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AMP-activated Protein Kinase Regulates E3 Ligases in Rodent Heart

Kedryn K. Baskin, BA[1] and **Heinrich Taegtmeyer, MD, DPhil**[1,*]

[1]Department of Internal Medicine, Division of Cardiology, University of Texas Health Science Center, Houston, Texas

Abstract

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 MuRF1 in the heart.

Methods and Results—Nutrient deprivation, pharmacologic 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 transcription factor 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.

Keywords

AMPK; protein degradation; ubiquitin ligases; transcriptional regulation

Introduction

The heart adapts metabolically, functionally and structurally to changes in its environment. Any stress that reduces the intracellular [ATP]:[AMP] ratio activates the enzyme AMP-activated protein kinase (AMPK), resulting in an increased provision of energy through substrate metabolism and inhibition of energy consuming processes,[1] including protein

[*]Corresponding author: University of Texas Health Science Center, Houston, 6431 Fannin, MSB 1.246, Houston, TX 77030, USA. Phone: 713-500-6569, Fax: 713-500-0637, Heinrich.Taegtmeyer@uth.tmc.edu.

Disclosures
None.

synthesis.[2, 3] A role for AMPK in protein degradation in the heart has not been clearly defined, but recent reports have suggested a possible role for AMPK in protein degradation in skeletal muscle.[4, 5]

Intracellular protein degradation in cardiomyocytes is controlled by independent but interrelated processes: ubiquitin proteasome system (UPS)-mediated proteolysis and autophagy. While macroautophagy can degrade whole organelles,[6, 7] individual proteins are degraded through the UPS.[8] Ubiquitin ligases confer specificity to the system by the selective ubiquitination of target proteins which are then degraded by the proteasome. Two muscle-specific ubiquitin ligases, muscle atrophy F-box protein (MAFbx) or Atrogin-1, and muscle RING finger protein 1 (MuRF1), are consistently increased in models of skeletal muscle atrophy. Furthermore, mice lacking MAFbx/Atrogin-1 or MuRF1 subjected to atrophic stimuli show reduced levels of skeletal muscle atrophy.[9, 10] Both ligases also play a critical role in regulating cardiomyocyte size and heart muscle mass. The overexpression of MAFbx/Atrogin-1 in the heart blunts the development of cardiac hypertrophy in response to both physiologic and pathologic hypertrophic stimuli *in vivo*.[11, 12] *Vice versa* MuRF1 deficient mice display exaggerated cardiac hypertrophy in response to pressure overload,[13] while the overexpression of MuRF1 in cardiomyocytes prevents pharmacologically-induced hypertrophy.[14] Collectively these studies suggest that the proteins targeted for degradation by MAFbx/Atrogin-1 and MuRF1 are determinants of cardiomyocyte size.

Early studies in the heart *in vitro* and *in vivo* demonstrated that nutrient deprivation decreases protein synthesis and increases fractional rates of protein degradation.[15, 16] AMPK is activated during nutrient deprivation in order to provide energy to maintain normal cellular function.[1] Although it has recently been reported that nutrient deprivation induces autophagy in cardiomyocytes through AMPK,[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,[18] 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.

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.

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;[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.[19] Under these conditions, cell size is drastically reduced and molecular markers of atrophy (i.e. atrogenes, including ubiquitin ligases MAFbx/Atrogin-1 and MuRF1) are strongly induced.[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,[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-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) is a known pharmacological activator of AMPK and is readily taken up by cardiomyocytes. AICAR treatment increased MAFbx/Atrogin-1 and MuRF1 mRNA expression and protein levels in a dose-dependent manner (Figure 2A and B) while Compound C, a potent AMPK inhibitor, decreased MAFbx/Atrogin-1 and MuRF1 expression (Figure 2C). The phosphorylation status of acetyl-CoA carboxylase (ACC), a direct target of AMPK, was monitored as a marker of AMPK activity. Intraperitoneal (IP) injection of AICAR in mice is also sufficient to activate AMPK *in vivo*.[21, 22] Either acute or chronic AMPK activation increased MAFbx/Atrogin-1 and MuRF1 mRNA levels in the heart *in vivo* (Figure 2D). Additionally, two known targets of AMPK in skeletal muscle, GLUT4[23] and UCP3,[24] 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, while dnAMPK had no significant effect on mRNA expression but slightly decreased protein levels at an MOI of 50 (Figure 3A–C).

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[2, 3] and, as shown here, by enhancing protein degradation.

AMPK activation regulates the expression of MuRF1 *in vitro* through the transcription factor MEF2

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 MEF2 (myocyte enhancer factor 2) consensus binding sequence[25, 26] 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,[27, 28] 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.[29] We found that pharmacological or genetic activation of AMPK enhanced MEF2 transcriptional activity, while 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 aAMPK or dnAMPK. In agreement with our other results, we recovered the MuRF1 proximal promoter in MEF2 immunoprecipitates from myocytes expressing aAMPK, but not myocytes expressing the dnAMPK or empty virus control. In contrast, distal promoter sequences could not be amplified in MEF2 immunoprecipitates (Figure 4E). These data demonstrate that MuRF1 is transcriptionally regulated by AMPK through the transcription factor MEF2.

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[30] 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 using a β-galactosidase activity assay. AICAR treatment significantly increased β-galactosidase activity to the same extent as isoproterenol, used as a positive control[31] (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 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.

In order to investigate the physiological importance of the AMPK-MuRF1 axis *in vivo* we subjected wild type (WT) and MuRF1 knockout (−/−) mice to three 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 (EF) was decreased in WT mice in response to fasting, but loss of MuRF1 preserved EF (Figure 7B). Fractional shortening was also decreased after fasting in WT, but not MuRF1 −/− animals (Supplemental Table 1). MuRF1 gene expression was significantly increased in WT starved mice (Figure 7C). We also investigated two 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)[32] and

cardiac troponin I (TnI)[33] decreased in WT fasted mice (Figure 7D and E). 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 MuRF1 transcriptional regulation through the AMPK-MEF2 regulatory axis is summarized in Figure 8.

Discussion

We have presented evidence in support of the hypothesis that AMP-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”[34] to a specific situation when 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[35] 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 upregrulated in the heart in several settings of cardiac remodeling,[36, 37] 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[2, 3] and thus by decreasing protein synthesis, while at the same time AMPK activates autophagy.[17, 38, 39] While AMPK itself has been found to be regulated by the UPS,[40] whether AMPK regulates protein degradation through the UPS has not 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.[41] AMPKα2, the active subunit highly expressed in the heart,[42] is preferentially localized to the nucleus.[43] 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.[44] 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.[24, 45] The activation of PGC1α by AMPK leads to increased mitochondrial gene expression and mitochondrial biogenesis.[23] Additionally, the activation of AMPK in muscle increases GLUT4 transcription by increasing both PGC1α[23] and MEF2 transcriptional activity, the latter through inactivation of HDAC5.[46] The role of AMPK in transcription is only now coming into focus. Akin to its yeast homologue SNF-1,[47] AMPK phosphorylates histone 2B in mammalian cells[48, 49] 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.[50–52] TNFα increases the expression of MAFbx/Atrogin-1 and MuRF1 in cardiomyocytes[53] and in skeletal muscle[54] independent of Akt through Foxo4.[55] In C_2C_{12} myotubes[56] and *in vivo*[57] MAFbx/Atrogin-1 and MuRF1 expression is increased by

glucocorticoid stimulation, and the transcription factor C/EBP1 has been suggested to regulate MAFbx/Atrogin-1 transcription in a glucocorticoid-dependent manner in skeletal muscle.[58] The IκKβ/NF-κB pathway also regulates the transcription of MuRF1 in cachexia-induced muscle wasting.[59] More recently myogenin has been found to regulate transcription of both MAFbx/Atrogin-1 and MuRF1 in an HDAC-dependent manner.[60] 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,[61] and that the absence of MuRF1 spared muscle and heart from atrophy on the other hand.[62] MuRF1 targets EEF1G (a component of the elongation factor complex EF-1) for degradation.[63] It also targets key enzymes involved in ATP production (including aldolase a and pyruvate dehydrogenase).[64] 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 cardio-protective through several 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).[62] Furthermore, the upregulation of MuRF1 during starvation may degrade metabolic enzymes (like 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.[65] 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. [66, 67] It is tempting to speculate that MuRF1 interacts with phosphofructokinase because in muscle atrophy, MuRF1 is upregulated and phosphofructokinase is downregulated,[9] and both enzymes can localize to the M-line.[68, 69] Therefore, it is possible 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 three-fold higher than in the hearts from WT animals (Baskin KK and Taegtmeyer H, unpublished results).

A further point needs to 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.[70–73] 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.[35] 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 physiologic state.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

aAMPK	active AMPK adenovirus
dnAPMK	dominant negative AMPK adenovirus
ACC	acetyl-CoA carboxylase
ISO	isoproterenol
CC	Compound C
3-MA	3-methyladenine

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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 here that activation of AMPK regulates the transcription of two 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.

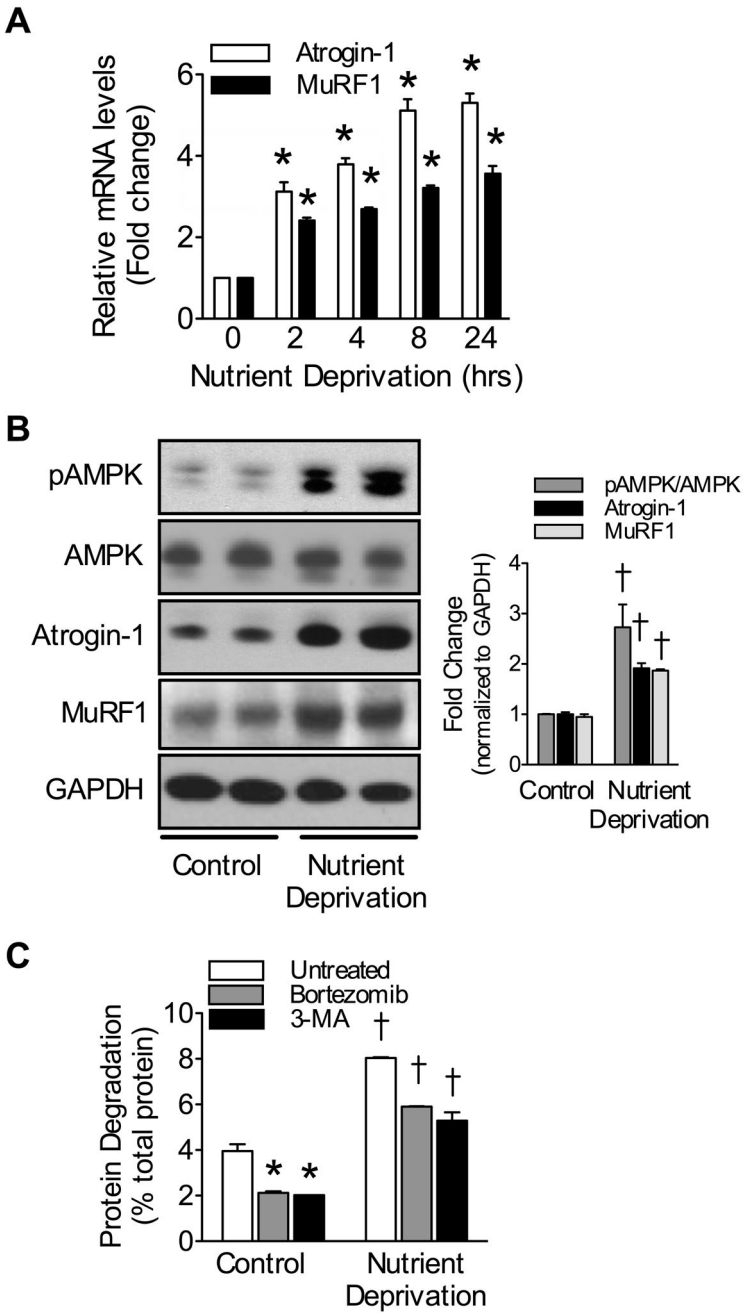

Figure 1.

Nutrient deprivation increases expression of ubiquitin ligases and enhances protein degradation.

(A) Atrogin-1 and MuRF1 mRNA expression in nutrient deprived NRVM. (B) Relative protein levels and quantification in NRVM after 24 hours of nutrient deprivation. (C) Protein degradation in NRVM after 24 hours of nutrient deprivation with 1μmol/L Bortezomib or 10μmol/L 3-methyladenine treatment. Data are mean ± SEM of 3 independent experiments performed in triplicate. *P< 0.01 vs control or untreated, †P< 0.01 vs control or complete nutrients.

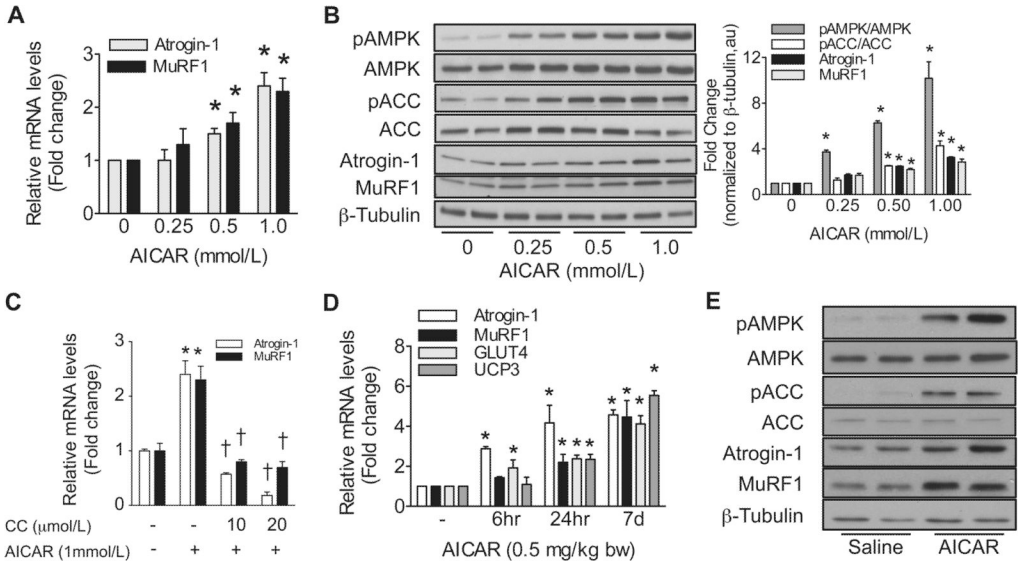

Figure 2. AMPK activation increases expression of ubiquitin ligases *in vitro* and *in vivo*

(A) Atrogin-1 and MuRF1 mRNA expression and **(B)** relative protein levels and quantification in NRVM after 24 hours of AMPK activation with AICAR treatment. **(C)** Atrogin-1 and MuRF1 mRNA expression in NRVM after 24 hours of AMPK activation and inhibition. **(D)** AMPK activation *in vivo* with AICAR treatment to increase Atrogin-1, MuRF1, GLUT4, and UCP3 mRNA expression in mouse heart after either acute (6 or 24 hours after injection) or chronic (one injection per day for 7 days) AICAR treatment (n=5–8, 0.5 mg/kg body weight (bw) AICAR). **(E)** Relative protein levels in mouse heart after 7 days of *in vivo* AICAR treatment. The *in vitro* data are mean ± SEM of 3 independent experiments, each performed in triplicate. *P< 0.01 vs untreated or saline, †P< 0.01 vs AICAR.

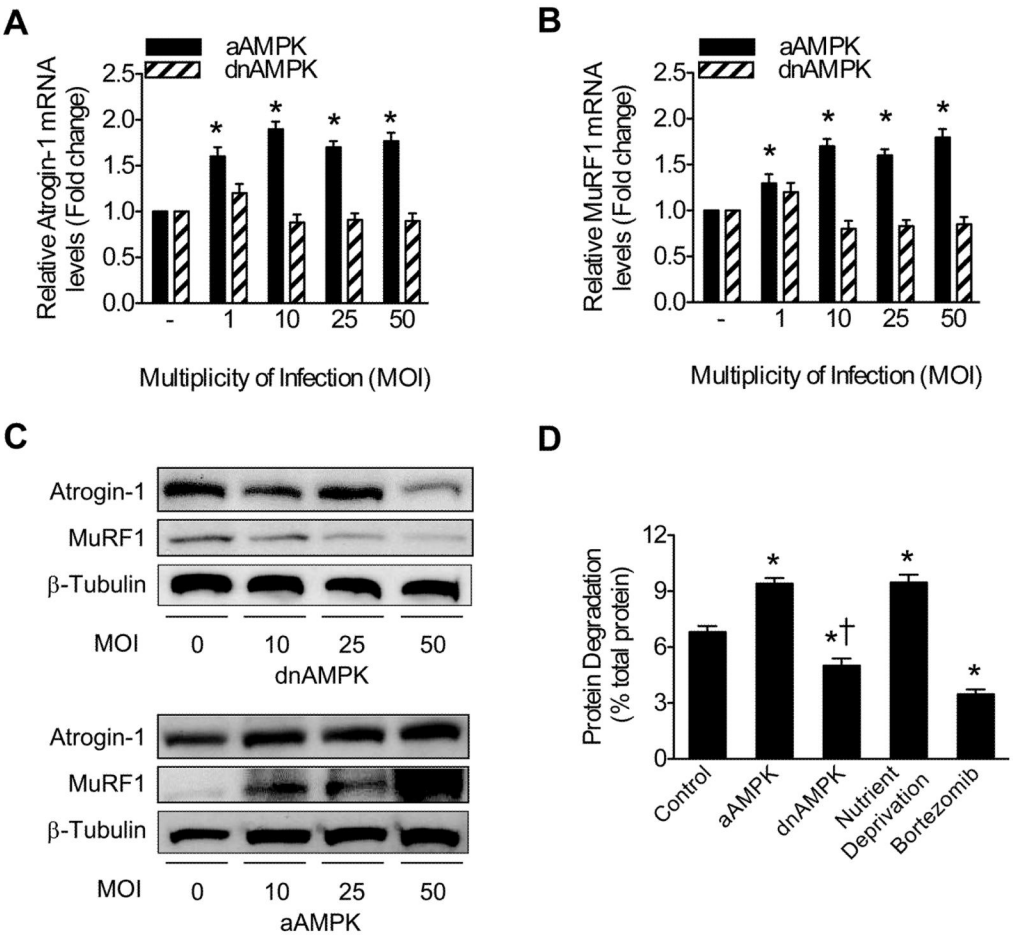

Figure 3. AMPK regulates expression of ubiquitin ligases and protein degradation in cardiomyocytes

(A) Atrogin-1 and **(B)** MuRF1 mRNA expression in NRVM 24 hours after adenoviral transduction of active (aAMPK) or dominant negative (dnAMPK) AMPK. **(C)** Atrogin-1 and MuRF1 protein levels in response to dnAMPK or aAMPK in NRVM 24 hours after adenoviral transduction. **(D)** Protein degradation in the presence of aAMPK or dnAMPK (MOI=10, 1μmol/L Bortezomib). Data are mean ± SEM of 3 independent experiments performed in triplicate. *P< 0.01 vs control, †P< 0.01 vs aAMPK.

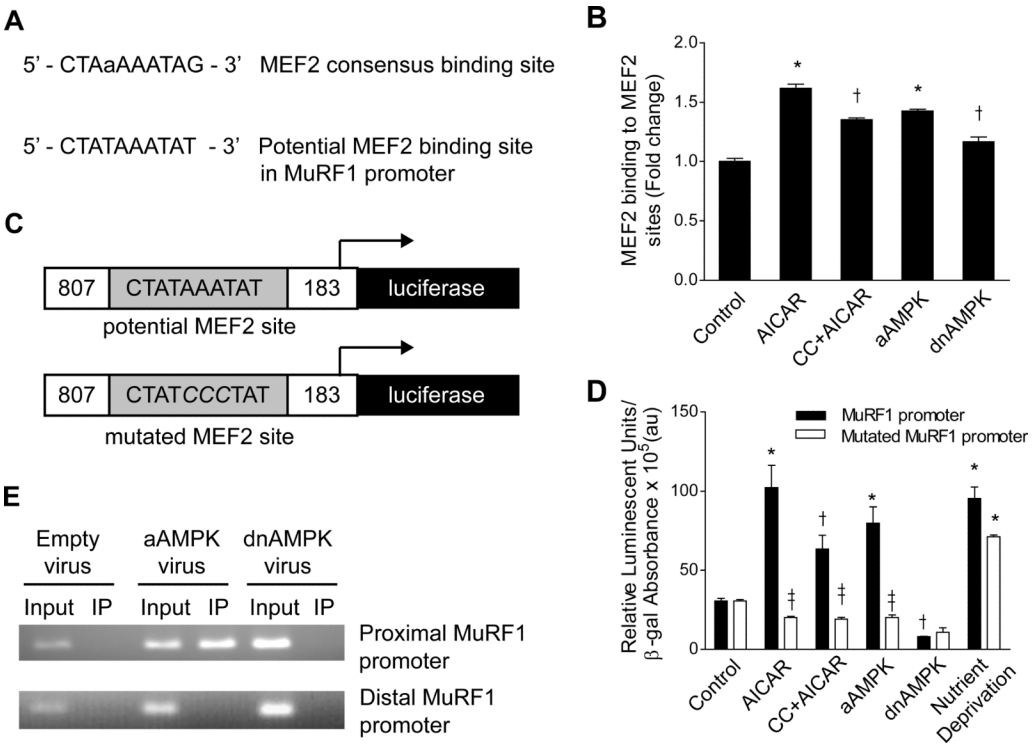

Figure 4. MuRF1 transcription is regulated by an AMPK-MEF2 dependent mechanism *in vitro*

(A) MEF2 consensus binding site and potential MEF2 binding site on MuRF1. **(B)** MEF2 transcriptional activity following activation or inhibition of AMPK in NRVM (1mmol/L AICAR, 20μmol/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 vs control, †P<0.01 vs AICAR or aAMPK, ‡P< 0.01 vs MuRF1 promoter.

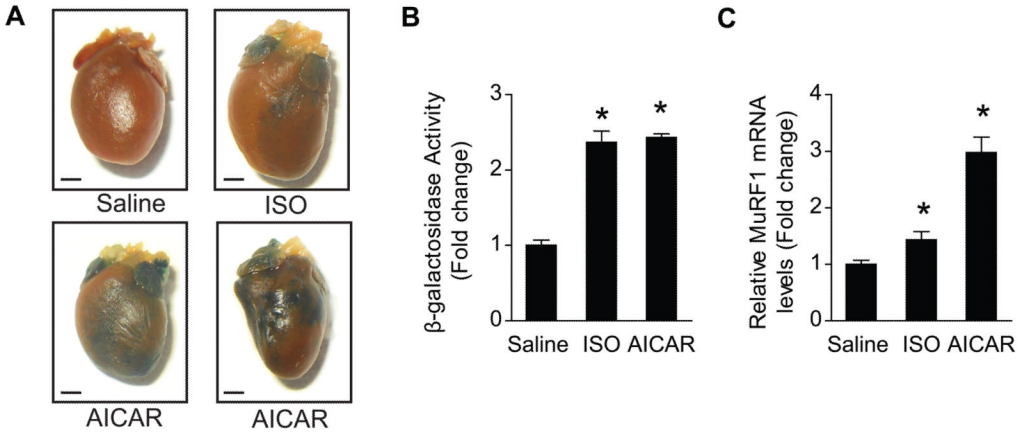

Figure 5. MEF2 transcriptional activity and MuRF1 transcription are regulated by AMPK *in vivo*

(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* (15μg/g bw Isoproterenol used as a positive control (ISO), 0.5mg/g bw AICAR, n=6–8). I*n vitro* data are mean ± SEM of 3 independent experiments performed in triplicate. Scale bars=1mm. *P< 0.01 vs saline.

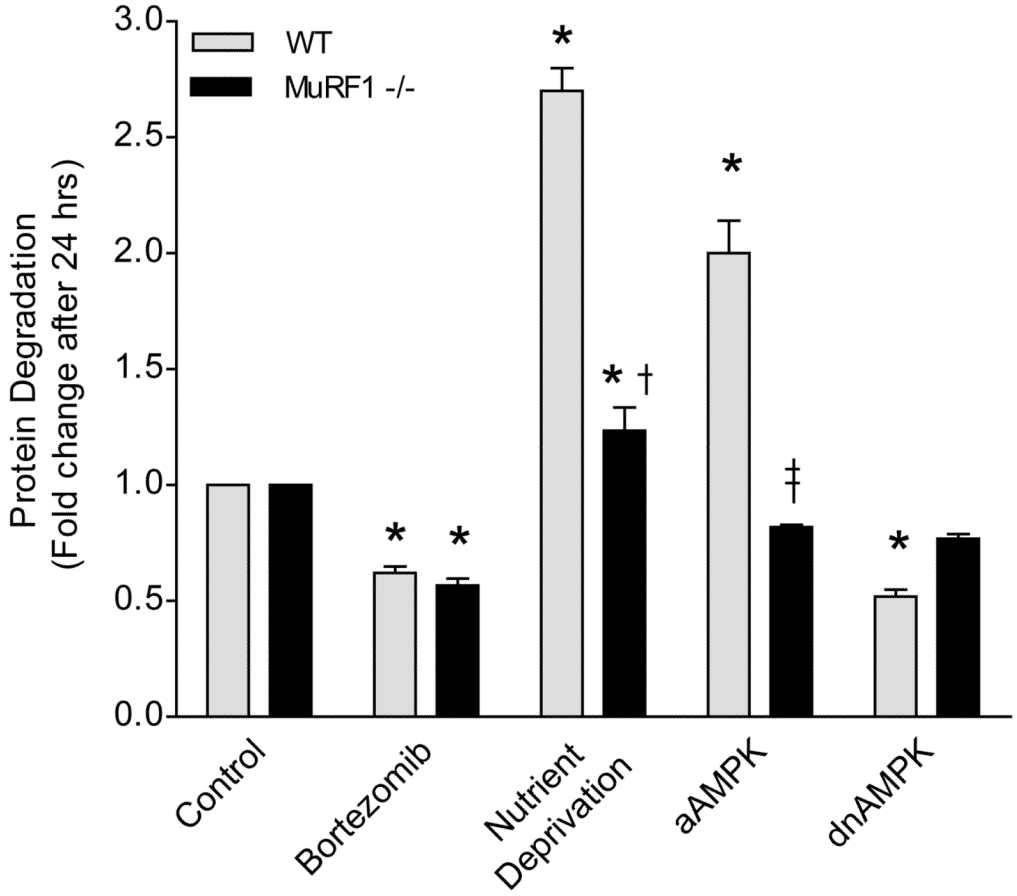

Figure 6. MuRF1 is required for AMPK-regulated protein degradation in cardiomyocytes

Protein degradation in adult mouse cardiomyoytes isolated from wild type (WT) or MuRF1 knockout mouse hearts 24 hours after treatment (1mmol/L Bortezomib) or adenoviral transduction (MOI=1). Data are mean ± SEM of 3 independent experiments performed in duplicate. *P< 0.01 vs control, †P< 0.01 vs Nutrient Deprivation, ‡P< 0.01 vs aAMPK.

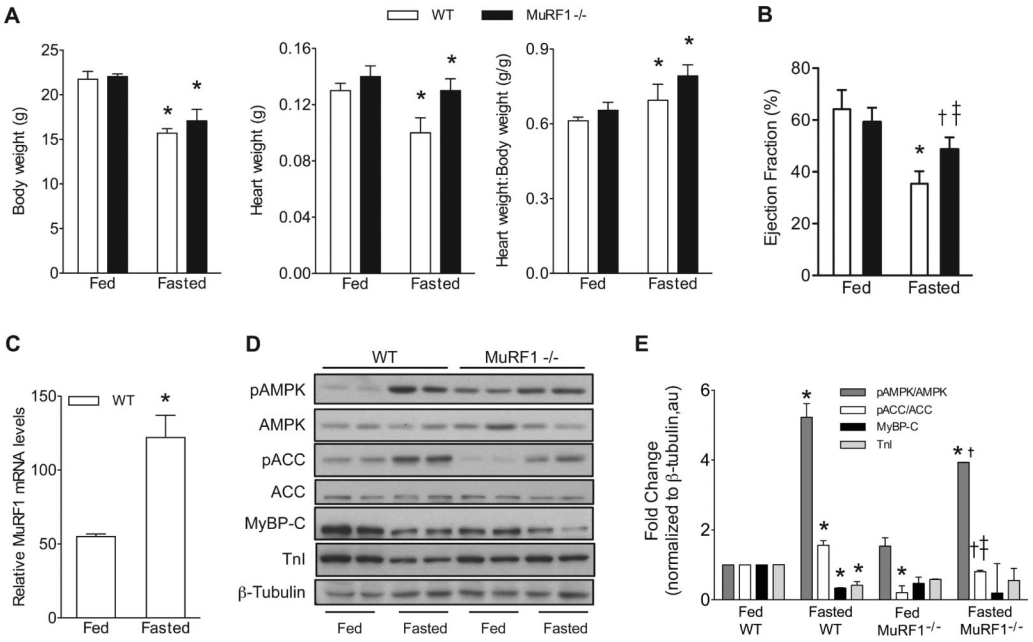

Figure 7. Absence of MuRF1 preserves cardiovascular function during nutrient deprivation

(A) Heart weight, body weight, heart weight to body weight ratios, **(B)** ejection fraction, **(C)** MuRF1 expression, **(D)** relative protein levels and **(E)** protein quantification in wild type (WT) or MuRF1 −/− hearts after 3 days of fasting (n=6–8). *P< 0.01 vs Fed, † P< 0.01 vs WT Fasted, ‡P< 0.01 vs MuRF1 −/− Fed.

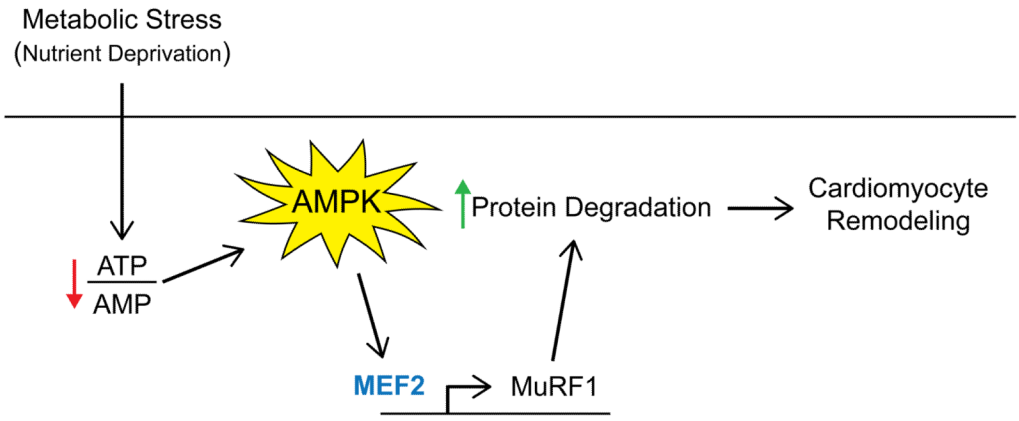

Figure 8. AMPK regulates MuRF1 transcription in a MEF2-dependent manner

AMPK regulates MuRF1 expression through the transcription factor MEF2. This leads to increased protein degradation in the cardiomyocyte and increased remodeling.