Essential role for UVRAG in autophagy and maintenance of cardiac function

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Aims Ultraviolet irradiation resistance-associated gene (UVRAG) is a tumour suppressor candidate that regulates cell autophagy and endocytosis. However, the in vivo function of UVRAG remains poorly understood. We sought to determine the physiological role of UVRAG in the heart.

Methods and results We characterized mice with disruption of the UVRAG gene by piggyBac (PB) transposon insertion. PB construct was inserted into intron 14 of the UVRAG gene and disruption of UVRAG transcript was confirmed by reverse transcript-polymerase chain reaction. Immunoblotting revealed that UVRAG was deficient in multiple tissues. Autophagic flux was attenuated in UVRAG-deficient (UVRAG−/−) mouse embryonic fibroblasts. In UVRAG-deficient hearts, autophagosomes were accumulated and autophagic flux, assessed as the increased protein abundance of LC3 II in chloroquine-treated animals, was impaired. UVRAG-deficient mice were viable, fertile, and developmentally normal. However, they developed age-related cardiomyopathy associated with compromised cardiac function. In addition, inflammatory response was enhanced in UVRAG-deficient hearts.

Conclusion Collectively, our findings suggest that UVRAG is essential for the regulation of autophagy and maintenance of cardiac function.

Keywords UVRAG • Autophagy • Cardiomyopathy • Autophagosome • Inflammation

1. Introduction

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved process by which cellular components are degraded by the lysosome pathway. Autophagy is initiated with the surrounding and sequestration of cytoplasmic components by isolation membrane (phagophore), which is elongated to form double-membrane vesicles called autophagosomes. Autophagosomes subsequently undergo a maturation process consisting of fusion with acidified endosome/lysosome, followed by lysosomal hydrolysis of sequestered cytoplasmic components, and the inner membrane of autophagosomes. Under normal conditions, autophagy is constitutively active at low levels and essential for the maintenance of basal homeostasis through degradation of damaged proteins and organelles such as mitochondria.1,2 Autophagy is up-regulated in response to multiple stresses, such as nutrient starvation, hypoxia, oxidative damage, growth factor deprivation, and mechanical stress.3–5 Dysregulation of autophagy has been implicated in multiple pathological conditions, including cardiovascular diseases.6–9

UVRAG, a putative mammalian ortholog of yeast Vps38, has been implicated in vacuolar protein sorting and retrograde transport.10 In mammalian cells, UVRAG promotes autophagosome formation by binding directly to Beclin 1–Vps34 complex and enhances Vps34 activity.11 UVRAG also interacts with Bif, which has been shown to promote Vps34 activation.12 In addition, UVRAG promotes autophagosome maturation and endocytic trafficking by recruiting a subset of Beclin 1–Vps34 complex.13,14 UVRAG has been shown to suppress cancer cell proliferation and growth by inducing autophagy.15 UVRAG also exerts tumour suppressor functions by maintaining chromosomal stability through autophagy-independent mechanism.16 Moreover, UVRAG suppresses apoptosis by regulating the subcellular localization of BCL2-associated X protein.17 It has recently been shown that UVRAG is required for organ rotation by regulating Notch endocytosis, but not autophagy in Drosophila.18 However, the physiological role of UVRAG in the heart remains to be determined.

In the present study, we report the characterization of mice with disruption of UVRAG via the piggyBac (PB) transposon system.19 We show that UVRAG deficiency causes autophagosome accumulation via...
impairment of autophagic flux both in mouse embryonic fibroblasts (MEFs) and the heart. In addition, disruption of UVRAG leads to age-related cardiomyopathy accompanied by enhanced inflammatory response. Our results suggest that UVRAG is essential for the regulation of autophagy and maintenance of cardiac function.

2. Methods

2.1 Animals
All the mice used in this study are on the FVB/NJ background. The animal experimentation was conducted in accordance with the Institutional Animal Care and Use Committee of Shanghai, who reviewed and approved all experimental protocols according to the Guide for the Care and Use of Laboratory Animals (Directive 2010/63/EU of the European Parliament). The PB-inserted UVRAG mouse line was kindly provided by Institute of Developmental Biology and Molecular Medicine of Fudan University. The PB transposon was inserted into intron 14 of UVRAG on mouse chromosome 7 and the direction of the insertion was opposite to the gene location. Mice were anaesthetized by isoflurane inhalation and euthanized by rapid cervical dislocation. Mice tissues were then dissected for immunoblotting, RT–PCR, quantitative real-time RT–PCR, and electron microscopy analysis.

2.2 Antibodies
Rabbit anti-p62 polyclonal antibody was purchased from Cell Signaling Technology, Inc. Rabbit anti-LC3 polyclonal antibody, mouse anti-LC3 monoclonal antibody, mouse anti-UVRAG monoclonal antibody, and rabbit anti-Beclin 1 polyclonal antibody were obtained from Santa Cruz. Rat anti-LAMP-1 monoclonal antibody, rat anti-LAMP-2 monoclonal antibody, rabbit anti-CD45 antibody and rat anti-cathepsin D polyclonal antibody, and rabbit anti-GAPDH polyclonal antibody were obtained from MBL. Rat anti-LAMP-1 monoclonal antibody, rabbit anti-LC3 polyclonal antibody, and rabbit anti-p62 polyclonal antibody were purchased from Cell Signaling Technology, Inc. Rabbit anti-LC3 polyclonal antibody, mouse anti-LC3 monoclonal antibody, and rabbit anti-UVRAG monoclonal antibody were from Sigma-Aldrich.

2.3 Cell culture
UVRAGFR+ matings were set up and the pregnant female mice on post-coital day 13.5 were euthanized by CO2 inhalation. Primary MEFs were generated from Day 13.5 embryos and cultured in DMEM (high glucose) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine.

2.4 Autophagic flux assessment
For in vitro autophagic flux assessment, MEFs were treated with vehicle or 100 nM bafilomycin A1 (Merck) for 4 h and cell lysates were prepared for the detection of LC3 II protein abundance. For in vivo autophagic flux assessment, mice were injected intraperitoneally with vehicle or 75 mg/kg chloroquine (Sigma-Aldrich) and the hearts were dissected 6 h after injection for the detection of LC3 II protein abundance.

2.5 Immunoblotting
Mice tissues were homogenized in lysis buffer containing Complete protease inhibitor mixture (Roche) and the tissue lysates were collected after centrifugation. The protein concentration was evaluated with Nanodrop 1000 Spectrophotometer (NanoDrop Technologies, Inc). Protein samples were separated on 12.5% SDS–PAGE gels and then transferred to nitrocellulose membranes (Bio-Rad). The membranes were then blocked for 1 h at room temperature with 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween 20 (TBST), probed with primary antibodies overnight at 4 C, washed with TBST three times, and incubated with secondary antibodies for 1 h at room temperature, washed again with TBST three times. Immunolabelled proteins were then revealed by using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

2.6 RT–PCR and quantitative real-time RT–PCR
Total RNA was prepared from left ventricular tissues of wild type (WT) and UVRAG-deficient mice using TRIzol Reagent (Life Technologies) and cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies). RT–PCR was performed to determine the disruption of UVRAG mRNA using the primers P1/P2 located within exons 14 and 15. GAPDH was used as an internal control. Expression of foetal genes was analysed by quantitative real-time RT–PCR using FastStart Universal SYBR Green Master (Roxy) system (Roche) and was normalized to GAPDH. Real-time RT–PCR was performed in triplicate for each sample. The primers for quantitative real-time RT–PCR were listed in Supplementary material online, Table S1.

2.7 Electron microscopy
Mice hearts were quickly dissected and left ventricular tissues were cut into 1 mm3 pieces, which were immersion-fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 2 h at 4 C and postfixed with 1% osmium tetroxide. The specimens were then conventionally processed for transmission electron microscopic study. MEFs were fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 2 h at 4 C and postfixed with 1% osmium tetroxide. Cells were then processed for ultrastructural analysis.

2.8 Histology and immunohistochemistry
For paraffin sections, mice were anaesthetized with isoflurane and the hearts were perfusion fixed with ice-cold 4% paraformaldehyde (Sigma-Aldrich) in PBS and later post-fixed overnight in the same fixative. Hearts embedded in paraffin were cut into serial 5 μm sections (Leica RM2235 rotary microtome) and stained with H&E or picrosirius red to evaluate morphology, cellular dimensions, and fibrosis. Immunofluorescence staining was performed on paraffin-embedded cardiac tissue sections according to standard protocol. For immunohistochemical analysis, heart sections were deparaffinized, rehydrated, and then underwent heat-induced antigen retrieval. Immunostaining was performed using the VECTASTAIN Elite ABC Kit (Vector Laboratories). Apoptotic cell death was determined by Terminal dUTP Nicked-End Labelling (TUNEL) assay on paraffin-embedded cardiac tissue sections using an ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore) according to the manufacturer’s instructions. Sections were counterstained with methyl green.

2.9 Cardiomyocyte cross-sectional area
The cross-sectional area of cardiomyocytes was measured by H&E staining with image analysis software (Image J) and calculated as the mean of 120–150 cells from randomly selected fields.

2.10 Echocardiography
Echocardiography was performed as described previously.20 In brief, the mice were anaesthetized by inhaled isoflurane (2.5% for induction and 1% for maintenance) and the depth of anaesthesia was determined by monitoring the righting reflex and heart rate. Two-dimensional echocardiographic imaging was conducted using a Vevo 770 platform (VisualSonics, Inc., Toronto, ON, Canada). M-mode images were used for ventricular measurement. Measurements were carried out at least in triplicate.

2.11 Statistics
All values were expressed as mean ± SEM. Student’s t-test was used to compare the difference between two groups and one-way ANOVA followed by Bonferroni’s method was used for post hoc pairwise multiple comparisons. Significance was considered with P < 0.05.
3. Results

3.1 Genetic characterization of UVRAG<sub>PB/PB</sub> mutant mice

The mouse UVRAG gene was localized to chromosome 7 and consisted of 15 exons separated by 14 introns. The PB transposon was inserted into intron 14 of the UVRAG gene (Figure 1A). UVRAG heterozygous (UVRAG<sup>PB/+</sup>) male mice were crossed with heterozygous female mice to generate homozygous mutant (UVRAG<sup>PB/PB</sup>). PCR using primers GL/PB/GR as indicated was performed to determine the genotypes of the offspring at 2 weeks of age (Figure 1B). Out of 265 progeny obtained from UVRAG heterozygote parents, the genotype frequencies were 67 UVRAG<sup>+/+</sup> (25%), 136 UVRAG<sup>PB/+</sup> (52%), and 62 UVRAG<sup>PB/PB</sup> (23%) (Figure 1C), suggesting that UVRAG<sup>PB/PB</sup> mice were born at Mendelian ratios. The transcript of UVRAG was not amplified by RT–PCR with a forward primer on exon 14 and reverse primer on exon 15 in UVRAG<sup>PB/PB</sup> (Figure 1D), indicative of disruption of endogenous transcript of UVRAG gene by the PB insertion. Consistent with this finding, immunoblotting analysis of total protein from diverse tissues revealed that UVRAG was deficient in UVRAG<sup>PB/PB</sup> mice (Figure 1E). Despite the deficiency for UVRAG, the adult UVRAG<sup>PB/PB</sup> mice were viable, fertile, and developmentally normal.

3.2 Disruption of UVRAG causes impairment of autophagy

We first assessed autophagy in the hearts from mice deficient for UVRAG. Immunoblotting showed the enhanced protein abundance of LC3 II in the hearts from UVRAG-deficient mice at 10 months of age (Figure 2A and B), suggestive of accumulation of autophagosomes. Moreover, UVRAG-deficient hearts showed a significant increase in endogenous LC3 immunofluorescence staining and LC3-positive puncta (Figure 2C and D). In addition, ultrastructural analysis revealed the accumulation of autophagic vacuoles in UVRAG-deficient hearts (Figure 2E), confirming that UVRAG deficiency led to the accumulation of autophagosomes.

To determine whether accumulated autophagosomes were caused by enhanced autophagosome formation or defective autophagosome removal, we first assessed the autophagic flux in MEFs. MEFs from WT and UVRAG-deficient mice were treated with bafilomycin A1, an autophagosome–lysosome fusion inhibitor. Immunoblotting showed that the protein abundance of LC3 II was significantly increased in UVRAG-deficient MEFs compared with WT MEFs. However, the increase in LC3 II protein levels in UVRAG-deficient MEFs was not further enhanced by bafilomycin A1 treatment (Figure 2F and G, Supplementary material online, Figure S1), suggesting that autophagic flux was impaired in UVRAG-deficient MEFs. We then performed transmission electron microscopy to examine the autophagic vacuoles. UVRAG-deficient MEFs showed clustered double-membrane autophagosomes and the complex vacuoles with multiple layers of membrane (Figure 2H), suggesting that autophagosome–lysosome fusion or degradation of autolysosomal contents was impaired. Taken together, these data demonstrated that autophagic flux was attenuated in UVRAG-deficient MEFs.

We next analysed autophagic flux in UVRAG-deficient hearts. p62, a marker for autophagic flux, was significantly increased in UVRAG-deficient hearts compared with WT controls (Figure 2I and J), indicative of impaired autophagic flux in UVRAG-deficient hearts. We then treated...
UVRAG-deficient mice and WT mice with chloroquine, an inhibitor of autophagosome–lysosome fusion. Six hours after injection, the hearts were dissected for the analysis of LC3 II protein abundance. Consistent with the results observed in MEFs, LC3 II protein levels were significantly increased in UVRAG-deficient hearts. However, the increase in LC3 II protein levels in UVRAG-deficient hearts were not further enhanced significantly by chloroquine treatment (Figure 2K and L), suggesting that accumulated autophagosomes were caused by defective autophagosome removal due to impaired autophagic flux. Collectively, these data clearly showed that autophagic flux was impaired in UVRAG-deficient hearts.

Impaired autophagic flux can occur from a decreased number of lysosomes. To evaluate the number of lysosomes in UVRAG-deficient hearts, we performed immunoblotting and immunohistochemistry of lysosome-associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2), two lysosomal markers. LAMP-1 and LAMP-2 protein levels were significantly increased in UVRAG-deficient hearts (Figure 3A–D and F), suggesting that impaired autophagosome clearance was associated with increased lysosome numbers, which was consistent with previous findings.21,22 Interestingly, cathepsin D, a lysosomal protease, was not significantly increased in UVRAG-deficient hearts (Figure 3E and F). Taken together, these results suggested that UVRAG deficiency...
attenuated autophagic flux in the heart possibly due to suppression of autophagosome–lysosome fusion.

3.3 UVRAG-deficient mice develop age-related cardiomyopathy

Autophagy is required for cardiac homeostasis. Thus, we investigated the impact of UVRAG deficiency on cardiac remodelling and function. No significant difference in the heart-to-body weight ratio was detected between UVRAG-deficient mice and corresponding WT controls (Supplementary material online, Table S2). No abnormality of cardiac structure was observed in UVRAG-deficient mice at 2 and 6 months of age compared with WT controls by histological analysis (data not shown). However, by 10 months of age, UVRAG-deficient hearts appear to be larger and the cross-sectional area of individual UVRAG-deficient cardiomyocytes was significantly increased compared with WT controls (Supplementary material online, Figure S2, Figure 4A–C). Interstitial cardiac fibrosis was enhanced in UVRAG-deficient hearts compared with WT controls (Supplementary material online, Figure S2, Figure 4A–C). Interstitial cardiac fibrosis was enhanced in UVRAG-deficient hearts compared with WT controls (Supplementary material online, Table S3). In addition, ejection fraction (EF) and per cent fractional shortening (%FS) were attenuated in UVRAG-deficient mice (Figure 5D and E, Supplementary material online, Table S3), suggestive of impaired cardiac function. These results demonstrated that disruption of UVRAG led to age-related cardiomyopathy with compromised cardiac function.

3.4 UVRAG deficiency increases inflammatory response in the heart

Autophagosome accumulation is associated with cardiac inflammation, which is a major factor in heart disease. To determine whether inflammation was induced in UVRAG-deficient hearts, we performed quantitative real-time RT–PCR to evaluate the expression of pro-inflammatory cytokines in heart tissues. Interleukin-6 (IL-6) and tumour necrosis factor-α...
TNFα were significantly enhanced in UVRAG-deficient hearts compared with WT controls (Figure 6A and B). No significant difference in interleukin-1β (IL-1β) mRNA expression was detected in the hearts between UVRAG-deficient mice and WT controls (Figure 6C). These results indicated that inflammatory response was increased in UVRAG-deficient hearts. To confirm the enhancement of inflammation in UVRAG-deficient hearts, we performed immunohistochemical staining for inflammatory cell markers. CD45 and CD45R, markers for leucocyte and B cell, respectively, were markedly increased in UVRAG-deficient hearts (Figure 6D), suggestive of intense inflammatory cell infiltrate.

4. Discussion

In this study, using a mouse model with disruption of the UVRAG gene, we have explored the role of UVRAG in autophagy and cardiac structure and function. The major findings are as follows: (i) UVRAG disruption causes autophagosome accumulation via impairment of autophagic flux; (ii) UVRAG deficiency leads to cardiomyopathy accompanied by compromised cardiac function; (iii) inflammatory response is enhanced in UVRAG-deficient hearts. Taken together, these results suggest that UVRAG plays an essential role in autophagy and maintenance of cardiac function.

Previous in vitro studies have demonstrated that UVRAG plays an important role in autophagy by promoting autophagosome formation and autophagosome–lysosome fusion.11,13,14 Using a mouse model with disruption of UVRAG by PB insertion, we have explored the role of UVRAG in autophagy and cardiac function. The abundance of LC3 II protein was markedly increased in the hearts deficient for UVRAG, indicative of increased autophagosomes. The accumulation of autophagosomes in UVRAG-deficient hearts was confirmed by LC3 immunofluorescence staining and transmission electron microscopy. Autophagic flux was impaired in UVRAG-deficient hearts as indicated by increased p62 protein levels. Characterization of autophagic flux by assessing autophagosome clearance mediated by lysosomes demonstrated that autophagosome accumulation was caused by impairment of autophagosome removal. Therefore, the evidence we have collected clearly shows that...
UV-RAG disruption causes accumulation of autophagosomes via impairment of autophagic flux in vivo. However, the role of UVRAG in autophagosome formation in vivo remains to be determined.

UV-RAG-deficient mice developed age-related cardiomyopathy, which is at least in part attributable to impairment of autophagy as a previous study using cardiac-specific Atg5 knockout mice has shown that inhibition of autophagy induces age-related cardiomyopathy. However, the cardiac phenotypes we observed in UVRAG-deficient mice are less severe than those in Atg5-deficient mice. For instance, Atg5-deficient mice show increased dimension of left ventricular chamber and attenuated cardiac function at 6 months of age. In addition, Atg5-deficient mice begin to die at 6 months of age. In UVRAG-deficient mice, although heart chamber was enlarged and cardiac function was compromised at 10 months of age, no abnormality was detected at 6 months of age. Moreover, no UVRAG-deficient mice died before 10 months of age. This discrepancy can be explained as follows. First, autophagy is not completely blocked in UVRAG-deficient mice. Loss of autophagy leads to neonatal lethality as shown in Atg5 and Atg7-deficient mice. However, UVRAG-deficient mice were viable and developmentally normal, suggesting that UVRAG disruption partially suppresses autophagy. Secondly, in addition to the impairment of autophagic pathway, UVRAG deficiency may disrupt endocytic pathway and impact cardiac structure and function. Thirdly, UVRAG deficiency in non-cardiac organs may contribute to the observed phenotypes in the heart. Finally, mouse strains may have effect on the phenotypes observed as the UVRAG-deficient mice we used are on the FVB/N background, which is different from cardiac-specific Atg5-deficient mice.

We found that Beclin 1 protein level was down-regulated in UVRAG-deficient hearts (Supplementary material online, Figure S3). Beclin 1 interacts with Vps34 to form a core complex, which binds to UVRAG, ATG14, Bif, and Rubicon to regulate autophagosome formation and maturation. In beclin 1 heterozygous mice, protein levels of UVRAG, ATG14, and Vps34 are significantly reduced, indicating that components of the complex are co-dependent upon each other. Consistent with this notion, protein levels of Beclin 1 were decreased by ~50% in UVRAG-deficient hearts. The impact of reduction in Beclin 1 on autophagosome formation remains to be determined.

Impairment of autophagic flux can be caused by decreased lysosome number. In UVRAG-deficient hearts, the number of lysosomes was not reduced but rather increased as evidenced by enhanced protein levels of lysosomal markers such as LAMP-1 and LAMP-2. The increase in lysosome abundance may be caused by less lysosome consumption due to impairment of autophagic flux. In addition, UVRAG deficiency may lead to up-regulation of other genes in autophagy–lysosome pathway and enhancement of lysosome biogenesis, which is mainly controlled by transcription factor EB. Interestingly, unlike LAMP-1 and LAMP-2, cathepsin D was not increased in UVRAG-deficient hearts, indicating that the lysosomes are defective in function. The mechanisms by which cathepsin D is not up-regulated along with other lysosomal markers in UVRAG-deficient mice, which may contribute to the development of age-related cardiomyopathy, remain to be determined.
Inflammation, which has long been implicated in the pathogenesis of cardiovascular diseases, was enhanced in UVRAG-deficient hearts. Increase in inflammation was already detectable in mice at 6 months of age (data not shown), when cardiac structure and function were normal, suggesting that inflammatory response plays a causal role in cardiomyopathy in UVRAG-deficient mice. Inflammation may be caused by impaired autophagy as recent studies have demonstrated that mitochondria DNA escaped from autophagy causes inflammation and heart failure. In addition, impairment of autophagic flux due to blockade of autophagosome removal may lead to the ejection of accumulated autophagosomes out of cells with release of matrix metalloproteinases and induction of inflammation. Moreover, impairment of autophagic flux may cause leakage of lysosomes through cell membrane, which, in turn, stimulate inflammation. Lastly, the failure of the cardiac cells to remove autophagosomes efficiently may lead to the release of the autophagosome contents to the extracellular space, which may trigger programmed necrosis.

In summary, these findings suggest that UVRAG plays an essential role in autophagy and maintenance of cardiac function. As impairment of autophagy contributes to the pathogenesis of a variety of diseases, UVRAG could be a molecular target for protecting against these diseases.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

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**References**


**Figure 6** Inflammatory response is increased in UVRAG-deficient heart. Quantitative real-time RT–PCR analysis of inflammatory cytokines such as IL-6 (A), TNFα (B), and IL-1β (C) in WT and UVRAG-deficient hearts; (D) Immunohistochemistry of inflammatory cell markers CD45 and CD45R in WT and UVRAG-deficient hearts. Scale bar: 40 μm. +/+ , WT mice; PB/PB, UVRAG-deficient mice. n = 3. *P < 0.05 vs. WT.


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