Myostatin Regulates Energy Homeostasis in the Heart and Prevents Heart Failure

Nadine Biesemann, Luca Mendler, Astrid Wietelmann, Sven Hermann, Michael Schäfers, Marcus Krüger, Thomas Boettger, Thilo Borchardt, Thomas Braun

Rationale: Myostatin is a major negative regulator of skeletal muscle mass and initiates multiple metabolic changes, including enhanced insulin sensitivity. However, the function of myostatin in the heart is barely understood, although it is upregulated in the myocardium under several pathological conditions.

Objective: Here, we aimed to decipher the role of myostatin and myostatin-dependent signaling pathways for cardiac function and cardiac metabolism in adult mice. To avoid potential counterregulatory mechanisms occurring in constitutive and germ-line–based myostatin mutants, we generated a mouse model that allows myostatin inactivation in adult cardiomyocytes.

Methods and Results: Cardiac MRI revealed that genetic inactivation of myostatin signaling in the adult murine heart caused cardiac hypertrophy and heart failure, partially recapitulating effects of the age-dependent decline of the myostatin paralog growth and differentiation factor 11. We found that myostatin represses AMP-activated kinase activation in the heart via transforming growth factor-β–activated kinase 1, thereby preventing a metabolic switch toward glycolysis and glycogen accumulation. Furthermore, myostatin stimulated expression of regulator of G-protein signaling 2, a GTPase-activating protein that restricts Gaq and Gas signaling and thereby protects against cardiac failure. Inhibition of AMP-activated kinase in vivo rescued cardiac hypertrophy and prevented enhanced glycolytic flow and glycogen accumulation after inactivation of myostatin in cardiomyocytes.

Conclusions: Our results uncover an important role of myostatin in the heart for maintaining cardiac energy homeostasis and preventing cardiac hypertrophy. (Circ Res. 2014;115:296-310.)

Key Words: AMP-activated protein kinases ■ biochemistry ■ cardiomegaly ■ heart failure ■ metabolism ■ myostatin

Myostatin, also named growth and differentiation factor (GDF) 8, is a member of the transforming growth factor-β superfAMILY of growth factors and serves as a major negative regulator of skeletal muscle growth. Inactivation of myostatin by targeted deletion and by naturally occurring mutations leads to a vast increase of muscle mass in several species, including mice and human beings. Myostatin also plays a decisive role in the regulation of metabolic processes. Constitutive myostatin knockout mice show reduced adiposity, increased insulin sensitivity, and are resistant to obesity. Inactivation of myostatin in 2 genetic models of obesity led to a partial suppression of fat accumulation and normalization of glucose metabolism.

The primary site of myostatin expression is skeletal muscle, although myostatin is also produced in significant amounts in fat tissue and the heart. Interestingly, myostatin is strongly upregulated under different pathological conditions of the heart (eg, myocardial infarction, hypertrophy, and heart failure), arguing for a specific role in cardiac pathophysiology. Furthermore, a recent study revealed that GDF11, which is highly similar to myostatin/GDF8, reverses age-related cardiac hypertrophy, suggesting a role of GDF family members in the restriction of compensatory cardiac growth. Yet, germ-line inactivation of myostatin yielded conflicting results, describing no effect on cardiac hypertrophy or function or reduced ejection fraction and eccentric hypertrophy. Similarly, lineage-specific deletion of myostatin in cardiac progenitor cells revealed no differences in cardiac size and function in young mice, whereas constitutive overexpression of myostatin in the heart and in the heart and skeletal muscle reduces cardiac mass and cardiomyocyte proliferation without effects on cardiac systolic function.

Original received April 13, 2014; revision received May 7, 2014; accepted May 7, 2014. In April 2014, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.38 days.

From the Department of Cardiac Development and Remodeling, Max-Planck-Institute for Heart and Lung Research, Bad Nauheim, Germany (N.B., L.M., A.W., M.K., T. Boettger, T. Borchardt, T. Braun); Institute of Pharmacy and Biochemistry, Johannes Gutenberg University, Mainz, Germany (N.B.); Institute of Biochemistry, Faculty of General Medicine, University of Szeged, Szeged, Hungary (L.M.); and European Institute for Molecular Imaging, University of Münster, Münster, Germany (S.H., M.S.).

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi: 10.1161/CIRCRESAHA.115.304185/-/DC1.

Correspondence to Dr Thilo Borchardt or Dr Thomas Braun, Department of Cardiac Development and Remodeling, Max-Planck-Institute for Heart and Lung Research, Ludwigstrasse 43, D-61231 Bad Nauheim, Germany. E-mail Thilo.Borchardt@mpi-cn.mpg.de or Thomas.Braun@mpi-cn.mpg.de

© 2014 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org DOI: 10.1161/CIRCRESAHA.115.304185
To date, no study has addressed the consequences of acute myostatin inhibition in the adult heart, which would uncover potentially distinct functions of myostatin in the heart during development and postnatal life. Also, critically missing from previous studies is the role of myostatin in the regulation of myocardial metabolism and the resulting impact on cardiac hypertrophy and heart failure. The importance of metabolic changes for cardiac hypertrophy and heart failure is widely acknowledged, but the mechanisms that direct the switch to a fetal metabolic pattern during cardiac hypertrophy and heart failure are poorly understood. Here, we identify myostatin as an important regulator of myocardial metabolism and growth, which suppresses AMP-activated kinase (AMPK) activity in cardiomyocytes via transforming growth factor-β-activated kinase 1 (TAK1) to prevent acquisition of a fetal metabolic pattern, thereby stabilizing the metabolic status of adult cardiomyocytes and restricting cardiac hypertrophy. We describe that conditional inactivation of myostatin in the adult murine heart leads to enhanced glycolysis, augmented glycogen storage, and cardiac hypertrophy, resulting in heart failure and increased lethality.

Methods

Generation and Treatment of Transgenic Mouse Lines

All procedures were performed in accordance with the guidelines for animal experimentation of the local authorities. All animals were kept on the identical C57BL/6J background. For all experiments, male mice were used. The conditionally active Mstnfl/fl allele was generated by insertion of loxP elements upstream and downstream of exons 1 and 2 of the mouse myostatin gene using established techniques. To overexpress myostatin conditionally, we created CAGG-Mstn mice by random integration of a flox-βgeo-flox-myostatin-ires-EGFP cassette into the genome of embryonic stem cells. Cre recombination was achieved by intraperitoneal administration of tamoxifen for 5 consecutive days. To inhibit AMPK, mice were injected either with 20 mg/kg compound C (Tocris Biosciences, Bristol, UK) every second day or with 15 mg/kg 9-d-arabinofuranosyladenine (Ara-A) (Sigma, Steinheim, Germany) daily for a period of 10 days intraperitoneally following administration of tamoxifen. For AMPK-activation, mice were injected either with 20 mg/kg compound C (Tocris Biosciences, Bristol, UK) every second or with 15 mg/kg 9-d-arabinofuranosyladenine (Ara-A) (Sigma, Steinheim, Germany) daily for a period of 10 days intraperitoneally following administration of tamoxifen.

Physiological Measurements

Cardiac MRI was performed using a 7.0-T Bruker Pharmascan, equipped with a 300 mT/m gradient system, using a custom-built circularly polarized birdcage resonator and the IntraGate self-gating tool (Bruker, Ettlingen, Germany) following standard protocols. The skeletal muscle content was measured by MRI with a linear polarized 1H volume resonator. After several positioning scans, a coronal multislice multiecho spin-echo sequence was recorded and analyzed with the IntraGate self-gating software (BioRad, Germany) daily for a period of 10 days intraperitoneally following established protocols.

ELISAs, RT-PCR, and Microarray Analysis

For treatment with small molecules, cardiomyocytes were stimulated with different reagents as indicated. RNA was isolated according to standard protocols using the Trizol reagent (Invitrogen, Karlsruhe, Germany) and subjected to RT-PCR analysis using the Bio-Rad iCYCLER iQ5 Real-time PCR machine with acidic ribosomal protein as internal standard as described before. Serum concentration of myostatin was analyzed using a myostatin ELISA (R&D Systems, Minneapolis, MN). For microarray analysis, samples were processed according to the Affymetrix manual and hybridized to Affymetrix Mouse Gene 1.0 ST Arrays. Raw data were analyzed with the Affymetrix Expression Console using the robust multi-array average analysis algorithm. Enrichment analysis was performed with the R package ResA3. ResA3 uses resampling algorithms to determine the statistical significance of gene ontology annotations.

Western Blot Analysis

Ventricular tissue was ground in liquid nitrogen and lysed by standard procedures. Lysate (10–20 µg) was separated by SDS-PAGE and incubated with different antibodies. Immunoreactive proteins were visualized on a VersaDoc (BioRad) and quantified with the QuantityOne software (BioRad).

Morphological Analysis

To analyze the cardiomyocyte area, cryosections were fixed and incubated with tetramethylrhodamine-labeled lectin from Triticum vulgaris (wheat germ agglutinin; Sigma-Aldrich, Steinheim, Germany). Cryosections from gastrocnemius were incubated with anti-dystrophin (Abcam, Cambridge, UK) and the corresponding secondary antibody. Cardiomyocyte area was analyzed out of 4 random areas (×20 images) per tissue block (n=3 per mouse model) using the ImageJ software. Periodic acid Schiff staining was performed following the instruction of the manufacturer (Sigma, Steinheim, Germany).
Results

Deletion of Myostatin in Adult Cardiomyocytes Leads to Heart Failure and Increased Lethality

To analyze the role of myostatin in the postnatal heart, we generated a mouse strain in which exons 1 and 2 of myostatin were flanked by loxP sites. Induction of myostatin deletion in adult cardiomyocytes using aMyHC-MCM mice resulted in reduction of myostatin expression >80% 10 days after tamoxifen administration (Figure 1A). To our surprise, cardiomyocyte-specific deletion of myostatin caused a 26.5% increase in lethality after 10 days, whereas tamoxifen-treated control animals (aMyHC-MCM) and mice transiently overexpressing myostatin in cardiomyocytes (aMyHC-MCM/CAGG-Mstn) maintained a normal survival curve (Figure 1B). MRI measurements revealed normal heart functions in tamoxifen-treated aMyHC-MCM control mice and in aMyHC-MCM/Mstn<sup>fl/fl</sup> mice before tamoxifen administration (Figure 1C and 1D; Table). In contrast, we observed severe atrial and ventricular dilation and fibrosis of the right atrium 10 days after tamoxifen administration in aMyHC-MCM/Mstn<sup>fl/fl</sup> mice (Figure 1D; Online Figure IIA). Left ventricular function was severely impaired as indicated by >50% reduction of the ejection fraction, decreased stroke volume, and increased end-systolic volume (Table; Online Movie I). Furthermore, left ventricular mass and wall thickness were increased (Table). Midventricular sections of tamoxifen-treated aMyHC-MCM/Mstn<sup>fl/fl</sup> mice disclosed that the increase in left ventricular wall thickness 10 days after tamoxifen administration was caused by a 30% increase in cardiomyocyte cross-sectional area (Figure 1H–1J). Quantitative RT-PCR and Western blot analysis showed a strong induction of cardiac stress marker atrial natriuretic peptide and brain natriuretic peptide expression 10 days after deletion of myostatin (Online Figure IA and IB), which returned to baseline after 6 weeks (Online Figure IB). To monitor potential effects of the inactivation of myostatin on electric conduction, we implanted ECG transmitter in aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> mice. aMyHC-MCM/Mstn<sup>fl/fl</sup> mice displayed a severe prolongation of the PR interval starting 3 days after induction of myostatin deletion, which reached a maximum (64 ms) after 5 days (Online Figure II) indicating atrioventricular conduction defects. No evidence for arrhythmias or atrial fibrillation was found.

We expected that cardiac functions of aMyHC-MCM/Mstn<sup>fl/fl</sup> mice would further deteriorate with time. However, additional MRI measurements using the same group of tamoxifen-treated aMyHC-MCM/Mstn<sup>fl/fl</sup> mice revealed a major improvement of cardiac functions after 6 weeks, essentially leading to a complete restoration of cardiac contractility (Figure 1D; Table; Online Movie I). We reasoned that reversal of the phenotype might be caused by compensatory mechanisms, which are activated by extended loss of myostatin in cardiomyocytes. In fact, we observed a strong upregulation of myostatin expression in ventricular tissue starting 21 days after the initial tamoxifen treatment, which was still prominent after 120 days (Figure 1F). To identify the source of myostatin expression in hearts carrying mutated myostatin alleles in cardiomyocytes, we isolated cardiomyocyte and noncardiomyocyte fractions (Online Figure III) and measured myostatin expression by quantitative RT-PCR. Our data indicated that the increase of myostatin expression in the heart after inactivation of myostatin in cardiomyocytes was caused by noncardiomyocytes (Figure 1A and 1G; Online Figure IV), indicating a strong bias to maintain local myostatin levels. This conclusion was also supported by an increase of myostatin concentrations in the serum after deletion of myostatin in adult cardiomyocytes (Online Figure VA). In contrast to the loss of myostatin, transient overexpression of myostatin using aMyHC-MCM/CAGG-Mstn mice did not cause decline of contractility after 10 days and 6 weeks but significantly reduced left ventricular wall thickness arguing for beneficial effects of increased, short-term myostatin expression for the heart (Figure 1E; Table; Online Movie I). To analyze whether the loss of myostatin in adult cardiomyocytes had systemic effects (eg, on the skeletal musculature), we investigated the skeletal muscle content and skeletal muscle architecture by MRI and morphological examination, respectively. No changes in body weight, skeletal muscle mass, myocyte area, or myostatin expression were detected (Online Figure VB–VI).

Because deletion of myostatin in adult cardiomyocytes led to heart failure, we wanted to know whether constitutive deletion of myostatin in the cardiomyogenic lineage led to a similar phenotype. Analysis of aMyHC-Cre/Mstn<sup>fl/fl</sup> mice, in which the myostatin gene was deleted during development, showed a reduced survival rate at 4 to 5 months compared with aMyHC-Cre mice (Online Figure VIB), dilation of the left ventricle, left ventricular and right ventricular systolic dysfunction, and increased expression of cardiac stress markers (Online Figure VIC, VIE, and VIF; Online Table I; Online Movie II). However, this phenotype was less severe with deletion of myostatin in adult animals (Figure 1D; Table; Online Movie I) and did not result in cardiac hypertrophy (Online VID; Online Table I). In contrast, extended overexpression of myostatin during development using aMyHC-Cre/CAGG-Mstn mice showed no cardiac phenotype at 4.5 months of age (Online Tables I and Online Figure VIC; Online Movie II).

Myostatin Represses AMPK by Activation of TAK1 to Regulate Cardiac Metabolism and Restrict Hypertrophy

To gain further mechanistic insights into the function of myostatin in the heart, we performed DNA microarray analysis of hearts from aMyHC-MCM/Mstn<sup>fl/fl</sup> and control mice 10 days after tamoxifen-induced gene deletion. As expected, we detected numerous deregulated genes that reflect pathological changes in myostatin-deficient hearts (Online Figure...
Figure 1. Inactivation of myostatin (Mstn) in adult cardiomyocytes increases lethality and leads to heart failure and ventricular hypertrophy. A, Quantitative myostatin reverse transcription polymerase chain reaction (RT-PCR) analysis in aMyHC-MCM and aMyHC-MCM/Mstnfl/fl cardiomyocytes (n≥3). B, Survival curve (n=15 [aMyHC-MCM], n=34 [aMyHC-MCM/Mstnfl/fl], and n=8 [aMyHC-MCM/CAGG-Mstn]). C to F, Representative MRI 4-chamber views of aMyHC-MCM (C), aMyHC-MCM/Mstnfl/fl (D), and aMyHC-MCM/CAGG-Mstn (E) mice. Scale bar, 10 mm. Arrows indicate dilated right atria and left ventricle. F, Quantitative RT-PCR (qRT-PCR) analysis of myostatin expression in aMyHC-MCM and aMyHC-MCM/Mstnfl/fl ventricles (n=3). G, Quantitative myostatin RT-PCR of noncardiomyocytes from aMyHC-MCM and aMyHC-MCM/Mstnfl/fl mice (n≥3). H to J, Wheat germ agglutinin staining of aMyHC-MCM (H) and aMyHC-MCM/Mstnfl/fl (I) hearts. Scale bar, 50 μm. J, Analysis of cardiomyocyte cross-sectional area. Mean area was detected by analysis of 4 different areas per heart (n=3). Values represent means±SEM. *P<0.05, **P<0.01. d indicates days; NCM, noncardiomyocytes; and w, weeks.
VIIA). More interestingly, gene ontology term analysis in myostatin-mutant hearts using the ResA3 tool revealed significant changes in genes involved in the regulation of glucose and glycogen metabolism as well as of genes involved in fatty acid oxidation (Online Figure VIII). We hypothesized that myostatin inactivation suppresses energy-consuming pathways and activates a fetal metabolic pattern, which reflects the heart’s attempt to cope with heart failure and hypertrophy.

To further prove the myostatin-dependent reconfiguration of energy metabolism, we performed real-time measurements of the extracellular acidification rate, a parameter of glycolysis, in isolated cardiomyocytes from tamoxifen-treated aMyHC-MCM and aMyHC-MCM/Mstnfl/fl mice using the Seahorse XFe analyzer. Deletion of myostatin-mutant hearts using the ResA3 tool revealed significant changes in genes involved in the regulation of glucose uptake and glycogen deposition, which provides an endogenous reservoir of substrates for glycolysis (Figures 2H and 5C–5F). Next, we performed an [18F]-fluorodeoxyglucose positron emission tomographic analysis in living animals to avoid any potential in vitro artifacts and to demonstrate unambiguously that myostatin affects myocardial metabolism in the beating heart. [18F]-fluorodeoxyglucose is a radionuclide-labeled glucose analog, which is taken up by glucose transporters and trapped in the cell after phosphorylation by hexokinase. Our measurements revealed a significant increase of glucose uptake and glycolytic flux in the heart after deletion of myostatin in adult cardiomyocytes (Figure 2I–2K).

To investigate the molecular cause of enhanced glycolysis and glycogen deposition, we examined the activity of AMPK, which is a key regulator of energy homeostasis in the heart.28 Interestingly, inactivation of myostatin in adult cardiomyocytes of aMyHC-MCM/Mstnfl/fl mice resulted in a 1.9-fold increased phosphorylation and activation of AMPK-α 10 days after tamoxifen administration (Figure 3A). Correspondingly, we found increased phosphorylation of the AMPK targets acetyl-CoA carboxylase and glycogen synthase (Figure 3A). To further study the regulation of AMPK by myostatin, we analyzed myostatin-overexpressing aMyHC-Cre/CAGG-Mstn mice. Interestingly, we detected a strong reduction of AMPK and acetyl-CoA carboxylase phosphorylation in myostatin-overexpressing cardiomyocytes (Figure 3B). Furthermore, we found that treatment of adult mouse ventricular cardiomyocytes with recombinant myostatin for 10 minutes resulted in a strong inhibition of AMPK phosphorylation (Figure 3C and 3D), further corroborating myostatin-dependent suppression of AMPK activity in cardiomyocytes. Activation of AMPK with AICAR (5-aminoimidazole-4-carboxamide-1-β-d-ribofuransyl 5′-monophosphate) increased the glycolytic flux, whereas inhibition of AMPK activity with compound C decreased the glycolytic flux in wild-type cardiomyocytes, mimicking the effects of AMPK activation after inactivation of myostatin (Online Figure IX).

To delineate the intracellular signaling pathways that mediate cellular myostatin responses, we inhibited TAK1, which besides Smads is the main signal transducer of the myostatin receptors activin A receptor type IIB and activin A receptor type IIA (type II receptors) and activin receptor–like kinase (ALK) 4 and ALK5 (type I receptors).29 Treatment of adult mouse ventricular cardiomyocytes with (5Z)-7-oxooxazol, a TAK1 inhibitor, resulted in reduced phosphorylation of the TAK1 target p38, demonstrating efficiency of TAK1
Figure 2. Acute deletion of myostatin switches cardiomyocytes to a glycolytic metabolism. A to E, Real-time analysis of extracellular acidification rate (ECAR, because of glycolysis) and oxygen consumption rate (OCR, because of mitochondrial respiration) of isolated cardiomyocytes from aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> mice 10 days after tamoxifen administration, measured in Seahorse extracellular flux assay. A, C to E, Glucose-starved cardiomyocytes from aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> mice 10 days after tamoxifen administration were treated with 10 mmol/L glucose, 5 μmol/L oligomycin (Oligo, ATP synthase inhibitor), and finally 100 mmol/L 2-deoxy glucose (2-DG). ECAR was measured in real-time (n≥3, 20 replicates per mouse). A, Glycolytic flux. C, ECAR in glucose-starved cardiomyocytes. D, Glycolysis (glucose-induced response, blue). E, Maximal glycolytic capacity (oligomycin-induced response, green). B, Cardiomyocytes from aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> mice 10 days after tamoxifen administration were treated with 5 μmol/L oligomycin, 0.1 μmol/L carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and 5 μmol/L antimycin. OCR was measured in real time with the Seahorse XFe 96 analyzer (n≥3, 20 replicates per mouse). F to G, Lactate content in noncultivated cardiomyocytes (F, n=3) and serum (G, n=4) from aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> mice 10 days after tamoxifen administration. H, Glycogen content in freshly isolated cardiomyocytes from aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> mice 10 days after tamoxifen administration (n=3). I to K, Glucose uptake in aMyHC-MCM (I) and aMyHC-MCM/Mstn<sup>fl/fl</sup> (J) mice 10 days after tamoxifen administration measured by [18F]-fluorodeoxyglucose (FDG)- positron emission tomography. I and J, Representative image slices. K, Glucose uptake determined by percentage of injected dose (%ID) of [18F]-FDG (n≥5). Values represent means±SEM. *P<0.05, **P<0.01.
inhibition. Inhibition of TAK1 reversed the effects of myostatin on AMPK activity (Figure 3C), and treatment of adult cardiomyocytes with the ALK4/ALK5/ALK7 inhibitor SB505124 rescued the inhibitory effect of myostatin on AMPK activity (Figure 3D), suggesting that myostatin inhibits AMPK activity via ALK4/ALK5 and TAK1. We concluded that myostatin represses AMPK activity in adult cardiomyocytes, thereby conserving the metabolic status and restricting cardiac hypertrophy (Figure 5K).

Because our data suggested a crucial role of myostatin-dependent control of AMPK activity in the regulation of cardiomyocyte metabolism, we investigated whether inactivation of myostatin alters the interaction of AMPK with its partners. Coimmunoprecipitation of AMPK and its binding partners in cardiomyocytes followed by mass spectrometry uncovered 6 different isoforms of AMPK in the heart, as well as several novel potential interaction partners (Figure 4B and 4C). Importantly, we observed major changes in the AMPK interactome after inactivation of myostatin, whereas the composition of AMPK isoforms did not change (Figure 4A and 4C). The majority of deregulated interaction partners were part of metabolic pathways. Deletion
Figure 4. Loss of Myostatin changes the interaction of AMP-activated kinase (AMPK) with key metabolic proteins. A to E, Immunoprecipitation of AMPK-α in cardiomyocytes of aMyHC-MCM and aMyHC-MCM/Mstnfl/fl mice 10 days after tamoxifen administration (n=2). AMPK interaction partners were identified by mass spectrometry. A, Volcano plot of proteins interacting with AMPK in aMyHC-MCM and aMyHC-MCM/Mstnfl/fl cardiomyocytes. Differences between interaction partners and significance of regulation (−log10 P value) were assessed using a 2-sided t test. Deregulated genes involved in metabolism are shown in green; genes involved in cardiac hypertrophy and growth are shown in red. B, Network of proteins interacting with AMPK-α assembled with the STRING software. The different AMPK isoforms are highlighted in the black box. C, Differences of protein concentrations and P value of AMPK isoforms between aMyHC-MCM and aMyHC-MCM/Mstnfl/fl cardiomyocytes. D, Validation of data by biological replicates. Log10 ratios of biological replicates were plotted against each other in a scatter plot yielding a Pearson Correlation r of 0.977 (aMyHC-MCM) and 0.975 (aMyHC-MCM/Mstnfl/fl). E, Enrichment analysis of AMPK-α interaction partners from aMyHC-MCM and aMyHC-MCM/Mstnfl/fl cardiomyocytes using the ResA3 tool. The gene ontology term, number of corresponding terms (n), P value, and a 7-figure summary are shown. The summary diagram contains the minimum and maximum (outer whiskers), p10 percentile and p90 percentile (inner whiskers), first and third quartiles (box), median (black line in box), and mean (light line in box). PRKAG indicates γ subunit of AMPK.
of myostatin decreased the interaction of AMPK with all major enzymes involved in β-oxidation (acyl-CoA dehydrogenases, enoyl-CoA hydratases, acetyl-CoA acetyltransferases, β-hydroxacyl-CoA dehydrogenases and enoyl-CoA isomerases) and increased binding to glycolytic enzymes (eg, hexokinase; Figure 4A and 4E). In addition, we found increased binding of proteins involved in cell growth and differentiation (Figure 4A and 4E).

To further validate the central role of AMPK in mediating effects of myostatin on myocardial metabolism and hypertrophy, we treated aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> mice with the AMPK inhibitor compound C. Compound C treatment significantly inhibited AMPK activation after acute myostatin deletion (Online FigureXA) and completely normalized cardiac hypertrophy as indicated by reduction of the left ventricular mass and wall thickness (Figure 5A; Online FigureXB). Because compound C reduces cardiac contractility, it was not possible to analyze additional parameters of cardiac function in compound C–treated myostatin-mutant mice (Online Figure XC and XD). However, administration of compound C reduced atrial natriuretic peptide and brain natriuretic peptide expression in aMyHC-MCM/Mstn<sup>fl/fl</sup> mice to baseline levels (Figure 5B; Online FigureXE), inhibited increased glycolysis and extracellular acidification rate after glucose starvation (Figure 5I and 5J; Online Figure XF and XG), and prevented enhanced glycosgen deposition in aMyHC-MCM/Mstn<sup>fl/fl</sup> hearts (Figure 5G and 5H), confirming that deletion of myostatin activates glycolysis and glycogen accumulation via AMPK. Similar experiments were performed using another AMPK inhibitor, Ara-A, yielding essentially the same results (Online FigureXIB–XIH).

**Hypocontractility of Myostatin-Mutant Adult Hearts Is Associated With Downregulation of Rgs2**

Myocardial contractility is primarily controlled by Ca<sup>2+</sup> and cAMP signaling and not by AMPK, which is a main regulator of cellular metabolism. Because inhibition of AMPK did not improve impaired cardiac contractility, we analyzed additional signaling pathways potentially affected by loss of myostatin. Gene ontology term analysis of transcriptional profiles of mutant hearts revealed significant changes in the expression of genes regulating G-protein signaling pathways and cellular responses to calcium (Figure 6A). We also found a clear increase of protein kinase A (PKA) phosphorylation, along with its direct target CAMKII (calmodulin-dependent protein kinase II), in aMyHC-MCM/Mstn<sup>fl/fl</sup> hearts 10 days after tamoxifen administration (Figure 6B). Because activation of PKA increases intracellular calcium, we examined typical targets of calcium signaling and detected a strong up-regulation of protein kinase C (PKC-α/βII) and PKD (protein kinase D) phosphorylation (Figure 6C). In addition, we saw upregulation of calcineurin and myocyte enhancer factor 2A (MEF2A; Figure 6C), prominent inducers of mechanical dysfunction and hypertrophy. Increased PKC-α activity has been demonstrated to reduce cardiac contractility and promote heart failure, suggesting that the decreased contractility after deletion of myostatin in adult hearts is mediated by increased PKC-α phosphorylation. Interestingly, after 6 weeks, all upregulating signaling pathways returned to baseline when the decrease of myostatin expression in the heart is overcompensated by noncardiomyocytes (Figure 7A). To validate these findings, we again used our myostatin-overexpressing aMyHC-Cre/CAGG-Mstn mice. We found a decline of PKA, PKC-α/βII, and phospholamban phosphorylation (Figure 6D), strongly suggesting that myostatin signaling suppresses PKA phosphorylation and thereby also subsequent phosphorylation events.

Next, we wanted to learn more about myostatin-mediated regulation of Ca<sup>2+</sup> and cAMP signaling. Guided by our DNA microarray analysis, we performed quantitative RT-PCR experiments and detected a downregulation of regulator of G-protein signaling 2 (Rgs2) (Figure 7B), which inhibits β-adrenergic<sup>34</sup> and Gq-mediated signaling<sup>35</sup> and is required to prevent cardiac hypertrophy and heart failure in response to moderate pressure-overload.<sup>36</sup> Treatment of adult mouse ventricular cardiomyocytes with myostatin stimulated RGS2 protein expression (Figure 7C) in the presence of (5Z)-7-oxozenoic acid, indicating that myostatin exerts its effect on Rgs2 by a TAK1-independent pathway. Interestingly, the mouse Rgs2 promoter carries a binding site for forkhead activin signal transducer 1, which interacts with Smads raising the possibility that myostatin activates Rgs2 transcription via its Smad2 and Smad3 signaling branch. To prove this hypothesis, we performed p-Smad3 ChIP (chromatin immunoprecipitation) using aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> hearts 10 days after Tamoxifen administration. Deletion of myostatin clearly diminished binding of p-Smad3 to the Rgs2 promoter (Figure 7D), indicating that myostatin activates Rgs2 transcription via Smad3 and thereby restricts Gq signaling (Figure 7E).

**Discussion**

Initially, we were surprised that inactivation of myostatin in adult cardiomyocytes led to major changes in cardiac metabolism, including glycosgen accumulation, cardiac hypertrophy, >50% reduced ejection fraction, and a dramatic increase in lethality, because germ-line deletion of myostatin uncovered only minor effects on cardiac function.<sup>11–14</sup> However, previous studies uncovered a correlation of reduced myostatin activity with early lethality in cattle<sup>37</sup> and demonstrated that inactivation of myostatin in laminin-deficient dyW mice leads to early postnatal death.<sup>38</sup> We assume that inactivation of myostatin in adult lineage–specific inactivation of myostatin induced excessive expression in noncardiomyocytes of the heart, which rescued several aspects of the heart phenotype. Interestingly, administration of GDF11, a paralog of myostatin with 90% sequence homology,<sup>9</sup> rescues cardiac hypertrophy in aging mice, confirming the importance of myostatin (GDF8)/GDF11-dependent signaling for the regulation of cardiac hypertrophy.<sup>10,39</sup> In contrast to deletion of myostatin in adult cardiomyocytes, constitutive lineage–specific mutants seem to follow a different path avoiding excessive expression in noncardiomyocytes of the heart at the expense of incomplete
Figure 5. Myostatin (Mstn) mediates cardiac hypertrophy and glycogen accumulation via AMP-activated kinase (AMPK). A to J, aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> mice were treated with tamoxifen and with or without the AMPK inhibitor compound C. A, MRI analysis of left ventricular mass (LV mass) of aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> hearts with and without compound C treatment (n≥7 without compound C, n=3 with compound C). Mice were measured before and 10 days after tamoxifen administration. B, Quantitative atrial natriuretic peptide (ANP) reverse transcription polymerase chain reaction (RT-PCR) in aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> ventricles 10 days after induction of the knockout (KO) with and without compound C (n≥3). C to H, Representative periodic acid Schiff staining of aMyHC-MCM (C, E, G) and aMyHC-MCM/Mstn<sup>fl/fl</sup> (D, F, H) hearts 10 days (d) and 6 weeks (w) after tamoxifen administration with (G, H) and without compound C treatment (C to F). Glycogen appears pink. Scale bar, 20 μm. I and J, Real-time analysis of extracellular acidification rate (ECAR, because of glycolysis) of isolated cardiomyocytes from controls and aMyHC-MCM/Mstn<sup>fl/fl</sup> mice 10 days after tamoxifen administration with and without compound C treatment, measured in Seahorse extracellular flux assay. Glucose-starved cardiomyocytes were treated with 10 mmol/L glucose, 5 μmol/L oligomycin (Oligo, ATP synthase inhibitor), and finally 100 mmol/L 2-deoxy glucose (2-DG; n≥3 without compound C, n=3 with compound C, 20 replicates per mouse). I, Glycolytic flux. J, ECAR in glucose-starved cardiomyocytes. K, Model of the signaling mechanism: Mstn inhibits AMPK activity via its receptor and transforming growth factor-β–activated kinase 1 (TAK1). Acute deletion of Mstn causes increased glycogen content, glycolysis, and cardiac hypertrophy via AMPK. Used pharmacological inhibitors are shown. Values represent means±SEM. *P<0.05, **P<0.01. LV mass indicates LV mass end diastolic.
compensation. Apparently, the early loss of myostatin during development favors a slower, less disruptive adaptation, allowing to cope with the pathogenic situation. Germ-line mutants, which cannot activate myostatin in noncardiomyocytes, have to rely on different means to prevent detrimental effects of the loss of myostatin. It seems likely that the function of myostatin in germ-line mutants is compensated by GDF11 (or other members of the GDF family).1 In fact, previous studies revealed functional redundancy between myostatin and GDF11 because double knockout mice display more

Figure 6. Deletion of Myostatin in adult cardiomyocytes activates Gaq and β-adrenergic signaling. A, Enrichment analysis of MicroArrays from aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> ventricles (n=3) with ResA3. Shown are gene ontology (GO) term, number of corresponding terms (n), P value, and a 7-figure summary. The summary diagram contains the minimum and maximum (outer whiskers), p10 percentile and p90 percentile (inner whiskers), first and third quartiles (box), median (black line in box), and mean (light line in box). Selected GO terms are shown. B, Western blot analysis and quantification of p-protein kinase A (PKA; Thr197), PKA, and phosphorylated calmodulin-dependent protein kinase II (p-CAMKII; Thr286) levels in aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> ventricles after 10 days (n=3). C, p-protein kinase C (PKC)-α/βII (Thr638/641), p-protein kinase D (PKD; Ser744/748), calcineurin, and myocyte enhancer factor 2A (MEF2A) levels in aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> ventricles after 10 days (n=3). D, p-PKA C (Thr197), p-phospholamban (Ser16), and p-PKC-α/βII (Thr638/641) in aMyHC-Cre and aMyHC-Cre/CAGG-Mstn ventricles (n≥3). Values represent means±SEM; *P<0.05, **P<0.01, ***P<0.001. d indicates days; and RALA, Ras-related protein A.
Figure 7. Deletion of myostatin (MSTN) in adult cardiomyocytes inhibits regulator of G-protein signaling 2 (Rgs2) expression via Smads. A, Western blot analysis of p-protein kinase C (PKC)-α/βII (Thr638/641), phosphorylated calmodulin-dependent protein kinase II (p-CAMKII), calcineurin, and myocyte enhancer factor 2A (MEF2A) levels in aMyHC-MCM and aMyHC-MCM/Mstnfl/fl hearts after 10 days and 6 weeks. B, Quantitative Rgs2 reverse transcription polymerase chain reaction (RT-PCR) analysis in aMyHC-MCM and aMyHC-MCM/Mstnfl/fl ventricles (n≥3). C, Analysis of Rgs2 in cultured cardiomyocytes. Murine adult ventricular cardiomyocytes were stimulated with dimethyl sulfoxide (DMSO; 1 hour), 30 ng/mL MSTN (30 minutes), and 5 µmol/L (5Z)-7-oxozeanol (Oxo; 1 hour). Cells were preincubated with 5 µmol/L (5Z)-7-oxozeanol for 30 minutes and then stimulated with 30 ng/mL MSTN for 30 minutes. A-tubulin served as loading control (n=3). D, Quantitative RT-PCR (qRT-PCR) of the forkhead activin signal transducer 1 (Fast1) binding site (Smad-interacting protein) and a control region on the Rgs2 promoter from chromatin immunoprecipitation (ChIP) with pSmad3 antibody. IgG served as control. ChIPs were performed on aMyHC-MCM and aMyHC-MCM/Mstnfl/fl ventricles 10 days after tamoxifen administration (n=3). The data show the average of 2 ChIPs and are normalized to input. E, Model of the signaling mechanism: MSTN stimulates Rgs2 expression via Smads. Deletion of myostatin reduces Rgs2 expression, thereby preventing Rgs2-dependent inhibition of Gaq and protein kinase A (PKA), which leads to activation of PKC, calcineurin, PKA and CAMKII, PKD, calcineurin, and CAMKII activate MEF2A, resulting in induction of cardiac stress program. Values represent means±SEM. *P<0.05, **P<0.01. Ctrl indicates control; d, days; and RALA, Ras-related protein A.
severe developmental defects than single GDF11 knockouts.\(^4^0\) Furthermore, activin A receptor type IIB knockout mice show severe cardiac abnormalities,\(^4^1\) highlighting the importance of the myostatin–activin A receptor type IIB signaling axis for maintenance of cardiac function.

Previous attempts to determine the function of myostatin in the heart by genetic loss-of-function analysis have some limitations: (1) constitutive myostatin mutants develop a strong hypermuscularity, which will have major effects on the cardiovascular system. (2) Compensatory circuits that are activated after acute loss of myostatin function will be over-ruled by slowly emerging adaptive responses when myostatin expression is inactivated during development. Accordingly, Nkx2.5-Cre/Mstn\(^{\text{fl/fl}}\) mice, which have more time to establish compensatory circuits compared with our aMyHC-Cre/Mstn\(^{\text{fl/fl}}\) mutants, do not show a cardiac phenotype probably because Nkx2.5 is expressed before mature cardiomyocytes form.\(^4^5\) It is also important to point out that heart functions of lineage-restricted, aMyHC-Cre/Mstn\(^{\text{fl/fl}}\) mutants become compromised in aging mice, which were not analyzed in previous studies, which relied on 8- to 10-week-old mice.\(^1^5\) (3) The strong pressure to compensate the loss of myostatin makes it necessary to monitor individual animals continuously before and after myostatin deletion, which is only possible with an inducible mutation.

One of the most evident molecular changes in hearts of aMyHC-MCM/Mstn\(^{\text{fl/fl}}\) mice was the strong activation of AMPK, which is a key regulator of energy homeostasis in the heart stimulating glycolysis and glycogen accumulation. Activation of AMPK accompanied by enhanced glycolysis is seen in several pathological conditions, such as myocardial ischemia, hypertrophy, or left ventricular pressure overload.\(^1^9,3^0,4^2\) Our findings reveal that myostatin signaling represses AMPK and changes the interaction of AMPK with key metabolic enzymes as well as with proteins involved in cardiac hypertrophy. The resulting switch in the metabolic program explains the increased glucose uptake, glycolytic capacity, and augmented glycogen storage in aMyHC-MCM/Mstn\(^{\text{fl/fl}}\) hearts. AMPK seems also to play a major role in the development of cardiac hypertrophy in myostatin-mutant mice because treatment with 2 different AMPK inhibitors rescued cardiac hypertrophy. It is tempting to speculate that the increase of myostatin expression after myocardial infarction reflects a negative feedback loop, which balances AMPK activity.

Interestingly, AMPK activation was mediated by TAK1, a well-known signal transducer of the myostatin receptors ALK4/ALK5, and not by Smads, which are assumed to elicit most of the intracellular actions of myostatin.\(^4^3\) The suppression of AMPK by TAK1 is surprising because TAK1 was recently described to phosphorylate and activate AMPK.\(^4^4\) However, other reports demonstrated that overexpression of an active form of TAK1 in neonatal cardiomyocytes does not activate AMPK, although inhibition of TAK1 activity by overexpression of a dominant-negative form prevented AMPK phosphorylation, which might indicate cell type-specific modes of action.\(^4^5\) Activation of AMPK has also been seen in skeletal muscle and white adipose tissue of myostatin mutants confirming our observations.\(^4^6,4^7\) Because inhibition of AMPK in vivo with compound C and Ara-A prevented cardiac hypertrophy and glycogen accumulation, we concluded that phosphorylation of AMPK via TAK1 was responsible for major parts of the heart phenotype. Additional support for this conclusion comes from transgenic mice with impaired AMPK-\(\alpha\) activity, which causes a reduction in heart size.\(^4^8\) Interestingly, patients and mice with PRKAG2 mutations resulting in activation of AMPK-\(\alpha\) develop glycogen storage cardiomyopathy and cardiac hypertrophy, mimicking aspects of the myostatin heart phenotype. The PRKAG2 cardiomyopathy is attenuated by genetic inhibition of AMPK-\(\alpha\),\(^2^9,4^9\) which resembles the reversal of increased glycolysis, and glycogen accumulation in myostatin mutants after inhibition of AMPK in vivo.

In contrast to the myostatin–TAK1–AMPK signaling axis, activation of Rgs2 was apparently achieved by myostatin-dependent interaction of Smad3 with forkhead activin signal transducer 1 on the Rgs2 promoter. We assume that down-regulation of Rgs2 in myostatin mutants, which leads to increased Gq and \(\beta\)-adrenergic signaling, resulted in increased phosphorylation of PKC-\(\alpha\) and calmodulin-dependent protein kinase II and increased MEF2A expression, which has been linked to heart failure and hypertrophy as well as decreased contractility and sudden cardiac death.\(^3^2,3^3,5^0\) The fact that Rgs2-deficient mice develop cardiac hypertrophy and heart failure in response to pressure-overload\(^1^6\) further emphasizes a critical role of the myostatin–Rgs2 axis in the development of heart failure.

Short-term overexpression tended to improve cardiac contractility and inhibit cardiac hypertrophy. Therefore, myostatin might be seen as an antihypertrophic and cardioprotective factor, which is required to maintain a mature aerobic energy metabolism. This would be in line with a recent study on GDF11,\(^1^0\) which reverses age-dependent cardiac hypertrophy. Loss of Myostatin facilitates insulin-independent increase in glycolysis and cardiac hypertrophy via the energy sensor AMPK. Clearly, additional research is necessary to elucidate the complex network linking cardiac metabolism and hypertrophy.\(^1^9\)

Sources of Funding

This work was supported by the DFG (Deutsche Forschungsgemeinschaft; Excellence Cluster Cardio-Pulmonary System, Br1416, and SFB [Sonderforschungsbereich] TR81), the German-Israeli Fund, the LOEWE (Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz) Center for Cell and Gene Therapy, the German Center for Cardiovascular Research, the German Center for Lung Research, the Universities of Giessen and Marburg Lung Center, and the Interdisciplinary Centre of Clinical Research (IZKF core unit PIX [Preclinical Imaging eXperts]), Münster, Germany.

Disclosures

None.

References


What Is Known?

- Loss of myostatin leads to hypermuscularity and resistance to obesity.
- Myostatin is upregulated in the heart under pathological conditions.
- Glycolysis increases in hypertrophied and failing hearts, but the underlying mechanisms are poorly understood.

What New Information Does This Article Contribute?

- Induced deletion of myostatin in the adult heart leads to cardiac hypertrophy and failure.
- Myostatin prevents metabolic switch to glycolysis by inhibiting the energy sensor AMP-activated kinase.
- Myostatin activates regulator of G-protein signaling 2, an inhibitor of Gq signaling, thereby protecting against heart failure.

Myostatin is upregulated under several pathological conditions in the human heart, but its function in the heart is not fully understood. In the present study, we demonstrate that induced deletion of myostatin in adult cardiomyocytes causes cardiac hypertrophy and changes the metabolic profile, resulting in increased reliance on glucose and glycolysis. We identified several different signaling pathways in cardiomyocytes affected by the loss of myostatin, leading to activation of AMP-activated kinase and reduced expression of regulator of G-protein signaling 2. Pharmacological inhibition of AMP-activated kinase reversed the increase in glycolysis and attenuated cardiac hypertrophy in myostatin mutants. Interestingly, the phenotype caused by induced deletion of myostatin in adult cardiomyocytes differs from constitutive inactivation during development probably because of the initiation of compensatory processes. Short-term upregulation of myostatin in disease conditions seems to improve oxidative capacity and efficiency and to maintain mature aerobic energy metabolism. Systemic pharmacological inhibition of myostatin signaling in adults might have adverse effects on the heart, arguing for a restriction of long-term myostatin inhibition to skeletal muscle to exploit its therapeutic potential.
Myostatin Regulates Energy Homeostasis in the Heart and Prevents Heart Failure
Nadine Biesemann, Luca Mendler, Astrid Wietelmann, Sven Hermann, Michael Schäfers, Marcus Krüger, Thomas Boettger, Thilo Borchardt and Thomas Braun

Circ Res. 2014;115:296-310; originally published online May 7, 2014;
doi: 10.1161/CIRCRESAHA.115.304185

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
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:
http://circres.ahajournals.org/content/115/2/296

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/05/07/CIRCRESAHA.115.304185.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplemental Material

Material and Methods

Generation and treatment of transgenic mouse lines

All procedures were performed in accordance with the guidelines for animal experimentation of the local authorities. All animals were kept on a pure and identical background. For all studies, male mice, which were kept under the same conditions are used.

The conditionally active Mstn<sup>fl/fl</sup> allele was generated by insertion of loxP elements upstream and downstream of exons 1 and 2 of the mouse Myostatin gene using established techniques. Homologous recombination in ES cells was confirmed by Southern Blotting and positive ES cell clones were used to generate chimeric mice via blastocyst injection. Heterozygous offspring was crossed with mice expressing Flp recombinase to remove the selection cassette<sup>1</sup>. Mstn<sup>fl/fl</sup> mice were maintained on a C57Bl6/J background. To conditionally overexpress Myostatin we created CAGG-Mstn mice by random integration of a flox-ßGeo-flox-Myostatin-ires-EGFP cassette into the genome of ES cells. The cassette was generated by inserting a full-length Mstn-ires2-EGFP fragment into the vector pCALL<sup>2</sup> harbouring a ßgeo (lacZ/neoR fusion) gene flanked by loxP elements under the control of the CAGG-promoter. Myostatin expressing ES cell clones with a single cassette integration were screened for high level LacZ expression followed by blastocyst injection. Tissue specific and tamoxifen dependent deletion or overexpression of Myostatin was achieved by crossing Mstn<sup>fl/fl</sup> and CAGG-Mstn mice to aMyHC-Cre<sup>3</sup> and aMyHC-MCM mice<sup>4</sup>. Cre recombination was achieved by intraperitoneal administration of tamoxifen (100mg/kg, Sigma, Steinheim, Germany) for 5 consecutive days. To inhibit AMPK aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> mice were injected either with 20mg/kg Compound C (Tocris Biosciences, Bristol, UK) every second day, or with 15mg/kg Ara-A (Sigma, Steinheim, Germany) daily, over a period of 10 days intraperitoneally following established protocols<sup>5</sup>.

Physiological measurements

Cardiac MRI was performed on a 7.0 T Bruker Pharmascan, equipped with a 300mT/m gradient system, using a custom-built circularly polarized birdcage resonator and the IntraGate<sup>TM</sup> self-gating tool (Bruker, Ettlingen, Germany) following standard protocols. Multiple contiguous short-axis slices consisting of 6 slices were acquired for coverage of the left ventricle and 10 slices for complete coverage of the left and right ventricle.

Skeletal muscle content was measured with a 60 mm inner diameter linear polarized <sup>1</sup>H volume resonator for RF pulse transmission and signal reception (Bruker Biospin). Localizer images were acquired using a spin-echo sequence and corrections of slice angulation were performed. RARE-(Rapid Acquisition with Relaxation Enhancement) sequences (TR=2500 ms, TE=36.7 ms slice thickness=1 mm) in axial and coronal orientation were used to determine the exact position of the lower part of the mouse body. Afterwards a coronal MSME-(Multi-Slice-Multi-Echo) spin-echo-sequence with an echo time (TE=8.6 ms), repetition time (TR=453 ms), a field of view (FOV=7x7 cm<sup>2</sup>), matrix size (MTX=512x256) and a slice thickness of 1 mm was recorded. For volumetric quantification of muscle tissue an ImageJ plugin was used. Anatomical landmarks were defined by the operator and used to derive tissue-specific signal intensity thresholds and to define the region of interest for intensity-sensitive region segmentation. The resulting tissue voxel volumes inside the region of interest were determined and returned as cubic millimeters. In both measurements, mice were measured under volatile isoflurane (1.5-2%) anaesthesia. Body temperature was maintained at 37°C by a thermostatically regulated water flow system.

PET imaging was performed to assess cardiac glucose metabolism in vivo using F-18 labeled Fluordeoxyglucose (FDG). The non-fasted animals were anesthetized by inhalation
of isoflurane (1.5%) and then intravenously injected with FDG (~10 MBq) in 100 μL of saline solution one hour prior to each PET scan. The mouse was placed on a heating pad to maintain body temperature within the reference range. All vital parameters were measured with a dedicated system (BioVet, Spin Systems Pty Ltd.). PET list mode data were acquired for 60 minutes using the 32-module quadHIDAC scanner (Oxford Positron Systems, Weston-on-the-Green, UK) adapted to small animal imaging 6. Quantification of segmental tracer uptake and volumes of the left ventricle were performed using an automated 3-dimensional contour detection algorithm developed in-house and validated against MRI 7.

Bioenergetic profiling

Cardiac metabolism was analyzed using the Seahorse XFe® 96 analyzer (Seahorse Bioscience, North Billerica, USA) employing the protocol of Readnower et al. 8. After isolation, 6000 adult mouse cardiomyocytes were seeded per well overnight on a XF96 cell culture plate (Seahorse Bioscience, North Billerica, USA). On the following day, cell metabolism was analyzed either with the XF glycolysis stress test kit for analysis of glycolytic function or the XF Mito stress test kit for analysis of mitochondrial function according to manufacturers protocol (Seahorse Bioscience, North Billerica, USA). For analysis of glycolysis, plating media was changed into glucose-free medium (DMEM, 143mM NaCl, 200mM L-glutamine, pH 7.4) 1 hour prior to the measurement. Next, cardiomyocytes were treated with a saturating concentration of glucose (10mM), an ATP synthase inhibitor (Oligomycin, 5µM) and 2-deoxy glucose. The extracellular acidification rate, which mainly depends on glycolysis, was analyzed by the Seahorse Extracellular Flux Analyzer in real time. For analysis of mitochondrial function, plating media was changed into XF assay medium (unbuffered DMEM, 2mM glutamax, 5mM glucose, 0.1mM pyruvate, pH 7.4) 1h prior to the measurement. Afterwards, cardiomyocytes were treated with and without 5µM Oligomycin, 0.1µM FCCP (uncoupling agent) and 5µM Antimycin (complex III inhibitor). The oxygen consumption rate (OCR) was determined in real time. Measurements were done in 20 replicates per mouse (n=3). The protocol was based on baseline OCR/ECAR measurements [3x(3min mix, 3min measurement)], followed by injection of port A and OCR/ECAR measurement [2x(3min mix, 3min measurement)], followed by injection of port B and C and identical measurements. Data were corrected to baseline (medium only).

Lactate and glycogen content were analyzed in 100.000 non-cultured cardiomyocytes or serum from aMyHC-MCM and aMyHC-MCM/Mstnfl/fl mice with a colorimetric assay according to manufacturer’s protocol (BioVision, Milpitas, USA). Background reading (without enzyme) was subtracted from the lactate/glycogen reading for measurement of cardiomyocytes.

Chromatin immunoprecipitation (ChIP), AMPK co-immunoprecipitation and mass spectrometry analysis

Ventricles from aMyHC-MCM and aMyHC-MCM/Mstnfl/fl 10 days after Tamoxifen administration were cut into small pieces and crosslinked with 1% formaldehyde for 10min. Nuclei were isolated in cell lysis buffer (5mM HEPES, 85mM KCl, 0.5% NP-40) and re-suspended in nuclei lysis buffer (50mM Tris-HCl (pH 8.1), 10mM EDTA (pH 8.0), 1% SDS) before chromatin was sheared by sonification using the Bioruptor (Diagenode, Dennville) to obtain fragments of 200-500 base pairs. Chromatin samples were diluted 5-10fold in IP dilution buffer 9 and pre-cleared for 2h at 4°C with protein A beads (Roche, Basel, Switzerland). Afterwards the solution was incubated with anti-pSmad3 antibody (4µl, Cell Signaling, Danvers, USA) or anti-IgG antibody (2µl, Diagenode, Dennville, USA) overnight. Antibody binding beads (Diagenode, Dennville, USA) were added for 2h and washed several times as described 9. Precipitated chromatin was eluted in TE, cross-links were reversed with 10% chelax at 95°C for 10 minutes and the protein was digested by proteinase K. ChIP experiments were analyzed by quantitative RT-PCR. ChIP experiments were repeated at least three times and data were normalized to the input.

For MS/MS analysis of AMPK-immunoprecipitations cardiomyocytes from aMyHC-MCM and aMyHC-MCM/Mstnfl/fl mice were used following standard protocols. Cardiomyocytes were resuspended in RIPA buffer and incubated on a rotator for 30 minutes at 4°C. After
centrifugation, 1mg protein from the supernatant was incubated with or without anti-AMPK-α antibody (Cell Signaling, Danvers, USA) 1 hour at 4°C with constant rotation. Afterwards, 30µl protein-G agarose beads slurry were added and incubated overnight with constant rotation. After several washing steps, the beads were resuspended with LDS sample buffer and reducing agents and loaded on a gradient SDS-Gel. Afterwards, gel bands were excised and subjected to in-gel digestion and MS/MS analysis according to Jedrusik-Bode et al. 10. Only interaction partners that were enriched in comparison to control reactions without antibody were used for further analysis.

**RT-PCR and microarray analysis**

Hearts were dissected and washed with ice cold PBS. After removal of the atria, the ventricles were separated in three horizontal pieces for morphological, RNA and protein analysis. Adult murine ventricular cardiomyocytes and non-cardiomyocytes were isolated according to a protocol by O’Connel et al.11. For treatment with pharmacological substances cells were incubated over night at 37°C and cardiomyocytes were stimulated with the following substances: 30ng/ml recombinant Myostatin (R&D Systems, Minneapolis, USA), 10µM SB203580 (Merck, Darmstadt, Germany), 5µM (5Z)-7-Oxozeanol, 1µM SB505124, 2mM AICAR and 10µM Compound C (all from Tocris Biosciences, Bristol, UK). We always used cardiomyocytes from one wildtype heart for all different stimulation conditions. Individual experiments were repeated at least three times.

RNA was isolated from ventricular tissue, cardiomyocytes or non-cardiomyocytes according to standard protocols using the Trizol reagent (Invitrogen, Karlsruhe, Germany) and subjected to RT-PCR analysis using a Bio-Rad iCycler iQ5 Real-time PCR machine with ARP (acidic ribosomal protein) as internal standard as described before 12. Quantitative Real-Time PCR was performed in triplicates. Sequences for primer sets are available upon request from the authors. For microarray analysis samples were processed according to the Affymetrix manual and hybridized to Affymetrix Mouse Gene 1.0 ST Arrays. Raw data were analyzed with the Affymetrix Expression Console using the RMA analysis algorithm. Statistical analysis was performed using Arraystar 5.0. Data are deposited at ArrayExpress (Accession No.: E-MEXP-3744).

**Western Blot analysis**

Ventricular tissue was pestled in liquid nitrogen and lysed by standard procedures. 10-20µg of lysed ventricular tissue or cardiomyocytes were separated by SDS-PAGE and incubated with different antibodies. Antibodies recognizing p-AMPK-α (Thr172), AMPK-α, p-Acetyl-CoA Carboxylase (Ser79), p-PKA C (Thr197), p-PKC-α/β2 (Thr638/641), p-Glycogen Synthase (Ser641), p-CamkII (Thr286), p-PKD (Ser744/748), Calcineurin A, MEF2A, p-p38 (Thr180/Tyr182), p38, p-TAK1 (Thr187), p-MKK3/6 (Ser189/207), p-Hsp27 (Ser82) and p-Smad3 (Ser423/425) were from Cell Signaling (Danvers, USA). Antibody against RalA was from BD (Franklin Lakes, USA). Antibodies recognizing ANP were from Millipore (Billerica, USA), anti-p-Phospholamban (Ser16) from Santa Cruz (Santa Cruz, USA), anti-Rgs2 from Abcam (Cambridge, UK) and anti-α-tubulin from Sigma (Steinheim, Germany). Immunoreactive proteins were visualized on a VersaDoc (BioRad) and quantified with the QuantityOne software (BioRad).

**Morphological analysis**

Midventricular parts of the heart were cut into 10µm thick sections using a Leica CM 1950 cryostat. To analyze the cardiomyocyte area, sections were fixed and incubated with TRITC-labeled Lectin from Triticum vulgaris (Sigma, Steinheim, Germany). Cardiomyocyte area was analyzed out of four random areas (20x images) per tissue block (n=3 per mouse model) using the ImageJ software. PAS (Periodic Acid-Schiff) staining was performed according to manufacturers protocol (Sigma, Steinheim, Germany).
Statistical Analysis

Data are reported as mean ±SEM. Statistical analysis was performed using GraphPad Prism (version 5.0, GraphPad Software Inc.). Two groups were compared using the Student’s t-test, three or more groups using one-way ANOVA followed by Bonferroni’s multiple comparison test. Survival curves were analyzed with the Mantel-Cox test. Values of p<0.05 were considered as statistically significant.

Supporting Information References

Online Figures

Online Figure I: Deletion of Myostatin in cardiomyocytes activates the cardiac stress program. (A) Quantitative RT-PCR of ANP, Acta1 and BNP expression in aMyHC-MCM and aMyHC-MCM/Mstn^{fl/fl} ventricles 10 days after induction of the knockout (3 independent experiments, n≥3) (B) Western Blot analysis and quantitation of ANP levels in aMyHC-MCM and aMyHC-MCM/Mstn^{fl/fl} ventricles (3 independent experiments, n≥3). Values represent means ±SEM; *P<0.05, **P<0.01.
Online Figure II: Deletion of Myostatin leads to atrial fibrosis and impaired atrioventricular conduction. (A) Representative images of aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> hearts 10 days after tamoxifen administration. Arrows indicate dilated and fibrotic atria. (B) Representative ECGs of aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> mice before and 5 days after tamoxifen administration. (C-F) Telemetric recording of PR interval (C), heart rate (D), QRS interval (E) and QTc interval (F) in aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> mice before and after tamoxifen treatment (n=2). Every data point is the average of two analyzed timepoints measured at a 1 hour interval. Values represent means ±SEM; *P<0.05, **P< 0.01.
Online Figure III: The cardiomyocyte fraction shows a high purity. (A-C) Quantitative RT-PCR of aMyHC (A), PECAM (B) and Col1a1 (C) expression in ventricles, cardiomyocytes (CM) and non-cardiomyocytes (NCM) from aMyHC-Cre mice (3 independent experiments, n≥3). Values represent means ±SEM; *P<0.05, **P<0.01, ***P<0.001.
Online Figure IV: Under physiologic conditions the majority of cardiac Myostatin is generated by cardiomyocytes. Quantitative Myostatin RT-PCR analysis in ventricles and cardiomyocytes (CM) from aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> mice 6 weeks after administration of Tamoxifen (3 independent experiments, n=3). Values represent means ±SEM; *P<0.05.
Online Figure V: Short-term deletion and overexpression of Myostatin in the heart is not affecting skeletal muscle mass. (A) Myostatin ELISA to analyze the serum concentration of Myostatin in aMyHC-MCM and aMyHC-MCM/Mstn^{fl/fl} mice 10 days after Tamoxifen administration (n≥4). (B) Quantitative Myostatin RT-PCR analysis in gastrocnemius from aMyHC-MCM and aMyHC-MCM/Mstn^{fl/fl} mice 10 days after Tamoxifen administration (3 independent experiments, n=3). (C) Gastrocnemius weight normalized to body weight of aMyHC-MCM, aMyHC-MCM/Mstn^{fl/fl} and aMyHC-MCM/CAGG-Mstn mice 10 days after Tamoxifen administration (n=3). (D, F) MRI analysis of the skeletal muscle content. (D) Representative MRI images analyzed with an ImageJ plugin, highlighting the skeletal muscle content in brown. Shown are aMyHC-MCM, aMyHC-MCM/Mstn^{fl/fl} and aMyHC-MCM/CAGG-Mstn mice before and 10 days after Tamoxifen administration. (F) Skeletal muscle content normalized to body weight of aMyHC-MCM, aMyHC-MCM/Mstn^{fl/fl} and aMyHC-MCM/CAGG-Mstn mice before and 10 days after Tamoxifen administration (n≥3). (E) Body weight of aMyHC-MCM, aMyHC-MCM/Mstn^{fl/fl} and aMyHC-MCM/CAGG-Mstn mice before, 10 days and 42 days after Tamoxifen administration (n≥7). (G-I) Dystrophin staining of gastrocnemius from aMyHC-MCM (G) aMyHC-MCM/Mstn^{fl/fl} (H), and aMyHC-MCM/CAGG-Mstn (I) mice 10 days after Tamoxifen administration. Scale bar: 50µm. Values represent means ±SEM; **P<0.01.
Online Figure VI: Germ line deletion of Myostatin in cardiomyocytes leads to systolic dysfunction. (A) Quantitative Myostatin RT-PCR analysis in ventricles, cardiomyocytes (CM), and non-cardiomyocytes (NCM) (n≥3) of aMyHC-Cre, aMyHC-Cre/Mstn<sup>fl/fl</sup> and aMyHC-Cre/CAGG-Mstn mice. (B) Survival curve of aMyHC-Cre (n=15), aMyHC-Cre/Mstn<sup>fl/fl</sup> (n=13) and aMyHC-Cre/CAGG-Mstn (n=19) mice. (C) Representative MRI four chamber views of aMyHC-Cre, aMyHC-Cre/Mstn<sup>fl/fl</sup> and aMyHC-Cre/CAGG-Mstn mice. Scale bar: 10mm. Arrow indicates dilated left ventricle. (D) Representative WGA (20x) staining of aMyHC-Cre, aMyHC-Cre/Mstn<sup>fl/fl</sup> and aMyHC-Cre/CAGG-Mstn midventricular heart sections. (E) Quantitative RT-PCR analysis of expression of ANP, BNP, Acta1, bMHC and aMHC in aMyHC-Cre and aMyHC-Cre/Mstn<sup>fl/fl</sup> ventricles (3 independent experiments, n≥3 for each experiment). (F) ANP levels in aMyHC-Cre and aMyHC-Cre/Mstn<sup>fl/fl</sup> ventricular samples (3 independent experiments, n≥3). Values represent means ±SEM; *P<0.05, **P<0.01.
Online Figure VII: Deletion of Myostatin in adult cardiomyocytes induces genes correlated with heart failure and hypertrophy. MicroArray analysis of aMyHC-MCM and aMyHC-MCM/Mstn\textsuperscript{fl/fl} ventricular samples (n=3). Heart failure and hypertrophy markers (A) and TGF/GDF family members are shown (B, C). Values represent means ±SEM; *P<0.05, **P<0.01; ***P<0.001.
Online Figure VIII: Acute deletion of Myostatin switches cardiomyocytes to a glycolytic metabolism. Enrichment analysis of MicroArrays from aMyHC-MCM and aMyHC-MCM/Mstnfl/fl ventricles (n=3) with ResA3. Shown are GO term, number of corresponding terms (n), p-value and a seven-figure summary. The summary diagram contains the minimum and maximum (outer whiskers), p10-percentile and p90-percentile (inner whiskers), first and third quartiles (box), median (black line in box) and mean (light line in box). Selected GO terms are shown.
Online Figure IX: AMPK affects the glycolytic flux in primary cardiomyocytes. Real-time Seahorse Extracellular Flux assay of the extracellular acidification rate (ECAR) reflecting the rate of glycolysis of primary cardiomyocytes isolated from wildtype mice. Cardiomyocytes were stimulated with AICAR (AMPK activator, 2mM) or Compound C (AMPK inhibitor, 10µM) 10 minutes before the measurement. During the measurement, glucose starved cardiomyocytes were treated with 10mM glucose, 5µM oligomycin (Oligo, ATP synthase inhibitor) and finally 100mM 2-deoxy glucose (2-DG), (n=4, 6 replicates per mouse).
Online Figure X: Inhibition of AMPK with Compound C blocks cardiac hypertrophy and increased glycolysis in aMyHC-MCM/Mstn<sup>fl/fl</sup> mice. (A-G) aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> mice were treated with tamoxifen and with or without an AMPK inhibitor (Compound C, 20mg/kg mouse). (A) Western Blot analysis and quantitation of p-AMPK levels in aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> cardiomyocytes 10 days after Tamoxifen administration with and without Compound C treatment (3 independent experiments, n=3). (B-D) MRI analysis of the left-ventricular wall thickness enddiastolic (LV wall thickness ED, B), stroke volume (SV, C), and endsystolic volume (ES volume, D) of aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> hearts with and without Compound C treatment (n≥7 without Compound C, n=3 with Compound C). Mice were measured before and 10 days after tamoxifen administration and values normalized to the corresponding control. (E) Quantitative RT-PCR analysis of BNP expression in aMyHC-MCM and aMyHC-MCM/Mstn<sup>fl/fl</sup> ventricles 10 days after induction of the knockout with and without Compound C (3 independent experiments, n≥3). (F-G) Real-time Seahorse Extracellular Flux assays of the extracellular acidification rate (ECAR) reflecting the rate of glycolysis of cardiomyocytes isolated from control and aMyHC-MCM/Mstn<sup>fl/fl</sup> mice 10 days after Tamoxifen administration with and without Compound C treatment. Glucose starved cardiomyocytes were treated with 10mM glucose, 5µM oligomycin (Oligo, ATP synthase inhibitor) and finally 100mM 2-deoxy glucose (2-DG), (n≥3 without Compound C, n=3 with Compound C , 20 replicates per mouse). (F) Glycolysis (glucose-induced response). (G) Maximal glycolytic capacity (Oligomycin-induced response). Values represent means ±SEM; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Online Figure XI: Inhibition of AMPK with Ara-A blocks cardiac hypertrophy and increased glycolysis in aMyHC-MCM/Mstnfl/fl mice. (A-H) aMyHC-MCM and aMyHC-MCM/Mstnfl/fl mice were treated with tamoxifen and with or without an AMPK inhibitor (Ara-A, 15mg/kg mouse). (A) Western Blot analysis and p-AMPK levels in aMyHC-MCM and aMyHC-MCM/Mstnfl/fl cardiomyocytes 10 days after Tamoxifen administration with and without Ara-A treatment (n=3). (B-E) MRI analysis of the left-ventricular mass enddiastolic (LV mass ED, B), left ventricular wall thickness enddiastolic (LV wall thickness ED, C), stroke volume (SV, D), and endsystolic volume (ES volume, E) of aMyHC-MCM and aMyHC-MCM/Mstnfl/fl hearts with and without Ara-A (n=7 without Compound C, n=3 with Ara-A). Mice were measured before and 10 days after tamoxifen administration. (F-G) Real-time Seahorse Extracellular Flux assays of the extracellular acidification rate (ECAR) reflecting the rate of glycolysis of cardiomyocytes isolated from control and aMyHC-MCM/Mstnfl/fl mice 10 days after Tamoxifen administration with and without Ara-A treatment. Glucose starved cardiomyocytes were treated with 10mM glucose, 5µM oligomycin (Oligo, ATP synthase inhibitor) and finally 100mM 2-deoxy glucose (2-DG), (n≥3 without Ara-A, n=3 with Ara-A, 20 replicates per mouse). (F) Basal ECAR in glucose starved cardiomyocytes. (G) Glycolysis (glucose-induced response). (H) Lactate content in 100.000 non-cultivated cardiomyocytes (n=3) from aMyHC-MCM and aMyHC-MCM/Mstnfl/fl mice 10 days after Tamoxifen administration with and without Ara-A treatment. Values represent means ±SEM; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Online Table I: Comparative MRI analysis of aMyHC-Cre, CAGG-Mstn, aMyHC-Cre/Mstn<sup>fl/fl</sup> and aMyHC-Cre/CAGG-Mstn mice. Male mice were 4 months of age (n=3 CAGG-Mstn, n≥9 aMyHC-Cre, aMyHC-Cre/Mstn<sup>fl/fl</sup> and aMyHC-Cre/CAGG-Mstn). All measurements represent means ±SEM. EF: ejection fraction; SV: stroke volume; LV mass: left-ventricular mass; EDV: enddiastolic volume; ESV: endsystolic volume; LV wall thickness ED: enddiastolic left-ventricular wall thickness. Significant changes are highlighted in red. Data from the right ventricle are shaded in gray. *P<0.05, **P<0.01 vs. aMyHC-Cre mice; ΔP<0.05 vs. CAGG-Mstn mice.
Legends for Online Movies

**Online Movie I: Deletion of Myostatin in adult cardiomyocytes leads to heart failure.** Representative MRI four chamber movies of hearts of aMyHC-MCM (1st row), aMyHC-MCM/Mstn"fl/fl" (2nd row), and aMyHC-MCM/CAGG-Mstn (3rd row) mice. The MRI movies were taken before (1st column), 10 days (2nd column) and 6 weeks (3rd column) after tamoxifen administration and show the same mouse of each respective genotype at different time points.

**Online Movie II: Constitutive deletion of Myostatin in cardiomyocytes leads to left-ventricular dysfunction.** Representative MRI four chamber movies of hearts from aMyHC-Cre, aMyHC-Cre/Mstn"fl/fl", and aMyHC-Cre/CAGG-Mstn mice (from left to right).