UBC9-Mediated Sumoylation Favorably Impacts Cardiac Function in Compromised Hearts

Manish K. Gupta, Patrick M. McLendon, James Gulick, Jeanne James, Kamel Khalili, Jeffrey Robbins

**Rationale:** SUMOylation plays an important role in cardiac function and can be protective against cardiac stress. Recent studies show that SUMOylation is an integral part of the ubiquitin proteasome system, and expression of the small ubiquitin–like modifier (SUMO) E2 enzyme UBC9 improves cardiac protein quality control. However, the precise role of SUMOylation on other protein degradation pathways, particularly autophagy, remains undefined in the heart.

**Objective:** To determine whether SUMOylation affects cardiac autophagy and whether this effect is protective in a mouse model of proteotoxic cardiac stress.

**Methods and Results:** We modulated expression of UBC9, a SUMO E2 ligase, using gain- and loss-of-function in neonatal rat ventricular cardiomyocytes. UBC9 expression seemed to directly alter autophagic flux. To confirm this effect in vivo, we generated transgenic mice overexpressing UBC9 in cardiomyocytes. These mice have an increased level of SUMOylation at baseline and, in confirmation of the data obtained from neonatal rat ventricular cardiomyocytes, demonstrated increased autophagy, suggesting that increased UBC9-mediated SUMOylation is sufficient to upregulate cardiac autophagy. Finally, we tested the protective role of SUMOylation-mediated autophagy by expressing UBC9 in a model of cardiac proteotoxicity, induced by cardiomyocyte-specific expression of a mutant α-B-crystallin, mutant CryAB (CryABR120G), which shows impaired autophagy. UBC9 overexpression reduced aggregate formation, decreased fibrosis, reduced hypertrophy, and improved cardiac function and survival.

**Conclusions:** The data showed that increased UBC9-mediated SUMOylation is sufficient to induce relatively high levels of autophagy and may represent a novel strategy for increasing autophagic flux and ameliorating morbidity in proteotoxic cardiac disease. (Circ Res. 2016;118:1894-1905. DOI: 10.1161/CIRCRESAHA.115.308268.)

**Key Words:** autophagy ■ crystallin ■ heart disease ■ hypertrophy ■ transgenic mice

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Post-translational modifications (PTMs) play an important role in the fine-tuning of protein function after biogenesis. SUMOylation is a reversible PTM in which a small ubiquitin–like modifier (SUMO) protein is covalently attached to a lysine residue of the target protein. The SUMO system was described ≈20 years ago and categorized as a member of the ubiquitin PTM family. Subsequent data have clearly shown that SUMOylation is a versatile and dynamic PTM, regulating several important cellular functions, including transcription, cell division, protein stability and translocation, signal transduction, protein–protein interactions, and chromatin segregation. Early studies suggested that SUMO-modified proteins were present in the nucleus and played a crucial role in nuclear organization and function. Subsequent data extended SUMOylation’s function to the cytoplasm, as well as to the cell membrane and mitochondria. Four different isoforms of SUMO (SUMO 1–4) have been reported. SUMO 1 shares only 50% identity with the other isoforms, whereas SUMO 2 to 4 share 86% homology to one another. SUMOylation is an ATP-dependent reaction where cascades of enzymes activate the SUMO protein, which then attaches activated SUMO (SUMO-Gly-Gly) protein to the ε-amino group of a lysine residue. Analogous to the ubiquitination pathway, SUMOylation is mediated via 3 key enzymes: SUMO E1 (the heterodimer SAE1 and SAE2), SUMO E2 conjugating enzyme (UBC9), and several E3 ligating enzymes. In addition, several SUMO-specific proteases help in the removal of SUMO protein from the substrate, recycling the moiety for subsequent rounds of SUMOylation.

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Protein quality control (PQC) is critical for maintaining cardiomyocyte health. Cardiomyocytes have developed 2 broad strategies for maintaining PQC. A majority of cardiomyocyte protein is degraded via the ubiquitin proteasome system, as well as autophagy. Although autophagy is essential for maintaining cardiomyocyte health, it is insufficient to handle the high turnover rate of cardiomyocyte proteins. Cardiomyocytes have developed further strategies to maintain PQC via SUMOylation and other PTMs, as described in this article.
pathway. The targeted protein is ubiquitinylated by a cascade of ubiquitin enzymes, E1, E2, and E3. The ubiquitinylated protein is then degraded through the proteasome, and the released ubiquitin molecules are recycled.12 Although related, SUMOylation is distinct from ubiquitination and early on was thought that SUMOylation could compete with that process.13 However, subsequent data showed that SUMOylation is an essential component of the cellular PQC system with SUMOylated protein able to be subsequently ubiquitinated before degradation.14

Autophagy constitutes another, separate arm of PQC, in which misfolded or damaged cellular proteins, organelles, or protein aggregates are engulfed in a double-membrane structure and degraded by lysosomes.15 These various entities are often subjected to multiple PTM events, including ubiquitination, phosphorylation, acetylation, and O-GlcNAcylation.16–18 Several components integral to autophagy also undergo PTM by SUMOylation.20–22 Several components integral to autophagy also undergo PTM by SUMOylation.20–22 Several components integral to autophagy also undergo PTM by SUMOylation.20–22 These suggestive data prompted us to explore possible linkages between SUMOylation and autophagy in the heart.

Initial studies showed that SUMOylation enhanced PQC activity in cardiomyocytes and could augment aggregated protein clearance from the cells as well.22 We showed that up-regulating expression of UBC9, a SUMOylation E2 enzyme, enhanced expression of several proteins that reside in the endoplasmic reticulum.22 These proteins can function as chaperones and are involved in protein folding and the endoplasmic reticulum–associated protein degradation process. Herein, we find that increased SUMOylation in cardiomyocytes upregulates autophagy. Cardiomyocyte-specific expression of UBC9 in transgenic mice results in increased SUMOylation and autophagic flux and, in a proteotoxic model of cardiac disease, enhanced removal of misfolded or aggregated protein and significantly improved cardiac function.

Methods

Transgenic Mouse Generation

The UBC9 coding region was amplified from mouse heart cDNA and the cloning sequence confirmed. For the generation of cardiomyocyte-specific transgenic mice, the UBC9 coding sequence was cloned into the α-myosin heavy chain promoter vector at the Sal I site (Online Figure I).23 A hemagglutinin-tag epitope (MYPPDYVDPYA) was introduced at the N terminus for easy detection of the UBC9 protein. The construct was microinjected into fertilized eggs of FVBN mice to generate multiple founder lines. Real-time polymerase chain reaction (PCR) as well as Western blotting with hemagglutinin (HA)-tag or UBC9 antibody confirmed expression of UBC9 protein in the transgenic hearts. Transgenic mice expressing mutant CryAB (CryAB1270K) have been described.14 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.

Cell Culture

Neonatal rat ventricular cardiomyocytes (NRVCs) were isolated as described.25 In brief, left ventricles (LVs) from 1- to 2-day-old Harlan Sprague–Dawley rats were collected and digested with 0.05% trypsin overnight at 4°C. Cardiomyocytes were separated from the fibroblasts by preplating the digested cell suspension. NRVCs were plated in 10 cm² plates and 2-well chamber slides at a density of 1.5×10⁶ and 1×10⁶ cells, respectively. Initially, cells were grown in DMEM (Gibco) containing 10% fetal bovine serum, glutamine and 1x antibiotic/antimyotic (Gibco) for 24 hours. Cells were further grown in 2% fetal bovine serum DMEM for all experiments. Adenoviral transduction was done in serum-free DMEM for 2 hours and the media replaced with 2% fetal bovine serum DMEM. UBC9 expression was decreased or ablated by siRNA as described earlier.26 Cardiomyocytes were transfected with control siRNA or UBC9 siRNA using Lipofectamine 2000. Cells were harvested after 48 hours of transduction and analyzed using Western blotting or immunocytochemistry.

Adenovirus

Recombinant adenovirus was generated as described.26 The mouse SUMO1 cDNA was amplified from cardiac cDNA and the octapeptide, DYKDDDDK introduced at the N terminus for detection via a FLAG antibody. SUMO1 adenovirus was generated using the adenovirus-packaging kit (Agilent Technologies). Expression of recombinant protein was confirmed in cardiomyocytes transduced with Ad-SUMO1. Generation of Ad-β-GAL has been described.25

Protein Extraction and Western Blot Analysis

NRVCs were washed twice with PBS and lysed in RIPA buffer containing protease and phosphatase inhibitor (Roche). Lysed cells were sonicated briefly to disrupt the nucleic acids. Lysates were cleared by centrifugation at 10000g and the supernatant used for the protein expression analysis. Snap-frozen ventricular tissues were minced and homogenized in RIPA buffer with a Bead Beater (Bertin Technologies) and lysates cleared by centrifugation at 10000g for 30 minutes at 4°C. Supernatants were kept for the protein expression analysis. Protein concentration was determined using a Protein Assay Kit (Bio-Rad). Protein samples were prepared by dissolving protein in 1x Laemmli buffer (Sigma), and the samples were heated at 100°C for 5 minutes. Proteins were resolved in SDS-PAGE and then transferred to PVDF membrane by electrophoresis. Membranes were blocked with blocking buffer for 1 hour at room temperature followed by overnight incubation in primary antibody at 4°C as described.27 Membranes were then washed 3× with 0.1% Tween 20 in TBS, after which they were incubated with a secondary antibody conjugated with alkaline phosphatase for 2 hours. Signals were detected using an ECF detection kit (GE Healthcare). Blots were scanned with a STORM 820 fluorescent scanner (GE Healthcare). The following antibodies were used for immunoblotting, mammalian target of rapamycin, p-MTOR, HA-tag, microtubule-associated protein 1A/1B-light chain 3 (LC3) II, β-actin, UBC9 (Cell signaling), Beclin (Santa Cruz), vacuolar protein-sorting (Vps)34 (Abcam), GAPDH, Tnl (Millipore), α-B-crystallin (CryAB; Enzo Life Sciences), and secondary antibody conjugated with alkaline phosphatase (Santa Cruz Biotechnology).

RNA Isolation and Expression Analysis by Real-Time PCR

RNA was isolated from snap-frozen tissues using TRIzol reagent (Life Technologies). Tissues were minced and homogenized using the Bead Beater (Bertin Technologies). Isolated RNA was decontaminated from DNA by DNeasy treatment and purified using the RNaseasy...
Mini kit (Qiagen). cDNA was prepared from the isolated RNA using a SuperScript III First-Strand Synthesis SuperMix reagent kit (Invitrogen). Gene expression was analyzed by quantitative real-time PCR via a Taqman probe with a CFX-96 real-time PCR machine. Data were normalized to either β-actin or GAPDH. Autophagy-related gene expression was analyzed using the Autophagy PCR array kit (SA Biosciences).

**Autophagy During Nutrient Starvation**

NRVCs were grown in DMEM with 2% fetal bovine serum and treated with adenovirus as described. After 48 hours, the cells were incubated in either DMEM glucose-containing (4.5 g/L) or glucose-depleted media for another 8 hours. To assess the effect of nutrient starvation on the induction of cardiac autophagy in vivo, 12-week-old mice were fasted for 24 hours with unrestricted access to water. The hearts were isolated, and expression of activated LC3 (LC3-II) protein was detected by Western blot as a marker of increased autophagic activity. LC3 levels were also estimated by calculating the green fluorescent protein (GFP)-LC3 puncta in GFP-LC3 mice as a control, or UBC9 mice crossed with GFP-LC3 mice (provided by J. Sadoshima, Hill, UT SW). Hearts were perfused with perfusion buffer (4% paraformaldehyde in 1× PBS) and fixed in the perfusion buffer for another 12 hours at 4°C. The tissues were placed in 15% sucrose in 1× PBS (4 hours) and 30% sucrose in 1× PBS (12 hours) before embedding them in OCT solution. Embedded tissues were stored at −80°C. Five-micron sections were stained with DAPI. Slides were mounted with Vectashield Hard Set (Vector Labs). Autophagy, as represented by green puncta, was quantitated using confocal microscopy.

**Autophagy Flux Assay**

Autophagic flux was detected in NRVCs as well as in mouse hearts. NRVCs were transduced with Ad-GFP–LC3 (gift of J. Sadoshima) along with Ad-UBC9, Ad-SUMO1, or Ad-β-gal for 48 hours. Cells were fixed and green puncta quantitated using confocal microscopy. To measure autophagic flux, NRVCs were treated with Bafilomycin A1 at 50 nmol/L or a vehicle control (DMSO) for 4 hours. Cellular autophagy was also estimated by Western blots of protein derived from NRVCs transduced with Ad-UBC9 or Ad-SUMO1 for 48 hours. Autophagic flux was measured in vivo by injecting Bafilomycin A1 (intraperitoneal) as described. After 2 hours, the mice were euthanized and hearts were analyzed for LC3-II using Western blots. Autophagic flux in CryABR120G hearts was determined by crossing the transgenic and nontransgenic controls. Experiments were repeated multiple times. All statistical analyses were done with SPSS or SAS software. Statistical significance for the Kaplan–Meier data (Figure S) was determined using the LIFETEST Procedure in SAS. Analyses between the experimental groups used Student t test or 1-way ANOVA when the multiple groups were compared. Values of P<0.05 were considered significant.

**Results**

**SUMOylation Is a Critical Sensor of Nutrient Starvation**

To begin to explore the potential interactions between SUMOylation and autophagy, we initiated studies in which we perturbed autophagic flux and determined the effects of that treatment on SUMOylation. Starvation is well established to effectively induce or increase autophagy and, consistent with previous data, when we subjected NRVCs to nutrient depletion for 8 hours, autophagic markers such as LC3-II were upregulated (Online Figure II A–IIC). These NRVCs also showed significantly increased levels of SUMOylation. We subsequently extended these observations to the whole animal by subjecting a cohort of mice to nutrient deprivation for 24 hours (Methods section of this article). Measurements of both LC3-II and SUMOylation levels in cardiac tissue after 24 hours of starvation showed that, once again, both were elevated (Online Figure IID–IFE). The correlations prompted us to test the hypothesis that SUMOylation and autophagy could be mechanistically related.

**SUMOylation Induces Autophagy and Results in Increased Autophagic Flux**

Building on our earlier observations that enhanced expression of UBC9 can upregulate SUMOylation, we used gain-and loss-of-function approaches to modulate SUMOylation levels and determine the effects on autophagy. Although it is clear that autophagy is subject to many layers of controls, the effects of specific PTMs on the process in general and SUMOylation, in particular, are not understood. The effect of SUMOylation on autophagy was monitored using the fusion protein GFP–LC3 construct, a widely used reporter protein for autophagy. As part of the autophagic process, LC3-II protein sequesters with the autophagic vesicle and appears as green puncta, which can be visualized using fluorescence microscopy. UBC9 expression significantly increased visible puncta formation in the NRVCs (Figure 1A and 1B), both in the absence and in the presence of Bafilomycin A1, an autophagy inhibitor that is used to determine if autophagic flux is indeed being affected. Bafilomycin A1 treatment did unambiguously show a positive, dose-dependent effect of UBC9 expression on autophagic flux (Figure 1B), and this was accompanied by the upregulation of the autophagic marker LC3-II (Figure 1C and 1D). To confirm these data, we induced autophagy in the NRVCs via nutrient deprivation and showed that increased UBC9 protein expression significantly augmented the number of GFP–LC3-II puncta both in the absence and in the presence of Bafilomycin A1, further demonstrating that UBC9 can be an important positive regulator for autophagy (Figure 1E and 1F).

Finally, we affected the degree of SUMOylation by expressing SUMO1 protein directly. As was the case for UBC9, expression of SUMO1 protein significantly increased LC3-II protein and the number of GFP–LC3 puncta in NRVC (Online Figure III). Autophagic flux, as measured in the presence of Bafilomycin A1, was also significantly increased (Online Figure III), further suggesting that SUMOylation can affect autophagy in the NRVCs.

We then explored the consequences of decreasing UBC9 expression by knockdown using siRNA. Decreasing UBC9 protein significantly decreased the expression of the autophagy marker protein LC3-II and upregulated the protein p62, which helps transport ubiquitinated protein to the proteasome and autophagosome (Figure 2A and 2D). Interestingly, the protein Beclin-1 was significantly decreased as a result of UBC9 knockdown (Figure 2C and 2D): Beclin-1 plays a critical role...
in driving multiple steps in autophagy.\textsuperscript{36} The level of ubiquitinated protein was also increased significantly (Figure 2E and 2F), further suggesting the importance of UBC9 expression in modulating this critical PTM in cardiomyocytes. We confirmed the effects of UBC9 knockdown in nutrient starved cells as well. As is the case under normal nutrient conditions, autophagy was significantly downregulated and, even under nutrient deprived conditions, UBC9 knockdown largely or completely abrogated the autophagic response (Figure 2G and 2H).

**UBC9-Transgenic Mice Have Increased Levels of Cardiac SUMOylation and Autophagy**

We wished to determine if the results observed in the NRVC could be recapitulated in vivo and so, we developed multiple lines of transgenic mice that expressed enhanced levels of UBC9 in the cardiomyocytes only. Cardiomyocyte-specific expression was driven using the $\alpha$-myosin heavy chain promoter (Online Figure IA). Transgene expression was confirmed by Western blots with UBC9 and HA-tag antibodies and all 3 UBC9-transgenic lines seemed to be healthy without any detectable morbidity and the young adults showed no alterations in cardiac mass or hemodynamics (Online Figure ID–IG). Cardiac morphology was unaltered in the 4-month-old transgenic mice, with no apparent fibrosis or hypertrophy (Figure 3).

As expression of UBC9 led to a significant increase in the level of SUMOylated proteins in the NRVCs,\textsuperscript{22} we subsequently analyzed the cardiac protein complement in the UBC9-transgenic mice. SUMOylated protein was significantly increased in the transgenic hearts (Figure 4A and 4B). Accumulating data indicate that interactions between SUMOylation and autophagy are important for maintaining cellular homeostasis.\textsuperscript{37–39} Therefore, we wished to determine if the enhanced SUMOylation in the UBC9-transgenic hearts had any effects on cardiac autophagy and, as a first step, defined cellular levels of proteins that are normally associated with active autophagic flux, including the lipidated form of LC3-II, which we found was upregulated (Figure 4C and 4D). Upregulation...
is apparently selective, however, as the adaptor protein, p62, which contains both ubiquitin and LC3 binding sites, was not significantly upregulated in the hearts (Figure 4D). To gain a better understanding of the overall autophagic response, we used PCR arrays and analyzed a panel of transcripts encoding critical proteins in autophagy. Consistent with the increased autophagic flux, we found significant upregulation of genes underlying autophagic activity in the UBC9-transgenic hearts (Figure 4E), suggesting that increasing cardiomyocyte UBC9 expression affected autophagy in the hearts.

To test if UBC9 overexpression led to increased SUMOylation of a critical regulator of the autophagic process, we looked at Vps34, a class III PI3-kinase involved in autophagosome formation in the control and transgenic hearts. Vps34 is positively regulated by SUMOylation, and this modification is required for autophagosome formation.\(^{19}\) Therefore, we immunoprecipitated Vps34 from heart lysates prepared from UBC9-transgenic mice. Immunoblotting for Vps34 confirmed successful pull down of Vps34, and probing the blots with an antibody to detect SUMO1 confirmed increased SUMOylation of Vps34 in transgenic mice compared with NTG control mice (Figure 4F). These data confirm that UBC9 directs SUMOylation specifically to autophagy-essential proteins.

We subsequently examined SUMOylation’s ability to directly mediate autophagy in vivo by crossing the UBC9-transgenic mice with reporter mice expressing a GFP-labeled marker of autophagosomes, GFP–LC3.\(^{40}\) Green puncta, indicators of autophagosome formation, were quantitated in cardiac sections derived from the double-transgenic animals with and without Bafilomycin A1 treatment to accurately reflect autophagic flux. Increased SUMOylation mediated by UBC9 expression led to increased GFP–LC3 puncta formation.

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**Figure 2.** Knockdown of UBC9 inhibits autophagy in neonatal rat ventricular cardiomyocytes (NRVCs). A, Western blots and their quantitation (B) showing that decreased UBC9 expression leads to decreased levels of autophagic flux in NRVCs as indicated by decreased levels of microtubule-associated protein 1A/1B-light chain 3 (LC3) II in UBC9-siRNA cells compared with control siRNA–treated cells in both the presence and the absence of bafilomycin (n=5 per treatment). C and D, Western analyses show that knockdown of UBC9 decreases Beclin-1 levels and increases p62 protein (n=3 per treatment). \( ^{**}P<0.01, \) significant difference between Ctrl vs UBC9-siRNA treated NRVCs. Values were normalized to GAPDH. E, Representative images show that UBC9 knockdown increases the level of ubiquitinated proteins (green indicates ubiquitin positive, whereas red [cMyBP-C antibody] identifies NRVCs). F, Ubiquitin staining was quantitated using MetaMorph (n=4 wells/group). \( ^{**}P<0.01, \) significant difference between Ctrl vs UBC9-siRNA treated NRVCs. G and H, UBC9-siRNA treated NRVCs were unable to respond to starvation by increasing autophagy. NRVCs were treated with Ctrl-siRNA or UBC9-siRNA, along with Ad-GFP–LC3 and incubated for another 48 hours. Cells were fixed, stained, and green puncta counted under the microscope. (n=4 wells/group). \( ^{**}P<0.001.\)
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(Figure 5A and 5B), confirming that cardiac autophagy was upregulated in the hearts. We then explored whether UBC9 expression compromised the autophagic response to nutrient deprivation by measuring both LC3-II levels in the UBC9-transgenic hearts (Figure 5C and 5D) and green puncta in the double transgenics (Figure 5E and 5F). Starvation induced autophagy above the levels observed as a result of UBC9 expression alone, confirming that the autophagic response was not compromised by increased SUMOylation.

**UBC9 Expression Decreases Morbidity in Desmin-Related Cardiomyopathy**

Mutations in the intermediate filament protein, desmin, and in the associated small heat shock protein chaperone, CryAB, can cause desmin-related cardiomyopathy (DRM). We have modeled this disease extensively in mice by expressing both mutated desmin constructs and a mutated CryAB (CryABR120G) that is causative for human DRM. The CryABR120G hearts show significant deficits in multiple limbs of their PQC mechanisms with both proteasomal and autophagic protein clearance compromised. We previously showed that upregulating autophagy via either voluntary exercise or by cardiomyocyte-specific expression of a rate limiting protein in the autophagic pathways, Atg7, had beneficial effects on the disease’s natural history in this model. We wished to determine if modulation of the SUMO pathway could also decrease morbidity in the CryABR120G model as this would confirm that altering SUMOylation levels under conditions in which autophagy was compromised could physiologically affect on a proteotoxic disease model.

The UBC9-transgenic mice were crossed with the CryABR120G animals and the double-transgenic animals selected for further analyses in which they were compared with the CryABR120G mice at 5 months, when heart disease is apparent. Increased SUMOylation seemed to be beneficial, with hypertrophy significantly decreased (Figure 6A and 6B) both in terms of overall mass and on a cardiomyocyte basis as well, as defined by staining of the cells with wheat germ agglutinin (Figure 6C and 6D). Fibrosis was also significantly attenuated (Figure 6E).

One of the hallmarks of CryABR120G DRM is the accumulation of large, proteinaceous aggregates in the cardiomyocytes. We have shown that upregulating autophagic flux promotes the clearance of these aggregates and this correlates with decreased morbidity as well. To confirm that UBC9-induced stimulation of autophagy would have a similar effect on cell proteotoxicity, we measured the extent of aggregate formation in the single- and double-transgenic hearts. Aggregated protein was detected by immunostaining with CryAB antibody and cardiomyocytes identified with troponin I antibody. The double-transgenic mice (UBC9×CryABR120G) hearts have significantly reduced aggregate levels (Figure 7A and 7B), and this reduction is correlated with decreased cell death as well (Figure 7C). As expected, the reduction in aggregate content was mirrored by an overall reduction in total cardiac CryAB (Figure 7D).

All of these data, if physiologically relevant, should result in decreased symptoms of heart failure. Using echocardiography, we found the cardiac status to be substantially improved in the double transgenics when compared with the CryABR120G hearts, with improved LV systolic dimension and LV fractional shortening (Figure 7E). We subsequently confirmed that UBC9 overexpression in the CryABR120G mice actually increased autophagy by crossing the double-transgenic animals into the transgenic line that expressed the tfLC3 construct so that we could differentiate between the autophagosomes (yellow) and the autolysosomes, which represent the final step in autophagic breakdown (red; Figure 8A–8C). The data showed that autolysosome numbers, as represented by red puncta, were significantly increased in the triple-transgenic animals, confirming that autophagy was upregulated. We then carried out survival curves using 2 independently derived lines of UBC9 mice (lines 799 and 801). UBC9 overexpression had no effect on lifespan.
with the 2 lines showing no statistically significant differences (Figure 8D). Subsequently, we used line 801 to cross into the CryABR120G background; the double-transgenic mice had a substantially higher probability of survival in early adulthood as well, compared with the CryABR120G animals (Figure 8D). These data are consistent with a protective role for SUMOylation in a proteotoxic model of hypertrophy and heart failure.

Discussion

Cellular proteins in general and cardiac proteins in particular are invariably modified by specific cotranslational and post-translational processes. PTM can modulate protein maturation, translocation, protein–protein interactions, half-life and protein function.44,45 Phosphorylation,46 glycosylation,47 ubiquitination,48 and potentially, NEDDylation2 can be critical regulators of the protein’s location, function, and interactions, allowing a cell to modulate its protein complement in response to the constantly changing demands of the intracellular and extracellular environments. Most PTMs are dynamic in nature and tightly regulated under normal physiological conditions, with the overall PTM profile quickly adapting to changes in the extracellular environment, such as nutrient scarcity, physiological stress, or deregulation of cellular homeostasis. Herein, we have explored the role of one of these PTMs, SUMOylation and its relationship to other PQC systems in the cardiomyocyte in a disease model of proteotoxicity.24

Among the several known PTM proteins, SUMO family members are particularly interesting as they are highly conserved throughout evolution and, while showing less than 20% homology to ubiquitin, the proteins are structurally similar and the process of SUMOylation shares striking similarities to that of ubiquitination.8,13 SUMOylation of cardiac proteins

![Figure 4](Figure4.png)

**Figure 4.** Expression of UBC9 upregulates total cardiac SUMOylated protein and autophagic markers. **A,** Western blot showing SUMOylated protein in nontransgenic (NTG) and UBC9-transgenic (TG) hearts. **B,** Quantitation of SUMOylated protein level in NTG and UBC9-TG mice (n=4). Values were normalized to actin. **C,** Western blot showing levels of selected proteins associated with autophagy. **D,** Quantitation shows that microtubule-associated protein 1A/1B-light chain 3 (LC3) II is significantly upregulated at 4 months (n=4). Values were normalized to GAPDH. **E,** Gene expression associated with increased autophagy was analyzed via a polymerase chain reaction array (4 mo, n=4). ***P<0.001, significant difference between NTG and UBC9-TG mice. **P<0.01, significant difference between NTG and UBC9-TG mice. *P<0.05, significant difference between NTG and UBC9-TG mice. **F,** UBC9 expression leads to increased SUMOylation of a target protein, vacuolar protein-sorting (Vps)34. Vps34 was immunoprecipitated and the SUMOylation levels subsequently tested on Westerns using SUMO1 antibodies. i.p. indicates intraperitoneal; and SUMO, small ubiquitin–like modifier.
has recently been reviewed, and SUMO1 modification of cardiac SERCA2a can result in increased activity and improved cardiac contractility in heart failure. The 4 SUMO isoforms play distinct functions in the cell. SUMO1 adds 1 SUMO group and can modulate protein−protein interaction, protein translocation, and stability. The SUMO2−3 isoforms perform poly-SUMOylation PTMs and can modulate protein degradation. Recent studies suggest that the SUMO2−3 isoforms can also target proteins for poly-ubiquitination and subsequent degradation via the proteasome. SUMO4 differs from the other isoforms in that it has proline rather than glutamine at residue 90 and is not processed and conjugated at

Figure 5. Autophagy is upregulated in UBC9-transgenic (TG) hearts. A and B, TG UBC9 expression increases green fluorescent protein (GFP)-microtubule-associated protein 1A/1B-light chain 3 (LC3) puncta in the heart. C, Western blot and (D) quantitation of activated LC3 (LC3-II) levels in nontransgenic (NTG) and UBC9-TG hearts before and after 24 hours of starvation. Values were normalized to GAPDH. E, Sections derived from GFP−LC3 and GFP−LC3×UBC9 hearts show increases in autophagic vacuoles (green puncta) in the UBC9-TG material under both normal and starved conditions. F, Quantitation of data in E (n=4). **P<0.01, ***P<0.001 significant difference between NTG and UBC9-TG mice.

Figure 6. Cardiac hypertrophy in mutant CryAB (CryABR120G) is attenuated in UBC9-transgenic (TG) mice. A−C, Analysis of heart tissue sections by hematoxylin and eosin staining (top), Masson trichrome staining (middle), and cell membrane staining with wheat germ agglutinin to determine cell size (bottom). D, Quantitation of cell size and (E) fibrosis (n=6). ***P<0.001, significant difference between CryABR120G mice and CryABR120G×UBC9-TG crosses. All mice were 5 months old. NTG indicates nontransgenic.
baseline. It can, however, be hydrolyzed under certain stress conditions. This can lead to the subsequent modification of its targeted substrate proteins. In this study, we explore the intersections between SUMOylation and autophagy, another limb of the cell's machinery for dealing with unwanted proteins and aggregates.

We found that cardiac-specific expression of UBC9 increases overall cardiomyocyte SUMOylation. UBC9 can function as a transcriptional coregulator as well as a SUMO E2 ligase. SUMOylation is essential for normal cardiac development and knockdown of SUMO protein causes abnormal heart development and premature death. Decreased levels
of UBC9 also lead to accumulation of aggregated proteins and changes in cell metabolism.\textsuperscript{58} Our data show that cardi-ac-specific expression of UBC9 protein results in increased SUMOylation levels that are accompanied by increased basal autophagic flux. Cardiomyocyte-specific UBC9 protein expression significantly increased the expression of multiple transcripts encoding autophagy proteins as well.

Recent studies suggest that SUMO PTM can increase the solubility of aggregated proteins and subsequently reduce their cellular toxicity.\textsuperscript{59} In a mouse model of DRM generated by expression of CryAB\textsuperscript{R120G}, granulofilamentous aggregates play a major role in the resultant cardiomyocyte pathology. Upregulation of the cells’ clearance pathways resulted in decreased morbidity and significantly increased lifespan.\textsuperscript{30} Autophagy is a critical cellular process whose activity is closely linked to overall cellular homeostasis. It functions in protein degradation, organelle turnover, and recycling of cellular components under both normal and stress conditions.\textsuperscript{60,61} If autophagy is compromised in a proteotoxic disease such as DRM, cellular aggregate content of the cell increases and leads to cell death and augmented cardiac disease.\textsuperscript{30,62} Autophagy is a multi-step process consisting of initiation, elongation, and fusion. Each step is regulated by a cascade of proteins, whose function can be also regulated by PTMs, such as phosphorylation, acetylation, and ubiquitination.\textsuperscript{16,18,63}

Although we show that changes in SUMOylation are correlated with the beneficial effects of increased autophagy, we have not, in this article, directly shown the mechanistic under-pinnings. In future studies, one could define the ability of UBC9 rescue through autophagy-independent effects to test necessity. We also do not yet have a clear understanding of what might be the most critical target or targets of SUMOylation in the regulation of autophagy. The data in Figure 4E underlie the broad scope of the autophagic response. Beclin 1, a key player at multiple levels in autophagy, clearly is responsive to UBC9 expression (Figure 2). In cell culture, SUMOylation can regulate the function of Vps34, a key component of autophagic vesicle formation and autophagosome–lysosome fusion. Vps34 SUMOylation at lysine840 induces Vps34 activity, leading increased to increased lipid kinase activity.\textsuperscript{19} In another study carried out with yeast α-synuclein, the protein found in the intracytoplasmic inclusions known as Lewy bodies in Parkinson disease,\textsuperscript{64,65} Shahpasandzadeh et al\textsuperscript{61} found that impaired SUMOylation of α-synuclein resulted in almost complete prevention of

![Image](http://circres.ahajournals.org/Downloadedfrom)
autophagy-mediated aggregate clearance. Consistent with these data, our results show that, in NRVCs, SUMOylation knockdown decreases autophagic flux, leading to increased aggregate content and decreased function. Conversely, in the heart, upregulation of SUMOylation (Figure 4) upregulates autophagic flux and improved cardiac function in the face of a proteotoxic insult. Thus, SUMOylation plays a critical role in modulating autophagy. Upregulating the SUMO pathway by UBC9 overexpression seems to be largely benign, with no hypertrophy, fibrosis, altered cardiac morphology, or hemodynamics apparent. Nor did we observe any increases in mortality in the first year of life (Figure 8). Importantly, in the context of proteotoxic disease, increasing the pathway’s activity can be therapeutic, reducing the proteotoxic load and prolonging both normal cardiac function and lifespan in the face of chronic disease. Our data support the hypothesis that manipulating SUMOylation levels in the heart may present the opportunity for therapeutic intervention under proteotoxic conditions.

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Disclosures

None.

References

Numerous data from multiple experimental avenues highlight the importance of autophagy, as being able to mediate critical aspects of normal heart function as well as the heart's ability to respond to outside and internal stressors. It is therefore important to understand how and what modulates autophagy, particularly in the context where it is compromised, such as when the heart experiences a general, proteotoxic environment. We explored the potential of modulating autophagy using the protein post-translational modification known as SUMOylation, where small ubiquitin–like modifier (SUMO) groups are added to selected proteins. A protein's SUMOylation can have pleiotropic effects, and this post-translational modification can affect protein quality control. We used loss- and gain-of-function approaches in both cardiomyocyte cell cultures and transgenic mice to explore the effects of UBC9 downregulation and overexpression, as this protein, a SUMOylation E2 enzyme, can modulate overall SUMOylation activity. The data show that increased UBC9-mediated SUMOylation is sufficient to induce relatively high levels of autophagy and, in the context of a proteotoxic heart, is cardioprotective. No toxic effects of increased SUMOylation were noted in normal mice and modulating this pathway's activity may represent a novel strategy for increasing autophagic flux and ameliorating morbidity in proteotoxic cardiac disease.

Novelty and Significance

What Is Known?
- The process of cellular self-digestion, autophagy, is critical for normal cell function and is often altered in cardiovascular disease.
- The post-translational protein modification process of SUMOylation seems to be important in cardiac protein quality control as well.

What New Information Does This Article Contribute?
- By upgrading the process of SUMOylation, we were also able to increase autophagy, showing a potential link between the 2 processes.
- Upregulation of SUMOylation, in a cardiac proteotoxic environment where autophagy is compromised, resulted in cardioprotection and was accompanied by increased autophagic flux.


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UBC9-Mediated SUMOylation Favorably Impacts Cardiac Function in Compromised Hearts

Manish K Gupta, PhD, Patrick M McLendon, PhD, James Gulick, MS, Jeanne James, MD, Kamel Khalili, PhD and Jeffrey Robbins, PhD

From The Heart Institute, Department of Pediatrics, The Cincinnati Children’s Hospital Medical Center, Cincinnati, OH 45229, USA

Correspondence to Jeffrey Robbins PhD, Division of Molecular Cardiovascular Biology, Cincinnati Children’s Hospital Medical Center, MLC 7020, 240 Albert Sabin Way, Cincinnati, OH 45229-3039. Tel: (513) 636-8098; Fax: (513) 636-5958. E-mail: jeff.robbins@cchmc.org
Online Figure I. Transgenic expression of UBC9 in the heart. A, Schematic diagram showing the UBC9 construct. B, mouse cDNA was prepared from hearts (n=3/line) and UBC9 expression determined by RT-PCR. C, UBC9 expression levels were analyzed in cardiac protein extracts derived from 3 TG lines (650, 799, 801) by Western blots with HA-tag antibody. D-G, Echocardiographic analysis of heart function shows that UBC9 expression did not affect cardiac function. All mice were 4 months old; n=4 in Panels C-F. FS; fractional shortening, LVIDs (d); LV internal diameter end systole and diastole, respectively
Online Figure II. Starvation increases SUMOylated protein levels. A, Western blot showing LC3 II and SUMOylation levels in NRVCs cultured in DMEM media minus sera and glucose (starved state). B, C, Histograms quantitating the autophagy marker protein LC3 II and SUMOylated protein (n=3). D, Western blot showing SUMOylation levels in cardiac tissue at 0 hour fasting and 24 hour fasting. E, F Histograms quantitating the autophagy marker protein LC3 II and SUMOylated protein (n=3). **P<0.01, significant difference between 0 hour and 24 hours. ***P<0.001, significant difference between 0 hour and 8 hours.
Online Figure III. SUMO1 expression levels correlate with upregulated markers of autophagy in NRVC. **A**, NRVCs were infected with Ad-β- gal (30 MOI) or Ad-SUMO1 (30 MOI) for 3 days and LC3 II, a marker of autophagy, was analyzed by Western blots. Cells were treated with either Bafilomycin A1 or DMSO for 4 hours. SUMOylated protein levels were then quantitated by Western analyses using SUMO1 antibody. **B**, Quantitation of LC3 II expression (n=5). **C**, NRVCs were co-infected with Ad-GFP-LC3 (10 MOI) in the presence of Ad-β-gal (30 MOI) or Ad-SUMO1 (30 MOI) for 3 days. Cells were fixed and stained for GFP-containing puncta, which indicate autophagic vacuoles. DAPI was used to stain nuclei. *P<0.05 **D**, Quantitation of green puncta (n=4 wells/group). ***P<0.001