Loss of autophagy protein ATG5 impairs cardiac capacity in mice and humans through diminishing mitochondrial abundance and disrupting Ca\(^{2+}\) cycling

Senka Ljubojević-Holzer\(^{1,2,†}\), Simon Kraler \(^{1,†}\), Nataša Djalinac\(^{1}\), Mahmoud Abdellatif \(^{1}\), Julia Voglhuber \(^{1,2}\), Julia Schipke \(^{3}\), Marlene Schmidt \(^{4}\), Katharina-Maria Kling\(^{3}\), Greta Therese Franke\(^{3}\), Viktoria Herbst\(^{4}\), Andreas Zirlik \(^{1}\), Dirk von Lewinski \(^{1}\), Daniel Scherr\(^{1}\), Peter P. Rainer \(^{1}\), Michael Kohlhaas \(^{4}\), Alexander Nickel\(^{4}\), Christian Mühlfeld\(^{3}\), Christoph Maack\(^{4}\), and Simon Sedej \(^{1,2,5,*}\)

\(^{1}\)Department of Cardiology, Medical University of Graz, Auenbruggerplatz 15, 8036 Graz, Austria; \(^{2}\)BioTechMed Graz, Mozarthaus 12/I, 8010 Graz, Austria; \(^{3}\)Institute of Functional and Applied Anatomy, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany; \(^{4}\)Department of Translational Research, Comprehensive Heart Failure Center (CHFC), University Clinic Würzburg, Am Schwarzenberg 15, Haus A15, 97078 Würzburg, Germany; and \(^{5}\)Institute of Physiology, Faculty of Medicine, University of Maribor, 2000 Maribor, Slovenia

Received 23 January 2021; editorial decision 17 March 2021; accepted 19 March 2021; online publish-ahead-of-print 22 March 2021

Time for primary review: 15 days

Aims

Autophagy protects against the development of cardiac hypertrophy and failure. While aberrant Ca\(^{2+}\) handling promotes myocardial remodelling and contributes to contractile dysfunction, the role of autophagy in maintaining Ca\(^{2+}\) homeostasis remains elusive. Here, we examined whether Atg5 deficiency-mediated autophagy promotes early changes in subcellular Ca\(^{2+}\) handling in ventricular cardiomyocytes, and whether those alterations associate with compromised cardiac reserve capacity, which commonly precedes the onset of heart failure.

Methods and results

RT–qPCR and immunoblotting demonstrated reduced Atg5 gene and protein expression and decreased abundancy of autophagy markers in hypertrophied and failing human hearts. The function of ATG5 was examined using cardiomyocyte-specific Atg5-knockout mice (Atg5\(^{-/-}\)). Before manifesting cardiac dysfunction, Atg5\(^{-/-}\) mice showed compromised cardiac reserve in response to \(\beta\)-adrenergic stimulation. Consequently, effort intolerance and maximal oxygen consumption were reduced during treadmill-based exercise tolerance testing. Mechanistically, cellular imaging revealed that Atg5 deprivation did not alter spatial and functional organization of intracellular Ca\(^{2+}\) stores or affect Ca\(^{2+}\) cycling in response to slow pacing or upon acute isoprenaline administration. However, high-frequency stimulation exposed stunted amplitude of Ca\(^{2+}\) transients, augmented nucleoplasmic Ca\(^{2+}\) load, and increased CaMKII activity, especially in the nuclear region of hypertrophied Atg5\(^{-/-}\) cardiomyocytes. These changes in Ca\(^{2+}\) cycling were recapitulated in hypertrophied human cardiomyocytes. Finally, ultrastructural analysis revealed accumulation of mitochondria with reduced volume and size distribution, meanwhile functional measurements showed impaired redox balance in Atg5\(^{-/-}\) cardiomyocytes, implying energetic unsustainability due to overcompensation of single mitochondria, particularly under increased workload.

Conclusion

Loss of cardiac Atg5-dependent autophagy reduces mitochondrial abundance and causes subtle alterations in subcellular Ca\(^{2+}\) cycling upon increased workload in mice. Autophagy-related impairment of Ca\(^{2+}\) handling is progressively worsened by \(\beta\)-adrenergic signalling in ventricular cardiomyocytes, thereby leading to energetic exhaustion and compromised cardiac reserve.
1. Introduction

Macroautophagy (hereafter referred to as autophagy) is a key protective process necessary for cardiac homeostasis. Under physiological conditions, constitutive autophagy maintains structure and function of the heart by eliminating damaged molecules and dysfunctional organelles from the cytoplasm, which might otherwise elicit harmful effects to cardiac cells, including long-lived cardiomyocytes. Autophagy-related proteins (ATG) are essential in this process, among which ATG5 is indispensable for the early phase of autophagosome formation. Mice with cardiomyocyte-restricted Atg5 deletion display increased ubiquitination, mitochondrial aggregates and disorganized sarcomere structure in cardiomyocytes. Despite early structural deteriorations within Atg5 null cardiomyocytes, cardiac function is remarkably normal until early adulthood. Hence, it has been argued that a mechanism, such as the Atg5/Atg7-independent alternative autophagy and/or chaperone-mediated autophagy may compensate for Atg5 deficiency to limit cardiac damage. However, such cardioprotective mechanism efficiently operates against adverse remodelling only temporarily, because cardiomyocyte-specific Atg5-deficient mice develop left ventricular hypertrophy that shortly thereafter progresses to heart failure, resulting ultimately in premature death.

Mechanistically, long-term absence of basal autophagy is associated with reduced mitochondrial respiratory function and increased oxidative stress, which predisposes exhausted cardiomyocytes to apoptotic cell death and contractility decline. In addition, the absence of essential autophagy-related proteins may also cause perturbations of other interrelated cellular mechanisms, which in conjunction with damaged mitochondria initiate and drive adverse remodelling and contractile dysfunction. Notably, disturbed Ca\textsuperscript{2+} handling is one such early abnormality during adverse myocardial remodelling that causes heart failure. In this regard, autophagy-deprived hearts exhibit compromised energy and redox balance due to increased number of ill-functioning mitochondria. Such perturbations may cause shifts in ionic gradients and attenuate excitability by deregulating the energy-demanding process of intracellular Ca\textsuperscript{2+} homeostasis, especially under increased workload. Besides cytoplasmic and mitochondrial Ca\textsuperscript{2+} changes, altered nucleoplasmic Ca\textsuperscript{2+} signalling could be critical to functional remodelling of cardiomyocytes, which lack autophagy, as activation of nuclear Ca\textsuperscript{2+}-mediated hypertrophic gene program affects protein expression, including components of...
the excitation–contraction coupling machinery. To this end, however, it remains unknown whether absence of cardiac autophagy alters subcellular Ca\(^{2+}\) cycling at early stages of remodelling that precede the development of heart failure.

In this study, we examined the ATGS expression at the transcriptional and protein levels in non-failing, hypertrophic and failing human hearts. We then used cardiomyocyte-specific Atg5-deficient mice before they develop heart failure to identify the specific role of ATGS in subcellular Ca\(^{2+}\) cycling under normal and stressed conditions. Finally, we functionally assessed the translational potential of our findings in hypertrophied human cardiomyocytes.

2. Methods

A detailed description of methods is available in Supplementary material online.

2.1 Ethics

Animal experiments were performed in accordance with European ethical regulation (Directive 2010/63/EU) and approved by the responsible Austrian government agency (BMBWF: 66.010/0038-WFN/3b/2017; BMBWF-66.010/0208-V/3b/2018). Procedures involving human samples were approved by the Ethical Committee of the Medical University of Graz (28-508 ex 15/16), and performed in accordance with principles outlined in the Declaration of Helsinki. Informed written consent was not feasible to obtain because the patients were not able to give the informed consent as a result of their underlying medical condition. The requirement for informed consent was waived by the ethical committee. Patients’ characteristics are described in Supplementary material online, Table S1. Donor hearts were explanted post-mortem together with other organs. Upon ice-cold cardioplegia, cardiac biopsies were harvested from the left ventricular free wall, quickly frozen in liquid nitrogen and stored at -80°C for future analysis.

2.2 Mice

Cardiomyocyte-specific Atg5 knock-out mice (Atg5\(^{-/-}\); B6-129S4-Cre/+; male mice; N = 52) were generated from Atg5\(^{-/-}\) mice crossed with heterozygous knock-in mice that express Cre\(^+\) recombinase driven by the cardiomyocyte-specific α-myosin light chain (MLC2a-Cre) gene promoter. We used Atg5\(^{-/-}\)/+ male mice between 12 and 15 weeks, because at this age they present a normal cardiac phenotype despite a complete block of autophagy. Age-matched Atg5\(^{+/+}\) male littermates served as controls (N = 45).

2.3 Haemodynamic measurements and cardiac stress testing

Haemodynamic assessment was performed according to the established protocol. Mice were sedated with isoflurane (5% for induction and 1–2% for maintenance), intubated, and mechanically ventilated (Harvard Mini-Vent. type 845, Harvard Apparatus). A pressure-conductance catheter (SPR-839; Millar Instruments) was inserted into the right carotid artery and advanced into the LV, where pressure signals were recorded (MPVS ultra, Millar Instruments), and analysed offline (LabChart 8 Pro, AD Instruments). Cardiac parameters were measured at baseline and after intraperitoneal isoprenaline administration (2 μg/kg body weight) to evaluate cardiac reserve capacity. After assessment of haemodynamic parameters that were averaged from 10 consecutive beats with ventilation suspended at end-expiration, mice were euthanized by decapitation.

Hearts were rapidly frozen in liquid nitrogen and stored at -80°C for molecular analysis.

2.4 Treadmill running coupled to indirect calorimetry

A subset of mice (N = 16) underwent peak effort testing on a motorized treadmill coupled to a calorimetric unit (TSE Systems, Germany). Mice were subjected to a ramp exercise protocol with an initial velocity of 3 m/min followed by a constant acceleration of 3 m/min\(^2\) at 0° inclination. The exercise ended at the maximal exhaustion, which was defined as the mouse’s inability to maintain running speed despite being in contact with the electrical grid for five consecutive seconds. Maximal oxygen consumption (VO\(_{2}\)max) and run distance were determined at the point at which oxygen uptake reached a plateau during exhaustive workout/exercise.

2.5 Real-time quantitative PCR (RT–qPCR)

Total purification of mouse and human RNA and measurements of RNA yield and quality were performed as described previously. cDNA was synthesized using the QuantiTect Reverse Transcription kit (for mouse; Qiagen, Hilden, Germany) and the HighCapacity cDNA RT Kit (for human; Thermo Fisher Scientific, Wilmington, USA), respectively. RT–qPCR was performed with CDNA at a concentration of 10 ng per 10 μL reaction volume. TaqMan Gene Expression Assays were used for all qPCR experiments following the protocol outlined in Supplementary material online, Tables S2 and S3. Primer efficiency correction was performed with GenEx 5.4.4. Software (MultiD, Sweden). RT–qPCR data analysis was done using the 2^(-ΔΔCT)-method.

2.6 Cardiomyocyte isolation

Mice were heparinized (500 IU/kg body weight), and anaesthetized with 5% isoflurane prior to cervical dislocation. Hearts were excised and the aorta was rapidly cannulated. Mouse and human ventricular cardiomyocytes were isolated by a standard liberase-based dissociation procedure using Langendorff perfusion protocol as described previously.

2.7 Subcellular Ca\(^{2+}\) imaging and cardiomyocyte shortening

Intracellular Ca\(^{2+}\) stores were visualized in cardiomyocytes loaded with 10 μM Mag-fluo-4/AM (Life Technologies, Grand Island, NY, USA) using a confocal imaging system (Zeiss LSM 510 Meta). Ca\(^{2+}\) transients were recorded in cardiomyocytes loaded with 5 μM Indo-1/AM or 8 μM Fluo-4/AM (both Thermo Fisher Scientific, Vienna, Austria). Indo-1-loaded and field-stimulated cardiomyocytes were subjected to a stress protocol to simulate an increased physiological workload. Cells were first paced at 0.5 Hz (baseline) followed by administration of 30 nM isoprenaline and a subsequent increase of the stimulation frequency to 5 Hz for 3 min. Stimulation rate was then set back to 0.5 Hz and isoprenaline was washed out. Ca\(^{2+}\) signals were acquired using a customized IonOptix system (Ionomix, Dublin, Ireland). In a subset of dye-free cardiomyocytes that were subjected to the stress protocol, contraction was assessed using a SarcLen Sarcomere Length Acquisition Module (MyoCam-S, Ionomix, Dublin, Ireland) at the acquisition rate of 240 Hz. At least 10 consecutive beats were averaged and analysed using IonWizard Software (Ionomix, Dublin, Ireland). Simultaneous imaging of nucleoplasmic and cytoplasmic Ca\(^{2+}\) transients was done in Fluo-4/AM-loaded cardiomyocytes using confocal microscopy as described. A subset of cardiomyocytes was acutely exposed to 10 nM isoprenaline to
measure the response to β-adrenergic stimulation. Frequency-dependent changes in Ca\(^{2+}\) cycling were assessed by gradually increasing stimulation rate from 1 to 4 Hz (mouse) or from 0.25 to 1 Hz (human). Cytoplasmic and nucleoplasmic fluorescence signals were transformed into calibrated [Ca\(^{2+}\)]. \(^{18}\) Ca\(^{2+}\) spark were recorded in line-scan mode at a sampling rate of 1.54 or 1.92 ms per line within 30 s after the cessation of stimulation at 4 Hz, and analysed with ImageJ plugin SparkMaster (NIH, Bethesda, USA). \(^{19}\) Ca\(^{2+}\) spark frequency was normalized to cell length and recording time, meanwhile RyR2-mediated Ca\(^{2+}\) leak was calculated as described previously.\(^{20}\)

2.8 Mitochondrial redox state measurements

The mitochondrial redox state of NAD(P)H/NAD(P)\(^+\) and FADH\(_2\)/FAD was quantified as the autofluorescence of NAD(P)H and FAD, which was recorded in the same cell using excitation/emission wavelengths of 340/450 nm for NAD(P)H and 485/525 nm for FAD. Calibration was performed at the end of every experiment with the mitochondrial uncoupler FCCP (5 \(\mu\)M) and the complex IV inhibitor N-cytochrome b (4 \(\mu\)M). Alternatively, the mitochondrial membrane potential (\(\Delta w_{m}\)) was determined with 100 \(\mu\)M TMRM using excitation/emission wavelengths of 540/605 nm. For the semi-quantitative assessment of \(\Delta w_{m}\), TMRM fluorescence was determined before and after the administration of 5 \(\mu\)M FCCP and 1.25 \(\mu\)M oligomycin, which is known to completely dissipate \(\Delta w_{m}\).\(^{21}\)

2.9 Respiration measurements in isolated cardiac mitochondria

Freshly isolated cardiac mitochondria were used for high-resolution respirometry measurements that were performed as described previously.\(^{21}\) Oxygen consumption was assayed at 37°C with an Oxygraph-2k high-resolution respirometer and DatLab software was used for data acquisition and analysis (Oroboros Instruments, Innsbruck, Austria). Two mitochondrial respiration protocols were used to quantify oxygen consumption rate upon supplementation of pyruvate (for carbohydrate metabolism) or fatty acids (for \(\beta\)-oxidation) as a fuel. In the ‘carbohydrate’ protocol, measurements of complex I and II activity were performed with 5 \(\mu\)M Na-pyruvate and 5 \(\mu\)M Na-malate. The metabolites were added as reduced substrates after initially recording residual oxygen consumption resulting in leak respiration, followed by increasing concentrations of ADP (0.03, 0.1, 0.3, 1 \(\mu\)M). Complex I was inhibited by 0.5 \(\mu\)M rotenone to prevent reverse electron flux. Finally, oxygen consumption coupled to ADP phosphorylation was inhibited by adding 1.25 \(\mu\)M oligomycin, followed by titration with 10 \(\mu\)M DNP to determine uncoupled respiration state 3 u. In the ‘fatty acid’ protocol, respiration was measured using 1 \(\mu\)M carnitine, 3 \(\mu\)M malate, 10 \(\mu\)M palmitoyl-CoA, 10 \(\mu\)M oleoyl-carnitine. Similar to the ‘carbohydrate’ protocol, increasing amounts of ADP, 1.25 \(\mu\)M oligomycin or 10 \(\mu\)M DNP were added. Mitochondrial membrane potential was simultaneously probed using 1 \(\mu\)M TMRM and Smart Fluo-Sensor Green, as described before.\(^{21}\)

2.10 Immunocytochemistry

Immunocytochemistry was performed using antibodies against SERCA2a (A010-20, Badrilla), RyR2 (MA3-925, Thermo Fisher Scientific) and phospho-Thr286-CaMKII (ab32678, Abcam).\(^{9}\) Western blotting and detailed immunostaining analyses were used to validate the specificity of antibodies. Cells pretreated with KN-93 served as a negative control for anti-phospho-Thr286-CaMKII antibody; meanwhile bona fide target staining was distinguished from the background by staining with a suitable secondary antibody alone.

2.11 Western blotting

Hearts were perfused with normal Tyrode solution containing or not 10 \(nM\) isoprenaline for 5 min, and were then flash frozen for further immunoblotting with primary antibodies recognizing ATG5, SERCA2a, phospholamban (PLB), Troponin I, phospho-PLB-Ser16, phospho-Troponin I-Ser23/24 and GAPDH (for details, see Supplementary material online).

2.12 Acute inhibition of autophagic flux

Autophagic flux in Atg5\(^{+/−}\) mice was blocked using leupeptin-based inhibition of LC3-II (microtubule-associated protein 1 light chain 3-II) turnover, as described previously.\(^{22}\) Atg5\(^{+/−}\) mice received intraperitoneal injections of leupeptin (40 mg/kg body weight, Sigma–Aldrich, Vienna, Austria) or vehicle (sterile 0.9% NaCl solution), and were sacrificed 50 min after the injection. Isolated cardiomyocytes were subjected to subcellular Ca\(^{2+}\) imaging and immunoblot analysis of LC3 and p62/SQSTM1.

2.13 Design-based stereology

Hearts were retrogradely perfused with 4% neutral-buffered formaldehyde and processed for the quantification of LV cardiomyocyte composition using electron microscopy and design-based stereology as described elsewhere.\(^{4}\)

2.14 Statistical analysis

Statistical testing was performed using GraphPad Prism 8 (GraphPad Software, CA). Data are presented as bar graphs with error bars showing mean and SEM, respectively, with single data points superimposed. Indicated sample size was determined based on the means and variance of the studied parameters in our previous work on disease mouse models and failing human hearts.\(^{9,10}\) Data residuals distribution was determined using Shapiro–Welch’s test, while homogeneity of variance was verified by Bartlett’s test. Comparisons between two groups were analysed using Student’s t-test or Mann-Whitney test, as appropriate. In case of multiple comparisons, ANOVA with Dunnett’s or Bonferroni’s post hoc test was applied. In case of Ca\(^{2+}\)-transient measurements at different stimulation frequencies or time-lapse/serial measurements, two-way repeated measures ANOVA was used. Significant factorial designs were followed by multiple comparisons using Sidak’s or Bonferroni’s post hoc test. Association of Atg5 gene and ATG5 protein expression levels to interventricular septum was tested using Pearson correlation. Differences were considered significant, if two-tailed P-value was less than 0.05.

3. Results

3.1 Reduced expression of ATG5 and autophagy markers in human hearts with compensated hypertrophy and heart failure

Given that acute pressure overload reduces ATG5 expression and overall pro-autophagic flux in mice,\(^{23}\) we assessed the clinical relevance of these findings by quantifying gene and protein expression of ATG5 in human left ventricular (LV) biopsies of non-failing, hypertrophied, and failing
hearts from donors, who underwent echocardiographic examination prior to heart extraction (Supplementary material online, Figure S1). Importantly, both Atg5 gene and protein expression were significantly reduced in hypertrophied and failing human hearts as compared to non-failing controls (Figure 1A and C). Furthermore, mRNA and protein expression of ATG5 inversely correlated with the thickness of the interventricular septum (IVS) from non-failing and hypertrophied human hearts (N = 7/16 hearts, respectively). (D) Regression analysis of ATG5 protein expression levels and thickness of the interventricular septum (IVS) from non-failing and hypertrophied human hearts (N = 10/17/11 hearts, respectively). (E) Representative original immunoblots and, (F) expression of the autophagy-related protein markers in non-failing (NF), hypertrophied (Hyp), and end-stage failing hearts (F) (N = 6/3/5 hearts, respectively). (A–F) Data show mean ± SEM. Indicated P-values were calculated using ANOVA followed by Dunnett’s post hoc test. (B) and (D) Association of Atg5 gene and ATG5 protein expression with IVS thickness was computed using Pearson correlation.

3.2 Atg5−/− mice exhibit reduced contractility and exercise capacity in response to stress

To assess the effect of myocardial Atg5 deficiency on cardiac function in vivo, we performed baseline and acute β-adrenergic stress testing in Atg5−/− mice before the development of congestive heart failure. These mice had inhibited cardiomyocyte autophagy (Supplementary material online, Figure S2), which was associated with a mild increase in hypertrophy, as determined by elevated heart-to-body weight ratio and myocardial Nppb gene expression (Figure 2A–C). At baseline, Atg5-deficient mice showed no cardiac abnormalities (Supplementary material online, Table S4), as also denoted by similar heart rates (Figure 2D) and systolic function, measured invasively by maximal LV pressure development rate (dP/dt max) (Figure 2E). Isoprenaline administration expectedly evoked increased heart rate and contractility in both groups. However, the increase in dP/dt max upon isoprenaline was lower in Atg5−/− mice than in control animals (Figure 2E), despite similar maximum heart rate development (Figure 2D), indicating reduced cardiac reserve capacity. To examine the outcome of such attenuated inotropic response in the physiological context of increased cardiac demand, we exposed Atg5-deficient mice to exercise tolerance testing. Atg5+/+ and Atg5−/− mice displayed a similar level of exhaustion as determined by comparable blood lactate concentrations (15 ± 6 and 17.3 ± 5.7 mM, respectively; P = 0.45). However, Atg5−/− mice reached exhaustion faster than control mice and, thus, had shorter maximum run distance (Figure 2F). Most
importantly, Atg5−/− mice had significantly reduced total workload (Figure 2G), which coincided with lower maximal aerobic capacity (Figure 2H), collectively indicating effort intolerance.

### 3.3 Preserved Ca²⁺ homeostasis in Atg5−/− cardiomyocytes during baseline and β-adrenergic stimulation

Since intracellular Ca²⁺ homeostasis is the major determinant of excitation–contraction coupling and, thus, determines cardiac performance, we evaluated the functional impact of impaired ATGS-dependent autophagy on intracellular Ca²⁺ handling by quantifying cytosolic and nuclear Ca²⁺ transients under normal and stressed conditions. At baseline, Indo-1 wide-field and Fluo-4 confocal imaging revealed similar intracellular Ca²⁺ concentrations ([Ca²⁺]) at diastole and systole, as also kinetic parameters in both groups (Figure 3A–F). As alterations in Ca²⁺ cycling occur rapidly during sympathetic activation, β-adrenergic stimulation might unmask subtle changes that are indiscernible at baseline. To that end, we investigated whether acute isoprenaline exposure alters intracellular Ca²⁺ handling in Atg5-deprived cardiomyocytes. While

**Figure 2** Atg5-deprived mice exhibit mild hypertrophy, reduced cardiac contractility and exercise capacity. (A) Photomicrographs of hearts from 12-week-old Atg5+/- (left) and Atg5−/− mice (right). (B) Heart weight-to-body weight ratio (HW/BW) and, (C) Nppb expression in Atg5+/- vs. Atg5−/− hearts (N = 10 vs. 9 mice for HW/BW, and N = 5 mice per group for Nppb expression). (D) Heart rate (bpm, beats per minute) and, (E) maximal positive pressure development (dP/dt max) in the left ventricle at baseline and upon intraperitoneal administration of isoprenaline (ISO, 2 μg/kg body weight) in Atg5+/- and Atg5−/− mice. N = 4/5 mice, respectively. (F) Maximal run distance, (G) workload and, (H) maximal oxygen consumption (VO2max) in Atg5−/− mice and their control littermates during peak effort testing on a motorized treadmill coupled to indirect calorimetry. N = 8 mice per group. (B–H) Data show mean ± SEM. Indicated P-values were calculated using Mann–Whitney test comparing Atg5−/− or ISO treatment to the respective control.
isoprenaline increased the amplitude and reduced the decay time of global Ca$^{2+}$ transients similarly in both groups (Figure 3A–F). Atg5$^{-/-}$ cardiac myocytes displayed reduced sarcomere length during diastole and systole, which coincided with normal fractional shortening and contractile kinetics (Figure 3C), suggesting an early onset of diastolic dysfunction in Atg5$^{-/-}$ mice. We showed previously that nucleoplasmic and cytoplasmic Ca$^{2+}$ homeostasis can be regulated independently, and that nucleoplasmic Ca$^{2+}$ signalling is an early contributor to the development of cardiac hypertrophy. However, at slow pacing rates Atg5$^{-/-}$ cardiomyocytes displayed preserved nucleoplasmic and cytoplasmic Ca$^{2+}$ homeostasis could be regulated independently.  

Figure 3 Impaired subcellular Ca$^{2+}$ cycling in response to increased workload in Atg5$^{-/-}$ cardiomyocytes. (A) Schematic representation of the stress protocol that simulates increased physiological workload by increasing stimulation frequency from 0.5 to 5 Hz during exposure to 10 nM isoprenaline (ISO). (B) Quantification of global calcium transients using Indo-1/AM. From left to right: Systolic and diastolic [Ca$^{2+}$]$_{c}$, amplitude, decay time (DT) and time-to-peak (ttp) in isolated cardiac myocytes subjected to a physiological stress protocol (A). Data show mean ± SEM. n = 28/18 cells from 3/2 Atg5$^{+/+}$/Atg5$^{-/-}$ mice, respectively. (C) Quantification of myocyte contractility in unstained cells using the stress protocol in (A). From left to right: Sarcomere length, fractional shortening (FS), relaxation time (RT) and time-to-peak (ttp). Data show mean ± SEM (n = 35/32 cells from 3/3 Atg5$^{+/+}$/Atg5$^{-/-}$ mice, respectively). (D) Original line-scan Fluo-4/AM fluorescence recordings (top) of intracellular Ca$^{2+}$ transients at baseline and upon acute administration of 10 nM isoprenaline (ISO) in a representative control (left) and Atg5-deficient (right) cardiomyocyte, and corresponding calibrated cytoplasmic (black) and nucleoplasmic (red) Ca$^{2+}$ transients (bottom). Scale bar, 20 μm. Mean values of cytoplasmic (E) and nucleoplasmic (F) Ca$^{2+}$ transient parameters displaying diastolic (dia) [Ca$^{2+}$]$_{c}$, Ca$^{2+}$ transient amplitude, time-to-peak (ttp), and time from peak [Ca$^{2+}$]$_{c}$ to 50% decline (DT50). Data show mean ± SEM (n = 24/20–22 cells from 5/4 Atg5$^{+/+}$/Atg5$^{-/-}$ mice, respectively). (G) Original line-scan confocal images of cytoplasmic and nucleoplasmic Ca$^{2+}$ transients at 1 and 4 Hz stimulation in a ventricular myocyte isolated from Atg5$^{+/+}$ (left) and Atg5$^{-/-}$ (right) mice. Note the area of increased fluorescence intensity displaying increased nuclear Ca$^{2+}$ (nuc). Scale bar, 20 μm. (H) Averaged original recordings of electrically stimulated Ca$^{2+}$ transients in the nucleus (red) vs. cytoplasm (black) of ventricular myocytes isolated from Atg5$^{+/+}$ (left) and Atg5$^{-/-}$ (right) mice in response to a gradual increase of stimulation frequency from 1 to 4 Hz. (I) Frequency-dependent changes in peak systolic amplitude in electrically stimulated Ca$^{2+}$ transient in the cytoplasm (left) vs. nucleus (right) of ventricular myocytes isolated from control and Atg5-deficient mice. (J) Cytoplasmic (left) and nucleoplasmic (right) Ca$^{2+}$ load was calculated as a time-integral area under Ca$^{2+}$ transient–time curve within 1 s in Atg5$^{+/+}$ and Atg5$^{-/-}$ cardiomyocytes. (H–J) Data show mean ± SEM (n = 19/25 cells from 3/3 Atg5$^{+/+}$/Atg5$^{-/-}$ mice, respectively). (B and C) and (I and J) P-values were calculated using Bonferroni’s or Sidak’s post hoc test (vs. Atg5$^{+/+}$ control), following significant two-way repeated-measures ANOVA. (E and F) P-values were calculated using Mann–Whitney test comparing Atg5$^{-/-}$ vs ISO to the respective control. Average data and group comparisons related to Figure 3 are summarized in Supplementary material online, Table S5.
cytoplasmic Ca\(^{2+}\) handling at baseline and upon isoprenaline administration (Figure 3D–F), indicating that subcellular Ca\(^{2+}\) handling was preserved during normal and stressed conditions despite the loss of Atg5-dependent autophagy.

### 3.4 Impaired subcellular Ca\(^{2+}\) cycling and signalling in Atg5\(^{-/-}\) mice at high-pacing frequencies

Unlike in vitro, β-adrenergic stimulation in vivo exerts multiple effects on the heart, including positive inotropy and chronotropy. Therefore, we assessed the impact of Atg5 deficiency at high heart rates by exposing cardiomyocytes to increasing pacing frequencies (1–4 Hz), but in the absence of isoprenaline. Notably, the frequency-dependent increase in peak systolic Ca\(^{2+}\) amplitude that was observed in Atg5\(^{+/+}\) cells was blunted in Atg5\(^{-/-}\) cardiomyocytes in both the cytoplasm and nucleus (Figure 3G–I). However, although the time-averaged cytoplasmic Ca\(^{2+}\) transient was identical between Atg5\(^{-/-}\) and Atg5\(^{+/+}\), time-averaged nucleoplasmic Ca\(^{2+}\) load was elevated in Atg5-deficient cells (Figure 3J).

Such increase in nucleoplasmic [Ca\(^{2+}\)] is known to activate Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) at high-pacing frequencies and stimulate cardiomyocyte growth,\(^{24}\) as indicated by increased cell and nucleus width-to-length ratio (Supplementary material online, Figure S3). Therefore, we opted to measure CaMKII phosphorylation in Atg5\(^{-/-}\) cardiomyocytes subjected to 1 and 4 Hz stimulation. Although the extent of CaMKII phosphorylation was comparable in the cytoplasm and nucleoplasm between Atg5\(^{-/-}\) and Atg5\(^{+/+}\) cells, CaMKII activation was more pronounced on the nuclear envelope of Atg5\(^{-/-}\) cardiomyocytes already at low pacing rates as compared to control cells (Figure 4D). At high frequencies, CaMKII phosphorylation was elevated also in the cytoplasm and nucleoplasm of Atg5\(^{-/-}\) cells (Figure 4A–C). To examine whether acute or chronic autophagy inhibition impairs subcellular Ca\(^{2+}\) handling, we then acutely blocked autophagic flux in Atg5\(^{+/+}\) mice, as determined by LC3 and p62 immunoblotting after blocking lysosomal function with intraperitoneal injection of leupeptin (Supplementary material online, Figure S4). Pharmacological autophagy inhibition failed to elicit direct changes in time-averaged Ca\(^{2+}\) transients in the cytoplasm and nucleoplasm. Therefore, loss of Atg5-dependent autophagy seems to induce adverse cardiac remodelling that involves increased CaMKII activity due to the imbalances in nuclear [Ca\(^{2+}\)].

### 3.5 Atg5 deficiency does not alter intracellular Ca\(^{2+}\) stores but reduces the phosphorylation of troponin I

Because sarcoplasmic reticulum (SR) is the major determinant of the Ca\(^{2+}\) transient in cardiomyocytes, we examined whether Atg5 deficiency alters the properties of the SR and, consequently, has an impact on the SR Ca\(^{2+}\) load and/or spontaneous SR Ca\(^{2+}\) release. To this end, we performed imaging of Ca\(^{2+}\) in intracellular stores, which revealed no organizational alterations or fragmentation of the SR or NE, and no obvious reduction in the ability of Mag-Fluo-4 to diffuse through the SR and NE lumen (Supplementary material online, Figure S5A), suggesting that both intracellular Ca\(^{2+}\) stores were efficiently connected in Atg5\(^{-/-}\) cardiomyocytes. This finding was corroborated with a similar SR Ca\(^{2+}\) load in Atg5\(^{-/-}\) and Atg5\(^{+/+}\) cardiomyocytes, as assessed by the peak amplitude of the caffeine-induced Ca\(^{2+}\) transient (Supplementary material online, Figure S5B). Loss of Atg5 also failed to increase RyR2-mediated SR Ca\(^{2+}\) leak measured as a product of Ca\(^{2+}\) spark frequency and Ca\(^{2+}\) spark mass (Supplementary material online, Figure S5C). These effects coincided with unaltered expression of SR Ca\(^{2+}\) ATPase 2a (SERCA2a) and phosphorylation of its main regulator phospholamban (PLB) between both groups, meanwhile phosphorylation of troponin I (TnI) at serines 23/24 was significantly reduced in Atg5\(^{-/-}\) mice as compared to controls (Figure 5A–D). To assess the effect of acute β-adrenergic stimulation on PLB and TnI phosphorylation, we then perfused a subset of mouse hearts ex vivo with 10 nM isoprenaline. As isoprenaline only moderately augments the beat rate of ex vivo perfused hearts,\(^{25}\) the effects of frequency-mediated β-adrenergic signalling in vivo are essentially blunted. Hence, this approach enabled us to study primarily the impact of protein kinase A (PKA)-dependent phosphorylation on PLB and TnI. Notably, isoprenaline-perfused hearts of Atg5\(^{+/+}\) and Atg5\(^{-/-}\) mice displayed increased PLB and TnI phosphorylation (Figure 5B, C, and D). However, despite fully preserved PLB phosphorylation, Atg5-deficient hearts showed a markedly attenuated increase in PKA-dependent phosphorylation of TnI at serines 23/24 (Figure 5E).

### 3.6 Reduced abundance of mitochondria leads to their functional overcompensation in Atg5\(^{-/-}\) mice

Both reduced TnI phosphorylation and blunted increase of Ca\(^{2+}\) transient amplitude at high-pacing frequencies contribute to the reduction of systolic function in Atg5\(^{-/-}\) mice under conditions of increased cardiac demand. This may result from mitochondrial abnormalities, such as reduced respiratory activity,\(^{26}\) and/or impaired cardiomyocyte composition. Therefore, we systematically assessed the structural characteristics and quantified ultrastructural composition of Atg5-deficient cardiomyocytes. Unlike in Atg5\(^{+/+}\) cells (Figure 6A and B), we observed accumulation of concentric layers of membrane sheaths as well as cisternae of membranes resembling SR (Figure 6C and D) in the sarcolemma of autophagy-incompetent cardiomyocytes. In addition, myofibrillar disarray and atypical aggregations of mitochondria were clearly distinguishable (Figure 6E and...
3.7 Human cardiomyocytes from hypertrophied hearts phenocopy impaired subcellular Ca\(^{2+}\) cycling in Atg5\(^{-/-}\) cardiomyocytes

Finally, we tested whether reduced ATGS expression in hypertrophied human myocardium (Figure 1A and C) is related to alterations in Ca\(^{2+}\) handling, which resemble those in Atg5\(^{-/-}\) mice. Indeed, human cardiomyocytes displayed blunted frequency-dependent increase of Ca\(^{2+}\) amplitude and increased nucleoplasmic Ca\(^{2+}\) load at higher pacing frequencies (Figure 7A–D). As \(\beta\)-adrenergic stimulation accelerates Ca\(^{2+}\) transient decay and improves frequency-dependent changes in Ca\(^{2+}\) cycling,\(^{25}\) we hypothesized that isoprenaline diminishes or even prevents the disturbances of Ca\(^{2+}\) cycling in hypertrophied cardiomyocytes exposed to increasing pacing frequencies alone. Isoprenaline expectedly provoked a robust increase of both nucleoplasmic and cytoplasmic Ca\(^{2+}\) transients in both non-failing and hypertrophied cardiomyocytes. Interestingly, the increase in peak amplitude was preserved in hypertrophied compared to control cells at high-stimulation frequencies only in non-selective \(\beta\)-adrenergic agonist, isoprenaline, induced only a moderately positive inotropic response in autophagy-deficient mouse hearts during increased cardiac demand. Reduced positive inotropy in Atg5\(^{-/-}\) mice coincided with compromised cardiopulmonary functional reserve capacity during exercise. Our finding that reduced cardiac reserve and exercise intolerance in Atg5\(^{-/-}\) mice preceded the onset of heart failure.

4. Discussion

Beta-adrenergic receptor signalling is the fundamental physiological mechanism underlying acute modulation of Ca\(^{2+}\) handling and contractility in cardiomyocytes.\(^{27}\) However, sympathetic hyperactivity is a hallmark of heart failure development,\(^{28}\) and previous work demonstrated that autophagy protects mice against the development of heart failure induced by chronic \(\beta\)-adrenergic stress.\(^{30}\) Our results demonstrate that the non-selective \(\beta\)-adrenergic agonist, isoprenaline, induced only a moderately positive inotropic response in autophagy-deficient mouse hearts during increased cardiac demand. Reduced positive inotropy in Atg5\(^{-/-}\) mice coincided with compromised cardiopulmonary functional reserve capacity during exercise. Our finding that reduced cardiac reserve and exercise intolerance in Atg5\(^{-/-}\) mice preceded the onset of heart failure...
might be clinically relevant as exercise capacity is known to be a major predictor of all-cause mortality. In addition, our study demonstrates that a failure to reinstate or increase basal autophagy in the heart, and possibly also in other tissues (e.g. skeletal muscle), could be associated with the low ability of some individuals to sustain or enhance cardiac reserve in response to exercise. Of note, physical activity is a non-pharmacological intervention that consistently re-establishes autophagic flux and mitochondrial quality control in experimental heart failure. This underscores the importance of basal autophagy in cardiac response to acute exercise, and extends previous reports on skeletal muscle function, in which Gene deletion was shown to compromise muscle mass, force generation and mitochondrial function. Indeed, isoprenaline-mediated β-adrenergic stress unmasked cardiac decompensation associated with exercise intolerance, a hallmark of heart failure, arising from cardiomyocyte abnormalities, including alterations in myofilament properties (e.g. TnI) and mitochondrial inability to maintain redox balance under acute stress. In line with this notion, aged hearts display a decline in autophagic activity, altered redox state, attenuated response to sympathetic stimulation, and reduced contractility. In the absence of β-adrenergic stimulation, however, high-frequency pacing stunted the amplitude of Ca^{2+} transients, while it augmented nucleoplasmic Ca^{2+} load and CaMKII activity, especially in the nuclear region, suggesting that null cardiomyocytes rely considerably on β-adrenergic signalling during acute stress.

Cardiomyocyte contraction and relaxation depend on the conversion of rise and fall of cytosolic [Ca^{2+}] into force production. Increases in contraction and relaxation under conditions of elevated workload are primarily determined by a combination of increased SERCA2a activity
Figure 7 Impaired subcellular Ca\textsuperscript{2+} cycling in response to increased workload in hypertrophied human cardiomyocytes. (A) Original line-scan confocal images of cytoplasmic and nucleoplasmic Ca\textsuperscript{2+} transients recorded at 0.25 and 1 Hz stimulation in a ventricular myocyte isolated from non-failing (left) and hypertrophied (right) human hearts. Scale bar, 10 µm. (B) Averaged original recordings of electrically stimulated Ca\textsuperscript{2+} transients in the nucleus (red) vs. cytoplasm (black) of ventricular myocytes isolated from non-failing (left) and hypertrophied (right) human hearts in response to gradual increase of stimulation frequency from 0.25 to 1 Hz. (C) Frequency-dependent changes in peak systolic amplitude in electrically stimulated Ca\textsuperscript{2+} transient in the cytoplasm (left) vs. nucleus (right) of ventricular myocytes isolated from control and hypertrophied human hearts. (D) Cytoplasmic (left) and nucleoplasmic (right) Ca\textsuperscript{2+} load calculated as a time-integral area under Ca\textsuperscript{2+} transient-time curve over 1 s in control and hypertrophied cardiomyocytes. (C and D) Data show mean ± SEM of n = 7 cells from N = 3 hearts per group, P-values were calculated using Sidak’s post hoc test (vs. non-failing at 0.25 Hz), following significant two-way repeated-measures ANOVA. (E) Original line-scan Fluo-4/AM fluorescence recordings (top) of intracellular Ca\textsuperscript{2+} transients at baseline and upon acute application of 10 nM isoprenaline (ISO) in a representative control (left) and hypertrophied (right) cardiomyocyte, and corresponding calibrated cytoplasmic (black) and nucleoplasmic (red) Ca\textsuperscript{2+} transients (bottom). Mean values of cytoplasmic (F) and nucleoplasmic (G) Ca\textsuperscript{2+} transient parameters displaying diastolic (dia) [Ca\textsuperscript{2+}], Ca\textsuperscript{2+} transient amplitude, time-to-peak (ttp), and time from peak (Tp) to 50% decline (DT\textsubscript{50}). Data show mean ± SEM of n = 5–6 cells from N = 3 hearts per group. Scale bar, 10 µm. Average data and group comparisons related to Figure 7 are summarized in Supplementary material online, Table S6.
that is regulated via phosphorylation of PLB, and TnI phosphorylation, which links Ca\(^{2+}\)-troponin C binding with the activation of crossbridge reactions with the actin filament. Increased phosphorylation of PLB augments the rate and the maximal Ca\(^{2+}\) uptake by increasing the affinity of SERCA2a for Ca\(^{2+}\) and its turnover, whereas TnI Ser-23/24 phosphorylation results in decreased Ca\(^{2+}\)-sensitive force production and accelerated myofilament relaxation.\(^{33,34}\) Notably, we found that Atg5\(^{-/-}\) mice exposed to ischemia showed attenuated positive inotropic responsiveness to β-adrenergic stimulation in vivo, which was associated with reduced in vivo TnI phosphorylation, but intact PLB phosphorylation. Although previous studies\(^{35-37}\) that proposed a predominance of the phosphorylation of TnI over that of PLB to mediate positive inotropy in response to ischemia may partly explain the reduced cardiac reserve in vivo, reasons for these differences are not entirely clear. At least part of the difference may reflect high pre-existing sympathetic activity during in situ assessment (e.g. by catheterization), whereas the use of isolated cardiomyocytes minimizes these influences. In support of this idea, Layland et al.\(^{38}\) demonstrated that reduced phosphorylation of TnI is linked to contractile dysfunction in conditions similar to those occurring in vivo but much less so in the unloaded cardiomyocyte.

ATG5-mediated autophagy failed to directly regulate the subcellular Ca\(^{2+}\) handling. Along these lines, the properties of the major intracellular Ca\(^{2+}\) store, SR, including its spatial organization, Ca\(^{2+}\) content and RyR2-mediated SR Ca\(^{2+}\) leak, were unchanged in Atg5\(^{-/-}\) cardiomyocytes. In this regard, it is important to mention that mammalian cells lacking Atg5, including Atg5\(^{-/-}\) cardiomyocytes, can still form a fraction of autophagosomes/autolysosomes and perform autophagy-mediated protein degradation via the Atg5/Atg7-independent non-canonical pathway.\(^{6}\) Interestingly, this alternative autophagy process was also proposed to play a predominant role in mediating mitochondrial degradation in the heart during energy deprivation (e.g. ischemia).\(^{39}\) Therefore, it is possible that the non-conventional form of autophagy acts as a compensatory mechanism that maintains, at least temporarily, the Ca\(^{2+}\)-regulatory machinery in young Atg5\(^{-/-}\) mice. However, Atg5\(^{-/-}\) cardiomyocytes displayed accumulated mitochondria aggregates due to impaired mitochondrial clearance,\(^{2,40}\) which intriguingly coincided with reduced relative mitochondrial mass. This seemingly counterintuitive finding results from decreased individual mitochondrial volume in Atg5\(^{-/-}\) cardiomyocytes and, albeit not measured in our study, can be attributed to balanced mitochondrial degradation vs. biogenesis. In support of this notion, Sun et al.\(^{41}\) recently reported that insufficient autophagy causes reduction in mitochondria content in the heart of a mouse model of lipopolysaccharide-induced sepsis, meanwhile enhanced autophagy in Beclin-1 transgenic hearts is associated with unexpectedly high mitochondrial abundance.

Apart from rapid Ca\(^{2+}\) regulation by β-adrenergic signalling, integrative subcellular changes in Ca\(^{2+}\) levels are critical for cardiac remodelling by modifying Ca\(^{2+}\)-dependent pathways mediating transcriptional regulation in cardiomyocytes. The selective increase in nucleoplasmic Ca\(^{2+}\) load at high-pacing frequencies in Atg5-deprived mice activated Ca\(^{2+}\)-dependent signalling involving CaMKII that is known to induce cardiac hypertrophy.\(^{9}\) Given that persistent overstimulation desensitizes the β-adrenergic signalling pathway, the progression of hypertrophy to heart failure in older Atg5\(^{-/-}\) mice is expected to depend at least in part, on sustained CaMKII activity. Activated nuclear CaMKII can trigger up-regulation of well-established marker genes linked to pathological hypertrophy, such as \(Nppb, Rcan-1, IL-6\) receptor, and transforming growth factor (TGF)-β1.\(^{24}\) Therefore, it is possible that CaMKII regulates Ca\(^{2+}\)-mediated gene expression that results from early anomalies in Ca\(^{2+}\) handling in Atg5-deficient mice. Importantly, future studies are warranted to unveil the mechanistic link between autophagy and CaMKII-dependent gene regulation.

Finally, the clinical relevance of our study is presented by the observation that cardiac expression of ATG5 at both transcriptional and protein levels was markedly reduced in hypertrophied and end-stage failing human hearts, while gene and protein expression of ATG5 inversely correlated with the extent of hypertrophy in donor hearts. Importantly, frequency-dependent and isoprenaline-induced changes of subcellular Ca\(^{2+}\) transients in hypertrophied human cardiomyocytes closely resembled those observed in Atg5\(^{-/-}\) ventricular myocytes. Hence, progressive loss of ATG5 protein and concomitant autophagy impairment parallels the development of hypertrophy and heart failure in humans. Along this line, early impairment of autophagy may cause energy deprivation in stressed myocardium, thereby initiating similar compensatory mechanisms followed by maladaptive events potentially resembling those observed in Atg5\(^{-/-}\) mice. However, although we present a large body of phenomenological evidence that points to a role for ATG5-induced autophagy in Ca\(^{2+}\) handling in cardiomyocytes, we acknowledge that a direct mechanistic link between impaired autophagy and Ca\(^{2+}\) cycling abnormalities requires further investigation.

In summary, this study demonstrates reduced mitochondrial abundance and capability to maintain redox balance in Atg5-deprived cardiomyocytes under stress. Functional alterations of the cytoplasmic and nucleoplasmic Ca\(^{2+}\) handling were associated with increased CaMKII activity and concomitant Ca\(^{2+}\)-mediated transcriptional regulation, and resulted in compromised cardiac functional reserve capacity, which precludes the development of heart failure in both mice and humans.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Authors’ contributions**

S.S. conceptualized the study; S.S. and S.L.-H. designed the study; S.L.-H., S.K., N.D., M.A., J.V., V.H., M.S., M.K., A.N., J.S., K.-M.K., G.F. and J.E. performed the experiments and analysed data; S.L.-H., M.A., C. Mühlfeld, C. Maack, and S.S. interpreted the data; A.Z., D.v.L., P.P.R. and D.S. characterized the patients or provided human cardiac tissue and data; S.L.-H. and S.S. wrote the manuscript. All authors revisited the work critically for important intellectual content and approved the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Acknowledgements**

The authors thank Noboru Mizushima (University of Tokyo, Japan) and Kenneth Chien (Harvard University, USA) for their generosity in providing Atg5\(^{lox/lox}\) mice and MLC2a-Cre\(^{+}\) mice, respectively. We are grateful for the excellent support by the animal facility staff of the Institute of Biomedical Research (IBF, Medical University of Graz) for animal wellbeing.

**Conflict of interest:** none declared.
Funding
This work was supported by the Austrian Science Fund (FWF) (grants P27637-B28 and I3301-MINOTAUR to S.S. and I3301-MINOTAUR to S.S.), and BioTechMed-Graz [Young Researcher Groups (YRG) to S.L.-H.]. S.K. was a recipient of the Medical University of Graz scholarship for talented students. M.A. is supported by research grants from the European Society of Cardiology and Austrian Society of Cardiology (Präsidienstipendium-ÖKG).

Data availability
The raw data are available from the corresponding author upon reasonable request.

References
1. Abdellatif M, Sedej S, Carmona-Gutierrez D, Madeo F, Kroemer G. Autophagy in cardiovascular aging. Circ Res 2018;123:803–824.

2. Nakai A, Yamaguchi O, Takeda T, Higuchi Y, Hikoso S, Taniike M, Omiya S, Mizote I, Matsamura Y, Ahasi M, Nishida K, Hori M, Mizushima N, Otsu K. The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Nat Med 2007;13:619–624.

3. Shiralabe A, Ikeda Y, Sciarretta S, Zablocki DK, Sadoshima J. Aging and autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Circ Res 2009;109:395–405.

4. Eisenberg T, Abdellatif M, Schroeder S, Prinsmissen U, Steckov S, Pennel T, Harger A, Schipke J, Zimmermann A, Schmidt A, Tong M, Ruckenstuhl C, Dammbruèc A, Gross AS, Wagner S, Sossalla S, Kreiner K, Lipp P, Müller C, Knopp A, Lorbek M, Hoth M, Kroemer G. Reversal of mitochondrial transhydrogenase causes oxidative stress in heart failure. Cell Metab 2015;22:472–484.

5. Haspel J, Shaik RS, Redgibo E, Nakahira K, Doliniy T, Engert JA, Choi AM. Characterization of macroautophagic flux in vivo using a leupeptin-based assay. Autophagy 2011;7:629–642.

6. Xie X, Bi HL, Lai S, Zhang YL, Li N, Cao HJ, Han L, Wang XH, Li H. The immunoprotaesan catalytic beta-subunit regulates cardiac hypertrophy by targeting the autophagy protein ATG5 for degradation. Sci Adv 2019;5:eavaa0495.

7. Nakai A, Yamaguchi O, Takeda T, Higuchi Y, Hikoso S, Taniike M, Omiya S, Mizote I, Matsamura Y, Ahashi M, Nishida K, Hori M, Mizushima N, Otsu K. Inhibition of autophagy in the heart induces age-related cardiomyopathy. Autophagy 2010;6:600–606.

8. Nishida Y, Ariyaka S, Fujitani K, Yamaguchi H, Mizuta T, Komase T, Komatsu M, Mizushima N, Otsu K. Inhibition of autophagy in the heart induces age-related cardiomyopathy. Autophagy 2010;6:600–606.

9. Bouchard C, Rankinen T. Individual differences in response to regular physical activity. Med Sci Sports Exerc 2001;33:1428–1438.

10. Bouchard C. Rankinen T. Individual differences in response to regular physical activity. Med Sci Sports Exerc 2001;33:1428–1438.

11. Lehnart SE, Maier LS, Hasenfuss G. Abnormalities of calcium metabolism and myo-}

12. Wettschureck N, Rutten H, Zywietz A, Gehring D, Wilkie TM, Chen J, Chien KR, Heinemann A, Sedej S, von Lewinski D, Bisping E. The role of stretch, tachycardia and sodium-calcium exchanger in induction of early cardiac remodelling. J Cell Mol Med 2020;24:8732–8743.

13. Sedej S, Heinzel FR, Walther S, Dybkova N, Wakula P, Grobboz J, Gronau P, Maier LS, Zos MA, Lai FA, Napolitano C, Priori SG, Kockskamper J, Bechara L, Rainer P, Heinemann A, Sedej S, von Lewinski D, Bisping E. The role of stretch, tachycardia and sodium-calcium exchanger in induction of early cardiac remodelling. J Cell Mol Med 2020;24:8732–8743.

16. Sedej S, Heinzel FR, Walther S, Dybkova N, Wakula P, Grobboz J, Gronau P, Maier LS, Zos MA, Lai FA, Napolitano C, Priori SG, Kockskamper J, Bechara L, Rainer P, Heinemann A, Sedej S, von Lewinski D, Bisping E. The role of stretch, tachycardia and sodium-calcium exchanger in induction of early cardiac remodelling. J Cell Mol Med 2020;24:8732–8743.
Translational perspective

Autophagy is a cytoprotective process ensuring homeostasis of the heart. Here, we report that maladaptive hypertrophy and heart failure associate with reduced expression of autophagy-related protein 5 (ATG5) in humans. In mice, ATG5-dependent autophagy disrupts subcellular Ca\textsuperscript{2+} homeostasis and Ca\textsuperscript{2+}-mediated transcriptional regulation, coinciding with impaired mitochondrial redox balance, especially during stress and increased cardiac demand. These effects translate into reduced cardiac functional reserve capacity and the development of premature heart failure. Hence, re-instating autophagy at early stages of cardiac remodelling may protect from heart failure through preserving mitochondrial abundance and nuclear Ca\textsuperscript{2+} homeostasis.