F-Box and Leucine-Rich Repeat Protein 22 Is a Cardiac-Enriched F-Box Protein That Regulates Sarcomeric Protein Turnover and Is Essential for Maintenance of Contractile Function In Vivo

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Rationale: The emerging role of the ubiquitin–proteasome system in cardiomyocyte function and homeostasis implies the necessity of tight regulation of protein degradation. However, little is known about cardiac components of this machinery.

Objective: We sought to determine whether molecules exist that control turnover of cardiac-specific proteins.

Methods and Results: Using a bioinformatic approach to identify novel cardiac-enriched sarcomere proteins, we identified F-box and leucine-rich repeat protein 22 (Fbxl22). Tissue-specific expression was confirmed by multiple tissue Northern and Western Blot analyses as well as quantitative reverse-transcriptase polymerase chain reaction on a human cDNA library. Immunocolocalization experiments in neonatal and adult rat ventricular cardiomyocytes as well as murine heart tissue located Fbxl22 to the sarcomeric z-disc. To detect cardiac protein interaction partners, we performed a yeast 2-hybrid screen using Fbxl22 as bait. Coimmunoprecipitation confirmed the identified interactions of Fbxl22 with S-phase kinase-associated protein 1 and Cullin1, 2 critical components of SCF (Skp1/Cul1/F-box) E3-ligases. Moreover, we identified several potential substrates, including the z-disc proteins α-actinin and filamin C. Consistently, in vitro overexpression of Fbxl22-mediated degradation of both substrates in a dose-dependent fashion, whereas proteasome inhibition with MG-132 markedly attenuated degradation of both α-actinin and filamin C. Finally, targeted knockdown of Fbxl22 in rat cardiomyocytes as well as zebrafish embryos results in the accumulation of α-actinin associated with severely impaired contractile function and cardiomyopathy in vivo.

Conclusions: These findings reveal the previously uncharacterized cardiac-specific F-box protein Fbxl22 as a component of a novel cardiac E3 ligase. Fbxl22 promotes the proteasome-dependent degradation of key sarcomeric proteins, such as α-actinin and filamin C, and is essential for maintenance of normal contractile function in vivo. (Circ Res. 2012;111:1504-1516.)

Key Words: cardiac myocytes ▪ cardiomyopathy ▪ F-box ▪ ubiquitin–proteasome system

Maintenance of cellular integrity and homeostasis requires tight control of the delicate balance of protein synthesis and degradation. This is of particular importance in heart and muscle tissue, in which a highly structured and compartmentalized cellular architecture has to be kept stable under conditions of permanent biomechanical stress. Several mechanisms ensure proper regulation of the proteolysis of cardiac proteins, including the ubiquitin–proteasome system (UPS), the calpain system, and autophagy, which mediates the controlled degradation of autosomal content. Of note, there is significant cross-talk between these systems, indicating an additional level of complexity in the regulation of protein turnover.
The UPS recognizes and targets (via ubiquitination) specific proteins for subsequent ATP-dependent degradation by the 26S proteasome. Ubiquitination via the UPS machinery is a complex process that requires the concerted action of many different enzymes. The essential 3 steps of the ubiquitination cascade are ubiquitin activation via the enzyme E1, ubiquitin conjugation via one of multiple E2 enzymes, and ubiquitin ligation by one of >100 E3s. The latter are of particular importance because E3 ligases confer substrate specificity to the complex, thereby providing a mechanism for fine-tuning the equilibrium of synthesis and degradation for many specific proteins. Polyubiquitination involving the lysine 48 residue of ubiquitin typically results in proteasomal degradation of the ubiquitinated substrates (canonical pathway). However, it is well-recognized that monoubiquitination or noncanonical polyubiquitination of target proteins (eg, via lysine 63 of ubiquitin) serves important roles in intracellular signaling such as regulation of transcription or activation of kinases.

The subgroup of E3 ligases that has been most widely characterized and that has been shown to be involved in a multitude of cellular functions is the S-phase kinase-associated protein 1 (Skp1)/Cullin/F-box (SCF) ubiquitin ligase complex, named for its principal components Skp1, Cullin, and an F-box protein. Within the SCF complex, F-box-containing proteins typically mediate substrate specificity via distinct protein–protein interaction domains and facilitate the transfer of ubiquitin from the respective E2 enzyme to the substrate, thereby determining its fate (degradation by the proteasome, altered signaling, or subcellular localization, and others).

A number of E3 ligases have been identified to be striated muscle-enriched or even muscle-specific, including Atrogin-1, the muscle-specific RING finger protein (MuRF)-family, or carboxyl terminus of Hsp70-interacting protein. Interestingly, many of the currently known targets of muscle-enriched E3 ligases are sarcomeric proteins, implying that the proper control of their quality as well as stoichiometry is of critical importance for myocyte integrity and function.

However, little is known about additional components that constitute and regulate the UPS in cardiomyocytes. A deeper understanding of the specific components of the cardiac proteasome appears to be of particular interest, because several heart diseases recently have been shown to involve dysregulation of the UPS, also. Although the activity of the proteasome itself is not consistently downregulated in diseased hearts, several mouse models of cardiac hypertrophy and failure reveal significant alterations in the amount of ubiquitinated proteins and the expression levels of several E3 ligases, including atrogin-1, MuRF1, Mdm2, and carboxyl terminus of Hsp70-interacting protein. Similarly, various reports indicate that human cardiomyopathy (eg, desminopathy or hypertrophic cardiomyopathy attributable to MyBP-C mutations) is associated with an increase of ubiquitinated cardiac proteins as well as malfunction of the cardiac UPS, resulting in the accumulation of misfolded or damaged proteins.

We report the first description of a previously uncharacterized cardiac-enriched F-box protein, termed F-box and leucine-rich repeat protein 22 (Fbxl22). Fbxl22 is a component of a novel bona fide SCF-E3 ligase, because it builds a complex with Skp1 and Cullin1 through its N-terminal F-box. Two important and abundant sarcomeric proteins, α-actinin-2 (ACTN) and filamin C (FLNC), were identified as substrates that are ubiquitinated in Fbxl22-dependent fashion. Consistently, overexpression of Fbxl22-facilitated proteasomal degradation of both ACTN and FLNC, whereas proteasome inhibition abrogated this effect. Conversely, targeted knockdown of Fbxl22 in zebrafish results in accumulation of α-actinin associated with severe contractile dysfunction and cardiomyopathy.

**Methods**

Detailed experimental procedures for cloning, bioinformatics, Northern blot analyses, Western blot analyses, immunofluorescence experiments, yeast 2-hybrid assays, tissue culture, immunoprecipitations, and reporter gene assays, as well as zebrafish injection procedures and fractional shortening measurements, are provided in the Online Data Supplement. Statistical analyses of the data were performed using ANOVA, followed by Student-Newman-Keuls post hoc tests. When appropriate, Student t test was used (2-sided, assuming similar variances). P < 0.05 was considered statistically significant. The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

**Results**

**Fbxl22 Is a Novel Cardiac-Enriched F-Box Protein**

To identify novel sarcomeric cardiac-enriched genes, we searched the expressed sequence tag databases for previously uncharacterized sequences predominantly found in cardiac cDNA libraries. The data extraction mainly relied on the T-STAG (tissue-specific transcripts and genes) and the Unigene (National Center for Biotechnology; http://www.ncbi.nlm.nih.gov/unigene) databases. Several of the newly identified expressed sequence tags corresponded to the human Unigene cluster Hs.656997 or the Mus musculus Unigene cluster Mm.297998. Expressed sequence tags in these clusters were significantly enriched for heart compared with other tissues (Online Figure IA). Subsequent bioinformatic analyses revealed identity with a predicted protein termed Fbxl22 (National Center for Biotechnology Information reference sequences: human [NM_203373, NP_976307], mouse [NM_175206, NP_780415]). These sequences were used as templates for the design of primers for the amplification of the complete open reading frames, which predicted a novel 241 (human)/236 (mouse) amino acid protein with a calculated molecular weight of 27 kDa (human) and 26 kDa (mouse), respectively. Northern blot analyses in murine tissues showed a cardiac-specific expression pattern with only weak bands in uterus and skeletal muscle (Figure 1A). Similarly, quantitative reverse-transcriptase polymerase chain reaction conducted on the hearts, several mouse models of cardiac hypertrophy and failure.
samples of a human cDNA library confirmed cardiac-specific/muscle-specific expression of Fbxl22 (Online Figure IB).

To next analyze the expression of Fbxl22 on the protein level, a Western blot of extracts from several mouse tissues was probed with a polyclonal antibody against Fbxl22. Again, a strong expression of Fbxl22 protein in mouse heart tissue at the predicted size of \( \approx 26 \text{kDa} \) was observed (Figure 1B). Fbxl22 is detected at \( \approx 1.6 \text{kb} \) (ladder in kb). B, Immunoblot of adult murine tissues with anti-Fbxl22 confirms predominant cardiac expression of Fbxl22 at \( \approx 26 \text{kDa} \). Bottom, Normalization with tubulin. C, Alignment of Fbxl22 sequences from several different species: homo sapiens, mus musculus, rattus norvegicus, bos Taurus, and danio rerio. The N terminus of Fbxl22 (AA1-113), which contains the F-box (AA1-44), is highly conserved across species, whereas the C terminus is less well-conserved. Identical amino acids are highlighted with darker shades of blue, and black bars below indicate the consensus sequence. D, Schematic structure of human Fbxl22 revealing the F-box motif (yellow) located in its highly conserved N-terminal part and exhibiting an aggregation of numerous leucine residues (19% of all amino acids). The less conserved C-terminal part of Fbxl22 contains several regions of low complexity (denoted in pink).

Fbxl22 Is Located at the Cardiac Z-Disc

Having shown a high expression level of Fbxl22 in the myocardium, we next aimed to determine its subcellular

localization by using immunostaining experiments. Staining of cultivated neonatal rat ventricular cardiomyocytes with anti-Fbxl22 and anti-calsarcin-1 antibodies revealed colocalization of both proteins at the level of the sarcomeric z-disc (Figure 2A). Likewise, immunostaining of isolated adult rat ventricular cardiomyocytes with anti-calsarcin-1 and anti-Fbxl22 confirmed a z-disc localization for Fbxl22 (Figure 2B, Online Figure II). Consistently, cryosections of intact murine heart again showed a z-disc specific signal (Figure 2C, Online Figure II), consistent with the notion that Fbxl22 is a component of the sarcomere.

**Fbxl22 Is a Novel Member of the Cardiac UPS**

Fbxl22 contains the F-box domain, which implies a role in the ubiquitin–proteasome machinery. Specifically, Fbxl22 may be a component of a novel cardiac-specific SCF–E3 ligase complex. To identify potential protein interaction partners, we performed a yeast 2-hybrid screen using Fbxl22 as bait (Figure 3A). Screening of a human cardiac cDNA library yielded 2 clones of Skp1, a well-characterized part of the SCF machinery known to interact with the F-box motif of several F-box proteins. To confirm this interaction in mammalian cells, human embryonic kidney (HEK)293 cells were transfected with hemagglutinin-tagged Fbxl22 and Myc-tagged Skp1. As shown in Figure 3C, coimmunoprecipitation studies confirmed a strong interaction of the 2 proteins. Moreover, the precipitated protein complex also contained endogenous Cullin1, another critical component of the SCF–E3 ligase machinery (Figure 3C). To also confirm this interaction in cardiomyocytes, we repeated this experiment with adenovirally overexpressed Fbxl22 in neonatal rat ventricular cardiomyocytes. Again, endogenous Skp1 could be coimmunoprecipitated by Fbxl22 (Figure 3D).

**Fbxl22 Directly Interacts With ACTN and FLNC**

The yeast 2-hybrid screen also yielded 2 sarcomeric proteins, α-actinin and FLNC (Figure 3A), which are known to be located at the cardiac Z-disc, consistent with the subcellular

Figure 2. F-box and leucine-rich repeat protein 22 (Fbxl22) is localized to the sarcomeric z-disc in neonatal and adult cardiomyocytes. A, Subcellular localization of Fbxl22 in neonatal rat ventricular cardiomyocytes (NRVCM). Staining of NRVCM with a Fbxl22 antibody (fluorescein isothiocyanate-green) reveals a strong signal at the sarcomeric z-disc, validated by colocalization with the known z-disc protein calsarcin-1 (Cy3-red). Scale bar, 5 μm. B, Subcellular localization of Fbxl22 in adult rat ventricular cardiomyocytes (ARVCM), which were stained with antibodies against Fbxl22 (green) and calsarcin-1 (red). When signals are merged, a colocalization at the sarcomeric z-disc is observed. Scale bar, 5 μm. C, Subcellular localization of Fbxl22 in the intact murine heart. Heart sections were stained with antibodies against Fbxl22 (green) and calsarcin-1 (red). Again, Fbxl22 was found to localize to the z-disc, confirming its subcellular localization in cardiac tissue. Scale bar, 5 μm.
localization of Fbxl22. In accordance with the interacting domains (Figure 3B), we engineered epitope-tagged expression constructs of ACTN and the C-terminal part of FLNC, respectively, and cotransfected these with Fbxl22. Subsequent coimmunoprecipitation and Western blot analyses validated the interactions of Fbxl22 with both ACTN and FLNC (Figure 3E and 3F). Consistently, confocal microscopy reveals colocalization of Fbxl22 with FLNC at the z-disc of isolated cardiomyocytes (Online Figure III). Because sequence analyses of Fbxl22 (Figure 1C and 1D) had revealed that its N terminus is highly conserved across different species, we speculated that this part of the protein might confer the interaction with the identified binding partners. Therefore, an expression construct encoding for the 113 aminoterminal amino acids of Fbxl22 was expressed in cardiomyocytes. Coimmunoprecipitation with an antibody against the epitope tag followed by immunoblotting with a Myc antibody confirmed the association of Fbxl22 and Myc-tagged Skp1. Reincubation with an antibody against Cullin1, another known member of the SCF complex, also coimmunoprecipitates with Fbxl22. Input controls for Skp1 (IB: Myc) and Fbxl22 (IB: HA) are shown in the middle panels. Coimmunoprecipitation of Fbxl22 with endogenous Skp1. Cardiomyocytes (neonatal rat ventricular cardiomyocytes [NRVCM]) were infected with V5-tagged Fbxl22 as indicated. Coimmunoprecipitation with V5 antibody followed by immunoblotting with Skp1 antibody confirmed the association of Fbxl22 and endogenous Skp1. Input controls for Skp1 and Fbxl22 are shown in the lower panels. Coimmunoprecipitation of Fbxl22 and FLNC. HEK cells were transfected with HA-tagged Fbxl22 and Myc-tagged ACTN as indicated. Immunoprecipitation with an antibody against the epitope tag followed by immunoblotting with an antibody against FLNC confirmed the association of Fbxl22 and FLNC. Input controls are shown in the lower panels. Coimmunoprecipitation of Fbxl22 and ACTN. Neonatal rat ventricular cardiomyocytes were infected with V5-tagged Fbxl22 as indicated. Immunoprecipitation with an antibody against the epitope tag followed by immunoblotting with an antibody against ACTN confirmed the association of Fbxl22 and ACTN. Input controls are shown in the lower panels.
acids was cloned and, again, HEK293 cells were transfected to perform coimmunoprecipitation experiments. This construct was sufficient to mediate the interaction with endogenous Skp1 (Online Figure IVA), consistent with the known role of F-box domains to bind to this component of the E3 ligase complex. Similarly, the N terminus of Fbxl22 strongly precipitated α-actinin (Online Figure IVB) and FLNC (Online Figure IVC), suggesting that these interactions also are mediated by the F-box containing and leucine-rich part of Fbxl22.

Next, we asked whether these interactions also occur in cardiomyocytes. We therefore performed a similar experiment with adenovirally overexpressed Fbxl22 in neonatal rat ventricular cardiomyocytes. Consistently, endogenous α-actinin could be coimmunoprecipitated by Fbxl22 (Figure 3G).

**Fbxl22 Facilitates Degradation of ACTN and FLNC**

After the identification of several sarcomeric proteins as binding partners for Fbxl22, we sought to investigate the potential function of these interactions and in which cellular processes Fbxl22 might be involved. Specifically, ACTN and FLNC might represent substrates for Fbxl22, because F-box proteins have been shown to target proteins to the proteasome.23,24

Thus, HEK293 cells were cotransfected with plasmids encoding for Fbxl22 and ACTN or FLNC, respectively. Western blot analyses confirmed a marked dose-dependent decrease of ACTN levels in the presence of Fbxl22 (Figure 4A). As shown in Figure 4B, densitometric analyses of several experiments (n=3) revealed a highly significant reduction of substrate levels by Fbxl22 by up to 66% (±16% SEM; **P<0.001**; Figure 4D). Similarly, cotransfection of FLNC and Fbxl22 again led to a significant decrease of filamin C (Figure 4C). Densitometric analysis revealed a 66% reduction of filamin levels in the presence of Fbxl22 (±12% SEM; **P<0.001**; Figure 4D).

Next, we investigated whether the same mechanism also is operative in cardiomyocytes. Again, upregulation of Fbxl22...
resulted in significant downregulation of endogenous α-actinin expression. Conversely, targeted knockdown of Fbxl22 using a synthetic adenovirally encoded microRNA led to accumulation of α-actinin in cardiomyocytes (Figure 4G and 4H). Of note, ACTN and FLNC mRNA levels were not significantly altered by Fbxl22 overexpression (Online Figure V). Taken together, these data suggest that Fbxl22 is sufficient to facilitate the degradation of at least 2 sarcomeric proteins, ACTN and FLNC, whereas its downregulation results in accumulation of its targets.

**Fbxl22-Mediated Degradation of ACTN and FLNC Is Proteasome-Dependent**

Proteasomal inhibition repeatedly has been demonstrated to attenuate SCF-mediated degradation of specific substrates. To provide further evidence of Fbxl22-facilitated proteasome-dependent degradation of ACTN and FLNC, we used the proteasome inhibitor MG-132 and repeated the experiments described. MG-132 completely prevented Fbxl22-mediated degradation of both ACTN (Figure 5A) and FLNC (Figure 5B), implying that Fbxl22 functions as a bona fide SCF-E3 ligase that mediates the proteasomal degradation of its sarcomeric substrates.

**Fbxl22 Promotes Ubiquitination of ACTN and FLNC**

Because SCF ligases are known to determine the fate of their respective substrates (eg, degradation, signaling) by specific ubiquitination, we hypothesized that the observed increased proteasomal degradation of ACTN and FLNC is associated with Fbxl22-mediated ubiquitination of these substrates. To validate this concept of ubiquitin-driven substrate degradation and to assess whether Fbxl22 and its associated proteins (Skp1, Cullin1) truly function as SCF-E3 ligase, we performed ubiquitination experiments.

Thus, HEK293 cells were transfected with plasmids encoding for Fbxl22, ubiquitin, and myc-tagged ACTN (Figure 6). In an in vitro ubiquitination assay, lysates from these cells then were incubated with ubiquitination buffer (containing MG-132, ubiquitin-aldehyde, dithiothreitol, and ATP) and incubated for 30 minutes at 37°C. Immunoblotting revealed a slurry of slower migrating, ubiquitinated ACTN in the presence of Fbxl22, which could be further enhanced by addition of ubiquitin (Figure 6A). Similarly, proteasome-inhibited HEK293 cells were transfected with plasmids encoding for Fbxl22, ubiquitin, and α-actinin or FLNC, respectively. Immunoprecipitation with Fbxl22 resulted in a marked increase in the amount of ubiquitinated substrates (Figure 6B and 6C), in line with the notion that Fbxl22 is an essential component of a novel cardiac-specific SCF-E3 ligase. Finally, cells again were transfected with Fbxl22 and Myc-tagged ACTN (but without additional ubiquitin), and were subjected to treatment with MG-132 or control (Online Figure VI). Fbxl22-facilitated degradation of ACTN (Online Figure VI, upper panel, lanes 1–2), whereas proteasome inhibition by MG-132 significantly attenuates this Fbxl22-dependent effect (Figure 5D, upper panel, lanes 3–4). Interestingly, ubiquitin precipitates of ACTN only accumulate in the presence of the proteasome inhibitor (Online Figure VI, lower panel, lane 4). To exclude an unspecific effect of Fbxl22 on ubiquitination, we assayed global ubiquitination levels in Fbxl22-depleted cardiomyocytes (neonatal rat ventricular cardiomyocytes) under starving conditions and in a growth-stimulated environment. Neither experiment showed a differential effect, consistent with the notion that Fbxl22 does not influence total ubiquitination (Online Figure VII). Taken together, these ubiquitination studies
confirm a dose-dependent increase in Fbxl22-dependent ubiquitination and subsequent degradation of the specific substrates ACTN and FLNC.

**Fbxl22 Is Downregulated on Cardiomyocyte Stress**
Next, we asked whether the expression level of Fbxl22 is differentially regulated under conditions of cardiomyocyte stress. Therefore, we first subjected neonatal rat cardiomyocytes to phenylephrine treatment, a potent inducer of hypertrophy and the fetal gene program (Figure 7A). Phenylephrine was sufficient to strongly induce atrial natriuretic factor expression (30.6×; \( P<0.001 \) versus control). In contrast, Fbxl22 was found to be significantly downregulated (0.23×; \( P<0.001 \) versus control). Similarly, analysis of mouse hearts subjected to pressure overload attributable to transverse aortic constriction revealed induction of atrial natriuretic factor (3.3×; \( P<0.001 \) versus control). Similarly, analysis of mouse hearts subjected to pressure overload attributable to transverse aortic constriction revealed induction of atrial natriuretic factor (3.3×; \( P<0.001 \) versus control). In contrast, Fbxl22 expression was again repressed (0.58×; \( P<0.001 \) versus control). Thus, Fbxl22 expression is subject to differential regulation on pharmacological and biomechanical stress, both in vitro and in vivo.

**Knockdown of Fbxl22 Leads to Progressive Reduction of Cardiac Contractility In Vivo**
To investigate the role of Fbxl22 in vivo, we inactivated zebrafish fbxl22 by injecting morpholino (MO)-modified antisense oligonucleotides directed against the translational start site (MO-fbxl22) into 1-cell-stage zebrafish embryos (Figure 8A). When injected with 2 ng of MO-fbxl22, 91.8±1.3 % of injected embryos (n=505; \( P<0.001 \); Figure 8B) had development of severely reduced cardiac contractility accompanied by signs of heart failure, such as pericardial edema (Figure 8A, Online Movie I), whereas cardiac performance of embryos injected with a standard MO-control was unaffected (n=101; Figure 8A, Online Movie II). To confirm that the fbxl22 morphant heart contractility phenotype is specific to the loss of fbxl22 function, we injected a second independent MO-modified antisense oligonucleotide directed against the splice donor site of exon 1 (MO2-fbxl22) of zebrafish fbxl22 into 1-cell-stage zebrafish embryos. Injection of 0.4 ng of MO2-fbxl22 leads to identical phenotypic characteristics compared with MO-fbxl22 morphant zebrafish embryos (n=505; \( P<0.001 \); Online Figure VIII), confirming specificity of the observed fbxl22 morphant heart failure phenotype. To quantify this observation, we assessed fractional shortening of the ventricular chamber in Fbxl22 morphant embryos at 48 and 72 hours postfertilization (hpf). As in embryos injected with MO-control, the 2 heart chambers–atrium and ventricle–contract rhythmically, sequentially, and vigorously in Fbxl22 morphant embryos by 36 hours of fertilization (hpf;
morphant hearts (Figure 8C). As revealed by whole-mount analyses, embryonic heart morphogenesis proceeds normally at the structural and molecular levels. As shown by histological analyses, we next analyzed Fbxl22 morphant hearts on the structural level. Although there were no apparent abnormalities in Fbxl22 morphant hearts, we observed a further enhanced accumulation of α-actinin in MG132-treated embryos compared with controls (3.7-fold upregulation; n=45 pooled embryos; Figure 8D), substantiating our previous findings in mammalian cells. To further confirm this finding, we next performed Fbxl22-knockdown experiments followed by incubation of the injected embryos with the proteasome inhibitor MG-132. As hypothesized, we observed a further enhanced accumulation of α-actinin in MG132-treated embryos compared with controls (3.7-fold upregulation; n=52 pooled embryos; Figure 8D). In contrast, several other potential target proteins such as myosin, cadiherin, α-actin, or tropomyosin did not reveal an upregulation on knockdown of Fbxl22 (Figure 8E), suggesting that Fbxl22 promotes degradation of target proteins in a selective fashion.

Discussion

Here, we provide the detailed molecular and functional characterization of a previously uncharacterized cardiac-enriched F-box protein, termed Fbxl22.

The F-box protein motif, a (loosely) conserved 40-amino acid sequence, initially has been identified in cyclin F, where it is required for the interaction with Skp1 and subsequent proteasome-dependent cyclin degradation. In subsequent studies, the F-box hypothesis has been confirmed and extended to multiple other proteins. In this concept, F-box-containing proteins are essential elements of the UPS, conferring substrate specificity to ubiquitin ligation. Consistently, we show that Fbxl22 also forms a complex with Skp1 and cullin, the principal components of an SCF-E3 ubiquitin ligase. Moreover, overexpression of Fbxl22 led to a dose-dependent ubiquitination and proteasomal degradation of its newly identified
targets, ACTN and FLNC. Conversely, proteasome inhibition with MG-132 markedly blunted these effects, again consistent with the notion that Fbxl22 is a novel component of a bona fide cardiac E3 ligase.

Similar to other tissues, the UPS increasingly has been recognized as a key mechanism in the control of protein turnover in the heart. Nonetheless, only a few other striated muscle-specific E3 ligases have been identified to date, including atrogin-1/
muscle atrophy F-box, the MuRF protein family members, carboxyl terminus of Hsp70-interacting protein, and ozz. Of these, only the MuRFs and, in particular, MuRF-1 also have been shown to target important sarcomeric proteins such as myosin-binding protein C and the myosin heavy chains. Like Fbxl22, MuRF3 also targets the muscle-specific filamin isoform, FLNC, suggesting a potential redundancy in the control of the intracellular levels of this essential muscle protein. The tight control of FLNC levels is further underscored by the existence of a ubiquitin-specific protease 25, which has been shown to deubiquitinate filamin.

In contrast, no other E3 ligase so far has been shown to mediate degradation of ACTN, which is another key component of the sarcomere, and especially of the sarcomeric z-disc. Fbxl22 is an attractive candidate in this regard because we could show that it not only facilitates actinin degradation but also is localized at the sarcomeric z-disc. Remarkably, most other muscle E3 ligases also have been shown to localize to the z-disc (atrogin-1, MuRFs, TRIM32), consistent with the emerging concept that this structure is a nodal point in intracellular signaling. In this regard, the UPS has been demonstrated not only to control turnover of key proteins in the heart and skeletal muscle but also to participate in diverse signaling pathways. For example, atrogin-1 has been ascribed an important role in regulating the phosphatase calcineurin as well as forerkhead proteins such as FoxO3, which in turn modify hypertrophic signaling. Similarly, the UPS regulates apoptosis via mdm2/carboxyl terminus of Hsp70-interacting protein-dependent degradation of p53. In skeletal muscle tissue, muscle atrophy is controlled by upregulation of the E3 ubiquitin ligases atrogin-1 and MuRF1. Conversely, muscle-specific ring finger 1-deficient mice are resistant to both skeletal muscle and cardiac atrophy.

Recently, the UPS also has been linked to human cardiomyopathy and heart failure. In most studies, failing hearts reveal an accumulation of ubiquitinated proteins, implying a general increase in proteasome activity. Moreover, impairment of the UPS has been linked to contractile dysfunction in experimental mouse models. Likewise, a general decrease in F-box proteins attributable to downregulation of CSN (COP9 signalosome) results in cardiac hypertrophy and severe heart failure.

Moreover, many individual components of the UPS, including several E3 ligases, have been shown to be dysregulated in heart failure. For example, atrogin-1 is upregulated in doxorubicin-induced cardiomyopathy, and both atrogin-1 and MuRF-1 are induced in experimental ischemic cardiomyopathy. Furthermore MuRF-1 and atrogin-1 have been implicated in hypertrophic cardiomyopathy, because both E3 ligases mediate the decay of truncated mutant myosin-binding protein C. Moreover, marked UPS dysfunction also has been demonstrated in desmin-related cardiomyopathy. Of note, both newly identified targets for Fbxl22-dependent degradation, ACTN and FLNC, have been linked to the pathogenesis of inherited cardiac and skeletal muscle diseases. ACTN has been shown to be a disease-causing gene for both dilated and hypertrophic cardiomyopathy, supporting the view of actinin as a key z-disc protein in the maintenance of cardiac integrity and function. Similarly, FLNC as the other Fbxl22 target identified in our study has been demonstrated to be a disease gene for myofibrillar myopathy. This striated muscle disease, predominantly affecting skeletal muscle but also the heart, is characterized by an accumulation of a mutated sarcomeric protein, typically proteins associated with z-disc such as FLNC, ZASP, or desmin. Thus, it is tempting to speculate that myofibrillar myopathy–associated filamin mutations may alter its Fbxl22-dependent degradation.

A potential role of Fbxl22 in cardiac diseases in general and cardiomyopathy in particular is supported by our finding that targeted ablation of Fbxl22 in zebrafish leads to considerable accumulation of α-actinin associated with severe contractile dysfunction in vivo. However, we could not detect actinin aggregates in zebrafish or cardiomyocytes on downregulation of Fbxl22 by immunofluorescence or electron microscopy. Thus, the exact mechanism responsible for cardiomyopathy in Fbxl22-knockdown zebrafish still remains unclear. It is conceivable that other potential mechanisms beyond mere (toxic) cellular accumulation of actinin and filamin may play a role, (eg, the upregulation of other still unknown Fbxl22 targets). Of note, despite the downregulation of Fbxl22, cardiomyocyte architecture as well as maturation were largely intact, suggesting that the observed cardiomyopathy is not merely secondary to structural alterations. Moreover, it remains to be established whether the accumulation of α-actinin is merely associated with cardiomyopathy or if a true causal relationship exists.

Finally, we found Fbxl22 markedly downregulated on cardiomyocyte stress, both in vitro and in vivo. Thus, it will be interesting to explore whether differential regulation of Fbxl22 is required for the stress response of cardiomyocyte and development of hypertrophy. Interestingly, small hairpin-RNA-mediated downregulation of FLNC inhibits cardiomyocyte hypertrophy. It is therefore conceivable that upregulation of FLNC and other key sarcomeric proteins is necessary for cardiomyocyte hypertrophy. Nevertheless, our current data cannot definitely answer these questions. Gain-of-function and loss-of-function experiments under conditions of increased cardiomyocyte stress will be required to ultimately define the role of Fbxl22 in vivo and, in particular, in cardiac disease.

In summary, we characterized a novel cardiac-specific F-box protein termed Fbxl22, which recruits 2 important sarcomeric z-disc proteins, ACTN and FLNC, for UPS-dependent degradation. Downregulation of Fbxl22 in vitro and in vivo results in a dysbalance of myocardial α-actinin content and impairment of contractile function. Additional in vivo experiments as well as analyses of Fbxl22 in patient samples will help to clarify whether this novel cardiac E3 ligase also participates in cardiac signaling pathways and the pathogenesis of cardiomyopathy.

Acknowledgments

We thank Ulrike Oehl and Jutta Kebs for excellent technical assistance, as well as Hiltraud Hosser for performing the ultrastructural analyses. M. Treier (MDC, Berlin) kindly provided the HIS-ubiquitin construct. The gift of the FLNC antibody by P. van der Ven and D. Fürst is highly appreciated.

Sources of Funding

N.F. and W.R. were supported by grants of the Bundesministerium für Bildung und Forschung (BMBF/NGFNplus).
Disclosures

None.

References


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**Novelty and Significance**

**What Is Known?**

- The ubiquitin–proteasome system plays an important role in maintaining myocyte integrity and homeostasis by tightly controlling protein degradation. F-box proteins as part of S-phase kinase-associated protein 1-Cullin-F-box–E3 ligases provide a substrate-specific mechanism for this machinery; however, little is known about cardiac-specific components of the ubiquitin–proteasome system.

- The cardiac z-disc is a key structure required for cardiomyocyte stability and cell signaling. Defects in this structure and several sarcomeric proteins have been linked to the pathogenesis of multiple forms of cardiomyopathies.

**What New Information Does This Article Contribute?**

- The cardiac-enriched F-box protein termed F-box and leucine-rich repeat protein 22 (Fbxl22) is located at the cardiac z-disc and interacts with key sarcomeric proteins.

- Fbxl22 forms a previously unknown S-phase kinase-associated protein 1-Cullin-F-box–E3 ligase that facilitates ubiquitination and degradation of alpha-actinin-2 and filamin C.

- Knockdown of Fbxl22 leads to accumulation of alpha-actinin-2 and results in marked impairment of cardiac function associated with severe cardiomyopathy in zebrafish.

Cardiomyocyte homeostasis depends on tight regulation of protein synthesis and degradation. However, the mechanism and molecular components that mediate turnover and degradation of cardiac proteins are still incompletely understood. In this study, we identified the previously uncharacterized cardiac-enriched F-box protein Fbxl22, which is located at the cardiac z-disc and directly interacts with S-phase kinase-associated protein 1, alpha-actinin-2, and filamin C. Moreover, it facilitates the proteasomal degradation of its sarcomeric binding partners. Conversely, knockdown of Fbxl22 is associated with increased levels of alpha-actinin-2 and filamin C and results in significant impairment of cardiac function. Finally, Fbxl22 expression levels were found to be significantly downregulated in models of cardiac stress. These findings enhance the understanding of substrate-specific protein degradation in cardiomyocytes and may shed new light on the role of ubiquitin–proteasome system-dependent z-disc protein maintenance in physiological signaling as well as cardiomyopathy.
F-Box and Leucine-Rich Repeat Protein 22 Is a Cardiac-Enriched F-Box Protein That Regulates Sarcomeric Protein Turnover and Is Essential for Maintenance of Contractile Function In Vivo

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Circ Res. 2012;111:1504-1516; originally published online September 12, 2012;
doi: 10.1161/CIRCRESAHA.112.271007

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Data Supplement (unedited) at:
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**EST-Screen**

With the focus being on bioinformatically classified F-box proteins (and E3-ligases) an electronic screen of the EST-database was performed. The aim was to identify the F-box protein with the highest degree of cardiac-specific expression. The data extraction mainly relied on the T-STAG (Tissue-Specific Transcripts and Genes) and the Unigene databases.

**Cloning of Fbxl22**

DNA expression plasmids encoding for Fbxl22 were generated using standard PCR methods on human, mouse and rat cardiac cDNA, employing the Gateway Cloning System (Invitrogen). In these experiments, the following gene-specific primers were utilized:

humanFbxl22-F (5’-GCTGGCACCATGCACATAACCCAGCTCAAC-3’)
humanFbxl22-R (5´- GCTGGGTCGCCTCAGTCGCACACCCGGAG-3´) (with stop codon)
humanFbxl22-RnoS (5´-GCTGGGTCGCCGTCGCACACCCGGAG-3´) (without stop codon)
ratFbxl22-F (5’-GCTGGCCACCATGCACATAACCCAGCTCAAT-3’)
ratFbxl22-RnoS (5´-GCTGGGTCGCCACCTGTTGCACACCTGCA-3´) (without stop codon)

For integration of the specific recombination site into the PCR-products, a second PCR was performed using two universal primers: attB-F (5’-GGGGACAAGTTTGTACAAAAAAGCTGGCACC-3’) and attB-R (5’-GGGGACCACTTTGTACAAGAAAGCTGGGTCGCC-3’) and 1 μl of the initial PCR-reaction as template. The PCR-product containing the recombination-specific attachment sites (att sites) was then recombined into the pDON201 entry vector. For recombinant protein expression, Fbxl22 cDNA was subsequently shuttled into Gateway compatible expression plasmids in order to obtain expression constructs encoding for amino-terminal (HA, MYC) or C-terminal (V5, His) fusion tags. All expression constructs were sequenced and tested for mutations prior to use.

**Cloning of synthetic Fbxl22 knockdown microRNAs:**

Knockdown Oligonucleotides miFbxl22_TOP (TGC TGT GCA GGA GGA AAT CAT TAA CCG TTT TGG CCA CTG ACT GAC GGT TAA TGT TCC TCC TGC A) and miFbxl22_Bottom (CCT GTG CAG GAG GAA CAT TAA CCG TCA GTC AGT GGC CAA AAC GGT TAA TGA TTT CCT CCT GCA C) were designed using Invitrogen’s BLOCK-iT™ RNAi Designer and subsequently cloned into the pcDNA™6.2-GW/EmGFP-miR vector according to manufacturer’s instructions. This construct was used as template for the recombination into the pDON201 entry vector which itself served as template for the generation expression constructs. As negative control we used the pcDNA™6.2-GW/EmGFP-miR plasmid, which can form a hairpin structure and is consecutively processed into a mature miRNA, yet is predicted not to target any known mammalian gene (sequence of the insert: 5’-GAA ATG TAC TGC GCG TGG AGA CGT TTT GCC TGG AGA CGT TTC GGC CAC TGA CTG ACG TCT CCA CGC AGT ACA TTT-3’).

**Generation of recombinant adenoviruses encoding for Fbxl22 or Fbxl22 knockdown miRNA**

Adenoviruses were generated using the appropriate Entry vectors in combination with the ViraPower™ Adenoviral Expression System (Invitrogen) according to manufacturer’s instructions. Ad-GFP encoding adenovirus served as control for overexpression experiments (Invitrogen).

**Northern blot analyses**

Northern blot procedures have been described previously. Briefly, multiple tissue northern blots (BioChain) containing mouse poly(A) RNA were incubated with 32P-dCTP-labeled (Rediprime II Random Prime labeling System, Amersham Biosciences) cDNA probes corresponding to the ORF of mouse Fbxl22 at 65°C for 12 hours. Subsequently, serial washes were conducted with 2 x SSC/0.1% SDS and 0.2 x SSC/0.1% SDS at 65°C. Autoradiography was performed at –80°C for 24-168 hours with an intensifying screen.

**Y2H library screening**

Automated yeast two-hybrid screens were performed as described. Specifically, human cDNA libraries from human heart and skeletal muscle (Clontech) as well as a library of individually cloned
full-length open reading frames from cDNAs of 5000 different genes (DKFZ Heidelberg) were screened to a minimal coverage of 5 million clones per library. To mate yeast strains harbouring the bait protein and the prey library, exponentially growing cultures of an O.D. (600nm) of 1 were combined, pelleted by centrifugation for 2 min at 2900rpm, and resuspended in an equal volume of YPD medium containing 20% PEG 6000. Mating mixes were incubated at 30°C with gentle agitation (100 rpm) for exactly 3 h, before washing and resuspending the cells in selective medium. For the generation of a high-quality dataset, interaction pairs were selected which were isolated at least twice, or where the bait interacted with two highly related preys, and which did not involve promiscuous preys.

**Tissue culture experiments**

HEK-293 and cos7 cells were maintained in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine and penicillin/streptomycin under standard tissue culture conditions (37°C/5% CO2). Cells were grown in 10cm dishes to 90% confluency and transfected using JetPEI (PolyPlus Transfection), according to the manufacturer’s instructions. Medium was changed to DMEM compl. 12h after transfection and cells were grown for an additional 36h. After washing the cells once with ice-cold 1xPBS, lysis was performed using RIPA-I-buffer supplemented with Protease inhibitor (Roche) and Phosphatase Inhibitor (Sigma). A 30min incubation period was followed by centrifugation (5' at 4°C and 12,000g) and transfer of the supernatant to new tubes. Protein concentrations were measured using Bradford Assay and 60-150µg of lysate (depending on assay) were mixed with 4xSDS-loading-buffer, boiled for 5' and loaded on SDS-PAGE-gel. MG-132 (Sigma) was dissolved in DMSO and administered at a final concentration of 10µM.

**Isolation and culture of neonatal rat ventricular cardiomyocytes (NRVCMs)**

NRVCM were isolated according to a previously published protocol 2. Briefly, hearts from 1-2 days old Wistar rats (Charles River) were excised and minced in ADS buffer (120 mmol/L NaCl, 20 mmol/L HEPES, 8 mmol/L NaH2PO4, 6 mmol/L glucose, 5 mmol/L KCl, 0.8 mmol/L MgSO4, pH 7.4). A series of digestion steps was carried out with an enzymatic solution containing collagense type II (0.5 mg/ml, Worthington) and pancreatin (0.6 mg/ml, Sigma-Aldrich) in sterile ADS buffer. A Percoll (GE Healthcare) gradient centrifugation step was applied to remove contaminating fibroblasts from cardiomyocytes. NRVCMs were resuspended and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FCS and penicillin/streptomycin under standard tissue culture conditions (37°C/5% CO2). Adenoviral overexpression of Fbxl22 was facilitated by infection with 20ifu of Adv-Fbxl22_V5, whereas knockdown of Fbxl22 was achieved employing 20ifu of Adv-miFbxl22. Lysates were acquired 48 hours after infection unless denoted otherwise. If proteasome inhibitor was used, MG-132 was applied at a concentration of 10µM at 24h before harvest.

Neonatal rat ventricular cardiomyocytes were stimulated with phenylephrine (PE, 100 µmol/L) for 24 h as indicated.

**Isolation and culture of adult rat ventricular cardiomyocytes**

Adult rat ventricular cardiomyocytes (ARVCM) were prepared as described before 4. Briefly, ARVCMs were isolated from Sprague-Dawley rats (~300g) using collagenase and plated on laminin-coated dishes. ARVCMs were cultured in a HEPES-modified medium 199 (M199, Sigma S7528, supplemented with 5mM taurine, 5mM carnitine, 5mM creatine, 5mM N-mercaptoproprionyl glycine, 0.1µM insulin, 10,000 U/ml penicillin and 10 mg/ml streptomycin, pH 7.25).

**Immunofluorescence microscopy**

The subcellular localization of Fbxl22 was determined in both neonatal and adult rat cardiomyocytes (NRVCM/ARVCM) as well as cryosections of murine WT-hearts using indirect immunofluorescence. Isolated NRVCM and ARVCM were grown for 48h/24h, fixed with methanol/aceton for 5 minutes and co-stained with antibodies against Fbxl22 (Abnova, Catalog #: H00283807-A01, 1:100), and calsarcin-1 (rabbit polyclonal 1:200) or Filamin C (rabbit polyclonal 1:50)5. Murine heart sections were fixed with methanol/aceton for 5 minutes and treated as described previously 6. Cryosections
were again incubated with anti-Fbxl22 and anti-calsarcin-1 or anti-Filamin C antibodies. Fluorescence labeling was carried out with secondary antibodies conjugated with goat anti-mouse fluorescein (1:200; Vector Laboratories Inc.) or goat anti-rabbit Cy3 (1:200; Dianova) for 60 min at RT. Vectashield medium with DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories) was used for mounting of the slides. All non-confocal analyses were performed using a Zeiss microscope (Axioskop 2 Plus), Confocal Images were acquired with an inverted laser scanning microscope (LSM 510; Carl Zeiss) using Plan-Apochromat 63x/1.40 oil objective for cells and anObjektiv EC Plan-Neofluar 40x/1.30 oil objective for for tissue sections. The images were further analysed using the ZEN lite 2011 software (Carl Zeiss).

Coimmunoprecipitation experiments

10cm dishes with HEK293 cells were transiently transfected with the respective plasmids by using JetPEI (PolyPlus Transfection) as described in the manufacturer’s manual. Potentially interacting proteins (fused to N-terminal or C-terminal tags) were coexpressed for 48 hours. The medium was then removed and cells were lysed in 1000μl of ice-cold ELB lysis buffer containing 50mM HEPES pH 7.0, 250mM NaCl, 1% NP40, 5mM EDTA, Protease Inhibitor Cocktail (Roche # 1 836 170) and Phosphatase Inhibitor Cocktail 1 + 2 (Sigma # P2850; P5726). Lysates were incubated on ice for 30 minutes. Cellular debris was removed by centrifugation (5min at 13,000 rpm at 4°C). 2000μg of the cleared protein extract were incubated with 50μl of Anti-HA-Agarose (Sigma # A2095) for 2 hours at 4°C with moderate agitation. After 4 cycles of washing with 1ml of lysisbuffer, protein complexes were eluted in 1x bead volume/1x SDS sample buffer. For Western blot experiments the beads were separated by 2min of centrifugation at 13,000 rpm at RT and the suprnatant containing the protein fraction was analyzed by immunoblotting using standard Western blotting techniques. Coimmunoprecipitation studies of endogenous interaction were performed with lysates from NRVCM which had been infected with 20ifu of an adenovirus overexpressing Fbxl22 and cultured for 48h; 20ifu of AdvGFP served as control. Processing of lysates and precipitation assays (V5-agarose conjugate, Biomol) as well as immunoblotting were done as described in the respective sections (anti-mouse monoclonal alpha-actinin, Sigma; anti-rabbit hSKP-1, CS - #2156).

Western blot analyses

In order to determine tissue specificity of Fbxl22 expression, protein extracts from several murine tissues were isolated and processed in RIPA-buffer (10 mM Tris-HCl pH 7.5, 15mM Na2EDTA; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS; Protease Inhibitor Cocktail (Roche # 1 836 170). After homogenisation, 150μg of the cleared supernatant were separated on a 12.5% PAA-Gel and transferred on a PVDF-Membrane. Subsequently, the membrane was probed with an anti-Fbxl22 antibody at a dilution of 1:300, followed by standard Western blotting procedures with the use of an ECL detection system (GE Healthcare RPN2106). Unless stated otherwise, antibodies were administered according to the manufacturer’s recommendations. Antibodies used include anti-Ubiquitin (SCBT, 1:200), anti-Fbxl22 (Abnova, Catalog # : H00283807-A01, 1:300), anti-V5 (Invitrogen), anti-Myc (Hybridoma, 1:5), anti-Myc poly (SCBT), anti-mouse monoclonal alpha-actinin (Sigma), anti-hCullin1 (Acris R1509), anti hSKP-1 (CS #2156), anti-Calsarcin-1 (rabbit polyclonal).

RNA isolation and purification.

Total RNA from NRVCMs was isolated using the TRIzol method (Invitrogen # 15596-018) according to the manufacturer’s protocol und resuspended in DEPC-treated water.

Quantitative real time PCR

For validation of gene expression data in human tissue, real-time PCR was applied utilizing the following primers: hqRFTbxl22_F (5′-CAG CTC CGA GAT GTG TTT GA-3′) and hqRFTbxl22_R (5′-AGT CGC GCT AAG CAG TCA TC-3′) as well as Universal Probe mix (Roche). For normalization of cDNA content, homo sapiens hypoxantine phosphoribosyltransferase 1 was used as a housekeeping gene control with the following primers: hqRTHPRT1F (5′-TGACCTTGATTATTTTGATACC-3′), hqRTHPRT1R (5′-CGAGCAAGACGTTCACTCC-3′).
For analysis of substrate mRNA-levels NRVC M subjected to adenoviral overexpression or knockdown of Fbxl22 were harvested after 48 hours and RNA was isolated as described before. cDNA was generated from total RNA using the Superscript III first strand kit (Invitrogen #18080-051). Transcriptional regulation of α-ACTN-2 and filamin C expression levels was analyzed employing the Platinum SYBR®GreenER™ qPCR SuperMix (Invitrogen #11760-500) in combination with the ABI Prism 7000 Sequence Detection System (Applied Biosystems) - Alpha-Actinin2_F 5’ - AGG AGG AAG AAT GGC CTG AT - 3’, Alpha-Actinin2_R 5’ - ACG AAG TTC CTC TGC CAA GA - 3’, FLNC_F 5’ - ACA ACA CAT TCC GAT GCA CA – 3’, FLNC_R 5’ – CCC TTG GTG AAC ACC TTG AA - 3’.

18S rRNA served as an internal standard - ratqRTr18S_F (5’-TCA AGA ACG AAA GTC GGA GG-3’), ratqRTr18S_R (5’-GGA CAT CTA AGG GCA TCA C-3’).

For analysis of ANF and Fbxl22-expression upon PE-mediated pharmacological stimulation cells were treated with PE for 24h beginning on day 2. Cells were harvested at 48 hours after preparation and cDNA was prepared as described above.

Ubiquitinylation studies

Studies of intrinsic ubiquitination were conducted with proteasome-inhibited (10 µM MG-132; Sigma) HEK cell lysates after transfection with V5-tagged hFbxl22, Myc-tagged alpha-actinin-2 or the C-terminal part of filamin C (AA 2083-2725) and HIS-tagged ubiquitin, as indicated. Precipitates were harvested after incubation with a polyclonal anti-ubiquitin antibody or polyclonal anti-V5 antibody (Sigma) for 4 hours, respectively. Immunoblotting was performed using standard technique and antibodies against Myc (Hybridoma 1:5), Ubiquitin (SCBT, 1:200), or V5 (Invitrogen). Where indicated an in vitro ubiquitination assay was performed: MG-132 treated lysates from cos7 cells that had been transfected with Fbxl22, ubiquitin and Myc-tagged α-actinin-2 were exposed to ubiquitination buffer (RIPA containing Protease Inhibitor Cocktail [Roche # 1 836 170] and Phosphatase Inhibitor Cocktail 1 + 2 [Sigma # P2850; P5726], MG-132 [10 µM, Sigma], ubiquitin-aldehyde [0.5 µM, Enzo Lifesciences], DTT [1 mM], Mg²Cl [2 µM] and ATP [5 mM]) and incubated for 30 minutes at 37°C. Immunoblotting of the array was performed as described above.

Transverse aortic constriction

Male C57BL/6N mice (age 9 to 10 weeks, n = 5) were exposed to chronic pressure overload by transverse aortic constriction (TAC). In preparation mice were subjected to anesthesia, intubation and ventilation before median sternotomy was performed; with the aorta visible a 7.0 prolene suture was placed distal of the brachiocephalic artery causing constriction of the aorta. The suture was tightened around a blunt 27-gauge needle. After removal of the needle the chest and overlying skin were closed. Acute and chronic mortality from the above described procedures did not exceed 5%. Sham-operated mice (n=5) underwent the same procedure but without aortic banding. At 30 days mice were sacrificed and hearts were explanted. In the following differential regulation of ANF- and Fbxl22-mRNA-levels was assessed by qRT-PCR using standard protocol.

Zebrafish strains and injection procedures

Care and breeding of zebrafish, danio rerio, was conducted as described [7]. The present study was performed after securing appropriate institutional approvals. It conforms with the Guide for the Care and Use of Laboratory Animals published by the “US National Institute of Health” (NIH Publication No. 85-23, revised 1996). Morpholino-modified antisense oligonucleotides were directed against the translational start-site of zebrafish Fbxl22 (MO-Fbxl22: 5’-CTTGATCTAGTTCTATGAGATGCAT-3’) and the splice-donor site of exon 1 (MO-Fbxl22-2: 5’-AAGGAAACACACTCACATGTTGCACAAAC-3’). Embryos at the 1-cell stage were injected with 2 ng MO-Fbxl22 or a standard control oligonucleotide (MO-control).
Sense-capped RNA was synthesized using the mM ESSAGE mMASCHINE system (Ambion) from pCS2-MTactn2. RNA was diluted (100 ng/μL or 200 ng/μL in 0.2 M KCl) and microinjected into one-cell stage embryos. Siblings from the same pool were injected using 0.2 M KCl as control.

**Zebrafish Histology, transmission electron microscopy, immunostaining, and immunoblotting**
Histological analysis and electron micrographs of zebrafish embryos were performed as described [7]. For immunoblotting, proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. Blots were probed with α-actinin (Sigma), tropomyosin (Sigma), MF20 [8], α-actin (Sigma), pan-cadherin (abcam) and β-actin (Sigma) antibodies and signals detected by chemoluminescence. For immunostainings, embryos were fixed in 4% paraformaldehyde.

**RNA in situ hybridization and functional assessment**
Whole-mount RNA in situ hybridization was used to detect expression of vmhc, amhc, cmlc2 and anf transcripts, as described [8]. Images and video films were recorded and digitized with a Zeiss microscope/MCU II. The functional assessment of cardiac contractility was carried out as described before [7]. Fractional shortening (FS) was measured with help of the zebraFS software (http://www.benegfx.de).

**Statistical analyses**
All results are shown as the mean +/- standard error of the mean (SEM) unless stated otherwise. Statistical analyses of the data were carried out using ANOVA followed by Student-Newman-Keuls post-hoc tests. If appropriate, Student’s t-test was employed (two sided, assuming similar variances). P values <0.05 were considered statistically significant. Real time PCR data analyses were carried out using the ΔΔct method.

Supplemental Material

Supplementary Figure I. Fbxl22 exhibits a cardiac-enriched expression profile.

Supplementary Figure II. Fbxl22 is localized at the cardiac z-disc (confocal microscopy).

Supplementary Figure III. Fbxl22 colocalizes with filamin C at the cardiac z-disc.

Supplementary Figure IV. The N-terminus of Fbxl22 is sufficient for the interaction with Skp1, α-actinin and filamin C.

Supplementary Figure V. Transcriptional levels of α-actinin and filamin C are not significantly regulated by Fbxl22-expression.

Supplementary Figure VI. Experiment illustrating Fbxl22- and proteasome-dependent substrate ubiquitination and degradation.

Supplementary Figure VII. Global ubiquitination in cardiomyocytes is unaltered by Fbxl22-knockdown

Supplementary Figure VIII. Phenotypic penetrance of Fbxl22 knockdown.

Supplementary Figure IX. Cardiac development in zebrafish embryos is unaffected by Fbxl22-knockdown

Supplementary Figure X. (Ultra-)structural analyses of zebrafish embryo skeletal muscle.

Supplementary Movie Legends

Supplementary Movie I. Contracting heart of a MO-Fbxl22-injected zebrafish embryo at 48hpf

Supplementary Movie II. Contracting heart of a MO-control-injected zebrafish embryo at 48hpf

Supplementary Movie III. Heart of a MO-Fbxl22-injected zebrafish embryo at 72hpf

Supplementary Movie IV. Flight response of Fbxl22 morphant embryos as compared to MO-control-injected zebrafish embryos
**Supplementary Figure I.** *Fbxl22* exhibits a cardiac-enriched expression profile.

**A**, EST-profile for murine *Fbxl22*. The EST database was screened for cardiac-enriched F-box proteins/E3-ligases. *Fbxl22* expression levels are highest in myocardial and uterine tissue.  

**B**, qRT-PCR for human *Fbxl22* RNA levels performed on a human cDNA-library provides further evidence of cardiac enrichment with expression levels being highest in adult and fetal heart tissue. Normalized for HRPT1 expression levels.
Supplementary Figure II. Fbxl22 is localized to the sarcomeric z-disc in isolated cardiomyocytes and whole heart cryosections

A, Subcellular localization of Fbxl22 in adult rat ventricular cardiomyocytes (ARVCM), which were stained with antibodies against Fbxl22 (green) and calsarcin-1 (red). When signals are merged, a colocalization at the sarcomeric z-disc is observed. Scale bar: 5µm. B, Subcellular localization of Fbxl22 in the intact murine heart. Murine heart sections were stained with antibodies against Fbxl22 (green) and calsarcin-1 (red). Again, Fbxl22 was found to localize to the z-disc, confirming its subcellular localization in cardiac tissue. Scale bar: 5µm
Supplementary Figure III. Fbxl22 colocalizes with FLNC in isolated cardiomyocytes.
Fbxl22 colocalizes with Filamin C (FLNC) in adult rat ventricular cardiomyocytes (ARVCM). Cells were stained with antibodies against Fbxl22 (green) and FLNC (red). When signals are merged, a colocalization at the sarcomeric z-disc is observed, confirming its potential for binding in the myocardium. Top panel gives an overview of some ARVCM, bottom panel gives a more detailed illustration from one of the cardiomyocytes. All images were obtained using confocal microscopy. Scale bar: 10µm (top panel), 5µm (bottom panel).
Supplementary figure IV. The N-terminus of Fbxl22 is sufficient for the interaction with Skp1, α-actinin and Filamin C.

A, Mapping of the interaction of Fbxl22 and Skp1 to the evolutionary conserved N-terminal part of Fbxl22. HEK cells were transfected with the HA-tagged N-terminus of Fbxl22 (AA 1-113). Immunoblotting of HA-precipitates with Skp1-antibody again detects native Skp1 (top) suggesting that the F-box containing N-terminus of Fbxl22 is sufficient to mediate this interaction. Input controls of the HA-tagged N-terminus of Fbxl22 are shown below.

B and C, Mapping of the interaction domain of Fbxl22 for α-ACTN-2 (B) and FLNC (C). HEK cells were transfected with the HA-tagged N-terminus of Fbxl22 (AA 1-113) and Flag-tagged α-ACTN-2 (B) or Myc-tagged FLNC (C), respectively. Western blot analyses of HA-precipitates with Flag-antibody/Myc-antibody demonstrate that the interaction of Fbxl22 and α-ACTN-2 (B) as well as Fbxl22 and FLNC (C) can also be mapped to the N-terminus of Fbxl22 (upper panels). To validate successful transfection of both the N-terminal fragment of Fbxl22 and the respective interacting proteins, HA-, Myc- and Flag-immunoblots of input controls were performed (bottom panels).
Supplementary figure V. Transcriptional levels of α-actinin and filamin C are not significantly regulated by Fbxl22-expression.

A and B, Adenoviral overexpression of Fbxl22 in NRVM does not mediate significant changes in mRNA-levels of alpha-actinin (A), p=0.83, and filamin C (B), p=0.1. C and D, Adenoviral knockdown of Fbxl22 in NRVM does not induce significant changes in mRNA-levels of alpha-actinin (C), p=0.56 and filamin C (D), p=0.57. A significant regulatory role of Fbxl22 in transcriptional control of alpha actinin and filamin C is not observed (A-D). All experiments are n=4.
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**IB: Myc**

**IP: Ubiquitin**

**IB: Myc**

**Supplementary Figure VI.** Experiment illustrating Fbxl22- and proteasome-dependent substrate ubiquitination and degradation.

HEK cells were transiently transfected with Myc-tagged α-actinin-2 and Fbxl22, either in the absence (lanes 1, 2) or presence (lanes 3, 4) of the proteasome inhibitor MG-132. Overexpression of Fbxl22 facilitates the degradation of α-actinin-2 (lane 1 and 2, *top panel*), while addition of MG-132 attenuates this effect (lanes 3 and 4, *top panel*). Immunoprecipitation of endogenous ubiquitin and subsequent immunoblotting with anti-myc (*bottom panel*), reveals a lack of ubiquitinated α-actinin-2 (lanes 1 and 2, *bottom panel*). In contrast, the presence of the proteasome inhibitor MG-132 leads to accumulation of ubiquitinated α-actinin-2 (lane 4, *bottom panel*).
Supplementary Figure VII. Global ubiquitination in cardiomyocytes is unaltered by Fbxl22-knockdown

Fbxl22-knockdown does not change total ubiquitination levels in cardiomyocytes. A knockdown of Fbxl22 leaves global ubiquitination levels in NRVM both in starving conditions (left 2 lanes) and in a growth-stimulated environment unchanged (lane 3+4). Proteasome inhibition in combination with knockdown of Fbxl22 does not influence total ubiquitination either (lane 5+6).
Supplementary Figure VIII. Phenotypic penetrance of Fbxl22-knockdown.

A, Knockdown of both total Fbxl22 and a splice-/exon-deficient, truncated fragment of Fbxl22 both result in a severe cardiac phenotype. B, More than 90% of embryos in each knockdown group exhibit distinct phenotypic aberrations in line with the described phenotype of a Fbxl22-knockdown. C, PCR demonstrating the efficacy of Fbxl22-knockdown and splice-modulation/truncation.
Supplementary Figure IX. Cardiac development in zebrafish embryos is unaffected by Fbxl22-knockdown
A - F, Whole-mount antisense RNA in situ hybridization of zebrafish morphants reveals normal expression levels of cardiac-specific myosin light chain 2 (cmlc2) and chamber-specific myosin heavy chains (vmhc, amhc). Furthermore chamber-restricted pattern of myosin heavy chains (vmhc, amhc) remains unaltered.
G - H, Analysis of atrial natriuretic factor (anf). ANF mRNA expression is not affected by knockdown of Fbxl22 in zebrafish embryos.
Supplementary Figure X. (Ultra-)structural analyses of zebrafish embryo skeletal muscle. 
A - B, Alpha-actinin immunofluorescence staining shows no significant alterations in the z-disc formation in Fbxl22 morphants as compared to controls. C - D, Ultrastructural analysis by transmission electron microscopy displays regular arrays of thick and thin myofilaments as well as z-disc formation. No ultrastructural abnormalities can be detected at 48 hpf.
Supplementary Movie Legends

Supplementary Movie I.
Contracting heart of a MO-Fbxl22-injected zebrafish embryo at 48hpf. In Fbxl22 morphant zebrafish embryos ventricular contractility is severely impaired. In addition, Fbxl22 morphant hearts display a pericardial edema. Lateral view, head to the left, ventral side downwards. A, atrium; V, ventricle

Supplementary Movie II.
Contracting heart of a MO-control-injected zebrafish embryo at 48hpf. In control zebrafish embryos both cardiac chambers vigorously contract with a regular rhythm and sequentially. Lateral view, head to the left, ventral side downwards. A, atrium; V, ventricle

Supplementary Movie III.
Heart of a MO-Fbxl22-injected zebrafish embryo at 72hpf. In Fbxl22 morphant zebrafish embryos ventricular contractility is almost completely abolished. In addition, Fbxl22 morphant hearts display a severe pericardial edema. Lateral view, head to the left, ventral side downwards. A, atrium; V, ventricle

Supplementary Movie IV.
When touch stimulated, MO-control-injected zebrafish embryos show normal flight response, whereas Fbxl22 morphant embryos are completely paralyzed.