Myomasp/LRRC39, a Heart- and Muscle-Specific Protein, Is a Novel Component of the Sarcomeric M-Band and Is Involved in Stretch Sensing

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Rationale and Objective: The M-band represents a transverse structure in the center of the sarcomeric A-band and provides an anchor for the myosin-containing thick filaments. In contrast to other sarcomeric structures, eg, the Z-disc, only few M-band–specific proteins have been identified to date, and its exact molecular composition remains unclear.

Methods and Results: Using a bioinformatic approach to identify novel heart- and muscle-specific genes, we found a leucine rich protein, myomasp (Myosin-interacting, M-band-associated stress-responsive protein)/LRRC39. RT-PCR and Northern and Western blot analyses confirmed a cardiac-enriched expression pattern, and immunolocalization of myomasp revealed a strong and specific signal at the sarcomeric M-band. Yeast 2-hybrid screens, as well as coimmunoprecipitation experiments, identified the C terminus of myosin heavy chain (MYH)7 as an interaction partner for myomasp. Knockdown of myomasp in neonatal rat ventricular myocytes (NRVCMs) led to a significant upregulation of the stretch-sensitive genes GDF-15 and BNP. Conversely, the expression of MYH7 and the M-band proteins myomesin-1 and -2 was found to be markedly reduced. Mechanistically, knockdown of myomasp in NRVCM led to a dose-dependent suppression of serum response factor–dependent gene expression, consistent with earlier observations linking the M-band to serum response factor–mediated signaling. Finally, downregulation of myomasp/LRRC39 in spontaneously beating engineered heart tissue constructs resulted in significantly lower force generation and reduced fractional shortening. Likewise, knockdown of the myomasp/LRRC39 ortholog in zebrafish resulted in severely impaired heart function and cardiomyopathy in vivo.

Conclusions: These findings reveal myomasp as a previously unrecognized component of an M-band–associated signaling pathway that regulates cardiomyocyte gene expression in response to biomechanical stress. (Circ Res. 2010;107:1253-1264.)

Key Words: myocytes ■ cardiac ■ stretch ■ serum response factor ■ M-band

The sarcomeres of cardiac and skeletal muscle represent the basic molecular unit for contractile force generation and transmission. The thin filaments of the sarcomere consist of actin fibers that are crosslinked at the Z-disc, whereas the thick filaments, mainly composed of myosin molecules, are attached to the M-band, which provides lateral stabilization. Beyond a mere mechanical function, these crucial sarcomeric structures and their molecular components have been implicated in the regulation of the dynamics of muscle contraction and the sensing of cardiomyocyte stress. Moreover, the sarcomeric Z-disc, as well as the M-band, has increasingly been recognized as a hub for signaling pathways that mediate diverse intracellular processes including cell growth and differentiation, as well as protein turnover and gene expression. At the level of the sarcomeric Z-disc, the conversion of structural and mechanical demands to gene transcription is mediated by several specific pathways, including calcium-dependent signaling via the calcineurin-
nuclear translocation of the transcription factor SRF.14 The protein turnover in response to biomechanical stress via show that an M-band signaling complex, consisting of titin, M-band has also been shown to play a critical role in mechanotransduction. Gautel and colleagues were able to

Finally, in vivo knockdown of myomasp/LRRC39 in ze-

Although several components of the M-band have already been identified years ago, including specific titin domains,17 the myomesin-1, -2, and -3,18–20 and obscurin,5 its exact molecular composition is still not completely resolved. Moreover, the small number of known M-band proteins suggests that additional components are yet to be discovered.

Thus, in an effort to identify novel sarcomeric proteins, we searched expressed sequence tag (EST) databases for previously uncharacterized genes with high abundance in cardiac and muscle cDNA libraries. Using this bioinformatic approach, we found an open reading frame encoding for a novel 335-aa protein “LRRC39,” which we termed myomasp (Myosin-interacting, M-band-associated stress-responsive protein). Here, we show that myomasp/LRRC39 is a highly cardiac-enriched protein that localizes to the sarcomeric M-band and directly binds to the C-terminal tail domain of myosin heavy chain (MYH)7. Knockdown of myomasp/ LRRC39 expression in cardiomyocytes results in the inhibition of SRF-dependent signaling and significant downregulation of MYH7 mRNA other M-band proteins such as myomesin-1 and -2. Conversely, the expression of stress-responsive genes such as GDF-15 and brain natriuretic peptide (BNP) is markedly induced on myomasp ablation. Finally, in vivo knockdown of myomasp/LRRC39 in ze-
calculated pattern, a strong expression in heart and skeletal muscle was observed, whereas other tissues revealed no significant signal. Consistently, hybridization of myomasp/LRRC39-specific cDNA probes with human or mouse multiple tissue Northern blots confirmed heart- and skeletal muscle-specific gene expression of both human and murine myomasp/LRRC39 (Figure 2B). Compared to quantitative real-time PCR (Figure 2A), the Northern blot analyses revealed a higher myomasp/LRRC39 expression in the heart than skeletal muscle tissue in mice (Figure 2B). To also analyze the expression of myomasp/LRRC39 on the protein level, a Western blot of several rat tissues extracts was probed with a polyclonal antibody against myomasp/LRRC39. Myomasp/LRRC39- and control-transfected HEK-293T cells served as positive and negative controls, respectively. Again, these experiments revealed a strong expression of myomasp/LRRC39 protein in rat heart and skeletal muscle tissue at the predicted size of approximately 39 kDa (Figure 2C). In addition to the expression pattern on RNA level, Western blot experiments also revealed a distinct protein expression in the kidneys.

Myomasp/LRRC39 Interacts With Myosin Heavy Chains

To identify potential protein interaction partners for myomasp/LRRC39, we performed yeast 2-hybrid experiments and screened human heart- and skeletal muscle-derived cDNA libraries. Among several potentially myomasp-interacting proteins, we found multiple clones encoding for the C termini of different myosin heavy chain genes (MYH1, MYH2, MYH6, MYH7) (Figure 3A). To confirm the interaction between MYH7 and myomasp/LRRC39, we conducted coimmunoprecipitation experiments in eukaryotic HEK-293T cells. These cells were transiently cotransfected with HA-tagged full-length myomasp/LRRC39, together with Myc-tagged full-length MYH7 or the respective empty vector controls. Results revealed an intense coprecipitation of myomasp with MYH7, whereas no specific band was observed with empty vector alone (Figure 3B). To map the myomasp/LRRC39-interacting domain of MYH7, we tested several MYH7 clones with different lengths for interaction with myomasp/LRRC39 in yeast. This experiment was of particular interest, because sarcomeric myosins are known to have...
an A-band localized head and neck region, responsible for actin–myosin interaction, and a C-terminal tail region crosslinked at the sarcomeric M-band. These mapping studies revealed that myomasp/LRRC39 interacts with the C-terminal 282 amino acids of MYH7, consistent with the notion that myomasp/LRRC39 could be involved in regulating and/or anchoring myosin heavy chain molecules at the sarcomeric M-band (Figure 3C).

Myomasp/LRRC39 Is a Novel Component of the Sarcomeric M-Band

Given the high expression levels of myomasp/LRRC39 in the myocardium and the protein–protein interaction with MYH7, we next aimed to determine its subcellular localization in isolated adult rat cardiomyocytes. A strong signal was detected at the sarcomeric M-band, as shown by coimmunostaining with a well-characterized N2A M-band–specific titin antibody (Figure 4A). Consistently, simultaneous staining of myomasp/LRRC39 in rat tissue extracts again confirmed the heart- and muscle-enriched expression and detects an additional band in kidney.

Figure 2. Cardiac- and skeletal muscle–enriched expression pattern of human and mouse myomasp/LRRC39. A and B, EST database–predicted muscle- and heart-enriched mRNA expression profile (left) confirmed by quantitative RT-PCR (right) (A) and additional Northern blot analyses (B) of multiple human and mouse tissues using myomasp/LRRC39-specific probes and primers. C, Western blot analysis of myomasp/ LRRC39 in rat tissue extracts again confirmed the heart- and muscle-enriched expression and detects an additional band in kidney.

region with its C terminus and to localize to the sarcomeric A-band. Simultaneous staining of MYBPC3 and myomasp/LRRC39 excluded an A-band localization of myomasp/LRRC39 (Figure 4A). In line with the subcellular localization in ARVCM, cryosections of intact rat heart and skeletal muscle tissues again revealed a sarcomeric M-band signal for myomasp (Figure 4B) and colocalization with the M-band–specific titin N2A domain (Figure 4B). Taken together, these data reveal that myomasp/LRRC39 is a novel component of the sarcomeric M-band.

Myomasp/LRRC39 Knockdown Reveals Differential Regulation of Sarcomeric and Stretch-Responsive Genes

The sarcomeric M-band has previously shown to be a hub for signaling molecules involved in cardiomyocyte gene regulation. To test whether myomasp/LRRC39 participates in the regulation of gene expression as well, we devised a knockdown experiment in neonatal rat cardiomyocytes, using an
adenovirally encoded synthetic microRNA (miMyomasp). Knockdown efficiency was >85% compared with an appropriate control virus expressing scrambled microRNAs (miNeg) (Online Figure II). Seventy-two hours after viral infection total RNA was isolated, and after quality control, amplification, and labeling, the cRNA was subsequently hybridized on Illumina Rat Sentrix-12 BeadChips. For comparison of the expression profile of myomasp/LRRC39 knockdown cardiomyocytes and control cells, the acquired data were processed and analyzed with Illumina Bead studio software. The distribution of the normalized differentially expressed genes (>4-fold) of 3 miMyomasp knockdown samples compared with 3 miNEG controls is shown in Online Figure I, A. Only those genes with a probability value of <0.005, which were up- or downregulated >4-fold and showing an array SD of >5-fold and a bead SD of >4-fold were taken into account for further analysis. By applying these selection criteria, 367 genes were found to be differentially expressed. A total of 129 genes were found to be upregulated after miMyomasp knockdown, whereas 238 showed a significant downregulation. The normalized data of the 367 genes that fulfilled the selection criteria are displayed in a heat map for each sample (Online Figure I, A). Hierarchical clustering between the sample groups revealed highly reproducible results and low variance (Online Figure I, B). Because myomasp/LRRC39 localizes to the M-band and interacts with MYH7, we focused our interest on the regulation of M-band–associated proteins. Of note, the microarray analyses of myomasp/LRRC39-deficient cardiomyocytes showed a highly significant reduction of MYH7B (−25.6-fold), as well as the M-band proteins myomesin 1 (−4.5-fold) and MYBPC3 (A-band). B, Consistent with the localization in ARCMs, staining of intact rat heart and skeletal muscle cryosections with myomasp/LRRC39 and titin-N2-A antibody showed colocalization at the sarcomeric M-band. Scale bar, 20 μm.
stretch-sensitive marker gene BNP revealed a marked upregulation of (6.3-fold increase, P<0.001; Figure 5B). Remarkably, one of the genes that displayed the most dramatic differential regulation (23.7-fold induction; Online Figure I, C) was the stress- and stretch-sensitive marker GDF15.28,29 The significant upregulation of GDF15 could be confirmed by quantitative real-time PCR and Western blot experiments (5.7-fold, P<0.01; Figure 5C). Because both GDF-15 and BNP are highly sensitive to mechanical stress,28–30 we asked whether myomasp/LRRC39 expression itself is also regulated in an in vitro model for stretch of cardiomyocytes (Figure 6A). Interestingly, static biaxial stretch of NRVCM for 24 hours led to a significant reduction in myomasp/LRRC39 mRNA expression (−78%, P<0.001). To further substantiate this finding, we analyzed C57BL6 mice which underwent transverse aortic constriction for 4 weeks (n=11) and compared them to sham-operated littermates (n=12; Figure 6B). Consistently, transverse aortic constriction in mice led also to a highly significant cardiac downregulation of myomasp/LRRC39 mRNA, as assessed by quantitative real-time PCR analysis (P<0.001).

Thus, downregulation of myomasp leads to suppression of the expression of other M-band–associated proteins including myomesin1 (MYOM1) and myomesin 2 (MYOM2). Conversely, myomasp deficiency results in the induction of the stretch-responsive genes BNP and GDF15. The marked downregulation of endogenous myomasp mRNA expression on biomechanical stress implies a role for myomasp/LRRC39 in the stretch signaling cascade.

**Downregulation of Myomasp/LRRC39 Inhibits SRF-Dependent Signaling**

It has previously been suggested that sarcomeric M-band proteins participate in the cardiomyocyte response to biomechanical stress via modulation of SRF-dependent signaling pathways.14 Thus, we tested the hypothesis that myomasp/LRRC39 might also participate in SRF-dependent transcriptional regulation. By measuring luciferase activity of NRVCMs coinfected with a reporter adenovirus (Ad) expressing luciferase under control of the SRF-responsive sm22 promoter (Figure 6C), we could show a significant and dose-dependent reduction of SRF activity on microRNA-mediated myomasp knockdown (~37.8% at 90 infectious units [ifu]; P<0.001; Figure 6D and 6E). Taken together, these data imply that myomasp/LRRC39 is involved in the regulation of M-band proteins via modulation of SRF-dependent gene expression.

**Functional Consequences of Myomasp/LRRC39 Knockdown In Vitro**

To elucidate the functional consequences of myomasp/LRRC39 downregulation on cardiomyocyte contraction and force generation, we used fibrin-based mini-engineered heart tissues (FBMEs) prepared as described in detail in the Online Methods section (Figure 7A). After 6 days of culture, FBMEs
where infected with 50-ifu AdmiMyomasp or 50-ifu control virus (AdmiNeg). After development of a spontaneous and synchronous beating pattern (8 to 10 days), force generation and fractional shortening of FBMEs were measured by video optical recordings on days 18 to 20. Infection efficiency and viral penetration of engineered heart tissue constructs was visually controlled by green fluorescent protein (GFP) expression (Figure 7B). FBMEs infected with 50-ifu AdmiMyomasp showed a significant reduction in force generation (70 versus 120 mN, \( P < 0.002 \), at a calcium concentration of 0.5 mmol/L; 100 mN versus 160 mN, \( P < 0.05 \), at 1.5 mmol/L calcium) and markedly impaired fractional shortening (4% versus 7%, \( P < 0.005 \), at 0.5 mmol/L calcium; 6% versus 8%, \( P < 0.05 \), at 1.5 mmol/L calcium content) compared with FBMEs infected with control virus (Figure 7C and 7D).

**Functional Consequences of a Myomasp/LRRC39 Knockdown In Vivo**

To further elucidate myomasp/LRRC39 function in vivo, we injected zebrafish embryos with morpholino (MO)-modified antisense oligonucleotides directed against the splice donor site of intron 2 of zebrafish myomasp/LRRC39. When injected with 4 ng of MO-Myomasp, 76.9% of injected embryos (n=267) (Figure 8A) revealed severe contractile dysfunction (Figure 8B; Online Movie 1), whereas heart function of embryos injected with a standard control MO was completely unaltered (Figure 8B; Online Movie 2). In myomasp morphants, ventricular and atrial fractional shortening significantly decreased to 6% and 11.6%, respectively (Figure 8C and 8D). Hence, to evaluate whether myomasp deficiency interferes with key steps of zebrafish heart development and thereby leads to the observed heart failure phenotype, we examined expression of cardiac chamber–specific proteins and mRNAs by immunostainings and antisense RNA in situ hybridization, respectively, as well as cardiac structure of myomasp morphants by histology. We found that loss of myomasp function does not interfere with crucial steps of cardiogenesis, such as heart tube looping, chamber demarcation, and the differentiation of ventricular and atrial cardiomyocytes (Figure 8E). Additionally, expression of cardiac genes such as the cardiac myosin light chain (cmlc2) and chamber-specific myosin heavy chains (vmhc, amhc, MF20, S46) were found to be expressed in the correct
heart chamber–restricted pattern and in normal amounts, demonstrating that myomasp deficiency does not lead to the misexpression of these contractile proteins or severe alteration in cardiogenesis, as observed in several other heart failure zebrafish mutants.31–33

To further assess whether impaired cardiomyocytes cytoarchitecture accounts for the contractile dysfunction of myomasp-deficient zebrafish embryos, we analyzed the ultrastructure of cardiac muscle cells by transmission electron microscopy. At 72 hours postfertilization, myomasp-deficient morphants show no clear ultrastructural defects in cardiac sarcomeres; however, no mature M-Band was definable neither in myomasp-deficient morphants nor in control MO-injected zebrafish. In contrast, deficiency of myomasp/LRRC39 leads to a marked narrowing of the skeletal muscle M-band accompanied “bare zone.” This A-band/M-band transition also appears “fuzzier” as compared with control MO-injected zebrafish. Other sarcomeric structures of myomasp-deficient morphants appeared completely unaltered. Arrays of thin myofilaments, as well as Z-discs, were found to be regular. Furthermore, the content and morphology of other cardiomyocytes components such as mitochondria and cell nuclei also appeared unaffected by the loss of myomasp function (Figure 8F).

Discussion

Here, we provide the detailed molecular characterization of a highly cardiac- and skeletal muscle–enriched protein, termed myomasp/LRRC39. Myomasp is a novel component of the sarcomeric M-band and is involved in SRF-dependent transcription and stress-responsive signaling. Moreover, myo-
masp/LRRC39 is required for proper cardiac function both in vitro and in vivo.

**Myomasp/LRRC39, a Leucine-Rich Repeat Protein**

Myomasp/LRRC39 contains a coiled-coil domain and 6 leucine-rich repeat domains and is highly conserved among species. Although we could not detect any closely related proteins in the database, leucine-rich repeat motifs are found in an increasing number of newly discovered proteins with diverse cellular functions, including cell adhesion, signal transduction, and regulation of gene expression. The primary function of the leucine-rich repeat domain is believed to provide a structural framework for protein–protein interactions. In 2007, Kim et al were the first to give insight into the importance of a muscle-specific, leucine-rich–containing protein, LRRC10. Using zebrafish as an in vivo model, they demonstrated that LRRC10-defective morphants markedly decreased over time. At 48 hours postfertilization, the expression of cardiac myosin proteins (MF20/S46), as well as mRNA expression of cardiac myosin light chain 2 (cmlc2), atrial myosin heavy chain (amhc), and ventricular myosin heavy chain (vmhc), is unaffected by the loss of myomasp function, as revealed by whole-mount immunostaining and antisense RNA in situ hybridization. Endocardial and myocardial cell layers are also unaltered in myomasp morphant zebrafish embryos, indicating no severe changes in cardiogenesis. In summary, histological analysis of zebrafish MO-myomasp knockdown showed no obvious alterations of cardiac structures. A indicates atrium; en, endocardium; my, myocardium; V, ventricle.

**Myomasp/LRRC39 Is a Novel Sarcomeric M-Band Protein**

In contrast to LRRC10, which is localized to T-tubuli and the Z-disc, myomasp/LRRC39 is exclusively detected at the sarcomeric M-band. Traditionally, the M-band has been viewed as a transverse structure providing mechanical an...
choring and alignment of the thick filaments of the sarcomere. Data from several studies indicate that regular M-band assembly is needed for proper packing of the thick filaments with appropriate distances to the thin filaments at the onset of contraction (termed “M-bridges”). A critical component in this model is the giant protein titin, which extends from the thin filaments at the Z-discs to the thick filaments at the M-band, thus providing mechanical cross-stabilization and adjustment of force imbalances between the filaments. Interestingly, the assembly and morphology of M-bands is variable in different muscle fibers. Fast muscle types have a 3-line M-band pattern; M1, -2, -4; whereas slow muscle cells show a 4-line pattern; M1, -2, -4, -6. In addition to titin, 2 other components of the M-band are thought to function as M-bridges, myomesin 1 and myomesin 2. Both proteins are aligned in antiparallel dimers to the central zone of myosins (M1 line) and to the C-terminal region of titin (M4 line). The nature of the M-line–bordering bare zone and the M6/M6 lines are not known at present. Differential expression levels of mature myomesin and an alternatively spliced embryonic ("EH")-myomesin isoform (initially described as skelemin) are thought to contribute to specific functional and structural features of different muscle types and developmental stages.

For example, a decreased amount of myomesin 2 in slow muscle fibers results in an increase of titin compliance and “fuzzy” sarcomeres. In contrast, fast muscle fibers display the highest content of mature myomesin and the lowest EH-myomesin level associated with a high order of M-band organization. Fast-type fibers therefore are thought to have the highest force generation capacity, whereas fuzzy, embryonic sarcomeres reveal less contractile efficiency but instead gain passive mechanical stability.

In this context, we could show that knockdown of myomasp/LRRC39 results in a significant reduction of myomesin 1 and -2 gene expression, which may explain the reduced force generation of myomasp-depleted engineered heart tissue and zebrafish hearts. Moreover, we could identify the C-terminal rod domain of MYH7 as an interacting partner for myomasp/LRRC39, thus representing a direct link to an important M-band structure. Of note, it has been shown that myomesins bind to both myosin and titin at the sarcomeric M-band. One might speculate that the knockdown of myomasp/LRRC39 may lead to a decrease in M-band crosslinking and stability and a reduced adaptive capacity to force imbalances or stretch, which may have led to an activation of stretch-responsive genes such as BNP and GDF-15.

**A Role for Myomasp/LRRC39 in Biomechanical Stress Signaling?**

Recently, a direct role for the M-band in intracellular signaling processes and mechanotransduction is emerging, providing another potential link between impaired M-band composition and altered cardiomyocyte gene expression. In this context, we observed a significant downregulation of endogenous myomasp/LRRC39 in stretched cardiomyocytes, a well-characterized model in which an upregulation of BNP and GDF15 has already been shown. This finding was further corroborated by analyzing C57BL6 mice which underwent transverse aortic constriction. In this in vivo model we also observed a significant downregulation of endogenous myomasp/LRRC39 mRNA, further supporting the notion that myomasp is involved in biomechanical stress signaling. Moreover, not the only the structural composition of the M-band may play a role in stretch sensing but the M-band itself could serve as a nodal point in signaling. In agreement with this hypothesis, Gautel and colleagues were able to show that mechanical stress plays a role in force-induced conformational changes in the kinase domain of titin. The activation of the kinase by stretch facilitates its interaction with Nbr1 and p62, followed by the binding of the ubiquitin ligase MURF-2. MURF-2 in turn interacts with the transcription factor SRF to inhibit its nuclear localization and thus its transcriptional activity. As a result the prohypertrophic response elicited by mechanical forces is attenuated. Similarly, we observed a suppression of SRF activity in cardiomyocytes on administration of increasing doses of myomasp/LRRC39 knockdown virus. The suppression of SRF-dependent transcription by downregulation of myomasp/LRRC39 might at least partly explain the reduced expression of myomesins and myosins as these genes are known to contain SRF-responsive CArG boxes in their promoter region. Yet, it is likely that competing signaling pathways exist because, for example, cardiac actin was not differentially regulated (data not shown).

Moreover, proper and stoichiometric protein composition of the M-band during stretch is needed to ensure a quick reaction to changes in sarcomeric force and/or enhanced stiffness/reduced compliance. Thus, an impaired M-band composition may induce stress-responsive pathways, independently of any direct effects on SRF signaling. Our data support a model where artificial myomasp knockdown in vitro partly mimics these effects of stretch, leading to the observed induction of immediate responsive genes like BNP and GDF-15.

Finally, the fact that myomasp-null zebrafish morphants show ultrastructural M-band alterations with a marked reduction of the A-band/M-band transition zone (bare zone) supports the concept that subtle structural perturbations may contribute to the observed functional defects. Regardless of the precise mechanism, our data underline the importance of proper M-band composition in general, and myomasp/LRRC39 expression in particular, in proper cardiomyocyte function.

**Myomasp/LRRC39 As a Candidate Gene for Cardiomyopathy**

Another important aspect with possible regulatory function is the protein-protein interaction between myomasp/LRRC39 and myosin/MYH7. MYH7 is connected to the M-band via its interaction with myomesin at the A-band/M-band transition zone. Mutations in the MYH7 gene are commonly found in patients experiencing hypertrophic and dilated cardiomyopathy. Most of these mutations affect the globular head region of the myosin molecule. In contrast, MYH7 mutations associated with skeletal muscle myosin storage myopathy with and without cardiomyopathy have almost exclusively been identified in the MYH7 C-terminal rod domain. Myomasp/LRRC39 directly interacts with the C-terminal coiled-coil myosin heavy chain tail region of MYH7. In addition, loss of myomasp/LRRC39 affects integrity of this
A-band/M-band transition zone in zebrafish skeletal muscle. Thus, myomasp might also be an attractive candidate gene for this disorder and/or involved in its pathophysiology. It would be of special interest to see whether patients carrying this myosin mutation reveal an altered M-band architecture or a differential localization of myomasp/LRRC39.

In conclusion, we identified a novel heart- and muscle-specific M-band protein termed myomasp/LRRC39, which interacts with MYH7 and negatively regulates stretch-sensitive genes. Downregulation of myomasp/LRRC39 alters the molecular M-band composition via modulation of SRF-dependent gene expression, as well as myocardial performance in vitro and in vivo.

Although we cannot rule out that structural alterations caused by abrogation of myomasp/LRRC39 result in changes in signaling and function, we interpret our results to support a role for myomasp/LRRC39 as a previously unrecognized component of an M-band–associated signaling pathway that regulates cardiomyocyte gene expression in response to biomechanical stress. Further in vivo gain- and loss-of-function experiments, as well as the analyses of myomasp/LRRC39 in patient samples, will help to clarify whether this novel sarcomeric protein also participates in the pathogenesis of cardiomyopathy.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- The sarcomeric contractile filaments are precisely crosslinked at the Z-disc and M-band to provide static stability. The Z-disc and M-band are structural hubs with additional signaling functions, integrating biomechanical strain with intracellular signaling pathways.
- Regular M-band assembly is needed for proper packing of the thick filaments with appropriate distances to the thin filaments at the onset of contraction.
- M-band-associated proteins have also been implicated in intracellular signaling processes and mechanotransduction.

What New Information Does This Article Contribute?

- We identified a cardiac-enriched transcript encoding for a novel component of the sarcomeric M-band, which we termed myomasp.
- Biomechanical strain in vitro, as well as in hearts subjected to aortic banding in vivo, led to marked downregulation of myomasp expression.
- Knockdown of myomasp/LRRC39 expression in vitro resulted in the inhibition of serum response factor (SRF)-dependent signaling and downregulation of M-band proteins myomesin-1 and -2, whereas the expression of stress-responsive genes such as GDF-15 and brain natriuretic peptide (BNP) is induced. In vivo knockdown of myomasp/LRRC39 in zebrafish resulted in reduced contractility, cardiomypathy, and perturbed ultrastructural M-band architecture.
- The Z-disc and the M-band are increasingly recognized as sarcomeric hubs with structural and signaling functions, integrating information on stretch with pathways controlling muscle growth and protein turnover. In this study, we found a novel cardiac-enriched protein, termed myomasp. Immunolocalization identified myomasp as component of the sarcomeric M-band, and interaction studies confirmed the C terminus of myosin heavy chain (MYH7) as an interaction partner. We found that myomasp mRNA expression was reduced in neonatal rat ventricular myocyte (NRVCVM) subjected to stretch or mice that underwent transverse aortic constriction. Myomasp knockdown in NRVCVM led to an upregulation of the stretch-sensitive genes GDF-15 and BNP, whereas expression of MYH7 mRNA and the M-band proteins myomesin-1 and -2 was reduced. Knockdown of myomasp suppressed SRF-dependent gene expression, consistent with observations linking the M-band to SRF-mediated signaling. Downregulation of myomasp in engineered heart tissue constructs decreased force generation and reduced fractional shortening. In vivo, myomasp-null zebrafish revealed reduced contractile function performance and ultrastructural M-band alterations, with marked reduction of the A-band/M-band transition zone. These observations support the concept that myomasp plays a critical role in maintaining M-band integrity, as well as regular contractile performance.
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Expanded material and methods

Cloning of human, mouse and rat myomasp/LRRC39:

The complete open reading frames of myomasp/LRRC39 were amplified from cardiac cDNA of the corresponding species employing the Gateway technology (Invitrogen). The following gene-specific primers were used, LRRC39F and LRRC39Rs for constructs with stop codon and LRRC39F and LRRC39Ros for constructs without stop codon, respectively:

humanLRRC39F (5´-GCTGGCACCATGACAGAAAATGTGGTTTGTACTG-3´)
humanLRRC39Rs  (5´-GCTGGGTCGCCTTATCCATCCGTATTTATGGAGAT-3´)
mouseLRRC39F (5´-GCTGGCACC ATGACAGAAAGTGCGGTTTGT-3´)
mouseLRRC39Rs (5´-GCTGGGTCGCCTCAATTACTTTCTCCATCAGAATGT-3´)
ratLRRC39F (5´-GCTGGGCACCATGACAGAAAGTGTGGTTTGTACC-3´)
ratLRRC39RS  (5´-GCTGGGTCGCCATTACTTTCTCCATCAGAATGTATAGAGC-3´)
humanLRRC39Ros (5-GCTGGGTCGCCTCCATCCGTATTTATGGAGATTG´-3)
mouseLRRC39Ros(5´-GCTGGGTCGCCATTACTTTCTCCATCAGAATGTATAGAGC-3´)
ratLRRC39Ros (5´-GCTGGGTCGCCTCCATCAGAATGTATAGAGC-3´)

For integration of the specific recombination site into the PCR-products, a second universal PCR was performed using attBFor (5´-GGGGACAAAGTTTGTACAAAAAAGCTGGCACC-3´) and attBRev (5´-GGGGACCACTTTTGTCACAAGAAAAGCTGGCACC-3´) primers and 1µl of the initial PCR-reaction as template. The PCR-product containing the recombination-specific attachment sites (att sites) was further recombined into the pDON201 entry vector.

For recombinant protein expression, rat myomasp/LRRC39 cDNA was subsequently shuttled into Gateway compatible expression plasmids in order to obtain expression constructs encoding for amino-terminal fusion tags (HA; MYC).
Cloning of synthetic LRRC39 knockdown microRNAs:

Knockdown Oligonucleotides miMyomasp_TOP
(TGCTGCTAGTGAGCAGTCCAATGCCTGTTTTGGCCACTGACTGACAGGCATTGCTGCTCACTAG) and miMyomasp_Bottom
(CCTGCTAGTGAGCAGCAATGCCTGTCAGTCAGTGGCCAAAACAGGCATTGGACTGCTCACTAGC) 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 GGC CAC TGA CTG ACG TCT CCA CGC AGT ACA TTT-3’).

Generation of recombinant adenoviruses encoding for myomasp/LRRC39 or myomasp/LRRC39 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-ß-galactosidase-V5 encoding adenovirus served as control for overexpression experiments (Invitrogen).

Northern blot analysis

Multiple tissue northern-blots (BioChain) containing mouse or human poly(A) RNA were hybridized overnight at 65°C with 32P-dCTP-labeled (Rediprime II Random Prime labelling System, Amersham Biosciences) cDNA probes corresponding to the ORF of mouse and human myomasp/LRRC39, respectively. 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 essentially performed as described¹, with the following differences: 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 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 YPDA containing 20% PEG 6000 in a 50mL centrifugation tube. 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-confidence 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.

**Coimmunoprecipitation experiments:**

Interacting proteins were transiently coexpressed in HEK293-T cells as N-terminal fusions (HA-myomasp + MYC-MYH7 / HA + MYC-MYH7 /). 48 hours after transfection, with jetPEI (PolyPlus Transfection) according to manufacturer instructions, the medium was removed and cells were lysed in 1000µl of ice-cold ELB lysis buffer (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 4°C. 3000µl of the cleared protein extract was incubated together with 50µl Anti-HA-Agarose (Sigma # A2095) for 2 hours at 4°C with moderate agitation. After 4 washes with 1ml lysisbuffer the protein complexes were eluted in 1 bead volume 1x SDS
sample buffer. For western blot experiments the beads were separated by 2min centrifugation at 13000 rpm / RT and the protein containing supernatant was analysed.

**RNA isolation and purification.**

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

**Quantitative real time PCR.**

CDNA was generated from total RNA using the Superscript III first strand kit (Invitrogen # 18080-051). Following primers were used for qPCR-validation of microarray results:

- **ratqRTrLRRRC39_F** (5’-ACA ATC CCT CTT GCT GTG C-3’)
- **ratqRTrLRRRC39_R** (5’-CCA TGC AGC CTG GAA TAT-3’)
- **ratqRTr18S_F** (5’-TCA AGA ACG AAA GTC GGA GG-3’)
- **ratqRTr18S_R** (5’-GGA CAT CTA AGG GCA TCA C-3’)
- **ratqRTrGDF15_F** (5’-AGC TGT CCG GAT ACT CAG TC-3’)
- **ratqRTrGDF15_R** (5’-GAG TCT CTT GGG TGC AAA TG-3’)
- **ratqRTrMYOM1_F** (5’-GGC CCA CAC TTC GCT GAG TA-3’)
- **ratqRTrMYOM1_R** (5’-TTG CCA CCT TGC ACT TCA AC-3’)
- **ratqRTrMYOM2_F** (5’-GAG AGG GCG AGA CGG TCA CAC T-3’)
- **ratqRTrMYOM2_R** (5’-CTC GAG GCT GTA CCC TCT TCA GA-3’)
- **ratqRTrMYH7B_F** (5’-CCT GCA GCC CTG CAT CGA CC-3’)
- **ratqRTrMYH7B_R** (5’-GCT TGG CCC TGA AGC TTG CGT-3’)
- **ratqRTrBNP_F** (5’-GCA GCA TGG ATC TCC AGA AGG-3’)
- **ratqRTrBNP_R** (5’-CTG CAG CCA GGA GGT CTT CC-3’)

For validation of gene expression data in human tissue, the universal probe® real-time PCR-system Roche applied science) was used with the following Primers **hqRTLRRRC39F** (5´-caacaaacttgaaacactcctgc-3´) **hqRTLRRRC39R** (5´-gcaagcatgtatttcatttcg-3´) and Universal ProbeLibrary probe: #46 (cat.no. 04688066001).
For normalization of cDNA content, homo sapiens hypoxanthine phosphoribosyltransferase 1 was used as a housekeeping gene control with the following probe and primers hqRTHPRT1F (5´-tgaccttgatttattttgcatacc-3´), hqRTHPRT1R (5´-cgagcaagacgttcagtcct-3´) and Universal ProbeLibrary probe: #73 (cat.no. 04688961001). For all other quantitative real-time PCR analyses, the Platinum SYBR®GreenER™ qPCR SuperMix (Invitrogen # 11760-500) was used in combination with the ABI Prism 7000 Sequence Detection System (Applied Biosystems).

**Sentrix BeadChip array Hybridisation**

Quality and integrity of total RNA was checked by gel analysis using the total RNA Nano chip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Berlin, Germany). Only samples with RNA index values greater than 8.5 were selected for expression profiling. RNA concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Biotin-labeled cRNA samples were prepared for hybridization on Illumina Rat Sentrix-12 BeadChip arrays (Illumina, Inc.) according to Illumina’s recommended sample labeling procedure based on the modified Eberwine protocol. In brief, 250 ng total RNA was used for cDNA synthesis, followed by an amplification/labeling step (in vitro transcription) to synthesize biotin-labeled cRNA according to the MessageAmp II aRNA Amplification kit (Ambion, Inc., Austin, TX). Biotin-16-UTP was purchased from Roche Applied Science, Penzberg, Germany. The cRNA was column purified according to TotalPrep RNA Amplification Kit, and eluted in 60 µl of water. Quality of cRNA was controlled using the RNA Nano Chip Assay on an Agilent 2100 Bioanalyzer and spectrophotometrically quantified (NanoDrop). Hybridization is performed at 58°C, in GEX-HCB buffer (Illumina Inc.) at a concentration of 100 ng cRNA/µl, unsealed in a wet chamber for 20h. Spike-in controls for low, medium and highly abundant RNAs were added, as well as mismatch control and biotinylation control oligonucleotides. Microarrays were washed twice in E1BC buffer (Illumina Inc.) at room temperature for 5 minutes. After blocking for 5 min in
4 ml of 1% (wt/vol) Blocker Casein in phosphate buffered saline Hammarsten grade (Pierce Biotechnology, Inc., Rockford, IL), array signals are developed by a 10-min incubation in 2 ml of 1 µg/ml Cy3-streptavidin (Amersham Biosciences, Buckinghamshire, UK) solution and 1% blocking solution. After a final wash in E1BC, the arrays are dried and scanned. **Microarray scanning and data analysis** Microarray scanning was done using a Beadstation array scanner, setting adjusted to a scaling factor of 1 and PMT settings at 430. Data extraction was done for all beads individually, and outliers were removed when > 2.5 MAD (median absolute deviation). All remaining data points are used for the calculation of the mean average signal for a given probe, and standard deviation for each probe was calculated. Data analysis was done by normalization of the signals using the cubic spline normalization algorithm with background subtraction, and differentially regulated genes are defined by calculating the standard deviation differences of a given probe in a one-by-one comparisons of samples or groups.²

**Western blot analysis**

In order to determine tissue specificity of myomasp/LRRC39 expression different protein extracts of rat tissues were prepared 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 supematant were separated on a 12,5% PAA-Gel and transferred on a PVDF-Membrane. The membrane was probed with an anti-human LRRC39 antibody (Abnova #H00127495-B01P ) at a dilution of 1 :750 according to the manufacturer's protocol followed by ECL detection (GE Healthcare RPN2106).

**Luciferase Reporter gene assays.**

16 hours after plating, neonatal cardiomyocytes were serum starved and infected with an adenoviral reporter gene construct carrying a firefly luciferase reporter under transcriptional control of the sm22-promotor, the myomasp/LRRC39 miRNA knockdown
virus as well as an adenoviral LacZ virus for normalization of the infection efficiency for 24 hours. To apply an identical virus load per condition we used an adenovirus encoding for a miRNA backbone but not carrying any predicted binding sites known to target any mammalian gene. After 24 hours of infection the sm22-promotor was induced by incubation of the cardiomyocytes in medium containing 10% FCS for additional 24 hours.

Luciferase assays were performed according to the manufacturer’s instructions using the Luciferase Assay System with Reporter Lysis Buffer (Promega # E4030). To normalize the infection efficiency a β-galactosidase assay was performed in a 0.1 mol/L sodium-phosphate-buffer (pH 7.3) with 1 mmol/L MgCl₂. For the colorimetric detection 2-Nitrophenyl-β-D-galactopyranoside (30µg/ml) was used as substrate. After incubation for 30 minutes a photometric analysis was performed.

**Immunofluorescence microscopy**

The subcellular localization of myomasp/LRRC39 was determined in adult rat cardiomyocytes (ARVCM) and cryosections of rat cardiac tissue using indirect immunofluorescence. Adult mouse and cardiomyocytes were prepared as described. ARVCM were fixed in 3.4% PFA for 10min at RT, 16h after isolation. Blocking and permeabilization were done in 5% BSA and 0,1% Triton X-100 (Sigma # T8787) for 1h at RT. Cryosections were blocked in 5% BSA but not permeabilized with Triton.

For detection, the cover slips or slides, respectively, were incubated with mouse polyclonal LRRC39 (Abnova # H00127495-B01P; 1:100) together with one of the following primary antibodies: polyclonal anti-calsarcin-1 (1:100), polyclonal anti-MYBPC3 (1:100), polyclonal anti-calsarcin-2 (1:200) at 4°C overnight.

Fluorescence labelling 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 Labboraties Inc.) was used for mounting of the slides.
Fluorescence micrographs were taken with Axioskop 2 Plus (Zeiss). Cell surface areas of cardiomyocytes were determined applying AxioVision Release 4.4 (Carl Zeiss Vision).

**Tissue culture experiments**

HEK-293T cells (large “T” transformed embryonic kidney cells) were maintained in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine and penicillin/streptomycin under standard cell culture conditions (37°C / 5% CO₂).

**Isolation and culture of neonatal rat ventricular cardiomyocytes (NRVMs).**

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 collagenase 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. NRVMs were resuspended and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FCS, penicillin/streptomycin and L-glutamine (PAA) for 24 h. After 24 h cells were either infected in serum-free medium or serum starved for 24 h before applying stimulants or inhibitors for the indicated time.

**Stretch experiments**

Cardiomyocyte stretch experiments were done as described previously⁶.

**Isolation and culture of adult rat ventricular cardiomyocytes (ARVMs).**

Adult rat cardiomyocytes (ARCM) were isolated from Sprague–Dawley rats as described ⁴-⁵.
Zebrafish Strain, Morpholino microinjection procedures, fractional shortening measurements

Care and breeding of zebrafish Danio rerio was done as described\(^7\). The present study was performed under institutional approvals which confirm to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIHPublication No. 85-23).

Morpholino-modified oligonucleotides were directed against the splice donor site of intron 2 (MO-myomasp/Lrrc39 = 5'- CGTTGTGCACGTACCTACCCACTGC -3') of zebrafish myomasp/Lrcc39. A standard control oligonucleotide (MO-control) (GENETOOLS, LLC) was injected at the same concentration as a negative control\(^8\). To inhibit pigmentation, 0.003 % 1-phenyl-2-thiourea was added to the embryo medium. Pictures and movies were recorded 24, 48 and 72 hours after fertilization.

To analyze contractile force of the myomasp morphant and control-morpholino injected embryos, we performed fractional shortening measurements with help of the zebraFS software application (www.benegfx.de).

Zebrafish histology, Transmission Electron Microscopy, antisense RNA in situ hybridization, and Immunostaining

Embryos were fixed in 4% paraformaldehyde and embedded in JB-4 (Polysciences, Inc.). 5µm sections were cut, dried, and stained with hematoxylin/eosin.

For transmission electron microscopy, zebrafish embryos were fixed overnight in 3% glutaraldehyde in 100mM cacodylic acid (pH 7.4) supplemented with 0.1% picrinic acid at 4°C, they were treated with OsO\(_4\) and dehydrated in a graded series of ethanol. Epon 812 was used for embedding. Subsequently, ultrathin sections (70nm) were obtained with an Ultracut E microtome (Leica), stained with uranyl acetate and Reynolds lead citrate and examined on a Philips EM 301 transmission electron microscope.
Whole mount antisense RNA in situ hybridization was carried out essentially as described\(^9\) using digoxigenin-labeled antisense probes for zebrafish \textit{cmlc2}, \textit{amhc} and \textit{vmhc}.

For whole-mount double immunostaining, embryos were fixed in Dent´s fixative and stained with monoclonal antibodies directed against atrial and ventricular meromyosin (MF20; DSHB) and against the atrial specific isoform of myosin heavy chain (S46; DSHB).

**Engineered heart tissues (”FBMES”)**

Unpurified heart cells were isolated from neonatal Wistar rats (postnatal day 0 to 3) by a fractionated DNase/Trypsin digestion protocol as previously described above. The resulting cell population was immediately subjected to fibrin-based mini engineered heart tissues (FBMEs) generation. Experimental performed as described in detail\(^{10}\). All procedures were reviewed and approved by Ethics Committee, University Hamburg.

Sylgard 184 silicone elastomer (Dow Corning) was used to produce silicone post racks in custom-made teflon casting molds. The two-component Sylgard 184 elastomer was handled according to manufactures instructions and degassed under vacuum conditions before casting. The racks contained 4 pairs of posts length/width of racks: 79 x 18.5 mm, length of posts 12 mm, diameter 1 mm, plate diameter 2 mm, distance (center-center) 8.5 mm. Teflon spacers for producing the casting molds had the following geometry: length 12 mm, width 3 mm, height 13.5 mm.

To generate FBMEs, a reconstitution mix was prepared on ice as follows (final concentration): 4.1x10\(^6\) cells/ml, 5 mg/ml bovine fibrinogen (Sigma F4753, stock solution: 200 mg/ml plus aprotinin 0.5 µg/mg fibrinogen in NaCl 0.9%), 100 µl/ml Matrigel (BD Bioscience 356235). 2xDMEM was added to match the volumes of fibrinogen and thrombin stock to ensure isotonic conditions. Casting molds were prepared by placing the teflon spacer in 24-well culture dishes and adding 1.6 ml 2% agarose in PBS.
(Invitrogen 15510-027) per well. After agarose solidification the spacer was removed and silicon post racks were placed onto the dishes with pairs of posts reaching into each casting mold. For each FBME 145 µl reconstitution mix was mixed briefly with 4.5 µl thrombin (100 U/ml, Sigma Aldrich T7513) and pipetted into the agarose slot.

For fibrinogen polymerisation the constructs were placed in a 37 °C, 7% CO₂ humidified cell culture incubator for 2 hours. 300 µl of cell culture medium was then added per well to ease removal of the constructs from agarose casting molds. The racks were transferred to new 24-well cell culture dishes. Constructs were maintained in 37 °C, 7% CO₂ humidified cell culture incubator. Media was changed on Mondays, Wednesdays and Fridays. EHT medium consisted of DMEM (Biochrom F0415), 10% horse serum (Gibco 26050), 2% chick embryo extract, 1% Penicillin/Streptomycin (Gibco 15140), insulin (10 µg/ml, Sigma Aldrich I9278), tranexamic acid (400 µm/ml, Sigma Aldrich 857653) and aprotinin (33 µg/ml, Sigma Aldrich A1153).

**Video optical analysis**

The setup for video optical recording consisted of a cell incubator unit with control of CO₂, humidity and temperature, and a glass roof for monitoring purposes. Light diodes were positioned underneath the cell culture dish. A Basler camera (Type A 602f-2) placed above the cell culture unit was positioned in XYZ direction (IAI Corporation) in a PC-controlled manner. Video optical recording and light exposure were synchronized to minimize heating of cell culture medium by light. Video optical analysis was performed with a customized software package by ctmv.de. The software is based on figure recognition of the contracting muscle strip at top and bottom ends in a fully automated manner. The distance between the ends of the muscle strip is determined during contractions and recorded over time. Based on post geometry, elastic modulus of Sylgard 184 (2.6 kPa) and delta of post distance (post deflection) force was calculated. Recorded contractions are identified by peak criteria (threshold value, minimum relaxation). Based on identified contractions, values for frequency, average force, fractional shortening, contraction – and relaxation time (T1, T2, respectively) were
calculated. Records of experiments are automatically generated with two levels of quality control: pictures are taken at the beginning and the end of each measurement. Contractions are recorded as force development over time. The effort to analyse contractility with this setup is limited to defining the x-, y-, z- coordinates of the camera for each FBME\textsuperscript{10}.

**Transverse aortic constriction in C57BL6 mice:**

Transverse aortic constriction (TAC) was performed in male C57BL6 mice (8-10 weeks old). Briefly the animals were anesthetized with ketamine (120 mg/kg i.p.) plus xylazine (15 mg/kg i.p.). The mice were orally intubated with 20-gauge tubing and ventilated (Harvard Apparatus) at 120 breaths per minute (0.2 ml tidal volume). The aortic constriction was created via a lateral thoracotomy at the second intercostal space. A suture (Prolene 6-0) was placed around the transverse aorta between the brachiocephalic and left carotid artery. The suture was ligated against a 27-gauge needle, the needle was removed leaving a discrete stenosis. The chest was closed and a pneumothorax evacuated. Sham animals underwent the same the procedure except ligation. After 4 weeks animals were sacrificed and RNA was harvested and after cDNA synthesis gene expression was assessed with quantitative realtime PCR. In these realtime experiments mouse- Rpl32 served as housekeeping gene:

Rpl32\textsubscript{e1} (5'-F GGTGGCTGCCATCTTACG-3')
Rpl32\textsubscript{e3} (5'-R CCGCACCCTTGGTCAATGC-3')
qPCR-LRRC39m F 730 (5'- CCTCCCACCAGAGCTCAGCA-3')
qPCR-LRRC39m R 834 (5'- TCGAGGGCGGCATGTCCA-3')
Statistical analyses.

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

Literature:

**Supplemental Figure I. Statistical microarray data analyses of myomasp/LRRC39 regulated genes.** (A) Scatter plot showing the log ratio of the means of myomasp/LRRC39 differentially regulated genes plotted against the log ratio of the means of the control hybridizations. Red dots represent significantly upregulated genes, blue dots downregulated genes (>4 fold each). (B) Heat map displaying the genes with > 4 fold differential expression. Each hybridization sample is plotted individually. Red colors represent upregulation, blue colors downregulation. (C) Table showing differential regulation of M-band associated proteins after myomasp/LRRC39 knockdown.

**Supplemental Figure II. Adenoviral knockdown of myomasp/LRRC39 in neonatal rat ventricular cardiomyocytes.** (A) Realtime PCR analysis showing a time dependent suppression of myomasp/LRRC39 mRNA after adenovirus mediated knockdown through infection with AdMiMyomasp compared to AdMiNeg infected cells. While adenovirus mediated knockdown for 24h revealed a moderate reduction of myomasp/LRRC39mRNA which not reached statistical significance, reduction of myomasp/LRRC39 mRNA after 72h was highly significant (-85%; p<0.01). (B) Representative immunoblot showing a markedly reduction of myomasp/LRRC39 protein expression through adenoviral infection with 50 ifu AdMiMyomasp and a AdMiMyomasp alternative virus compared to 50 ifu AdMiNeg for 72h.

**Supplemental Figure III. Morpholino knockdown of zebrafish myomasp/LRRC39 results in sarcomeric M-Band defects** Accessory panel with additional low (left panel) and high magnification EM images (right panel) of randomly selected zebrafish skeletal-muscle-sarcomeres comparing MO-myomasp knockdown zebrafish (upper panel) with control animals (bottom).

**Supplemental Figure IV. Myomesin 2 protein expression after knockdown of myomasp/LRRC39 in NRVM** Representative immunoblot probed with an anti-rabbit myomesin 2 antibody (Santa Cruz) showing a significant of target protein expression after adenoviral mediated knockdown of myomasp/LRRC9 in rat ventricular cardiomyocytes.
C

Regulation of M-band associated proteins after LRRC39 / Myomasp knockdown

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
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</table>

Supplemental Figure I
Supplemental Figure II

(A) Graph showing myomasp-mRNA expression (n-fold change) for various conditions:
- Ad miNEG 24h
- Ad miNEG 48h
- Ad miNEG 72h
- Ad miMyomasp 24h
- Ad miMyomasp 48h
- Ad miMyomasp 72h

Statistical significance:
- P = n.s
- P < 0.05
- P < 0.01

(B) Western blot images showing myomasp/LRRC and myomasp/V5 at 43 kDa.
Supplemental Figure III
Supplemental Figure IV