and UPS mediated protein degradation as playing important roles.

Our study suggests that UBC9 is a key enzyme of SUMOylation system and its enhanced expression can be beneficial in a proteotoxic environment, acting through multiple pathways (Online Figure VIII). We hypothesize that UBC9 may be a potential therapeutic target whose modulation could be productively explored in vivo, positively impacting on the proteasome functional insufficiency observed in cardiac proteotoxicity. Proteomic analyses indicate that proteasomal components can be SUMOylated as well, raising the possibility that proteasomal activity could be modulated directly by this post-translational process during stress. Our data justify further exploration of SUMOylation in cardiomyocytes. Studies defining the overall activity of the pathway, how it is affected by disease and whether its modulation can decrease developing heart failure are all underway in newly developed animal models.

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DISCLOSURES
None.
REFERENCES


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FIGURE LEGENDS

**Figure 1.** Expression of cardiac SUMOylation proteins differentially regulated in CryABR120G and in TAC induced hypertrophied hearts. **A, B,** Western blot analyses of UBC9, SUMO1, SUMO2/3 and CryAB protein in 14 week old normal control NTG (grey) and CryABR120G transgenic (R120G black) hearts; n=6. **C, D,** Western blot analyses of UBC9, SUMO1, SUMO2/3 and CryAB protein in normal, sham operated hearts and in hearts subjected to TAC and harvested after 2 weeks; n=8; Sham (grey) and TAC (black). **E, F,** SUMO1 conjugates are reduced in CryAB R120G hearts; n=3. *P<0.05, significant difference between NTG versus CryABR120G. **P<0.001, significant difference between sham versus TAC mice.

**Figure 2.** Expression and localization of UBC9 in cardiomyocytes. NRVCs were infected with Ad-UBC9 for 3 days at varying multiplicities of infection (MOI) as indicated. **A,** Expression analyzed via Western blots and **B,** quantitated (0-100 MOI). **C,** Immunohistochemical detection of UBC9 in NRVC (10 MOI). Cells were fixed and immunostained for UBC9 (green), TnI (red) and DAPI (nuclear staining; blue). *P<0.05, significant difference between β-gal (Control) versus UBC9 treated cells.

**Figure 3.** UBC9 expression reduces CryABR120G induced protein aggregate content in cardiomyocytes. NRVCs were co-infected with Ad-CryABR120G (10 MOI) in the presence of Ad-β-gal (30 MOI) or Ad-UBC9 (30 and 50 MOI; increasing amounts indicated by triangle) for 5 days. **A,** Cells were fixed and immunostained with CryAB (green), Tnl (red) and DAPI (nuclear staining; blue). **B,** Aggregates in the cardiomyocytes were quantitated using MetaMorph software; n=4/treatment. **C,** Fixed cells were also stained with ubiquitin antibody (green) and cardiac myosin binding protein C (red). **D,** Ubiquitin-containing aggregates were quantitated using MetaMorph software; n=4/treatment. ***P<0.0001, significant difference between β-gal versus UBC9 treated cells. **P<0.0001, significant difference between Ctrl versus β-gal and UBC9 treated cells. †P<0.001, significant difference between β-gal versus UBC9 treated cells.

**Figure 4.** UBC9 knockdown increases aggregate content in NRVCs. **A,** Western blot showing the effect of the two UBC9-siRNAs (10nM) on UBC9 expression. **B,** Quantification of the Western blot siRNA; n=3. **C,** NRVCs were treated with 10nM of the siRNAs for 24 hours and then infected with Ad-CryABR120G (10 MOI) for another 48 hours. Cells were fixed and stained for CryAB (green), phalloidin (red) and DAPI (blue). **D,** Aggregates in the cardiomyocytes were quantitated using MetaMorph software; n=4/treatment. *P<0.05, significant difference between scrambled (ctrl)-siRNA versus UBC9-siRNA’s. ***P<0.0001, significant difference between ctrl-siRNA versus UBC9-siRNA.

**Figure 5.** UBC9 enhances UPS performance. **A,** NRVC cells were co-infected with the proteasome activity reporter Ad-GFPu (5 MOI) in the presence of either Ad-β-gal (30 MOI) or Ad-UBC9 (30 MOI). **B,** Quantitation of the data in Panel A (n=6). **C,** UBC9 protein was knocked down by treatment of the cells for 24 hours with UBC9-siRNA2 (10nM). The cells were then infected with GFPu (5 MOI) and the protein collected for analysis after 72 hours. **D,** Quantitation of the data in Panel C (n=3). ***P<0.0001, significant difference between β-gal versus UBC9 and ctrl-siRNA versus UBC9-siRNA.

**Figure 6.** Proteasome activity is essential for UBC9’s effect on aggregate accumulation. **A,** NRVCs were co-infected with Ad-CryABR120G (10 MOI) in the presence of β-gal (30 MOI) or UBC9 (30 MOI) for 5 days. Cells were subsequently treated with the proteasomal inhibitors MG132 (5μM) or lactacystin (200nM) for 12 hours, the cells fixed and then stained with antibody for CryAB (green) and Tnl (red). **B,** Aggregate content was measured using MetaMorph software; n=4/treatment. *P<0.0001, significant difference between β-gal versus UBC9, β-gal MG132 treated, UBC9 MG132 treated, β-gal lactacystin treated, UBC9 lactacystin. †P<0.0001, significant difference between UBC9 with UBC9 MG132 treated and UBC9 lactacystin treated.
**Figure 7.** UBC9 accelerates removal of preexisting aggregates from cardiomyocytes. NRVCs were infected with either Ad- β-gal (30 MOI) or Ad-UBC9 (30 MOI) for 24 hours and then treated with the proteasomal inhibitor MG132 (5μM) for 12 hours. The inhibitor was removed from the medium and the cells were then cultured for the indicated times, aliquots collected and the cells fixed. Immunohistochemistry was performed with anti-ubiquitin (green) and anti-cardiac myosin binding protein C (red). Staining was quantified using MetaMorph software; n=4/treatment. **P<0.0001**, significant difference between β-gal control versus different treatment groups. ¶ P<0.01, significant difference between β-gal in the 12 hours wash versus UBC9 12 hours post wash.

**Figure 8.** UBC9 reduces PAO content. **A,** Cells were co-infected with Ad-CryABR120G (10 MOI) and Ad-β-gal (30 MOI) or Ad-UBC9 (30 MOI) for 5 days. Cells were fixed and stained with A11 antibody (green) and TnI antibody (red). **B,** A11 signal was quantitated with MetaMorph software; n=4/treatment. **C,** NRVC were infected with Ad- β-gal (30 MOI), UBC9 (30 MOI) alone or in combination with Ad-CryABR120G (10 MOI) for 5 days and cell toxicity measured using the adenylate kinase assay. **D,** TUNEL assay positive cells were visualized and **E,** quantitated using MetaMorph. **P<0.0001,** significant difference between β-gal versus UBC9 treated cells. **P<0.0001,** significant difference between β-gal versus R120G and β-gal +R120G treated cells.
Novelty and Significance

What Is Known?

- Altered or compromised mechanisms that regulate protein clearance are implicated in proteotoxicity in cardiomyocytes.
- Increasing cardiomyocyte’s ability to clear its misfolded and aggregated protein load might be beneficial in certain circumstances.
- Post-translational processes, such as ubiquitination, are major determinants of overall activity of cellular clearance mechanisms.

What New Information Does This Article Contribute?

- The post-translational process of SUMOylation is altered during the development of cardiac proteotoxicity and can be enhanced by cardiomyocyte-specific expression of SUMO conjugating enzyme UBC9.
- Expression of UBC9 in the background of a proteotoxic disease, induced by expression of the mutant chaperone αB crystallin R120G (CryABR120G), effectively augmented cardiomyocyte’s ability to clear its misfolded and aggregated protein load, including toxic pre-amyloid oligomers.
- Post-translational processes, such as SUMOylation can be effectively modulated to maintain protein degradative functions during proteotoxic challenges in the heart and these interventions might be beneficial in maintaining normal cellular function.

Proteotoxic processes play important functions in the developing pathology that both underlies and accompanies heart disease. Consequently, it is important to understand the processes that can decrease the burden of unfolded, misfolded and damaged proteins in the heart. Post-translational modifications such as ubiquitination are critical to targeting damaged proteins for degradation by the cell’s recycling machinery. These processes are compromised during the development of many proteotoxic diseases. We explored a relatively understudied post-translational modification process, SUMOylation, which is closely related to ubiquitination but is thought to play a more general role in cellular metabolism. We found that SUMOylation is, indeed, tightly controlled in the cardiomyocyte and is compromised during the development of cardiac pathology induced by increased proteotoxic load. We modulated SUMOylation activity in cardiomyocytes using both gain-and loss-of-function approaches. By upregulating SUMOylation in diseased cardiomyocytes, we were able to significantly reduce the proteotoxic load and increase survival rates. These data identify SUMOylation as having an important role in proteotoxic processes and open up a new set of therapeutic targets in proteotoxic cardiac disease.
Figure 2

(A) Western blot analysis showing expression levels of UBC9 (in kDa) at different MOI (multiplicity of infection) values (0, 10, 20, 30, 50, 100).

(B) Bar graph showing fold expression of UBC9 at the same MOI values. Significant differences are indicated by asterisks.

(C) Immunofluorescence images showing UBC9, TnI, and merged views under control and UBC9 conditions. Scale bar = 10 μm.
Figure 3

A

CryAB

UBC9

β-gal

Tnl

Merge

B

Aggregate (%)

R120G
β-gal
UBC9

+  
-  
-  

0  
5  
10  
15  
20  
25  
30  
35  
40  
45

***

C

R120G

Ctrl. cell

β-gal

UBC9

Ubiquitin

cMyBP-C

Merge

D

Ubiquitin (%)

Ctrl  
β-gal  
UBC9

0  
10  
20  
30  
40  
50  
60

***

†
Figure 8

A. Immunofluorescence images showing β-gal+R120G and UBC9+R120G treatments.

B. Bar graph showing PAO level (%).

C. Cell viability (% control) graph.

D. TUNEL and DAPI staining images for β-gal, UBC9, R120G, β-gal+R120G, and UBC9+R120G treatments.

E. Bar graph showing TUNEL positive cells (%).
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Supplemental Material

Sumo E2 Lgase UBC9 is Required for Efficient Protein Quality Control in Cardiomyocytes

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Running title: UBC9 in protein quality control

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Figure I, UBC9 expression enhances total NRVC SUMOylation and SUMOylation is decreased in CryAB$^{R120G}$ infected NRVC. 

**A**, Western blot shows total SUMOylation of NRVCs infected with Ad-β-gal (30 MOI) or Ad-UBC9 (30 MOI) for 3 days. 

**B**, Quantitation of the data in Panel A; n=3. 

**C**, NRVCs were treated with 10nM of the siRNAs for 72 hours and SUMOylated proteins detected by western blot. 

**D**, Quantitation of the data in Panel C, n=3. **P<0.01**, significant difference between β-gal versus UBC9 treated cells. **P<0.01**, significant difference between control (scrambled; ctrl)-siRNA versus UBC9-siRNA treated cells
Figure II. Modifying UBC9 protein levels does not change CryAB expression. A, NRVCs were co-infected with β-gal (30 MOI) or UBC9 (30 MOI) for 3 days and protein expression evaluated using Western blots. Neither levels of CryAB or selected ubiquitous proteins such as actin or GAPDH were affected by UBC9 overexpression. B, Quantitation of the data in Panel A, β-gal (grey) and UBC9 (black), n=3. C, NRVCs were treated with 10nM of the indicated siRNAs for 72 hours and protein determined as in Panel A. D, Quantitation of the data in Panel C; n=3
Figure III. Knockdown of UBC9 reduces cardiomyocyte viability. A, NRVCs were infected with varying amounts of Ad-UBC9 and cellular toxicity measured using the adenylate kinase assay. B, NRVCs were treated with 10nM of the siRNAs for 72 hours and then cellular toxicity measured using the adenylate kinase assay. **P<0.01, significant difference between ctrl-siRNA versus UBC9-siRNA. C, NRVCs were treated with 10nM of the siRNAs for 72 hours and cell death measured using the TUNEL assay. D, Quantification of data in Panel C. **P<0.01, significant difference between ctrl-siRNA versus UBC9-siRNA; n=4 for all panels except C.
Figure IV. Knockdown of UBC9 expression causes accumulation of ubiquitinated protein in the insoluble fraction. A-B, Western blot analysis shows total ubiquitination level of NRVCs both in the soluble (Sol.) and insoluble (Insol.) fractions derived from NRVCs infected with Ad-β-gal (30 MOI) or Ad-UBC9 (30 MOI) for 72 hours. C, Quantitation of the data in Panels A and B; β-gal (grey) and UBC9 (black); n=3. D, E, NRVCs were treated with 10nM of the siRNAs for 72 hours and ubiquitination assayed by Western blot with ubiquitin antibody. F, Quantitation of the data in Panels D and E; Ctrl-siRNA (grey) and UBC9-siRNA (black); n=3. **P<0.001, significant difference between Ctrl-siRNA versus UBC9-siRNA treated cells.
Figure V. In vitro proteasomal activity is unaffected by UBC9 expression. A, An in vitro assay for proteasome activity (Methods) shows the expected dependence upon exogenously provided substrate in the isolated system; n=3 B, In vitro proteasome activity was measured using 10 µg of crude proteasome isolated from NRVCs infected with Ad-β-gal (30 MOI) or Ad-UBC9 (30 MOI) for 3 days; n=3 C, Western blots show that expression levels of the basic proteasome subunits are unaffected by UBC9 expression. D, Quantitation of the data in Panel C, β-gal (grey) and UBC9 (black); n=3
Figure VI. Knockdown of UBC9 causes significant accumulation of ubiquitinated protein in the insoluble fraction in NRVC expressing CryAB<sup>R120G</sup>.

NRVCs were treated with 10nM of the siRNAs for 24 hours and then infected with Ad-CryAB<sup>R120G</sup> (10 MOI) for another 48 hours. Ubiquitinated protein level was detected on Western blots using ubiquitin antibody for both the soluble and insoluble fractions. B, Quantitation of the data in Panel A; Ctrl-siRNA (grey) and UBC9-siRNA (black), n=3

***<i>P</i><0.0001, significant difference between Ctrl-siRNA versus UBC9-siRNA treated cells
Figure VII. UBC9 directly targets elements of the unfolded protein response (UPR). A, Western blot and B, its quantitation show significant upregulation of a subset of UPR proteins; β-gal (grey) and UBC9 (black); n=3 C, Diagrammatic representation of vector used for the ER Stress Response Element (ERSE) promoter activity analysis. D, HEK 293T cells were transfected with vector alone (Vect.) a negative control with the ERSE reporter and UBC9 and activity measured using the dual glow luciferase assay;1 n=5; *P<0.05, significant difference between β-gal versus UBC9 treated cells. ***P<0.0001, significant difference between Vect. treated versus UBC9 treated cells.
Figure VIII. Summary and hypothesized impact of UBC9 in NRVCs. UBC9 plays multiple roles in cardiomyocytes. UBC9 can regulate cellular aggregate levels and impacts on proper protein folding and degradation through the UPS pathway. This function is to some extent related to SUMOylation levels in the cell and knockdown of UBC9 reduces SUMOylation and increases aggregated protein in the insoluble fraction. UBC9 also regulates UPR and ER homeostasis by regulating the ER resident protein levels in the cells.

References