Muscle RING-finger 2 and 3 maintain striated-muscle structure and function

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Abstract

Background The Muscle-specific RING-finger (MuRF) protein family of E3 ubiquitin ligases is important for maintenance of muscular structure and function. MuRF proteins mediate adaptation of striated muscles to stress. MuRF2 and MuRF3 bind to microtubules and are implicated in sarcomere formation with noticeable functional redundancy. However, if this redundancy is important for muscle function in vivo is unknown. Our objective was to investigate cooperative function of MuRF2 and MuRF3 in the skeletal muscle and the heart in vivo.

Methods MuRF2 and MuRF3 double knockout mice (DKO) were generated and phenotypically characterized. Skeletal muscle and the heart were investigated by morphological measurements, histological analyses, electron microscopy, immunoblotting, and real-time PCR. Isolated muscles were subjected to in vitro force measurements. Cardiac function was determined by echocardiography and working heart preparations. Function of cardiomyocytes was measured in vitro. Cell culture experiments and mass-spectrometry were used for mechanistic analyses.

Results DKO mice showed a protein aggregate myopathy in skeletal muscle. Maximal force development was reduced in DKO soleus and extensor digitorum longus. Additionally, a fibre type shift towards slow/type I fibres occurred in DKO soleus and extensor digitorum longus. MuRF2 and MuRF3-deficient hearts showed decreased systolic and diastolic function. Further analyses revealed an increased expression of the myosin heavy chain isoform beta/slow and disturbed calcium handling as potential causes for the phenotype in DKO hearts.

Conclusions The redundant function of MuRF2 and MuRF3 is important for maintenance of skeletal muscle and cardiac structure and function in vivo.

Keywords Protein homeostasis; Protein surplus myopathy; Heart failure; MuRF2; MuRF3; MAPKAPK

Introduction

Muscle-specific RING-finger (MuRF) proteins maintain cardiac and skeletal muscle structure and function.1–6 MuRF proteins belong to the tripartite motif-containing (TRIM) family of E3 ubiquitin ligases. All three MuRF family members MuRF1, MuRF2, and MuRF3 are predominantly expressed in the heart and skeletal muscle.5 MuRF1 is involved in skeletal muscle atrophy and cardiac hypertrophy.6,8 MuRF1 knockout mice are resistant to skeletal muscle atrophy7 and when subjected to chronic pressure overload develop exaggerated cardiac hypertrophy.6 MuRF2 is implicated in sarcomere formation.
because it transiently associates with microtubules, myosin, and titin during sarcomere assembly. MuRF2 also mediates signal transduction in cardiomyocytes as it translocates to the nucleus during mechanical inactivity where it decreases the abundance of the serum response transcription factor (SRF). MuRF3 binds to and stabilizes microtubules establishing a network resistant to depolymerization. MuRF3 knockout mice develop left ventricular dilation, heart failure, and cardiac rupture when subjected to myocardial infarction. However, no phenotype has been described for single germ line deletions of MuRF1, MuRF2, or MuRF3 under unstressed conditions, implying partially redundant functions for these highly homologous proteins under physiological circumstances. Earlier, we described a protein-surplus myosin storage myopathy in skeletal muscle and the heart of MuRF1 and MuRF3 double knockout (DKO) mice that supports this idea. Further support for redundancy comes from the phenotype of MuRF1 and MuRF2 DKO mice, because most of these mice die at birth. Surviving MuRF1 and MuRF2 DKO mice develop cardiac hypertrophy and a decrease in fast-MAPK and mediators of p38 MAPK signalling. The p38 stream targets of p38 mitogen-activated protein kinase and its family member protein kinase 3 (MAPKAPK3) to be enriched in DKO muscle.

The proteomics-based approach that identified MuRF2 and MuRF3 partially redundant functions in DKO muscle and vice versa, without involving MuRF1. Here, we tested the hypothesis that MuRF2 and MuRF3 have partially redundant functions in striated muscles in vivo. We generated MuRF2 and MuRF3 DKO mice and analysed the skeletal and cardiac muscle for altered structure and function.

To elucidate a potential signalling pathway possibly affected by MuRF2 and MuRF3-deficiency, we relied on a proteomics-based approach that identified MAPK-activated protein kinase 3 (MAPKAPK3) to be enriched in DKO muscle. MAPKAPK3 and its family member MAPKAPK2 are downstream targets of p38 mitogen-activated protein kinase (MAPK) and mediators of p38 MAPK signalling. The p38 MAPK pathway is involved in various striated muscle signalling events. In adult skeletal muscle, p38 MAPK and MAPKAPK2 are activated by muscle contraction, and MAPKAPK2/3 were reported to modify the skeletal muscle fibre type composition. In heart, p38 MAPK and MAPKAPK2/3 are highly abundant, and chronic p38 MAPK activation is involved in cardiac pathologies. Addition, MAPKAPK2/3-deficiency increased contractile activity in cardiomyocytes. Therefore, we investigated possible effects of MuRF2 and MuRF3-deficiency in soleus muscle and left ventricle on MAPKAPK2/3 protein levels and a potential interaction of MuRF2 and MuRF3 with MAPKAPK2/3.

Materials and methods

MuRF2 and MuRF3 knockout mice have been described previously. Animals were housed under standard conditions at 22 °C with a 12-hour light/12-hour dark cycle and maintained on commercial mouse chow and water ad libitum. MuRF2 and MuRF3 double heterozygous mice were interbred, and resulting control and DKO littermates were used for subsequent experiments. The following genotypes were used as controls: MuRF2+/+MucRF3+/+, MuRF2+/−MucRF3+/+, MuRF2+/−MucRF3+/−, and MuRF2+/−MucRF3−/−. The Landesamt für Gesundheit und Soziales approved the studies (LaGeSo, Berlin, Germany; permit number: G 0129/12). The studies followed the ‘Principles of Laboratory Animal Care’ (NIH publication No. 86-23, revised 1985), as well as the current version of German Law on the Protection of Animals. Muscle dissection, gene expression analyses, and protein purification were performed as recently published; for further details, please refer to the online Supporting Information.

Western blot analysis was performed on protein samples from skeletal muscles, hearts, and cells, as previously described. The following primary antibodies were used: anti-MuRF2 (polyclonal, rabbit, own production), anti-MuRF3 (polyclonal, rabbit, own production), anti-actin (monoclonal, mouse), anti-β/slow MyHC (clone NOQ7, monoclonal, mouse), anti-pan MyHCII (clone My32, monoclonal, mouse; all Sigma–Aldrich, Germany), anti-MAPKAPK2, anti-p38 MAPK (all: polyclonal, rabbit, Cell Signaling Technology Inc., Danvers, USA) antibody. Generation of anti-MuRF2 and anti-MuRF3 antibody is described in the online Supporting Information. Loading was controlled by using anti-glycereraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (clone 6C5, monoclonal, mouse, Millipore GmbH, Germany). Horseradish peroxidase (HRP)-linked IgG horse anti-mouse, goat anti-rabbit (both Cell Signaling Technology Inc., USA), or rabbit anti-goat (Abcam, UK) were used as secondary antibody. For further details on Western blot analysis, glutathione S-transferase (GST)-pulldowns, and co-immunoprecipitation please refer to the online data supplement. Analysis of MAPKAPK2, MAPKAPK3, and p38 followed our recently published work. Histological and immunohistochemical analyses were performed as described previously. Haematoxylin and eosin (H & E), metachromatic ATPase dye, or modified Gomori’s trichrome staining were performed as previously described. The following primary antibodies were used: anti-β/slow MyHC (clone NOQ7), anti-pan MyHCII (clone My32), and anti-Laminin (all Sigma–Aldrich, Germany). The following secondary antibodies were used: goat anti-mouse and goat anti-rabbit IgG (Alexa Fluor 488 nm or 555 nm, Invitrogen). Filamentous actin was stained with phalloidin–TRITC (Sigma–Aldrich, Germany). Images were acquired with a Leica CTR 6500 HS microscope, and Leica digital camera DFC 425 for histological analyses and Leica digital camera DFC 360 FX for fluorescence pictures. Analyses of images were performed with ImageJ software 1.42c (http://rsb.info.nih.gov/ij/). Myocyte cross-sectional area (MCSA) was measured from extensor digitorum longus from...
at least 100 myofibres per genotype and histological section of 9- to 22-week-old mice. Centralized nuclei were quantified in 60–270 myofibres per genotype and histological section from both soleus and extensor digitorum longus of 21- to 22-week-old mice. Fibre type composition was determined in 330–970 myofibres per genotype and histological section from both soleus and extensor digitorum longus of 21- to 22-week-old mice. Electron microscopy (16- to 22-week-old mice) and measurements of muscle force (14- to 27-week-old mice) were performed as previously described. For further details, please refer to the online Supporting Information.

Isolation of adult mouse ventricular cardiomyocytes from 15-week-old animals and measurements of single cell contraction and calcium transients were performed as described previously. Body composition was measured in 5-month-old male (n = 4) and female (n = 3) DKO mice and their control (n = 8 and n = 4, respectively) littermates using the LF90 II time domain NMR analyzer (6.5 mHz, Bruker Optics, USA). The mice were placed into the restraint tube, which was adjusted to minimize movements of the animal without impairing respiration. The tube together with the animal was placed into the LF90 II instrument, and fat mass, fat-free mass, and fluid were measured in triplicate. Echocardiography was performed on 8-week-old male DKO (n = 5) mice and their control littermates (n = 7) as previously described. The mice were anesthetized with 2% isoflurane and kept warm on a heated platform. Temperature and electrocardiogram were continuously monitored. Cardiac function and morphology were assessed with a VisualSonics Vevo 2100 High-Resolution Imaging System (VisualSonics, Toronto, Canada) with the use of a high-resolution (38 MHz) transducer. Left ventricular (LV) end-diastolic dimension (LDD) and end-systolic dimension (ESD), thickness of the left ventricular posterior wall (LVPW) in diastole and systole, left ventricular ejection fraction (EF), and cardiac output were measured. Langendorff heart experiments were performed as recently described. For further details, please refer to the online Supporting Information.

**Statistics**

Values are presented as mean ± SEM. Gene expression was normalized to stably expressed Hprt mRNA and calculated as relative change. Differences in morphologic, physiologic, and biochemical parameters between groups were analysed by Mann–Whitney U test or 2-sided Student’s t-test. Statistics were calculated with Microsoft Excel 2002 and Sigma Plot software 11.0. A P-value of less than 0.05 was considered as statistically significant.

**Results**

**General characteristics of muscle ring-finger 2 and muscle ring-finger 3 double knock-out mice**

MuRF2 and MuRF3 DKO mice display a protein aggregate myopathy in skeletal muscle

The soleus, extensor digitorum longus, gastrocnemius/plantaris, and tibialis anterior revealed pathological changes in DKO mice. Haematoxylin and eosin staining of histological sections showed intracellular protein aggregates surrounding an inner laying myofibre and adjacent to the membrane of the myofibres in all DKO skeletal muscles (Figure 2A, Figure S3a). Centralized nuclei were found in DKO myofibres of all muscles from male and female mice indicative for regeneration of the skeletal muscle (Figure 2A–C, Figure S3a). Quantitation of MCSA using H & E staining of histological cross-sections showed a higher amount of smaller myofibres in DKO extensor digitorum longus compared with littermate controls as an unspecific sign for myopathy (Figure 2D). Modified Gomori’s trichrome staining showed no increase in fibrotic tissue in skeletal muscle of DKO mice (Figure 2B, Figure S3b). In both H & E and modified Gomori’s staining, a demarcation between the inner laying myofibre and the accumulating protein was observed (Figure 2, Figure S3). We excluded the possibility that this structure was a membrane. The accumulations were contained within individual myofibres rather than the interstitium (Figure S4). By electron microscopy, the overall structure of the inner-laying myofibre core was normal in control and DKO soleus and extensor digitorum longus muscle (Figure 3A α–ι). However, the myofibre core showed Z-line streaming and myofibrils degenerating into the amorphous mass in DKO soleus and extensor digitorum longus (Figure 3A ε, ζ). This core structure was surrounded...
by mitochondria and an amorphous mass localized between the myofibre core and the sarcolemma (Figure 3A δ, ε). These observations reveal a protein storage myopathy affecting myofibres in DKO skeletal muscle with a preference for soleus and extensor digitorum longus.

**A fibre type shift towards slow/type I fibres occurred in MuRF2 and MuRF3 deficient muscle**

We next investigated whether or not protein accumulations were restricted to a specific fibre type. Protein accumulations were found in all types of myofibres and in all DKO but not in control muscles (Figure 3B). Notably, staining of protein accumulations by the ATPase dye resembled the staining of the specific fibre in which the accumulation occurred (Figure 3B). These data show a non-fibre type specific accumulation of myosin heavy chain (MyHC) protein. Most importantly, we observed slow/type I fibres in extensor digitorum longus of DKO mice where these fibres are usually not present (Figure 3B). Western blot analysis was used to test if myosin heavy chain 7 protein (MyHC-7), contained in slow/type I fibres, was increased in DKO extensor digitorum longus. Indeed, slow/type I MyHC protein was increased in protein lysates of DKO extensor digitorum longus (Figure 3C). Slow/type I myosin was found in both the supernatant and the pellet fraction indicating increased amounts of soluble and insoluble myosin in DKO muscle (Figure 3C). To confirm suspected accumulation of slow/type I and fast/type II myosin in their respective fibres, immunostaining of histological cross-sections from soleus and extensor digitorum longus using anti-slow and anti-fast myosin antibody was performed. We confirmed that slow and fast myosin accumulated in their respective fibres in soleus and extensor digitorum longus of DKO mice (Figure 4A). Quantification of fibre types showed a decrease in fast/type II and an increase of hybrid fibres (containing both slow and fast myosin) in soleus, and an increase in slow/type I fibres, as well as a decrease in fast/type II fibres in extensor digitorum longus of DKO mice (Figure 4B).

Increased expression in Myh7, encoding for slow/type I MyHC, and Myh3, encoding for embryonic MyHC, was found in soleus, extensor digitorum longus, gastrocnemius/plantar, and tibialis anterior of DKO mice (Figure 4C, Figure S5). In contrast, Myh2 and Myh1 expression (encoding type IIa and IIx MyHC, respectively) decreased in soleus and increased in extensor digitorum longus of DKO mice (Figure 4C), whereas...
Myh4 expression (encoding type Iib MyHC) remained unchanged in all muscles investigated (Figure 4C, Figure 5S). Thus, MuRF2 and MuRF3 are involved in myosin homeostasis in slow/type I and fast/type II fibres.

Contractile function of DKO muscle was reduced

We reasoned that protein accumulations in all myofibres and a shift in fibre type composition towards slow/type I fibres would decrease force development in muscle of DKO mice. Maximum force development was measured on isolated soleus and extensor digitorum longus of male and female DKO mice and compared to littermate controls. As expected, maximum force development of soleus and extensor digitorum longus of male (Figure 4D) and female (Figure 5E) DKO mice was reduced compared to controls. These data showed that MuRF2 and MuRF3 are important to regulate the function of skeletal muscle.

Cardiac structure and function were disturbed in DKO mice

We next investigated cardiac structure and function of DKO mice. First, absence of MuRF2 and/or MuRF3 in the heart in single knockout and DKO mice was confirmed by Western blot analyses using anti-MuRF2 and anti-MuRF3 antibody (Figure 5A). To analyse if a compensatory up-regulation of MuRF1 expression occurred in hearts of DKO mice, real-time RT–PCR was performed. MuRF1 mRNA expression was not increased in DKO animals indicating that no compensatory up-regulation of MuRF1 occurred in DKO hearts (Figure 5A). Relative heart and lung weights were unchanged in DKO mice compared to littermate controls (Figure 5B). Using H & E and modified Gomori’s trichrome staining of histological sections, neither protein accumulations nor interstitial fibrosis were found in hearts of DKO mice (Figure 5C). When using transmission electron microscopy, no ultrastructural changes were observed in DKO hearts (data not shown).
Echocardiography revealed an increased left ventricular end-systolic dimension (LVESD) and a decreased thickness of the left ventricular posterior wall (LVPW) at both systole and diastole in DKO animals. These changes were accompanied by a reduction in systolic function of the left ventricle with a decreased left ventricular ejection fraction and decreased cardiac output of DKO hearts (Figure 5D).

Figure 3 Double knockout (DKO) mice displayed a protein aggregate myopathy and a shift towards slow myofibres in extensor digitorum longus (EDL) muscle. (A) Electron microscopy of soleus (Sol) and EDL sections displayed accumulation of amorphous material in DKO myofibres. Asterisks indicate accumulating aggregates, arrow points to abnormal Z-line. Scale bar, 5 μm (α–δ), 2 μm (ε, ζ). (B) ATPase stain of Sol and EDL showed accumulations inside of slow/type I fibres (dark blue, white arrows) and fast/type II fibres (bright blue, black arrows) of DKO mice. Slow/type I fibres occurred in EDL of DKO mice (asterisk). Scale bar, 50 μm. (C) Immunoblotting of proteins from the soluble (supernatant) and particulate (pellet) fractions of EDL revealed accumulation of slow/type I myosin heavy chain protein in DKO muscle. Actin was used as a control.
Figure 4 Loss of muscle-specific RING-finger (MuRF)2 and MuRF3 leads to an increase in slow/type I fibres in skeletal muscle. (A) Immunohistochemistry of cross-sections from soleus (Sol, left panel) and extensor digitorum longus (EDL, right panel) using anti-laminin, anti-fast/type II myosin heavy chain, or anti-slow/type I myosin heavy chain antibody. Nuclei were stained with 4’,6-Diamidino-2-phenylindol (DAPI). Protein aggregations are indicated (arrows). Slow/type I fibres occurred in EDL of DKO mice (arrow). Scale bar, 25 μm. (B) Slow/type I and fast/type II MyHC containing fibres were quantified in Sol and EDL of control (n = 8–10) and DKO (n = 7–8) mice. Data are shown as mean ± SEM. **P < 0.01, ***P < 0.001. (C) Real-time RT–PCR analysis of myosin heavy chain (Myh) 1, 2, 3, 4 and 7 gene expression in Sol and EDL from control (n = 8–9) and DKO (n = 4) mice. Hypoxanthine guanine phosphoribosyl transferase (Hprt) expression was used as reference. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01. (D) Maximal force development of Sol and EDL from male 14- to 23-week-old control (n = 9 each) and DKO (n = 11–12) mice is shown. Specific force [mN/mm²] per stimulation frequency [Hz] is depicted. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
are important for maintenance of cardiac function. Next, we tested if reduced cardiac function was accompanied by an increased expression of cardiac stress markers. We also tested if genes involved in cardiac remodelling were increased in DKO hearts. We found an increased \textit{Myh7} expression in DKO hearts, whereas \textit{Myh6}, \textit{Nppa}, \textit{Nppb}, \textit{Ctgf}, \textit{Col1a1}, and \textit{Col3a1} remained unchanged (Figure 5E, Figure S7b).

At baseline, systolic left ventricular pressure (LVPsys, Figure 6A) and left ventricular developing pressure (LVPdevp, Figure 6B) were reduced in DKO compared with control hearts. However, following stimulation with the beta-receptor agonist isoproterenol, LVPsys and LVPdevp increased in both experimental groups (Figure 6A and 6B). We also calculated the derivatives of LVP dLVP/dt\textsubscript{max} (Figure 6C) and dLVP/dt\textsubscript{min} (Figure 6D) as indices of left ventricular contraction and relaxation velocity, respectively. dLVP/dt\textsubscript{min} was decreased whereas dLVP/dt\textsubscript{max} remained unchanged in DKO hearts (Figure 6C and 6D). Both indices increased in control and DKO hearts following isoproterenol treatment. However, left ventricular contraction (Figure 6C) and relaxation velocity (Figure 6D) in the response to isoproterenol was reduced in DKO hearts. In summary, DKO hearts showed reduced systolic and diastolic function at baseline and following stress.

\textbf{Systolic and diastolic function was reduced in double knockout cardiomyocytes}

We reasoned that the decrease in cardiac function in DKO mice occurred at the level of cardiomyocytes. To test this hypothesis, we analysed contraction and relaxation of adult cardiomyocytes from hearts of DKO and control mice. Cardiomyocytes from DKO mice were longer and wider resulting in an increased surface area compared with controls (Figure 7A). Using sarcomere length tracing, we measured peak sarcomere shortening as amplitude, maximum shortening velocity (+dL/dt), and maximum relaxation velocity (-dL/dt) of control and DKO myocytes. All these parameters were reduced in DKO cardiomyocytes (Figure 7B) indicative for a diminished systolic and diastolic function of DKO cardiomyocytes.

To evaluate whether or not diminished cardiomyocyte function was due to altered intracellular Ca\textsuperscript{2+}-availability, we...
measured Ca²⁺-transients simultaneously to sarcomere shortening. Ca²⁺-transient amplitudes were significantly decreased in DKO myocytes (Figure 7C). More specifically, diastolic Ca²⁺-concentration was reduced in DKO cardiomyocytes compared with controls (Figure 7C). When we calculated the velocities of increase and decay of Ca²⁺-transients in cardiomyocytes as maximum velocity of ratio increase (⁺dR/dt) and maximum velocity of ratio decay (⁻dR/dt), we found these parameters to be significantly decreased in DKO cardiomyocytes (Figure 7C). In summary, these data are indicative for a disturbed Ca²⁺-handling that might at least partially be responsible for decreased systolic and diastolic function of DKO hearts.

**MuRF family members physically interact, co-localize and stabilize each other in cardiac and skeletal muscle cell lines in vitro**

The cooperative function of MuRF proteins might be related to their ability to homodimerize and heterodimerize and to be localized to the same subcellular compartment or structure where they might fulfil similar functions. To investigate the molecular basis of functional redundancy of the MuRF family, we performed cell culture analyses. First, we visualized subcellular localization of MuRF proteins in the cardiac myoblasts cell line H9c2 transiently transfected with cDNA expression plasmids encoding MuRF1, MuRF2, and/or MuRF3. When solely expressed, MuRF1 and MuRF2 were predominantly cytoplasmic, and MuRF3 resided at microtubules. However, when MuRF1 and MuRF2 were co-expressed, both proteins occurred in thick tubule-like structures. Co-expression of MuRF1 or MuRF2 with MuRF3 led to co-localization of MuRF1 and MuRF2 with MuRF3 at microtubules, similar to what was seen for MuRF3 alone (Figure S8a). These data implicated that MuRF3 physically interacts with MuRF2 leading to recruitment of MuRF2 to microtubules. Second, to test if physical interaction was the cause for co-localization of MuRF2 and MuRF3, co-immunoprecipitation experiments followed by Western blot analyses were performed. These experiments showed that all MuRF proteins interact with each other (Figure S8b). Because MuRF proteins function as E3 ubiquitin ligases, we tested if co-expression of MuRF family members would decrease their protein content. First, expression plasmids encoding MuRF1, MuRF2, or MuRF3 were transfected in mouse skeletal myoblasts (C2C12-cells). Western blot analysis showed a weak signal of MuRF proteins when expressed individually (Figure S8c). However, co-expression of different MuRFs led to an increase in their

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**Figure 6** Double knockout (DKO) mice showed a decreased systolic and diastolic cardiac function. (A) Left ventricular systolic pressure (LVPsys) and left ventricular developing pressure (LVPdevp) were measured via an intraventricular balloon. At baseline, LVPsys and LVPdevp were reduced in DKO (n = 6) compared with control (n = 4) animals. Following stimulation with 50 nM isoproterenol (ISO), LVPsys and LVPdevp increased in both experimental groups. (B) Maximal (dLVP/dt, max) and minimal (dLVP/dt, min) derivatives of LVP, indices of contraction and relaxation velocity, respectively, were decreased in DKO (n = 6) compared with control (n = 4) mice. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
Protein amount indicating that they do not execute E3 ubiquitin ligase function to degrade their respective family members, but rather stabilize them (Figure S8c). In concert, these data demonstrated that physical interaction of MuRF2 and MuRF3 in myocytes mediates their co-localization and prevents them from being degraded. Our data also indicate that MuRF1 localizes to the same subcellular compartments as MuRF2 and MuRF3. However, if this subcellular localization enables MuRF1 to compensate for the loss of MuRF2 and MuRF3 remains to be proven.

Proteomic analysis indicated a reduction of proteins important for mitochondrial function and energy supply in DKO muscle.

To search for a potential mechanism by which the absence of MuRF2 and MuRF3 decreased muscle function, we performed mass-spectrometry on tissue lysates from DKO and control muscle. In total, we detected 37 710 peptides belonging to 3565 proteins in both control (n=3) and DKO (n=3) soleus. There were 2024 proteins identified in

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Figure 7 Decreased contraction and relaxation and impaired calcium handling in adult ventricular cardiomyocytes of double knockout (DKO) mice. (A) Quantification of length (left panel), width (middle panel), and area (right panel) of adult cardiomyocytes isolated from hearts of control (n=100 cells) and DKO (n=100 cells) animals indicated an increased size of cardiomyocytes in DKO mice. Data are presented as mean±SEM. **P < 0.01. (B) Peak sarcomere shortening as amplitude (left panel), maximum shortening velocity as +dl/dt (middle panel), and maximum relaxation velocity as -dl/dt (right panel) of adult control (n=135 cells) and DKO (n=130 cells) myocytes are shown. Cardiomyocytes were stimulated to contract at 5 Hz at 37°C, extracellular Ca2+-concentration was 1.25 mM. Data are presented as mean±SEM. **P < 0.01. (C) Intracellular Ca2+-transients of adult cardiomyocytes from control (n=76 cells) and DKO (n=62 cells) animals are shown as ratio transients. Graphs show fura-2 ratio amplitude (left panel), maximum velocity of ratio increase as +dr/dt (middle panel), and maximum velocity of ratio decay as -dr/dt (right panel). Cardiomyocytes were stimulated to contract at 5 Hz at 37°C, extracellular Ca2+-concentration was 1.25 mM; cells were fura-2 loaded. Data are presented as mean±SEM. **P < 0.01.
all six samples. We expected to identify an accumulation of proteins, which are potentially targeted by MuRF2 and MuRF3 for proteasomal degradation. Bioinformatics analyses of mass-spectrometry data identified proteins which are enriched and diminished in DKO muscle (Figure 8). As expected, GO term analysis of proteins increased in DKO muscle showed an enrichment of proteins predominantly involved in proteolysis, ubiquitin proteasome dependent protein degradation, and regulation of muscle function (Figure 8, Figure S9, Table S2). Importantly, among those proteins that were reduced in DKO muscle, we identified proteins important for mitochondrial function and cellular energy supply (Figure S9 and S10, Table S3).

**Mitogen-activated protein kinase-activated protein kinase 2 and mitogen-activated protein kinase-activated protein kinase 3 are enriched in DKO muscle**

For further analysis, we focussed on MAPKAPK2 and 3, two major final mediators of the p38 MAPK signalling cascade,14,15 which were enriched in DKO muscle. Western blot analysis from total tissue lysates revealed an increase in the MAPKAPK2 protein content in soleus and left ventricle of DKO mice (Figure 9A and 9B; left panels). A known interaction partner of MAPKAPK2 and MAPKAPK3 is p38 MAPK.14,15

To avoid unspecific background for the detection of MAPKAPK3 protein levels, we first performed p38-GST pulldown assays in total tissue lysates of soleus and left ventricle followed by immunoblotting using an anti-MAPKAPK3 antiserum as described previously.14,15 MAPKAPK3 protein levels were increased in DKO soleus and left ventricle compared with controls (Figure 9A and 9B; right panels). The anti-MAPKAPK2 antibody was used in this experiment as a positive control for equal enrichment of p38 MAPK interacting proteins and showed a similar increase in the amount of MAPKAPK2 protein after the p38-GST pulldown as detected by Western blot analysis in total tissue lysates (Figure 9A and 9B; left panels). In summary, MAPKAPK2 and MAPKAPK3 protein contents are increased in DKO soleus and left ventricle compared with control mice. The amount of total p38 MAPK protein remained unchanged in soleus and left ventricle in control compared with DKO mice (Figure 9A and 9B). In addition, real-time RT–PCR showed that MAPKAPK2 and MAPKAPK3 mRNA expression was not elevated in DKO soleus and left ventricle indicating that increased MAPKAPK2 and MAPKAPK3 protein contents were not due to their increased gene expression in the absence of MuRF2 and MuRF3 (Figure 9C). Further analyses using co-immunoprecipitation and co-localization experiments could not conclusively demonstrate that MAPKAPK2 and MAPKAPK3 interact with or co-localize with MuRF2 and MuRF3 [data not shown]. In concert, our data suggest that MuRF2 and MuRF3 are coordinately involved in maintenance of protein homeostasis in striated muscles primarily regulating protein degradation pathways and energy supply.

**Discussion**

The main finding of our work is that MuRF2 and MuRF3 are key factors for the maintenance of skeletal muscle and cardiac structure and function, because absence of both leads to myopathy in striated muscles. The observed phenotypes of DKO mice are summarized in Table 1. MuRF2 and MuRF3 avidly interact, co-localize to microtubules, and stabilize each other in vitro, which could serve as the molecular basis for their redundant function in myocytes. The interactions between MuRF2 and MuRF3 suggest a cooperative activity of both E3 ligases.

The MuRF2 and MuRF3 DKO mice developed a protein aggregate myopathy of the skeletal muscle. This finding resembles the phenotype of MuRF1 and MuRF3 DKO mice we reported earlier.4 We showed that MuRF1 and MuRF3 associate with, ubiquitinate, and mediate ubiquitin proteasome system (UPS) dependent myosin degradation.4 However, if MuRF2 is involved in myosin turnover and if this leads to myosin aggregates in MuRF2 and MuRF3 DKO mice was unknown. In addition, recent reports suggest E3 ubiquitin ligase independent functions of MuRF proteins.11 Others, and we, reported that MuRF2 and MuRF3 resulted in a myofibrillar phenotype in myocytes in vitro.12 We add to these data that the amount of MuRF2 and MuRF3 is not only balanced at the transcriptional but also at post-translational level, because MuRF2 and MuRF3 co-localize to microtubules and prevent their respective degradation. Redundancy of MuRF2 and MuRF3 is one explanation why MuRF2 and MuRF3 single knockout mice have no phenotype at baseline.4,5 We conclude that MuRF2 and MuRF3 function redundantly during sarcomere formation and that their absence leads to myosin aggregates in vivo. However, normally formed sarcomeres suggest additional factors compensating for the loss of MuRF2 and MuRF3 in DKO muscle during myogenesis.

MuRF1 is mainly expressed in fast/type II fibres,11 MuRF2 is predominantly expressed in slow/type I fibres, and MuRF3 is ubiquitously expressed.28 Up-regulation of MuRF1 paralleled by a predominant atrophy of fast/type II fibres during muscle atrophy28 underscores a functional relevance of
Figure 8 Proteomic analysis of lysates from double knockout (DKO) compared with control soleus muscle. (A) Volcano-plot. For each of the data sets, a t-test was used to calculate the $-\log_{10}(P$-value), which was plotted vs. the log-transformed means of the DKO/control ratios. Vertical lines indicate the top or bottom 30% most regulated proteins, while the horizontal line indicates the cut-off for the significance of the $P$-value. Proteins increased and decreased in DKO muscle are shown in red and green, respectively. (B) and (C) Heat-map of the Z-transformed $P$-values obtained by Gene Ontology (GO)-analysis of proteins upregulated in DKO muscle. The 30% upregulated (B) and 30% downregulated (C) proteins in DKO soleus were used for the GO analysis with the DAVID online tool. The obtained $P$-values of GO-term biological process that were significantly enriched ($P<0.05$) were log-transformed, Z-transformed, hierarchically clustered, and plotted as a heat map. Colour key indicates $P$-values.

this fibre type specific expression pattern. However, myosin aggregates occurred in both fast/type II and slow/type I myofibres of DKO muscle. These findings argue for a role of MuRF2 and MuRF3 in both fibre types, which seems to be MuRF1 independent. The latter is supported by the fact that myosin aggregates also occurred in fast/type II fibres were MuRF1 is preferentially expressed. MuRF1 and MuRF3 DKO, and MuRF2 and MuRF3 DKO (shown here), but not MuRF1 and MuRF2 DKO mice show a protein aggregate myopathy. Because myosin aggregates only occurred in the skeletal muscle of DKO mice lacking MuRF3, we believe that this phenotype is mediated by the absence of MuRF3. In contrast, based on the phenotype of MuRF1 and MuRF3 DKO and MuRF1 and MuRF2 DKO mice, MuRF1 appears to play a predominant role in both skeletal muscle and heart. More specifically, we found a severe myosin storage myopathy of both the heart and skeletal muscle in MuRF1 and MuRF3 DKO animals. Additionally, three-fourths of MuRF1 and MuRF2 DKO mice died around birth. However, the myopathic phenotype of MuRF2 and MuRF3 DKO mice was less severe. These data suggest that MuRF1 plays a predominant role in muscle and has non-overlapping functions with MuRF2 and MuRF3. Nevertheless, due to the myopathic phenotype of MuRF2 and MuRF3 DKO mice, it appears that MuRF1 cannot fully compensate for the loss of MuRF2 and MuRF3. Furthermore, absent MuRF2 and MuRF3 caused an increase in slow/type I and in hybrid fibres, as well as a decrease in fast/type II fibres. Possibly, this function is related to the regulation of myozin-1, as was recently shown in MuRF1 and MuRF2 DKO muscle. Of note, recently combined homozygous MuRF1 and heterozygous MuRF3 mutations were shown to cause a protein aggregate myopathy and cardiomyopathy in patients, which was reminiscent of the MuRF1 and MuRF3 DKO phenotype described here. Importantly, after comprehensive screening for mutations in several sarcomeric genes, our description of the MuRF1 and MuRF3 DKO mouse phenotype prompted the authors to search for mutations in the MuRF1 and MuRF3 genes. This analysis by Olive et al. now led to a new disease entity of skeletal muscle and cardiac protein aggregate myopathy. However, if combined MuRF2 and MuRF3 mutations occur in patients and are causative for protein aggregate myopathy and cardiomyopathy needs to be verified. Nevertheless, in those patients suffering from protein aggregate myopathies in whom mutations in sarcomeric genes were excluded, it appears to be reasonable to search for mutations in MuRF1, MuRF2 and MuRF3. MuRF2 and MuRF3 DKO mice exhibited cardiomyopathy with decreased systolic and diastolic function. Our data show that this phenotype occurred at the level of cardiomyocytes. First, no interstitial fibrosis was observed in MuRF2 and MuRF3 DKO mice, which often accompanies pathological cardiac remodelling and leads to a decrease in systolic and diastolic function. Second, beta/slow myosin expression was increased in DKO hearts. Normally, ventricular myocardium of rodents is mainly composed of alphaMhc/Myh6, accounting for more than 90% of all myosins. ATPase activity of alphaMhc/Myh6 is higher compared with beta/slow myosin, which allows greater economy in sarcomeric force generation. This leads to a higher contractile velocity of hearts expressing alphaMhc/Myh6 compared with hearts...
expressing beta/slow myosin. Accordingly, a decrease in systolic cardiac function was reported for transgenic mice overexpressing beta/slow myosin. Therefore, increased beta/slow myosin expression might have contributed to the reduced systolic and diastolic function of DKO cardiomyocytes and hearts. However, it is unknown why beta/slow myosin expression was increased in DKO hearts. Of note, MuRF1 and MuRF2 were shown to regulate myozenin-1 and myozenin-2, inhibitors of the calcineurin/nuclear factor of activated T-cells (NFAT) pathway. A downregulation of myozenin-1 and myozenin-2 was reported for MuRF1 and MuRF2 DKO mice possibly activating the calcineurin/nuclear factor of activated T-cells (NFAT) pathway. Activation of calcineurin could account for increased beta/slow myosin expression. Because calcineurin dephosphorylates calcium channels and phospholamban leading to a reduction in intracellular calcium and calcium uptake by the sarcoplasmic reticulum, its activation could also be responsible for the reduction in systolic and diastolic calcium transients in cardiomyocytes of DKO mice. This could also explain why MuRF2 and MuRF3 DKO hearts and cardiomyocytes did not respond differently when exposed to isoproterenol compared with controls. Normally, isoproterenol mediates phosphorylation of the L-type calcium channel and phospholamban in a protein kinase-A dependent manner leading to increased calcium currents and calcium load of the sarcoplasmic reticulum. However, activation of calcineurin opposes the effects of protein kinase-A. Although it is tempting to speculate that MuRF2 and MuRF3 are involved in regulation of calcineurin...
MAPKAPK3 DKO mice showed enhanced contractility and accelerated relaxation. Therefore, we assume that an increase in myosin heavy chain.

We reported that cardiomyocytes of MAPKAPK2 DKO mice, these findings are particularly important. Recently, we identified MAPKAPK2 and MAPKAPK3 to be elevated in skeletal muscle and hearts of DKO mice. In addition, proteins involved in mitochondrial function and energy supply were reduced in DKO mice, which could account for reduced muscle function in these mice. Because decreased cardiac function is not explained by protein aggregates in DKO mice, these findings are particularly important. Recently, we reported that cardiomyocytes of MAPKAPK2 and MAPKAPK3 DKO mice showed enhanced contractility and accelerated relaxation. Therefore, we assume that an increase in MAPKAPK2 and MAPKAPK3 in DKO hearts leads to an opposing phenotype. Because we also observed improved force parameters in MAPKAPK2/3 DKO soleus muscles, increased MAPKAPK2 and MAPKAPK3 protein levels in MuRF2 and MuRF3 DKO skeletal muscle might account for decreased muscle performance. Similarly, because of the observed positive effect of MAPKAPK2/3-deficiency on the oxidative energy metabolism, the increase of MAPKAPK2/3 protein levels in MuRF2 and MuRF3 DKO mice might, at least in part, account for reduced mitochondrial function and energy supply in DKO skeletal muscle. However, it remains to be elucidated how the absence of MuRF2 and MuRF3 leads to an increase in MAPKAPK2 and MAPKAPK3 because we did not observe changes in MAPKAPK2 and MAPKAPK3 gene expression and could not detect physical interaction between MAPKAPK2 and MAPKAPK3 with MuRF2 and MuRF3 in co-immunoprecipitation and co-localization experiments. Although physical interaction between MAPKAPK2/3 and their substrates are difficult to detect, the increase in MAPKAPK2/3 protein amounts could also be caused indirectly because of altered p38 MAPK-MAPKAPK2/3 signalling in MuRF2 and MuRF3 DKO mice. For example, because activated MAPKAPK2/3 shuttles from the nucleus to the cytoplasm, altered p38 MAPK-MAPKAPK2/3 signalling in DKO muscle might influence the duration time of MAPKAPK2/3 in one of the two cellular compartments and thereby affect MAPKAPK2/3 protein stabilities. Thus, further studies are needed to elucidate the mechanism of increased MAPKAPK2 and MAPKAPK3 contents in DKO muscle.

The p38 MAPK-MAPKAPK2/3 cascade not only regulates gene expression at the transcriptional and post-transcriptional level but also causes post-translational modifications leading to altered protein folding and degradation. For example, activated p38 MAPK was shown to interact with and phosphorylate E3 ubiquitin ligases, such as Siah2 and MIB1, resulting in increased ligase activity. In addition, MAPKAPK2 directly interacts with and phosphorylates the E3 ubiquitin ligase Hdm2/Mdm2 resulting in increased E3 ligase activity and protein degradation. Although it is tempting to speculate that such a relationship exists for MAPKAPK2/3, MuRF2, and MuRF3, a direct association between these proteins needs to be proven. It would also be interesting to investigate if the reduced phenotype in the skeletal muscle and heart of MuRF2 and MuRF3 DKO mice is directly associated with increased MAPKAPK2/3 protein levels, and could be abolished in compound MAPKAPK2/3 and MuRF2/3 quadruple KO mice.

We conclude that MuRF2 and MuRF3 are concomitantly involved in the regulation of structure and performance of the skeletal muscle and the heart. MuRF2 and MuRF3 play a role in fibre type specification of slow/type I fibres in extensor digitorum longus and soleus. Additionally, MuRF2 and MuRF3 affected mitochondrial function and energy supply in striated muscle. Accordingly, absence of MuRF2 and MuRF3 leads to a myopathic phenotype of the skeletal muscle and the heart.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web-site.

Appendix: Non-standard abbreviations

DKO: MuRF2 and MuRF3 double knockout mice

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Conflict of interest

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