AMP-Activated Protein Kinase Regulates E3 Ligases in Rodent Heart

Kedryn K. Baskin, Heinrich Taegtmeyer

Rationale: The degradation of proteins by the ubiquitin proteasome system (UPS) is required for the maintenance of cellular homeostasis in the heart. An important regulator of metabolic homeostasis is AMP-activated protein kinase (AMPK). AMPK activation inhibits protein synthesis and activates autophagy, but whether AMPK plays a role in regulating protein breakdown through the UPS in the heart is not known.

Objective: To determine whether AMPK enhances UPS-mediated protein degradation by directly regulating the ubiquitin ligases Atrogin-1 and muscle RING finger protein 1 (MuRF1) in the heart.

Methods and Results: Nutrient deprivation and pharmacological or genetic activation of AMPK increased mRNA expression and protein levels of Atrogin-1 and MuRF1 and consequently enhanced protein degradation in neonatal cardiomyocytes. Inhibition of AMPK abrogated these effects. Using gene reporter and chromatin immunoprecipitation assays, we found that AMPK regulates MuRF1 expression by acting through the myocyte enhancer factor 2 (MEF2). We further validated these findings in vivo using MEF2-LacZ reporter mice. Furthermore, we demonstrated in adult cardiomyocytes that MuRF1 is necessary for AMPK-mediated proteolysis through the UPS in the heart. Consequently, MuRF1 knockout mice were protected from severe cardiac dysfunction during fasting.

Conclusions: AMPK regulates the transcription of Atrogin-1 and MuRF1 and enhances UPS-mediated protein degradation in heart. Specifically, AMPK regulates MuRF1 through the transcription factor MEF2. The absence of MuRF1 in the heart preserves cardiac function during fasting. The results strengthen the hypothesis that AMPK serves as a modulator of intracellular protein degradation in the heart. (Circ Res. 2011;109:1153-1161.)

Key Words: AMPK • protein degradation • ubiquitin ligases • transcriptional regulation
been reported that nutrient deprivation induces autophagy in cardiomyocytes through AMPK,\textsuperscript{17} a role of AMPK in the cardiac UPS has never been considered. We have previously proposed that metabolic signals may trigger functional and structural remodeling of the stressed heart,\textsuperscript{18} and therefore we set out to test the hypothesis that AMPK regulates MAFbx/Atrogin-1 and MuRF1 in the heart.

### Methods

An expanded Methods section is available in the Data Supplement at http://circres.ahajournals.org.

### Statistical Analysis

Results are expressed as mean±SEM. Analysis was performed using 2-tailed, unpaired Student $t$ test or 1-way ANOVA with Tukey post hoc test. A value of $P<0.05$ was considered significant.

### Results

**Nutrient Deprivation Upregulates Markers of Protein Degradation in Cardiomyocytes**

Protein degradation is increased in the heart in response to nutrient deprivation, leading to cardiac atrophy\textsuperscript{15,16}; however, the mechanism(s) regulating this process are not entirely known. Molecular mechanisms of skeletal muscle wasting have been investigated before in cultured myoblasts deprived of nutrients.\textsuperscript{19} Under these conditions, cell size is drastically reduced and molecular markers of atrophy (ie, atrogenes, including ubiquitin ligases MAFbx/Atrogin-1 and MuRF1) are strongly induced.\textsuperscript{20} Akin to myoblasts, we found that nutrient deprivation increased the mRNA expression of both MAFbx/Atrogin-1 and MuRF1 in neonatal cardiomyocytes in a time-dependent manner (Figure 1A). The same was the case with glucose deprivation (data not shown). After 24 hours of nutrient deprivation, the protein levels of MAFbx/Atrogin-1 and MuRF1 were similarly increased, which correlated with increased AMPK activation (Figure 1B). Furthermore, in cardiomyocytes protein degradation was significantly increased during nutrient deprivation (Figure 1C). Treatment with either Bortezomib or 3-methyladenine decreased protein degradation in neonatal cardiomyocytes indicating involvement of both the UPS and macroautophagy, respectively. The effect of either inhibitor was incomplete, however, suggesting that both processes regulate protein degradation in the presence and absence of nutrients. Assessment of protein degradation with simultaneous inhibition of the UPS and autophagy was not possible as it resulted in cell death. These data indicate that akin to autophagy,\textsuperscript{17} the UPS also plays an important role in regulating protein degradation during nutrient deprivation.

**AMPK Regulates the Expression of Ubiquitin Ligases In Vitro and In Vivo**

We investigated the effect of direct AMPK activation and inhibition on MAFbx/Atrogin-1 and MuRF1 expression in cardiomyocytes. AICAR (5-aminimidazole-4-carboxamide-1-β-d-ribofuranoside) is a known pharmacological activator of AMPK and is readily taken up by cardiomyocytes (NRVM). AICAR treatment increased MAFbx/Atrogin-1 and MuRF1 mRNA expression (Figure 2A). The phosphorylation status of acetyl-CoA carboxylase, a direct target of AMPK, was monitored as a marker of AMPK activity. Intraperitoneal injection of AICAR in mice is also sufficient to activate AMPK in...
Either acute or chronic AMPK activation increased MAFbx/Atrogin-1 and MuRF1 mRNA levels in the heart in vivo (Figure 2D). Additionally, 2 known targets of AMPK in skeletal muscle, GLUT4 and UCP3, were also increased in the heart with AMPK activation (Figure 2D). To establish the specificity of the observed effects, we infected neonatal cardiomyocytes with adenoviral constructs to express either active AMPK (aAMPK) or dominant negative AMPK (dnAMPK). Increasing aAMPK MOI resulted in an upregulation of the mRNA expression and protein levels of MAFbx/Atrogin-1 and MuRF1, whereas dnAMPK had no significant effect on mRNA expression but slightly decreased protein levels at an MOI of 50 (Figure 3A through 3C).

We next investigated the consequence of AMPK activation and inhibition on proteasome-mediated protein degradation in cardiomyocytes. Analogous to the effects of nutrient deprivation, active AMPK increased protein degradation, which was suppressed by dnAMPK or by the proteasome inhibitor Bortezomib (Figure 3D). These data establish that AMPK is directly involved in cardiomyocyte remodeling, both metabolically and structurally, by inhibiting protein synthesis and, as shown here, by enhancing protein degradation.

**AMPK Activation Regulates the Expression of MuRF1 In Vitro Through Transcription Factor Myocyte Enhancer Factor 2**

To gain an understanding of how AMPK regulates the expression of MAFbx/Atrogin-1 and MuRF1, we performed in silico promoter analyses on both genes. These studies revealed a myocyte enhancer factor 2 (MEF2) consensus binding sequence upstream of the MuRF1 transcriptional start site (Figure 4A). Several reports have already shown that AICAR activation of AMPK induces MEF2 transcriptional activity in muscle, and we found the same to occur in neonatal cardiomyocytes. To assess MEF2 transcriptional activity in nuclear extracts from neonatal cardiomyocytes, we used the MEF2 TransAM assay. This ELISA-based method detects the binding of proteins within nuclear extracts to immobilized double stranded oligonucleotides containing MEF2 binding sites. We found that pharmacological or genetic activation of AMPK enhanced MEF2 transcriptional activity, whereas inhibition of AMPK decreased this effect (Figure 4B). To investigate whether MuRF1 transcription is
regulated by AMPK through MEF2, we conducted luciferase reporter gene assays. Vectors encoding the MuRF1 promoter, including the endogenous or mutated MEF2 binding site (Figure 4C), were cotransfected in H9c2 cells with a vector encoding β-galactosidase as an internal transfection control. Luciferase activity was increased by nutrient deprivation, AICAR treatment, or by transfection with aAMPK and conversely decreased by treatment with Compound C or by transfection with dnAMPK. Furthermore, MuRF1 transcription, regulated by AMPK activation, was abolished when the MEF2 binding site on the MuRF1 promoter was mutated (Figure 4D). These results indicate that AMPK promotes MEF2 association with the putative MEF2 binding site in the MuRF1 proximal promoter. To confirm this, we performed chromatin immunoprecipitation assays for endogenous MEF2 on extracts from cardiomyocytes infected with empty adenovirus or adenovirus expressing either aAMPK or dnAMPK. PCR assays on input and IP fractions amplified the MuRF1 promoter containing the putative MEF2 site (top panel) or a distal region of the MuRF1 promoter (bottom panel). Data are mean±SEM of 3 independent experiments performed in triplicate. *P<0.01 versus control, †P<0.01 versus AICAR or aAMPK, ‡P<0.01 versus MuRF1 promoter.

Figure 4. MuRF1 transcription is regulated by an AMPK-MEF2-dependent mechanism in vitro. A, MEF2 consensus binding site and potential MEF2 binding site on MuRF1. B, MEF2 transcriptional activity after activation or inhibition of AMPK in neonatal rat ventricular cardiomyocytes (1 mmol/L AICAR, 20 μmol/L Compound C (CC), aAMPK and dnAMPK MOI=10). C, MuRF1 promoter luciferase reporter vectors. D, Luciferase activity of the constructs transfected into H9c2 cells. E, MEF2 chromatin immunoprecipitation. Cardiomyocytes were infected with an empty adenovirus or adenovirus expressing either aAMPK or dnAMPK. PCR assays on input and IP fractions amplified the MuRF1 promoter containing the putative MEF2 site (−191 to −87, top panel) or a distal region of the MuRF1 promoter (−2094 to −1937, bottom panel). Data are mean±SEM of 3 independent experiments performed in triplicate. *P<0.01 versus control, †P<0.01 versus AICAR or aAMPK, ‡P<0.01 versus MuRF1 promoter.

MEF2 Transcriptional Activity and MuRF1 Transcription Are Regulated by AMPK in the Heart
To validate the role of AMPK in MEF2-regulated cardiac transcription of MuRF1 in vivo, we treated MEF2-lacZ reporter mice with AICAR. Consistent with our findings in isolated cardiomyocytes, cardiac MEF2 transcriptional activity was increased in response to AICAR treatment, as evidenced by increased LacZ staining (Figure 5A). We then quantified LacZ staining in cardiac protein extracts with the use of a β-galactosidase activity assay. AICAR treatment significantly increased β-galactosidase activity to the same extent as isoproterenol, used as a positive control (Figure 5B). Increased MEF2 transcriptional activity in response to AMPK activation augmented MuRF1 expression in vivo (Figure 5C). Collectively, the results demonstrate that AMPK regulates MEF2-mediated transcription of MuRF1 in vivo.

To determine the consequence of AMPK-regulated MuRF1, we investigated whether AMPK-mediated protein degradation through the UPS requires MuRF1. We isolated transcriptionally regulated by AMPK through the transcription factor MEF2.

Figure 5. MEF2 transcriptional activity and MuRF1 transcription are regulated by AMPK in vivo. A, MEF2 transcriptional activity, denoted as β-galactosidase staining, after 7 days of AMPK activation in whole hearts, and B, in heart protein extracts. C, MuRF1 expression in the heart in vivo (15 μg/g body wt isoproterenol used as a positive control (ISO), 0.5 mg/g body wt AICAR, n=6–8). In vitro data are mean±SEM of 3 independent experiments performed in triplicate. Scale bars=1 mm. *P<0.01 versus saline.
adult mouse cardiomyocytes from wild-type (WT) or MuRF1 knockout hearts and measured rates of protein degradation in vitro (Figure 6). Proteasome-mediated protein degradation was not altered in the absence of MuRF1 under normal conditions, as WT and MuRF1−/− cardiomyocytes were equally responsive to Bortezomib treatment. Similar to neonatal cardiomyocytes, nutrient deprivation increased protein degradation in WT adult cardiomyocytes. Under the same conditions, increases in protein degradation were less marked in MuRF1 deficient cardiomyocytes but were still higher than in controls. When active AMPK was expressed, protein degradation was greatly enhanced in WT myocytes, but it was not increased in the absence of MuRF1. When dnAMPK was expressed, protein degradation was decreased in WT and unchanged in MuRF1-deficient myocytes. Collectively, these data show that MuRF1 is required for AMPK-regulated protein degradation in cardiomyocytes.

To investigate the physiological importance of the AMPK-MuRF1 axis in vivo, we subjected WT and MuRF1−/− mice to 3 days of nutrient deprivation (starvation or fasting). As expected, 3 days of fasting significantly decreased body weight in both WT and MuRF1−/− mice; however heart weight was decreased only in WT mice (Figure 7A). Interestingly, ejection fraction was decreased in WT mice in response to fasting, but loss of MuRF1 preserved ejection fraction (Figure 7B). Fractional shortening was also decreased after fasting in WT but not MuRF1−/− animals (Online Table I). MuRF1 gene expression was significantly increased in WT fasted mice (Figure 7C). We also investigated 2 known protein targets of MuRF1 in the heart after 3 days of fasting. Indeed, the protein levels of both cardiac myosin-binding protein C (cMyBP-C) and cardiac troponin I (TnI) decreased in WT fasted mice (Figure 7D and 7E). MyBP-C is also degraded by Atrogin-1, and therefore it is not surprising that protein levels also trended to decrease in hearts from fasted MuRF1−/− mice. TnI levels, on the other hand, were not significantly decreased in fasted MuRF1−/− hearts, suggesting that degradation of cardiac TnI during fasting requires MuRF1. Together, these data suggest that AMPK-regulated MuRF1 in the heart during fasting is detrimental to structure and function by enhancing the degradation of specific MuRF1 targets in the heart. The proposed
AMPK activation on energy substrate metabolism. Studies have predominantly focused on the effects of AMPK in the heart until now. We have presented evidence in support of the hypothesis that AMPK regulates protein degradation in the cardiomyocyte and increased remodeling.

**Discussion**

We have provided evidence in support of the hypothesis that AMPK activated protein kinase regulates ubiquitin ligases in the rodent heart. The present work extends the long-established concept of the "dynamic state of body constituents" to a specific scenario in which the heart adapts to changes in its metabolic environment. Protein turnover (protein synthesis and degradation through the UPS and autophagy) constitutes a major line of defense for protein quality control of the cardiomyocyte and is a major mechanism of adaptation in the heart. It is therefore of interest to understand how protein degradation is regulated in the cardiomyocyte under various circumstances. We have previously shown that markers of the UPS are unregulated in the heart in several settings of cardiac remodeling, but how the markers themselves are regulated is not clear. It is known already that AMPK plays a role in cellular homeostasis in part by inhibiting the mTOR pathway and thus by decreasing protein synthesis, while at the same time AMPK regulates autophagy. Although AMPK itself has been found to be regulated by the UPS, whether AMPK regulates protein degradation through the UPS has not yet been investigated in the heart until now.

The role of AMPK in fuel homeostasis is well described, and studies have predominantly focused on the effects of AMPK activation on energy substrate metabolism. AMPKα2, the active subunit highly expressed in the heart, is preferentially localized to the nucleus. Hence, it is not surprising that AMPK also transcriptionally regulates metabolic gene expression. Although little is known about AMPK-regulated transcription in the heart, earlier reports in liver show that AMPK activation represses transcription by inactivating the transcription factors P300, HNF4-α, ChREBP, and TORC2. Consequently, the expression of genes involved in lipogenesis and gluconeogenesis is attenuated. AMPK can also activate transcription by enhancing CREB activity, thus increasing the expression of UCP3 and HKII. The activation of PGC1α by AMPK leads to increased mitochondrial gene expression and mitochondrial biogenesis. Additionally, the activation of AMPK in muscle increases GLUT4 transcription by increasing both PGC1α and MEF2 transcriptional activity, the latter through inactivation of HDAC5. The role of AMPK in transcription is only now coming into focus. Akin to its yeast homologue SNF-1, AMPK phosphorylates histone 2B in mammalian cells, suggesting that AMPK regulates entire transcriptional programs and not only transcription of individual genes. By providing evidence that AMPK regulates the transcription of ubiquitin ligases MAFbx/Atrogin-1 and MuRF1, key regulators of protein degradation in the heart, this study further expands the role of AMPK in both cellular homeostasis and transcriptional regulation in the heart.

Extensive analyses of the MAFbx/Atrogin-1 and MuRF1 promoters have not yet been reported, but independent studies have begun to elucidate the transcriptional regulation of both ligases. The expression of MAFbx/Atrogin-1 and MuRF1 is positively regulated by the transcription factor FoxO3A in the heart and is negatively regulated through Akt, which suppresses FoxO. TNFα increases the expression of MAFbx/Atrogin-1 and MuRF1 in cardiomyocytes and in skeletal muscle, independent of Akt through Foxo4. In C2C12 myotubes and in vivo, MAFbx/Atrogin-1 and MuRF1 expression is increased by glucocorticoid stimulation, and the transcription factor C/EBPα has been suggested to regulate MAFbx/Atrogin-1 transcription in a glucocorticoid-dependent manner in skeletal muscle. The IkB/NF-κB pathway also regulates the transcription of MuRF1 in cachexia-induced muscle wasting. More recently, myogenin has been found to regulate transcription of both MAFbx/Atrogin-1 and MuRF1 in an HDAC-dependent manner. Our findings now demonstrate that in the heart, AMPK contributes to the complex transcriptional regulation of ubiquitin ligases in the setting of nutrient deprivation and fasting.

The duration of fasting may be important, because we observed that prolonged fasting results in impaired contractile function of the heart (Figure 7). Our findings are in agreement with several reports showing that fasting decreased cardiac function in WT mice on the one hand, and that the absence of MuRF1 spared muscle and heart from atrophy on the other hand. MuRF1 targets EEF1G (a component of the elongation factor complex EF-1) for degradation. It also targets key enzymes involved in ATP production (including aldolase a and pyruvate dehydrogenase). These studies suggest that MuRF1 not only regulates protein degradation but also regulates protein synthesis and pathways of energy metabolism. Based on our in vivo findings, we now propose that during starvation, the absence of MuRF1 is cardioprotective through different mechanisms. First, MuRF1-independent mechanisms regulate protein degradation, but protein synthesis is not inhibited by MuRF1. Conversely, in WT hearts of fasted animals, the presence of MuRF1 is reflected in an imbalance of protein turnover (enhanced protein degradation and decreased protein synthesis). Furthermore, the upregulation of MuRF1 during starvation may degrade metabolic enzymes (such as aldolase a and the pyruvate dehydrogenase complex). Others have found that with fasting, intracellular glucose 6-phosphate and fructose 6-phosphate in the heart are increased, and glucose phosphorylation is decreased, most likely by allosteric inhibition of hexokinase. Glycogen deposition and citrate levels are also increased in the heart during fasting; the latter inhibits phosphofructokinase activity and therefore puts a brake on...
glycolysis. It is tempting to speculate that MuRF1 interacts with phosphorylase kinase because in muscle atrophy, MuRF1 is upregulated and phosphorylokinase is downregulated, and both enzymes can localize to the M-line. There is, therefore, the possibility that upregulation of MuRF1 during starvation leads to the downregulation of metabolic enzymes, decreased ATP production from glycolysis, and decreased cardiac function. Indeed, we observed that rates of glucose oxidation in hearts from MuRF1−/− animals are at least 3-fold higher than in the hearts from WT animals (Baskin K.K. and Taegtmeyer H., unpublished results).

A further point must be addressed. Beginning in 2002, a number of clinical studies have reported beneficial outcomes in diabetic heart failure patients treated with the AMPK activator metformin. Our experimental findings are not inconsistent with the clinical outcomes because in diabetes, in contrast to fasting, the heart is flooded with oxidizable fuel. AMPK-regulated protein degradation may be protective because of enhanced protein quality control.

We conclude that AMPK is a transcriptional regulator of ubiquitin ligases in heart muscle. Activation of AMPK results in increased rates of protein degradation and consequently leads to cardiomyocyte remodeling. Whether the remodeling is beneficial or detrimental may be dependent on the immediate cardiometabolic environment. We speculate that the activation of AMPK results in enhanced availability of intracellular amino acids for either ATP production or the synthesis of new proteins as the heart adapts to a new physiological state.

Acknowledgments

We thank Eric N. Olson and Rhonda Bassel-Duby for providing the desMEF2-LacZ reporter mice and Regeneron Pharmaceuticals, Inc., for providing the MuRF1 knockout mice. We also thank Meredith Rees for performing the echocardiographic measurements and Rebecca Berdeaux for assistance with the ChIP assays and careful review of the manuscript. Peter Razeghi and Romain Harnarcey provided critical comments and suggestions, and Roxy A. Tate helped with manuscript preparation.

Sources of Funding

These studies were supported in part by a grant from the National Heart, Lung, and Blood Institute (5R01HL061483-9) of the US Public Health Service. K.K.B. received a predoctoral fellowship from the American Heart Association, National Center (11PRE5200006).

Disclosures

None.

References

1100 Circulation Research  October 28, 2011


**Novelty and Significance**

What Is Known?

- AMP-activated protein kinase (AMPK), a key regulator of metabolic homeostasis, inhibits protein synthesis and activates autophagy in the heart.
- The ubiquitin proteasome system (UPS) maintains cellular homeostasis by degrading unnecessary and/or damaged proteins through key enzymes, including ubiquitin (E3) ligases.
- Two muscle-specific E3 ligases, Atrogin-1 and MuRF1, are critical regulators of cardiac size and mass.

What New Information Does This Article Contribute?

- Activation of AMPK in vitro and in vivo regulates the transcription of Atrogin-1 and MuRF1 in cardiomyocytes.
- AMPK regulates MuRF1 transcription through the transcription factor MEF2.
- MuRF1 is necessary for AMPK-mediated proteolysis through the UPS in the heart.
- MuRF1-deficient mice are protected from cardiac dysfunction during the metabolic stress of fasting.

The heart adapts both metabolically and structurally to changes in its environment. AMPK is an essential enzyme that regulates many adaptive processes. Not only does AMPK inhibit protein synthesis, but it also activates autophagy, lysosome-mediated protein degradation. Until now, the role of AMPK in proteasome-mediated protein degradation in the heart was not known. We show that activation of AMPK regulates the transcription of 2 ubiquitin ligases in the heart: Atrogin-1 and MuRF1. Specifically, AMPK regulates MuRF1 transcription through MEF2 in vitro and in vivo. Consequently, proteasome-mediated protein degradation is increased with AMPK activation. In cardiomyocytes, MuRF1 is necessary for AMPK-mediated proteolysis through the UPS. Excessive proteolysis, which can occur with long-term fasting, induces cardiac dysfunction. However, MuRF1-deficient mice are protected from cardiac dysfunction during fasting. Regulation of protein turnover is especially important in the terminally differentiated cardiomyocyte, where protein quality control is required to maintain normal contractile function. Excessive protein synthesis or degradation has physiological consequences. Therefore, it seems important to understand, in detail, how these processes are regulated.
AMP-Activated Protein Kinase Regulates E3 Ligases in Rodent Heart
Kedryn K. Baskin and Heinrich Taegtmeyer

Circ Res. 2011;109:1153-1161; originally published online September 15, 2011;
doi: 10.1161/CIRCRESAHA.111.252742

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/109/10/1153

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2011/09/15/CIRCRESAHA.111.252742.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

<table>
<thead>
<tr>
<th></th>
<th>Wild Type Fed</th>
<th>Wild Type Nutrient Deprived</th>
<th>MuRF1 -/- Fed</th>
<th>MuRF1 -/- Nutrient Deprived</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSd, mm</td>
<td>0.681 ± 0.078</td>
<td>0.608 ± 0.050</td>
<td>0.596 ± 0.087</td>
<td>0.760 ± 0.105</td>
</tr>
<tr>
<td>LVPW, mm</td>
<td>0.837 ± 0.092</td>
<td>0.752 ± 0.069</td>
<td>0.874 ± 0.122</td>
<td>0.956 ± 0.150</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>3.518 ± 0.203</td>
<td>3.677 ± 0.322</td>
<td>3.314 ± 0.276</td>
<td>3.650 ± 0.278</td>
</tr>
<tr>
<td>LVIDs, mm</td>
<td>2.544 ± 0.293</td>
<td>2.862 ± 0.0404</td>
<td>2.498 ± 0.214</td>
<td>2.778 ± 0.374</td>
</tr>
<tr>
<td>FS, %</td>
<td>34.28 ± 2.68</td>
<td>20.93 ± 2.47*</td>
<td>28.59 ± 2.51</td>
<td>25.72 ± 3.21</td>
</tr>
</tbody>
</table>

**Online Table I.** Nutrient deprivation reduces Fractional Shortening (FS) in WT but not in MuRF1 -/- mice. Cardiac function was assessed by M-mode echocardiography. IVSd, interventricular septum; LVPW, left ventricular posterior wall; LVIDd, left ventricular inner diameter in diastole; LVIDs, left ventricular inner diameter in systole; FS, fractional shortening. Measurement values in mm were averaged from at least four separate cardiac cycles. Data are mean ± SEM (n=6-8). *P<0.01 vs WT Fed.
Detailed Methods

Animals
We used different mouse models for in vivo experiments. In one set experiments were performed on 6-8 week old C57BL/6NCrl (Charles River, Wilmington, MA), or desMEF2-LacZ reporter mice. Male mice were injected intraperitoneally with 1mg/g body weight AICAR, 15mg/kg body weight isoproterenol, or an equal volume of 0.9% saline. In another set, MuRF1 -/- mice and their wild type littermates (8-10 weeks old) were used for in vivo nutrient deprivation experiments; mice had ad libitum access to water during the 3 day starvation period. MuRF1 -/- mice and their wild type littermates (8-10 weeks old) were also used to isolate adult cardiomyocytes for in vitro experiments. Additionally, neonatal Sprague-Dawley rats (1-to-2 days old) were obtained from Harlan (Houston, TX) and used for isolated neonatal cardiomyocyte experiments (see below). All experiments were performed according to the NIH guidelines and were approved by the Animal Welfare Committee of The University of Texas Health Science Center at Houston.

Analysis of Cardiac Function
Echocardiographic measurements were performed as previously described. Briefly, mice were anesthetized with 2% isoflurane, body fur was removed from the anterior thorax and upper abdominal area, and acoustic coupling gel was applied. Mice were taped in the supine position to a temperature-controlled board with all four limbs placed on copper electrodes with leads used for electrocardiographic monitoring. During imaging mice were maintained under 1% isoflurane using a nose cone. Analysis of cardiac function and structure was performed using a Vevo 770 ultrasound system (Visual Sonics). M-mode parasternal short-axis scans were used to quantify diastolic and systolic ventricular diameters. The calculations were performed on four different images of each mouse.

Cell Cultures
Neonatal rat ventricular myocytes (NRVM) were used for most in vitro experiments. In addition, adult mouse cardiomyocytes isolated from hearts of MuRF1 -/- mice and their wild type littermates were used for some of the pulse chase experiments, and H9c2 cardiomyocytes, a cell line derived from embryonic rat heart tissue (American Type Culture Collection, ATCC), were used for luciferase reporter assays. H9c2 cultures were maintained in accordance with ATCC recommendations.

NRVM were isolated from hearts of 1-2 day old Sprague-Dawley rats as previously described with minor modifications. Briefly, hearts were exposed through a thoracotomy, excised, minced, and digested through a series of agitated incubations in buffer containing collagenase and pancreatin. The cells collected after each digestion were pooled, and cardiomyocytes were separated from other cell types using percoll gradient centrifugation. Medium was changed 24 hours after plating and experiments were started 2-3 days after plating the cells to ensure homogenous cultures. All experiments were carried out on at least three separate NRVM isolations and each condition was performed in triplicate at minimum.

Adult mouse cardiomyocytes (AMCM) were isolated as described by O'Connell et.al. Briefly, hearts were excised from 2 month-old mice (wild type or MuRF1 knockout), placed in ice-cold saline, and the aorta was cannulated. Hearts were perfused with warm perfusion buffer for several minutes followed by perfusion with digestion buffer containing collagenase. Once digested, hearts were minced and myocytes from several hearts were pooled together and concentrated by centrifugation. After calcium reintroduction, cardiomyocytes were plated and cultured under 2%
carbon dioxide for several days. Experiments were started the day after isolation and were carried out on at least three separate isolations; each condition was performed in duplicate or triplicate.

**Protein Degradation Measurements**
NRVM or AMCM were incubated with 1 µCi/ml [14C]phenylalanine for 24 hours prior to adenovirus infection or 48 hours prior to treatments. Media were then switched to chase medium containing 2mM unlabeled phenylalanine to prevent reincorporation of [14C]phenylalanine. Aliquots of chase medium were collected over 24 hours, combined with 20% trichloroacetic acid (TCA) and incubated at 4°C overnight to precipitate proteins. Total protein in the cells was collected in 10% TCA and incubated at 4°C overnight. The acid-soluble radioactivity from the chase medium was used to denote the amount of protein degraded over time while total protein was used for normalization. Results are expressed as percent protein degradation or fold change over control. Protein degradation was calculated under several conditions including proteasome inhibition with Bortezomib and the inhibition of autophagy with 3-methyladenine (3-MA).8

**Chemicals and Antibodies**
AICAR (5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) was from Toronto Research Center (Toronto, ONT), Compound C was from EMD Biosciences, Inc (Cincinnati, OH), Bortezomib was from Selleck Chemicals LLC (Houston, TX), and 3-Methyladenine was from Sigma (St. Louis, MO). The following primary antibodies from Cell Signaling Technology (Danvers, MA) were used: AMPKα, phospho-AMPKα (Thr172), Acetyl CoA Carboxylase, phospho- Acetyl-CoA Carboxylase (Ser79), β-Tubulin (9F3). Histone deacetylase 5, phospho-histone deacetylase 5 (Ser498), and MuRF1 antibodies were from Abcam (Cambridge, MA), the Atrogin-1 antibody was from ECM biosciences LLC (Versailles, KY), and the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Fitzgerald Industries International (Acton, MA).

**Adenovirus Experiments**
Active and dominant-negative AMPKα2 recombinant adenovirus (Eton Bioscience Inc., San Diego, CA) were amplified in HEK293 cells and NRVM or AMCM were infected at the indicated multiplicity of infection (MOI). Four hours after infection medium was changed and experiments were performed. Cardiomyocytes were collect as described below.

**Luciferase Reporter Gene Assays**
A 1kB fragment of the rat MuRF1 promoter containing a putative MEF2 binding site was synthesized and cloned into the luciferase reporter plasmid pGL4.10 (Promega, Madison, WI). The transcriptional start site within the MuRF1 promoter was designated as nucleotide location 0 and the putative MEF2 binding site is located between nucleotides -194 and -184. The same construct with three A→C mutations in the putative MEF2 binding site was also cloned into the luciferase reporter plasmid pGL4.10 (Promega). Luciferase reporter constructs pGL4.10 (1µg) were cotransfected with a vector encoding β-galactosidase (1µg) into H9c2 cells using the polymer-based DNA transfection reagent, jetPEI™ (Polyplus-transfection Inc., New York, NY), according to the manufacturer’s instructions. Twelve hours post transfection treatments were initiated. Luciferase and β-galactosidase activities were measured using the Luciferase Assay System and β-galactosidase Enzyme Assay System (Promega). Data are reported as luciferase activity normalized to β-galactosidase activity.

**Gene Expression Analysis**
Total RNA was prepared from fresh-frozen heart tissue or NRVM using TRI Reagent® according to the manufacturer’s protocol (Molecular Research Center, INC., Cincinnati, OH). Total RNA was
quantified using the NanoDrop® ND-1000. Quantitative Real-Time RT-PCR was performed on 90ng total RNA per sample as previously described, except that cDNA standards were purchased from Integrated DNA Technologies (San Diego, CA). The nucleotide sequences for the assays used have been published previously. Gene expression results are expressed as fold change in mRNA levels compared to control.

Western Blot Analysis
NRVM were washed twice in ice-cold phosphate-buffered saline (PBS), collected in lysis buffer containing protease inhibitor cocktail tablets (Roche, Indianapolis, IN) and phosphatase inhibitor cocktails (Sigma), and passed through a 27-gauge needle to ensure membrane rupture. Heart tissue was ground and homogenized in the same buffer using a tissue homogenizer (Brinkman Polytron). Soluble protein was collected by centrifugation and quantified using the Bradford assay (Sigma). 15ng of total protein was resolved by SDS-PAGE and transferred to a PVDF membrane followed by antibody incubation. Western blot band intensity was determined and quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

MEF2 Activity Assay
The transcriptional activation of MEF2 was measured using a quantitative ELISA TransAM™ Kit per manufacturer’s instructions (Active Motif, Carlsbad, CA). NRVM were collected and the nuclear fraction was separated and resuspended in complete lysis buffer. Nuclear protein concentration was determined using the Bradford assay and 10μg total nuclear protein of each sample was used to measure MEF2 activity.

Chromatin Immunoprecipitation Assay (ChIP)
Twenty four hours after adenovirus infection cells were incubated with 1% formaldehyde to cross-link protein-DNA complexes. Nuclei were pelleted and immunoprecipitates of cross-linked complexes were prepared with a MEF2 antibody (Santa Cruz Biotechnology, Inc.). Immunoprecipitates were treated with proteinase K and then incubated at 65°C to release cross-links. DNA was purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation and was then analyzed with 25 cycles of PCR to amplify MuRF1 promoter sequences. Several different cycle numbers were employed to ensure linearity of amplification. PCR products were analyzed by 3% agarose gel with ethidium bromide.

Statistical Analysis
Results are expressed as means ± SEM. Analysis was performed using two-tailed, unpaired Student’s t test or one-way ANOVA with Turkey post hoc test. A value of P<0.05 was considered significant.
Supplemental References