CARDIAC MUSCLE RING FINGER 1 INHIBITS ADAPTIVE HYPERTROPHIC REMODELING INDUCED BY IGF-1, EXERCISE, AND THYROID HORMONE

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the curriculum of Molecular and Cellular Pathology

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ABSTRACT

Kristine Marie Wadosky: Cardiac muscle RING finger 1 inhibits adaptive hypertrophic remodeling induced by IGF-1, exercise, and thyroid hormone
(Under the direction of Monte S. Willis)

In the face of cardiac load, the heart responds by undergoing hypertrophy. Under pathological conditions, ventricular wall thickening cannot be maintained, impairing function (heart failure). In contrast, ventricular wall thickening in response to physiological stimuli does not impair cardiac function. Pathological and physiological hypertrophy are driven by distinct signaling pathways—while vasoactive factors induce the former, growth hormones induce the latter.

Understanding the mechanisms through which heart growth is adaptive has recently become a goal of cardiac research, in the hopes of using this information in the discovery of therapies that promote restoration of the myocardium following injury (since an effective treatment for heart failure has yet to be developed). The purpose of this dissertation work is to describe new mechanisms by which physiological cardiac hypertrophy is regulated. Signaling pathways activated in the heart following physiologic stimuli are receptor tyrosine kinases (RTKs) and nuclear receptors (NRs), of which the IGF-1/Akt and TRα pathways, respectively, are most understood. The work presented here establishes the striated muscle specific ubiquitin ligase, Muscle RING finger 1 (MuRF1) as a novel regulator of physiological cardiac hypertrophy through its ubiquitination of transcription factors.

MuRF1 inhibits cardiomyocyte growth in response to IGF-1 and aerobic exercise stimulation by poly-ubiquitinating and promoting the degradation of c-Jun, recently discovered to drive transcriptional expression of genes coding for members of the IGF-1 signaling cascade. In contrast, MuRF1 mono-ubiquitinates TRα, thereby inhibiting TRα transcriptional activity and thyroid hormone (TH)-dependent cardiac hypertrophy. Furthermore, it is established here that mono-ubiquitination of TRα by MuRF1 induces TRα accumulation in the cardiomyocyte nucleus where TRα interacts with
centrosome-associated protein 350 (CAP350). This inhibitory mechanism, while being established for other NRs, is completely novel for TRα and has never been described in the heart. Altogether, this dissertation contributes to the field of cardiac growth research by detailing the discovery of an inhibitor of not one, but two, signaling pathways that stimulate beneficial cardiac hypertrophy. Given that MuRF1 inhibits both IGF-1/Akt and TRα signaling, the activity of this ubiquitin ligase may be as efficacious target for new cardiac therapies by virtue of MuRF1’s widespread influence on the cardiomyocyte.
To my parents, without whom none of this would have been possible
ACKNOWLEDGMENTS

I would like to thank my advisor Monte S. Willis for keeping me focused while I completed my thesis work in his laboratory, aiding me in distilling my work into simple narratives, and providing me with numerous opportunities to publish. I would also like to thank our collaborator Cam Patterson for giving me access to his laboratory’s equipment and reagents, allowing my involvement in his laboratory’s meetings, and providing one-on-one feedback on my work. I would like to thank my committee for providing me with a fresh perspective each time we gathered. I would also like to thank the following faculty in the Department of Pathology and Laboratory Medicine for their additional support and mentorship: William B. Coleman, J. Charles Jeanette, and Li Qian.

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For the twelve years he has been part of my life, my partner Connor J Smith has been invaluable to me. During completion of this stage of my career especially, his dedication, patience, and unfettered support were immeasurable.

Most importantly, my parents, Andrew and Janet Wadosky, provided me with everything. Their household fostered me in an independent thinker at a young age. Questions of “Why?” were answered with egalitarian and thoughtful responses—imparting me with the confidence to continue asking. In my role as scientist, I am still asking “Why?” and the perseverance in finding an answer has driven my success. These values originate with my parents; my success is equally their success.
PREFACE

Chapter Two of this dissertation is comprised of the publication, “Muscle-RING finger-1 attenuates IGF-1-dependent cardiomyocyte hypertrophy by inhibiting JNK signaling,” of which Kristine Marie Wadosky is first author and that is in press for the *American Journal of Physiology Endocrinology and Metabolism*. The Appendix of this dissertation is comprised of the publication, “Regulation of the calpain and ubiquitin proteasome systems in a canine model of muscular dystrophy,” of which Kristine Marie Wadosky is first author and that has previously appeared in *Muscle and Nerve*. The original citation for the publication composing the Appendix of this dissertation is as follows: *Muscle Nerve* 44, no. 4 (October 2011): 553-62. Excerpts from a review article published by Kristine Marie Wadosky were used in Chapter Four: Discussion and Perspectives of this dissertation. The original citation for this publication excerpted in Chapter Four is as follows *Am J Physiol Heart Circ Physiol* 2012 Feb 1;302(3):H515-26.
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<th>Description</th>
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<tbody>
<tr>
<td>4E-BP1</td>
<td>4E-binding protein 1</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated kinase</td>
</tr>
<tr>
<td>ANF</td>
<td>Atrial natriuretic factor</td>
</tr>
<tr>
<td>AngII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine nucleotide translocator</td>
</tr>
<tr>
<td>API1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>AT1R</td>
<td>Angiotensin type 1 receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BARD1</td>
<td>BRCA1-associated RING domain 1</td>
</tr>
<tr>
<td>BNP</td>
<td>Brian natriuretic peptide</td>
</tr>
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<td>BRCA1</td>
<td>Breast cancer early onset 1</td>
</tr>
<tr>
<td>CaMK</td>
<td>Calcium/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>CAP350</td>
<td>Centrosome-associate protein 350</td>
</tr>
<tr>
<td>CHIP</td>
<td>Carboxyl terminus of Hsp70-interacting protein</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine kinase</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>DGC</td>
<td>Dystrophin-glycoprotein complex</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection fraction</td>
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<td>EFP</td>
<td>Estrogen responsive protein</td>
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<td>eIF2Bε</td>
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</tr>
<tr>
<td>eIF4E</td>
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</tr>
<tr>
<td>ET1</td>
<td>Endothelin 1</td>
</tr>
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<td>Abbreviation</td>
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<td>-------------</td>
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</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead Box-O proteins</td>
</tr>
<tr>
<td>FS</td>
<td>Fractional shortening</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GMEB1</td>
<td>Glucocorticoid modulatory element binding protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GRMD</td>
<td>Golden retriever muscular dystrophy</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>Hprt</td>
<td>Hypoxanthine phosphoribosyltransferase</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Heat shock protein 70</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
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<td>IGF-1R</td>
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<tr>
<td>IR</td>
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<tr>
<td>JNK</td>
<td>C-Jun N-terminal kinase</td>
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<tr>
<td>LXRα</td>
<td>Liver X receptor α</td>
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<tr>
<td>MAFBx</td>
<td>Muscle atrophy F-box</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylate transporter</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute 2</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>MT</td>
<td>Masson’s trichrome</td>
</tr>
<tr>
<td>MULAN</td>
<td>Mitochondrial ubiquitin ligase activator of NF-κB</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>Mammalian target of rapamycin complex 2</td>
</tr>
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<td>MKL1</td>
<td>Megakaryoblastic leukemia 1</td>
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<tr>
<td>MuRF1</td>
<td>Muscle ring finger protein 1</td>
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<tr>
<td>MyBP-C</td>
<td>Myosin binding protein-C</td>
</tr>
<tr>
<td>MyLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NCoR</td>
<td>Nuclear receptor corepressor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor–kappa B</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NR</td>
<td>Nuclear receptor</td>
</tr>
<tr>
<td>NRVM</td>
<td>Neonatal rat ventricular myocytes</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide dependent kinase-1</td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
</tr>
<tr>
<td>PGC1</td>
<td>Peroxisome proliferator-activated receptor γ coactivator 1</td>
</tr>
<tr>
<td>PLN</td>
<td>Phospholamban</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP3</td>
<td>phosphatidylinositol (3,4,5)-triphosphate</td>
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<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PPARα</td>
<td>Peroxisome proliferator-activated receptor α</td>
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<tr>
<td>PPARβ/δ</td>
<td>Peroxisome proliferator-activated receptor β/δ</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor γ</td>
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<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PSMA6</td>
<td>Proteasome subunit alpha type 6</td>
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</table>
PSMB4  Proteasome subunit beta type 4
PSME1  Proteasome activator subunit 1
RACK1  Receptor for activated protein C kinase 1
RNF4  RING finger protein 4
RNF6  RING finger protein 6
RNF8  RING finger protein 8
RT-PCR  Reverse transcript–polymerase chain reaction
RTK  Receptor tyrosine kinase
RXRα  Retinoid X receptor α
RYR1  Ryanodine receptor 1
SERCA2a  Sarcoplasmic reticulum Ca^{2+} ATPase 2a
SK actin  Skeletal muscle actin
SMCs  Smooth muscle cells
SRF  Serum response factor
Suc-LLVY-AMC  Succinyl–leucine–leucine–valine– tyrosine-4- methyl-7-courmarylamide
SUMO  Small ubiquitin-like modifier
TH  Thyroid hormone
T3  3,5,3’-triiodothyronine
T4  3,5,3’,5’-tetraiodothyronine
TAC  Transaortic constriction
Tbp  TATA box-binding protein
TL  Tibia length
TRα  Thyroid receptor α
TRβ  Thyroid receptor β
TRAF6  Tumor necrosis factor (TNF) receptor activated factor 6
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>TRE</td>
<td>Thyroid response element</td>
</tr>
<tr>
<td>TTC3</td>
<td>Tetra-tricopeptide repeat domain 3</td>
</tr>
<tr>
<td>UBC9</td>
<td>Ubiquitin-like protein SUMO 1-conjugating enzyme</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
</tr>
<tr>
<td>UNC4/5</td>
<td>Uncoordinated phenotype 4/5</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin proteasome system</td>
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CHAPTER 1: INTRODUCTION

Pathological and physiological cardiac hypertrophy have distinct characteristics

When the cardiac wall is exposed to increased mechanical and hemodynamic forces, cardiomyocytes counteract these forces by producing more sarcomeres, the smallest contractile unit responsible for force generation. Amassing additional sarcomeres increases the volume of individual cardiomyocytes and initiates ventricular wall thickening—stabilizing wall stress and maintaining overall cardiac output\(^1\).

Functionally, the heart’s response to ventricular wall thickening is largely dependent on the type of hypertrophic stimuli producing this response. Pathologic stimuli, such as hypertension, aortic stenosis, myocardial infarction and certain sarcomeric gene mutations, increase cardiac afterload by inducing the release of angiotensin II (AngII), endothelin 1 (ET1), norepinephrine (NE), and other vasoactive factors. These ligands bind to G protein-coupled receptors (GPCRs) and activate downstream mitogen-activated protein kinase (MAPK) cascades and G-protein-activated protein kinases. Initially this growth maintains cardiac function, but chronic excessive activation of these signaling cascades stimulates the production of reactive oxygen species and apoptotic programs in the cardiomyocyte and inflammation and extracellular matrix deposition at the tissue level. These cellular and structural changes adversely affect cardiac function by stiffening the ventricles, reducing capillary density, and impairing electrical stimulation of contraction and relaxation. Consequently, this cardiac damage progresses to heart failure. As a result of this maladaptive processes, this type of heart growth has been termed pathological cardiac hypertrophy\(^2\).

Increases in cardiac mechanical and hemodynamic forces also occur during postnatal growth and in response to exercise conditioning and pregnancy in adults\(^3\). In contrast to pathological cardiac hypertrophy, the myocardium’s response to increases in afterload induced by these stimuli is benign
— in that the morphological changes that occur in response to these physiological conditions do not cause cardiomyocyte apoptosis and necrosis, tissue fibrosis, or cardiac dysfunction, and in some cases, can enhance function. Additionally, in the case of either exercise or pregnancy, the cardiac hypertrophic remodeling is reversible once the stimulus is removed. Consequently, cardiac growth induced by these stimuli is termed physiological hypertrophy.

**Molecular mechanisms of physiological cardiac hypertrophy**

Multiple hormones and mechanical signals have been associated with promoting physiological heart growth downstream of post-natal growth signals, exercise, and pregnancy. While activation of physiological growth by extracellular signals compares to that of pathological growth, the types of receptors stimulated by physiological hypertrophy-inducing ligands contrast greatly with those that activate pathological hypertrophy—while pathological growth is largely attributed to the activation of GPCRs, physiological growth is primarily attributed to the activation of receptor tyrosine receptors (RTKs) and nuclear receptors (NRs).

RTKs are transmembrane proteins comprised of a hydrophobic region flanked by extracellular N-terminal and intracellular C-terminal domains. The binding of ligand to the extracellular domain of its cognate RTK induces receptor dimerization and subsequent auto-phosphorylation of the receptor’s intracellular domain by its partner. The activated RTK intracellular domain then binds to and phosphorylates several different substrates. While each RTK has a distinct set of substrates, most RTKs involved in myocardial signaling recruit phosphoinositide 3-kinase (PI-3K) to the plasma membrane, via PI-3K’s direct binding to the phosphotyrosine residues within the RTK dimer, as the initial step in the activation of the downstream kinase cascade.

In contrast to RTKs, NRs, as the name indicates, are localized to the cardiomyocyte nucleus when active, where they activate transcription by binding to their respective nucleotide motifs within the promoter regions of target genes. In the absence of ligand, a transcriptional corepressor, such as nuclear receptor corepressor (NCoR), binds to and inhibits the nuclear receptor. A conformational change induced by the ligand causes the corepressor to dissociate, releasing inhibition
and allowing the NR to homodimerize or heterodimerize with its coreceptors and recruit coactivators, if required, to the promoter\textsuperscript{12}. The composition of the active transcriptional complex, whether a homodimer or heterodimer, including a co-activator or not, is specific to each nuclear receptor\textsuperscript{12}. Also in contrast to RTKs, active NRs can be localized throughout the cell, achieving functions independent of transcriptional activation. These functions of certain NRs have been shown to be important for physiological growth in the heart\textsuperscript{7,11,13}.

The specific pathways that are known to induce physiological cardiac hypertrophy which have been most well-studied include the insulin-like growth factor-1 (IGF-1) and thyroid hormone (TH) signaling cascades. In addition, these cascades represent pathways initiated by both a RTK and NR (respectively) (Figure 1)\textsuperscript{2,3}. Considering that the basic information about the proteins, enzymatic cascades, transcriptional profiles, and regulatory processes involved for each of these signaling cascades has been established, the IGF-1 and TH pathways were focused on in this dissertation work so that an established and accepted mechanistic foundation was present to ensure that new experimental results could be interpreted accordingly.

**The IGF-1/Akt signaling cascade.** Exercise triggers the release of growth hormone (GH) from the pituitary gland that induces IGF-1 production in and subsequent release from the liver (Figure 1). While cardiomyocytes themselves transiently release IGF-1, circulating IGF-1 is the main source of IGF-1 for the cardiomyocyte, especially following exercise\textsuperscript{14-16}. IGF-1 binds to either its own receptor (IGF-1R) or the insulin receptor (IR), RTKs present at the cardiomyocyte plasma membrane (Figure 1)\textsuperscript{9}. Active IGF-1R or IR binds to and phosphorylates PI3K, which catalyzes the production of phosphatidylinositol (3,4,5)-triphosphate (PIP3) at the plasma membrane (Figure 1)\textsuperscript{2,9}. The presence of PIP3 induces phosphoinositide dependent kinase-1 (PDK1) translocation to the plasma membrane, where PDK1 phosphorylates Akt1 (Figure 1)\textsuperscript{2,9}, the main isoform of Akt in the heart (Akt2 is also present, but to a lesser extent), at T308\textsuperscript{17,18}. PDK1-dependent phosphorylation of Akt1 (hereafter referred to as Akt) is the crucial step in the IGF-1 pathway, since Akt is central to this kinase cascade\textsuperscript{9,19}. The T308 phosphorylated form of Akt is only partially active; therefore, the final
step in generating fully activated Akt is its phosphorylation by mammalian target of rapamycin C2 (mTORC2) at S473 (Figure 1). Akt’s importance in promoting physiological hypertrophy is illustrated by studies that have shown that hypertrophy induced by exercise is inhibited by knocking out Akt and hypertrophy without dysfunction is induced by acute cardiac-specific transgenic expression of Akt. In addition, inducing transcription of Akt by activating c-Jun (Figure 1) (via knocking out the c-Jun inhibitor sirtuin 6 (SIRT6)) in vivo, promotes cardiac hypertrophy.

Akt promotes cardiac growth mainly by phosphorylating substrates involved in both transcription and translation. Akt phosphorylates and inhibits Forkhead box O (FOXO) transcription factors (FOXO1 and FOXO3a) by promoting their nuclear export (Figure 1) and thereby prevents the transcription of the pro-atrophy gene expression profile induced by FOXOs. Glycogen synthase kinase-3β (GSK-3β), another protein which is inhibited by Akt-dependent phosphorylation, is itself an inhibitor of pro-growth transcription factors in the heart, including GATA4, nuclear factor of activated T cells (NFAT), myocardin, c-myc, c-Jun, and β-catenin; therefore Akt activates pro-growth transcription factors by inhibiting GSK-3β (Figure 1).

Akt promotes protein translation via multiple mechanisms. By inhibiting GSK-3β, Akt not only activates pro-growth transcription (as described above), but also activates translation since GSK-3β is also an inhibitor of eukaryotic initiation factor 2Bε (eIF2Bε). Akt also promotes the formation of the mTORC1 kinase complex, which stimulates protein translation in multiple ways. mTORC1 phosphorylates and activates p70S6 kinase, which subsequently activates ribosome biosynthesis by phosphorylating S6 and protein elongation by phosphorylating eukaryotic elongation factor 2 (eEF2) (Figure 1). mTORC1 also activates translation initiation by phosphorylating 4E-binding protein 1 (4E-BP1), releasing 4E-BP1’s inhibition of eukaryotic initiation factor (eIF4E) (Figure 1).

Finally, Akt improves cardiomyocyte contractility in addition to promoting growth. Cardiac-specific transgenic expression of constitutively active Akt not only induces growth on the level of the whole cardiomyocyte and individual myofibrils, but increases basal maximum left
ventricular (LV) dP/dt at baseline and at low concentrations of dobutamine\textsuperscript{24}. In addition, isolated cardiomyocytes from mice transgenically expressing Akt have increased rates of both contraction and relaxation, increased overall Ca\textsuperscript{2+} transients, and increased L-type Ca\textsuperscript{2+} channel current densities compared to wildtype cardiomyocytes\textsuperscript{31}. In that study\textsuperscript{31}, as well as others\textsuperscript{32,33}, increased expression of Akt was associated with increased expression of sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase 2a (SERCA2a), the primary channel responsible for Ca\textsuperscript{2+} reuptake into the sarcoplasmic reticulum at the end of each contraction cycle (Figure 1). Akt has also been shown to phosphorylate the SERCA2a inhibitor phospholamban (PLN), causing PLN to dissociate from and release its inhibition of SERCA2a (Figure 1), again allowing for enhanced Ca\textsuperscript{2+} influx into the SR\textsuperscript{32}. These studies show that Akt primarily affects cardiomyocyte contraction by improving sarcoplasmic reticulum Ca\textsuperscript{2+} loading, permitting for more efficient Ca\textsuperscript{2+} handling and excitation-contraction coupling.

**Activation of thyroid receptor (TR) by TH.** Signaling mechanisms activated by TH in the cardiomyocyte also induce physiological hypertrophy\textsuperscript{2,3,34}. The primary form of TH secreted by the thyroid gland is the pro-hormone 3,5,3\textsuperscript{',}5\textsuperscript{'}-tetraiodothyronine (T4), which is converted to 3,5,3\textsuperscript{'}-triiodothyronine (T3) by deiodinases (Figure 1)\textsuperscript{35}. Activity of deiodinases in the adult cardiomyocyte is marginal and only <7\% of total T3 is generated by the cardiomyocyte itself; therefore, the primary source of T3 for the cardiomyocyte is from the circulation\textsuperscript{35}. T3 requires plasma membrane transporters to enter the cell\textsuperscript{36}. The transporters responsible for T3 movement across the plasma membrane which have been most well-studied include two members of the monocarboxylate transporter (MCT) family, MCT8 and 10\textsuperscript{36,37}. These transporters are especially important to TH signaling in the cardiomyocyte, since the expression of MCT8 and 10 have recently been shown to be regulated by T3 in the heart in vivo (Figure 1)\textsuperscript{38}.

Once T3 has entered the cardiomyocyte, it can affect multiple signaling pathways\textsuperscript{6,13,39}. However, T3-dependent activation of the NR transcription factors thyroid receptors (TRs) (Figure 1) is the most well-established mechanism controlling TH-dependent cardiomyocyte growth\textsuperscript{3,11}. The TR isoforms expressed in the heart include TRα1 and 2 and TRβ1, where TRα accounts for 70\% and
TRβ1 accounts for 30% of total TR. Since TRα2 activity antagonizes T3-dependent activity and is only weakly activated by T3, TRα1 (hereafter referred to as TRα) is responsible for the bulk of T3-dependent activities in the cardiomyocyte. In the absence of T3 stimulation, a transcriptional corepressor, such as nuclear receptor corepressor (NCoR), binds to and inhibits TRα (Figure 1). A conformational change induced by T3 causes the corepressor to dissociate, releasing TRα inhibition and allowing the recruitment coreceptors, such as retinoid X receptor α (RXRα), and coactivators, such as peroxisome proliferator-activated receptor γ coactivator 1 (PGC1) (Figure 1). Together, the TRα complex binds the thyroid response element (TRE) in the promoter region of TH-regulated genes, activating or inhibiting transcription (Figure 1). Genes regulated by TRα include SERCA2a and PLN, which are activated and inhibited, respectively (Figure 1). The transcriptional profile promoted by TRα in its entirety is associated with favorable cardiac hypertrophy, where cardiac function is at least maintained, if not improved, and does not progress to heart failure (the primary characteristics of physiological growth).

TRα has several extranuclear actions which have significant effects on cardiomyocyte metabolism and growth. A truncated form of TRα, a 43kD protein (TRαp43) the translation of which is initiated by an internal methionine residue, localizes to the cardiomyocyte mitochondria where it activates transcription of mitochondrial genes (Figure 1); thereby promoting the activity of the respiratory chain and mitochondrial oxygen consumption. Another truncated form of TRα is also localized to the mitochondria (TRαp28), but it lacks the DNA-binding region and does not act as a mitochondrial transcription factor. Instead, TRαp28 binds to and activates adenine nucleotide translocator (ANT), the protein responsible for passage of ADP into the mitochondrial matrix, at the inner mitochondrial membrane. Via this interaction with ANT, TRαp28 instantaneously increases respiratory chain flux by feeding ADP substrate to ATP synthase (Figure 1). The overall effect of mitochondrial TRα isoforms (TRαp48 and TRαp28) is the increase in ATP production via oxidative phosphorylation that generates a greater pool of hydrolysable ATP within the cardiomyocyte. By doing so, the processes that promote physiological cardiac growth and
contractility (transcription, translation, and excitation-contraction coupling) proceed at a greater rate because of the readiness of energy substrates.

In addition to being localized to the cardiomyocyte nucleus and mitochondrion, active TRα is localized to the cytoplasm where its activity also promotes physiological growth as well\(^40\). TRα interacts with the p85 subunit of PI-3K, promoting the activation of PI-3K and the downstream IGF-1 signaling cascade (including Akt, mTOR, and p70S6K) (Figure 1)\(^44\). As described above, activation of this signaling cascade activates both transcription and translation, therefore providing a mechanism by which TRα activates overall transcriptional processes (independent of the activation of its own target genes) and protein synthesis (Figure 1)\(^44\). Furthermore, activation of PI-3K by TRα is a direct example of crosstalk between the IGF-1 and TH signaling pathways and represents the importance of comprehending these cascades not as distinct, but jointly promoting the final outcome of cardiac growth without pathological remodeling.

**The ubiquitin proteasome system\(^1\)**

The conjugation of ubiquitin is unique among post-translational modifications in that it involves the attachment of another polypeptide, the 76-amino acid polypeptide, ubiquitin\(^45,46\), instead of the addition of a functional group, such as a phosphate, acetate, lipid, or carbohydrate. In the first step of the ubiquitin conjugation cascade, free ubiquitin is covalently linked to the ubiquitin-activating enzyme (E1) in an ATP-dependent reaction (Figure 2, far left)\(^45,46\). Next, ubiquitin is transferred from the E1 enzyme to the ubiquitin-conjugating enzyme (E2) (Figure 2, middle). Finally, the interaction between the E2 and the ubiquitin ligase enzyme (E3) allows the E3 to initiate the transfer of ubiquitin or SUMO from the E2 to a lysine residue on the substrate (Figure 2, middle)\(^45,46\). The E3 is the pivotal component in both the ubiquitin conjugation pathways since it confers specificity to the system by directly interacting with the substrate. Therefore, identifying ubiquitin ligase-substrate pairs is integral to understanding the cellular functions affected by ubiquitin conjugation\(^45\). The most

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commonly described ubiquitination pattern is poly-ubiquitination, where the initial ubiquitin molecule is conjugated to the substrate, followed by the addition of subsequent ubiquitin molecules linked by their lysine located in the 48th amino acid position (Figure 2, right)\(^{45-47}\). When the lysines are serially connected to each other by this canonical lysine number 48, it is referred to as a K48 (K = lysine) poly-ubiquitin chain. This canonical K48 ubiquitination pattern is recognized by the 26S proteasome, targeting the modified protein for degradation (Figure 2, right)\(^{47-49}\). Other recognized ubiquitination patterns include mono-ubiquitination (the placement of only 1 ubiquitin on a substrate)\(^{46}\) and poly-ubiquitination via atypical [non-K48 linked (K63)] ubiquitin chains (Figure 2, right)\(^{46,50}\). Mono-ubiquitination and poly-ubiquitination via atypical ubiquitin chains have a wider effect on cellular function than polyubiquitination via K48, including translation, cell cycle regulation, signal transduction, protein trafficking, and protein-protein interactions\(^{45,46,51-53}\).

**Muscle RING Finger 1 (MuRF1): the discovery of a striated muscle-specific ubiquitin ligase**

**Discovery of MuRF proteins.** MuRF1 was initially identified in a yeast-two-hybrid screen using the transcription factor serum response factor (SRF) as bait\(^{54}\). Subsequent studies by this group illustrated that MuRF1 was exclusively expressed in striated muscle (skeletal and cardiac) and co-localized with sarcomeres in the mouse quadriceps\(^{54}\). Further work by another group identified other MuRF1 family members, MuRF2 and MuRF3, via a yeast-two-hybrid screen using MuRF1 as bait\(^{55}\). All MuRF family members are part of subclass of RING proteins called RING-B-box-coiled-coil\(^{54}\), containing an N-terminal RING domain, a MuRF family-specific conserved region, Zn-binding B-box motif, leucine-rich coiled coil motifs, and an acidic tail at the C-terminus, the last of which varies in length for each member\(^{55}\). MuRF1, MuRF2, and MuRF3 co-segregate into the same subgroup in an evolutionary tree comparison with other members of the RING protein family, despite being encoded by different genes on different chromosomes (mouse chromosome 1, 8, and 2, respectively)\(^{55}\). While expression patterns of each MuRF family member differ slightly, for instance, MuRF1 has been shown to be expressed in uterine smooth muscle\(^{56}\) and MuRF2 is expressed at low levels in the liver\(^{55}\). MuRF1, MuRF2, and MuRF3 are primarily expressed (and studied, for that
matter) in striated muscle. Of all the family members, MuRF1’s activity in both skeletal and cardiac muscle is most established.

Discovery of MuRF1’s connection to muscle atrophy. The first direct evidence of the importance of MuRF proteins to muscle physiology came about soon after their discovery, where it was shown that the transcript coding for MuRF1 was one of two transcripts most highly expressed after induction of skeletal muscle atrophy in vivo\(^\text{57}\). Unexpectedly, MuRF1/− mice did not show any differences in skeletal muscle and heart weights at baseline\(^\text{57}\). However, cutting the sciatic nerve of MuRF1/− animals, thereby causing denervation of the tibialis anterior and gastrocnemius muscles, showed that MuRF1/− skeletal muscles were heavier (+36%) compared to wildtype animals following this atrophy-inducing stimulus\(^\text{57}\). The sparing of MuRF1/− animals from denervation-induced skeletal muscle atrophy suggested that MuRF1’s expression was not merely correlative with atrophy induction but that MuRF1’s activity as a putative ubiquitin ligase was significant in the development of muscle atrophy\(^\text{57}\).

MuRF1’s interacting partners and substrates originally characterized in striated muscle. The first proteins identified to interact with MuRF1 include an array of sarcomeric proteins, including titin, myosin heavy chain (MHC), myosin binding protein-C (MyBP-C), myosin light chain (MyLC), troponins, nebulin, myotilin, and telethonin\(^\text{55,58}\). Comparison of immunoblot data for troponin T, MyLC 1, and MyLC2 between wildtype and MuRF1/− in cardiac tissue showed that higher molecular weight species (presumed to be ubiquitinated forms for these proteins) were reduced in MuRF1/−.\(^\text{58}\) While MuRF1’s homology to RING-domain-containing monomeric ubiquitin ligases suggested that it ubiquitinates proteins, the decrease of the potential ubiquitinated species of these sarcomeric proteins in MuRF1/− tissue provided evidence, albeit indirect, that MuRF1 is indeed a functional ubiquitin ligase\(^\text{58}\). The primary consequence of a protein’s ubiquitination is its degradation by the proteasome, by which 80-90% of intracellular proteins undergo proteolysis\(^\text{59}\). Therefore, further studies of MuRF1-dependent activity in muscle cells focused on determining the degree to which MuRF1 promotes the degradation of sarcomeric proteins. Indeed, denervation atrophy-
dependent decreases in MyBP-C, MyLC1, and MyLC2 protein levels were restored in the gastrocnemius of animals expressing MuRF1 without its RING domain (the region of MuRF1 conferring its potential ubiquitin ligase activity)\textsuperscript{60}. These data supplied further evidence to support the hypothesis that MuRF1 was an operating ubiquitin ligase, in addition to suggesting that the MuRF1-dependent degradation of sarcomeric proteins plays a role in the development of muscle atrophy. Confirmation of MuRF1’s ubiquitin ligase activity was secured when Kedar et al.\textsuperscript{61} showed that MuRF1 directly ubiquitinates cardiac troponin I (using \textit{in vitro} ubiquitination assays). This study not only provided definitive evidence of MuRF1’s activity as an ubiquitin ligase, but also showed that MuRF1 inhibits contraction of neonatal rat ventricular myocytes (NRVM), where maximal shortening and rate of shortening were decreased 25\% in cardiomyocytes with increased expression of MuRF1\textsuperscript{61}. These closing data are especially important because they introduced MuRF1’s significance in cardiomyocyte function and laid the groundwork for the study of MuRF1 in cardiac physiology.

\textbf{MuRF1 activity in the cardiomyocyte}

As described above, MuRF1 plays an active role in protein quality control for the sarcomere in striated muscle. In the cardiomyocyte specifically, MuRF1 targets troponin T, MyLC 1, MyLC2, troponin I, MyBP-C, βMHC, MHCIIa, and tropomyosin\textsuperscript{58, 61-64}, suggesting that it may maintain overall sarcomeric performance by directing degradation of proteins which become damaged during continuous cycles of excitation-contraction coupling. In addition to localizing to the sarcomeric M- and Z-lines\textsuperscript{54, 55}, MuRF1 localizes to multiple other regions of the cardiomyocyte and targets substrates that are imperative for metabolism, transcription, and growth signaling.

\textbf{MuRF1 and cardiomyocyte metabolism.} The amount of ATP required to sustain excitation-contraction coupling during each heartbeat is vast\textsuperscript{65}. The rapid movement of ATP throughout the cell in striated muscle is achieved by creatine kinase (CK) via a phosphotransferase system that transfers ATP from the mitochondrion to sites of utilization, maintaining cardiac function\textsuperscript{66}. MuRF1 ubiquitinates CK\textsuperscript{67} and mice with cardiac-specific transgenic expression of MuRF1 (MuRF1 Tg+) have decreased CK activity\textsuperscript{68}—showing that MuRF1 limits ATP
bioavailability in the cardiomyocyte. A recent study has also identified ATP synthase β and a mitochondrial specific heat shock protein (Hspd1) as MuRF1 ubiquitin substrates in cardiomyocytes\textsuperscript{64}, illustrating MuRF1’s importance to mitochondrial function and suggesting that MuRF1 can localize to the mitochondrion. Moreover, MuRF1 affects cardiomyocyte metabolism at the transcriptional level. Peroxisome proliferator-activated receptor (PPAR) family of NRs regulate the transcription of genes involved in lipid metabolism and promote fatty acid uptake and oxidation\textsuperscript{69}. Since fatty acid oxidation is the primary source of ATP for the heart, PPARs are crucial for energy production in the cardiomyocyte\textsuperscript{69}. MuRF1 has been found to mono-ubiquitinate PPARα, inhibiting PPARα transcriptional activity by nuclear export (instead of promoting PPARα degradation)\textsuperscript{70-72}. These results were consistent with the observation that MuRF1 Tg+ hearts are deficient in fatty acid oxidation\textsuperscript{68} and further work showed that PPARα transcriptional activity was inhibited by MuRF1 \textit{in vivo}\textsuperscript{70}.

**MuRF1 regulates transcription factors.** In addition to targeting PPARα, MuRF1 ubiquitinates multiple other transcription factors. As described above, MuRF1 was first identified in a screen for SRF-binding proteins\textsuperscript{54}. The direct effect of MuRF1 on SRF was discovered by our laboratory, where we showed that SRF-dependent luciferase activity was significantly decreased in cells expressing increased MuRF1\textsuperscript{73}. We have also shown that MuRF1 interacts with the transcription factor c-Jun and specifically promotes the degradation of phospho-c-Jun upon ischemia/reperfusion injury\textsuperscript{74}. Finally, we have recently reported that both MuRF1 and MuRF2 promote the transcriptional activity of E2F1 \textit{in vivo}\textsuperscript{75}. Direct chromatin immunoprecipitation of E2F1 from cardiac tissue show that E2F1 promoter occupancy was decreased in MuRF1-/−/MuRF2-/− double knockout mice, but not in single knockout mice, suggesting that MuRF1 and MuRF2 promote E2F1 activity redundantly; likely via the ubiquitination and proteasomal degradation of an inhibitor of E2F1\textsuperscript{75}. Another essential finding from this study was the spontaneous hypertrophic cardiomyopathy observed in MuRF1-/−/MuRF2-/− animals\textsuperscript{75}. Since these animals were born with this hypertrophic
defect, these data implicate MuRF1 and MuRF2 in developmental heart growth\textsuperscript{75}—providing initial evidence for MuRF1’s role in physiological hypertrophy.

**MuRF1 and cardiomyocyte hypertrophy.** MuRF1’s further role in growth signaling is illustrated via its interaction with receptor for activated protein C kinase 1 (RACK1). Upon activation of GPCRs by phenylephrine (PE), MuRF1 and RACK1 colocalize in the perinuclear region of the cardiomyocyte\textsuperscript{76}. MuRF1’s interaction with RACK1 inhibits the translocation of protein kinase C ε (PKCε) to the sarcolemma. This action of MuRF1 stops PKCε from interacting with focal adhesion kinase (FAK)\textsuperscript{76}, a kinase which governs the cardiomyocyte’s response to extracellular biomechanical stress signals\textsuperscript{77}. Together, these data suggest that MuRF1 inhibits cardiomyocyte growth, since PKCε and FAK are pro-growth kinases\textsuperscript{76}. Indeed, increased expression of MuRF1 inhibits growth induced by GPCR agonists PE, AngII, ET-1, and serum in NRVM\textsuperscript{76}. Since MuRF1 hinders cardiomyocyte growth downstream of GPCRs\textsuperscript{76}, most commonly associated with pathological growth signaling\textsuperscript{2}, these data indicate that MuRF1 inhibits pathological cardiac hypertrophy. Based on this evidence, our laboratory has shown that MuRF1-dependent inhibition of pathological cardiac hypertrophy is present \textit{in vivo} by showing that MuRF1/- mice have an exaggerated cardiac growth response to pressure-overload induced by transaortic constriction\textsuperscript{73}. These studies identify MuRF1 as a key regulator limiting cardiomyocyte growth in response to pathologic stimuli.

**Hypothesis for this dissertation work**

MuRF1 is known to inhibit pathological cardiac hypertrophy—shown both in isolated cardiomyocytes stimulated with GPCR agonists\textsuperscript{76} and in mice challenged with pressure overload\textsuperscript{73}. Given that MuRF1’s inhibits this form of cardiac hypertrophy, and especially since mice lacking both MuRF1 and MuRF2 undergo exaggerated developmental cardiac growth (a type of hypertrophy known to be physiological in nature) without a precipitating challenge, the overarching hypothesis for this dissertation work is that MuRF1 also inhibits physiological hypertrophy. IGF-1-, exercise-, and TH-dependent cardiac hypertrophy were chosen as models in to order to provide a thorough body of evidence for MuRF1-dependent effects on physiological hypertrophy. In addition, a wide array of
methods were utilized in this work—including *in vitro* and *in vivo* models of cardiomyocyte hypertrophy, fluorescent imaging, and molecular biology—to demonstrate MuRF1-dependent inhibition of physiological hypertrophy, establish the molecular mechanisms by which MuRF1 achieves limitation of this type of hypertrophy, and identify new substrates for MuRF1’s ubiquitin ligase activity.
CHAPTER 2: MUSCLE RING FINGER 1 ATTENUATES IGF-1-DEPENDENT CARDIOMYOCYTE HYPERTROPHY BY INHIBITING JNK SIGNALING

Introduction

MuRF1 is a muscle-specific ubiquitin ligase localized to multiple regions of the cardiomyocyte where it affects the function and stability of numerous proteins. MuRF1 localized to the sarcomeric M-line polyubiquitinates and directs the proteasomal degradation of a number of proteins, including troponin I, βMHC, and MyBPc. Cytoplasmic MuRF1 in cardiomyocytes interacts with the transcription factor c-Jun and specifically promotes the degradation of phospho-c-Jun upon ischemia/reperfusion injury. MuRF1 also localizes to the perinuclear region of the cardiomyocyte, where it interacts with RACK1 inhibiting the translocation of PKCε to focal adhesions following stimulation with GPCR agonists. In the nucleus, MuRF1 interacts with glucocorticoid modulatory element binding protein 1 (GMEB1), a nuclear transcriptional regulator.

These studies illustrate MuRF1’s widespread effect on cardiomyocyte structure, gene expression, and signaling. Our laboratory has further conveyed MuRF1’s key role in the myocardium by showing that MuRF1 inhibits pathological cardiac hypertrophy induced by pressure-overload in vivo. Mechanistically, we established that MuRF1 achieves hypertrophy attenuation by binding to and inhibiting the transcription factor SRF, an inducer of pro-hypertrophic gene expression. In addition, we have found that MuRF1 loss in the mouse heart blocks hypertrophy regression following the reversal of transaortic constriction (TAC)—indicating that MuRF1 is required for induction of cardiac atrophy. Furthermore, increased expression of MuRF1 has been shown to inhibit growth and expression of pathological hypertrophy markers in response to PE, AngII, ET-1, and serum in...
NRVM\textsuperscript{76}. Together, these studies have identified MuRF1 as a key regulator limiting cardiomyocyte growth in response to pathologic stimuli.

We hypothesized that MuRF1 also inhibits physiological cardiac hypertrophy, which is a process mediated by IGF-1 signaling. Physiological cardiac hypertrophy, such as that which occurs in an athlete’s heart in response to repetitive exercise, develops in response to IGF-1, a ligand synthesized and secreted by the liver in response to GH, that subsequently targets the cardiomyocyte\textsuperscript{3}. IGF-1 binds to its RTK receptor (IGF-1R) on the surface of cardiomyocytes, leading to the activation of the PI3K/Akt signaling cascade\textsuperscript{2}. Akt is a serine/threonine kinase central to the kinase cascade activated by IGF-1\textsuperscript{19}. Knockout of Akt inhibits cardiac hypertrophy in response to exercise\textsuperscript{20}, illustrating Akt’s importance in promoting physiological hypertrophy. Conversely, acute cardiac-specific transgenic expression of Akt has been shown to induce hypertrophy without dysfunction\textsuperscript{21}. Together, these studies show that Akt expression itself, in addition to its activation by through IGF-1 signaling, are imperative for the development physiological cardiac hypertrophy.

In this study, we examine the role of MuRF1 in physiological cardiomyocyte growth. We show that MuRF1 inhibits IGF-1-dependent growth in cardiomyocytes and limits the total protein expression of Akt, GSK3β, and mTOR. We go on to illustrate that the attenuation of IGF-1 signaling by MuRF1 requires c-Jun — where c-Jun activity is necessary for enhanced IGF-1 signaling observed when MuRF1 is knocked down. These data show for the first time that c-Jun can be regulated in an IGF-1-dependent manner in cardiomyocytes and provide new evidence supporting the role of c-Jun N-terminal kinase (JNK) signaling in cardiomyocyte growth and survival. Importantly, we establish that MuRF1 limits this newfound activity of c-Jun. Finally, we go on to show that MuRF1 acts to limit cardiac hypertrophy induced by voluntary wheel running exercise in the mouse—indicating that MuRF1-dependent limitation of IGF-1 signaling is applicable \textit{in vivo}.

\textbf{Materials and Methods}

\textbf{Cell culture, adenovirus transduction, and IGF-1 treatment.} HL-1 cells, a continuously proliferating cardiomyocyte cell line derived from atrial tumors, were maintained as previously
Before culturing cells, tissue culture vessels were coated with a mixture of 0.02% gelatin (w/v)/0.5% fibronectin (v/v) for at least 30 minutes. HL-1 cells were trypsinized using 0.05% trypsin with EDTA, seeded on the gelatin/fibronectin-coated dishes at a dilution between 1:5 to 1:2, and allowed to adhere overnight in Claycomb media (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% norepinephrine, and 1% L-glutamine. After culturing for at least 24 hours, media was changed to serum-free Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 1% penicillin/streptomycin and HL-1 cells were treated with adenovirus at the multiplicity of infections (MOIs) and for the times indicated. Transient knock-down of MuRF1 was achieved using recombinant adenoviruses expressing scrambled shRNA control (Adshscrambled) or shRNA-MuRF1 custom made by Vector Biolabs (Philadelphia, PA). shRNA sense sequence used for MuRF1 knockdown was GATCC-GCTCTGATCCTCCAGTACA-TTCAAGAGATGTACTGGAGGATCAGAGC-TTTTTTAGATCTA. Increased MuRF1 expression was achieved using previously described adenoviruses expressing GFP or myc-tagged MuRF1/bicistronic GFP. Following adenovirus transduction, cells were treated with IGF-1 (suspended in PBS) which was then added to serum-free DMEM at a final concentration of 10 nM.

**Isolation and culturing of NRVM.** NRVM were isolated using the Worthington Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corporation, Lakewood, NJ) according to manufacturer’s protocol. Briefly, dissected hearts were minced and digested using the provided trypsin solution overnight at 4°C. The next day, the digested tissue was oxygenated and warmed to 30°C, at which time trypsin inhibitor was added and incubated for 5 min. Cells were released using a standard plastic sterological pipette and triturate supernatant was filtered through the cell strainer provided. The filter was then washed twice with L15 media containing collagenase (provided), where filtrate was collected in the same tube as strained cells. Collagenase digestion was allowed to occur for 20 minutes at RT, after which cells were collected by centrifugation. Cardiomyocytes were reconstituted in Medium 199 (M199) supplemented with 15% FBS and 0.5% penicillin/streptomycin and seeded on 6-well dishes, coated with 40µg/mL fibronectin, at a concentration of 5x10^5 cells per
Cells were cultured for 48 hours, after which media was changed to M199 without serum. After 24 hours starvation, increased MuRF1 expression was achieved using previously described adenoviruses expressing GFP or myc-tagged MuRF1/bicistronic GFP in serum-free M199. Following adenovirus transduction, cells were treated with 10nM IGF-1 in PBS in serum-free M199.

**JNK Inhibitor treatment.** HL-1 cardiomyocytes were treated with the JNK inhibitor SP600125 as described previously. Briefly, cardiomyocytes were transduced with adenovirus, treated with SP600125 for 30 minutes, then challenged with IGF-1. At the concentration and the times indicated, SP600125 did not elicit any visible cell death or changes in cell density.

**Fluorescence microscopy.** HL-1 cells were cultured on optically active flexible membrane 6-well culture plates (Flexcell International, Hillsborough, NC) coated with gelatin/fibronectin. Following transduction with GFP-expressing adenovirus and IGF-1 stimulation, cells were fixed in 4% formaldehyde in PBS, membranes cut into 1 x 1 cm sections, and mounted onto glass slides with media containing DAPI. Fluorescent imaging was carried out using a Leica DMIRB Inverted fluorescence microscope (Leica Microsystems, Buffalo Grove, IL) and a Hamamatsu Orca ER camera (Bridgewater, NJ) with a 40X objective lens. Pixel/mm scale was set in ImageJ using an image of a 1mm graticule taken with the Hamamatsu Orca ER camera under the 40X objective lens of the Leica DMIRB Inverted fluorescence microscope. Cardiomyocyte area was determined using Image J software, in which a minimum of 200 cells in replicate wells in each experimental group were measured.

**RT-PCR determination of mRNA expression.** Total RNA was isolated from cells using the RNeasy kit according to the manufacturer’s protocols (Qiagen Valencia, CA). cDNA was made using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). One µl of cDNA product was then amplified on an ABI Prism 7500 Sequence Detection System in 12 µl final volume using the TaqMan® Universal PCR Master Mix. The PCR reaction mix included 0.6 µl of mouse specific Taqman® probes (Applied Biosystems) for MuRF1 (Mm01185221_m1), RYR1 (Mm01175211_m1), Igfbp5 (Mm00516037_m1), and MAPK13 (Mm00442488_m1).
(Hs99999901_s1) was used as a reference gene. Raw CT values were analyzed using the ΔΔCt method, where CT values were first normalized to 18S and then normalized to either vehicle control (RYR1, Igfbp5, and MAPK13) or adenovirus control (MuRF1). Fold change values (calculated by the formula 2(-ΔΔCT)) were used as final expression data.

**Antibodies and Immunoblot.** Cells were lysed in the presence of 2x protease inhibitor cocktail Roche Applied Science, Indianapolis, IN), 1x phosphatase inhibitor cocktail Roche Applied Science), and 0.2M glycerol-2-phosphate Sigma-Aldrich) and stored at -80C. Fifteen to twenty-five µg of protein was resolved on either 4-12% or 10% NuPAGE® gels (Life Technologies, Carlsbad, CA), transferred to Immobilon®-PVDF membrane (Millipore, Billerica, MA), blocked in 2% ECL blocking reagent (GE Healthcare Amersham™, Buckinghamshire, UK) in 1x TBST for 30 minutes, and incubated with primary antibody overnight at 4°C. After washing, HRP-linked secondary antibody was added to the PVDF membranes for 1 hour at RT. Signal was detected using ECL Prime, GE Healthcare Amersham™) and final immunoblot results were acquired using hyperfilm (GE Healthcare Amersham™). Densitometry analysis was performed using Quantity One 1-D Analysis Software (BioRad, Hercules, CA). Antibodies recognizing Akt, p-Akt (S473 and T308) (Catalog #9272, 9271, and 9275, respectively, 1:1000) and c-Jun, pc-Jun (S63, S73, and T91) were obtained from Cell Signaling Technology, Inc. (Catalog #9165, 9261S, 9164S, and 2303S, respectively, at 1:500). Antibodies recognizing GSK3α/β and pGSK3β (S9) (Catalog #44-610 and 44-600G, Invitrogen, Inc., Camarillo, CA) were used at 1:4000 and 1:1000 dilutions, respectively. Anti-MuRF1 was purchased from R&D Systems, Inc. (Catalog #AF5366, 1:250 dilution, Minneapolis, MN) and anti-β-actin was purchased from Sigma-Aldrich (Catalog #A5441, 1:4000).

**Animals.** MuRF1/- mice (129S/C57BL6) and α-MHC–MuRF1 (cardiac-specific) transgenic mice (DBA/C57BL6) ages 8-16 weeks were used as described. After 5 weeks of monitored wheel running exercise (or sham exercise), mice were euthanized, hearts flash frozen and stored at -80°C or perfusion fixed in 4% paraformaldehyde. All animal studies were approved by the
institutional care and use committee (IACUC) for animal research at the University of North Carolina at Chapel Hill.

**Unloaded voluntary wheel running and conscious echocardiographic analysis.** Female mice were assigned to either unloaded (no-resistance) wheel running or sedentary control groups as previously described\(^8^4\). Mice were randomly assigned to running or sham control groups and monitored in parallel by echocardiography on conscious mice using a Visual Sonics Vevo 770 and 2100 ultrasound biomicroscopy system at baseline, 2 weeks, and 5 weeks, as described previously\(^6^8, ^8^1\).

**Histology and cross-sectional cardiomyocyte area analysis.** Fixed cardiac tissue was processed for paraffin-embedding, sectioned, and stained with standard hematoxylin and eosin (H&E). H&E-stained slides were scanned using an Aperio Scanscope and exported using Aperio Imagescope software (Aperio Technologies, Vista, CA). Paraffin-embedded cardiac sections were stained with a TRITC-labeled lectin as previously described\(^7^3\) and cardiomyocyte cross-sectional area was measured using Image J software. A minimum of 15 random fields at 200X magnification in the left ventricle were imaged via fluorescent microscopy from at least 3 different hearts.

**Measurement of exercise statistics.** Exercise wheel use was measured using a Mity 8 Cyclocomputer (model CC-MT400), recording running time, average speed, and distance. Running statistics were recorded daily.

**RNA isolation and RT-PCR determination of mRNA expression from heart tissue.** Whole heart tissue was homogenized in RLT Plus Lysis buffer, containing β-mercaptoethanol at a 1:100 dilution, from Qiagen’s RNeasy Kit (Valencia, CA). Total RNA was isolated from whole heart lysate using the RNeasy kit according to the manufacturer’s protocols. cDNA was made using High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). One µl of cDNA product was then amplified on an ABI Prism 7500 Sequence Detection System in 12 µl final volume using the TaqMan® Universal PCR Master Mix. The PCR reaction mix included 0.6 µl of mouse specific Taqman® probes (Applied Biosystems) for either BNP (Mm00435304_g1), ANF
(Mm01255747_g1), skeletal muscle actin (Mm00808218_g1), Tbp (Mm00446971_m1), or Hprt (Mm01545399_m1). Raw CT values were analyzed according to the ΔCt method using multiple reference genes (Tbp and Hprt). ΔCt values for each gene were calculated by normalizing to the average CT value over all experimental groups. Reference gene correction factors for each animal were calculated by taking the geometric mean of Tbp and Hprt ΔCt values for that animal. Final expression data for each target gene was calculated by dividing the ΔCt value by the reference gene correction factor. Expression data for BNP, ANF, and skeletal muscle actin were averaged over three animals per experimental group.

**Statistics.** All statistical analysis was performed using Sigma Plot software (Systat Software, Inc., Chicago, IL). A two-way ANOVA test was used to determine the source of variation when data included two independent variables. For *in vivo* studies, the two variables were defined as genotype and exercise assignment group (sedentary or running). For *in vitro* studies, the two variables were defined as adenovirus and treatment. Interactions between the two variables were reported when significant (p<0.05) and the F-statistic (regression variable from the dependent variable mean/residual regression variation) and degrees of freedom (DF) were reported. Differences between specific groups were determined using a multiple comparison post-test via the Holm-Sidak method using the all-pairwise procedure. Significance level for all statistical analysis was set at p<0.05. For experiments using JNK inhibitor pre-treatment, a three-way ANOVA test was used to determine the source of variation because three independent variables (adenovirus, pre-treatment, and treatment) were identified. Interactions between all three variables, as well as pairwise interactions between variables, were reported when significant. When appropriate, the results of a Student’s t-test was performed to express differences between groups within one variable.

For *in vitro* cardiomyocyte area experiments, n-values were defined as the total number of measured cells from at least two separate slides from three independent wells where at least 200 cardiomyocytes in total were measured per treatment group. For *in vitro* RT-PCR experiments, n-values were defined as expression data for each gene within each treatment group from three
independent experiments. For *in vitro* immunoblot experiments, each treatment group is represented in three lanes, each lane from one independent experiment and n=1 was defined as one lane. For *in vivo* cardiomyocyte area experiments, n-values were defined as the total number of measured cells from separate 200X images, at least 2 slides per animal, where there were three animals per experimental group. A minimum of 180 cardiomyocytes in total over all three animals per experimental group were measured. For *in vivo* RT-PCR experiments, n-values were defined as expression data for three separate animals per each treatment group. Significance level for all statistical analysis was set at p<0.05.

**Results**

MuRF1 inhibits IGF-1-dependent cardiomyocyte hypertrophy by its effects on Akt signaling *in vitro*. To determine MuRF1’s role in IGF-1 induced cardiomyocyte growth, HL-1 cardiomyocytes were transduced with a GFP-adenovirus expressing scrambled shRNA (Adshscrambled) or shRNA specific for MuRF1 (AdshMuRF1) and challenged with IGF-1 (*Figure 3A*). Analysis of cellular surface area after IGF-1 challenge revealed that decreasing MuRF1 (AdshMuRF1) enhances cell growth compared to vehicle-treated controls cells (49.6% vs. 30.1%) (*Figure 3B*). In addition, there was a significant interaction between the adenovirus and treatment variables (F=3.879, DF=1), providing evidence that knockdown of MuRF1 (AdshMuRF1) and IGF-1 is required for this enhanced growth (*Figure 3A*). Conversely, increasing MuRF1 inhibits IGF-1 cardiomyocyte hypertrophy (*Figure 3C*). Compared to IGF-1-treated vehicle-treated cells (areas of which increased with IGF-1), IGF-1 stimulation did not result in significant growth in cardiomyocytes when MuRF1 expression was increased (AdMuRF1) (*Figure 3D*). In the same manner as HL-1 cardiomyocytes, we identified that increasing MuRF1 in NRVMs also completely inhibited IGF-1-dependent cardiomyocyte growth (*Figure 3E*), where statistical analysis showed that there was a significant interaction between variables (F=4.152, DF=1) (*Figure 3F*). Taken together, these studies illustrate MuRF1-dependent inhibition of IGF-1-dependent growth in both cardiac-derived cells and primary cardiomyocytes *in vitro*. 

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Since Akt is central in IGF-1 signal transduction in vivo, we investigated MuRF1’s regulation of Akt-associated regulated genes by qRT-PCR analysis. Mitogen activated kinase 13 (MAPK13), ryanodine receptor 1 (RYR1), and the insulin-like growth factor binding protein-5 (Igfbp5) mRNA levels were assayed (Figure 4) based on their differential expression in transgenic cardiac Akt mice at 14 days of age. After 18 hours of IGF-1 stimulation, a ~2 fold increase in MAPK13 and RYR1 expression was seen in HL-1 cells when MuRF1 expression was decreased 60% using AdshMuRF1 (Figure 4A). Specifically, statistical analysis showed that enhanced RYR1 expression required the interaction between MuRF1 knockdown (AdshMuRF1) and IGF-1 treatment (F=13.693, DF=1) (Figure 4A). Conversely, increased MuRF1 expression resulted in decreased MAPK13 expression (40%) and complete inhibition of IGF-1-driven RYR1 expression in HL-1 cells (Figure 4B). Furthermore, the combined effects of increased MuRF1 expression (AdMuRF1) and IGF-1 treatment led to significantly decreased Igfbp5 expression (F=5.576, DF=1) (Figure 4B). Together, these data support our proposed model that MuRF1 regulates IGF-1-dependent cardiomyocyte growth by regulating Akt activity.

MuRF1 inhibits total Akt, GSK3β, and mTOR protein levels in IGF-1 induced cardiomyocyte hypertrophy. Since the fully active form of Akt is dually phosphorylated at threonine-308 (T308) and serine-473 (S473) and phosphorylates downstream effector proteins, we first investigated how MuRF1 regulates Akt phosphorylation (Figure 5 and 6). We found that increasing MuRF1 resulted in a significant decrease in total Akt protein levels in vehicle-and IGF-1-treated cells, but not phosphorylated Akt (T308 and S473) (Figure 5A). Phosphorylation of Akt at T308 was also unaffected by increased MuRF1 expression (AdMuRF1) in the presence of IGF-1 in NRVM (Figure 6). While inhibition of phosphorylation of Akt at S473 in the presence of IGF-1 was observed with increased MuRF1 expression (F=14.887, DF=1) in NRVM (Figure 6), unchanged phosphorylation of Akt at T308 suggests that MuRF1’s primary role in inhibiting Akt activity is not via Akt’s phosphorylation. Similar to the inhibition of total Akt protein expression by MuRF1, the total protein expression of GSK3β, an immediate downstream effector of Akt, was significantly
decreased in vehicle-treated cells (Figure 4A). Phosphorylation (at S9) of GSK3β was also inhibited, where a significant interaction between increased MuRF1 expression and IGF-1 treatment was observed (F=12.258, DF=1) (Figure 5A), showing that signaling downstream of Akt is limited by MuRF1. Increased MuRF1 expression in NRVM was also required for inhibition of IGF-1-dependent phosphorylation of GSK3β at S9 (F=10.405, DF=1) (Figure 6), showing that MuRF1-dependent inhibition of Akt activity occurs both in cardiac-derived cells and primary cardiomyocytes.

We next investigated how decreasing MuRF1 affects total and phosphorylated Akt and GSK3β proteins in IGF-1 stimulated HL-1 cells. Surprisingly, we only identified a significant increase in p-Akt S473, where knockdown of MuRF1 was required for enhanced phosphorylation of Akt at S473 in the presence of IGF-1 (F=11.801, DF=1) (Figure 5B). p-Akt-T308 remained unchanged with MuRF1 knockdown in HL-1 cardiomyocytes stimulated with IGF-1 (Figure 5B), suggesting that the enhanced IGF-1-induced cardiomyocyte growth was not due to differences in the fully phosphorylated (active) form of Akt. Changes in only the S473-phosphorylated form of Akt in the presence of IGF-1, led us to hypothesize that the total protein expression of mTOR was being altered when MuRF1 is decreased, since mTOR complex 2 (mTORC2) phosphorylates Akt at S47319. Furthermore, inhibition of mTOR phosphorylation by increasing MuRF1 expression under basal conditions has been shown to occur in cardiomyocytes85. We found that IGF-1 treatment increased total protein levels of mTOR only in the presence of decreased MuRF1 (AdshMuRF1) (F=5.922, DF=1) (Figure 7). Interestingly, while MuRF1 knockdown induced increased mTOR phosphorylation at S2481 in vehicle-treated cardiomyocytes, IGF-1 treatment induced p-mTOR to be significantly decreased (F=21.990, DF=1) with the concomitant increase in total mTOR (Figure 7). Since phosphorylated mTOR is part of mTOR complex 1 (mTORC1)19, these data suggest that MuRF1 inhibits mTORC1 basally, consistent with recently published reports85, but that IGF-1 treatment may shift MuRF1’s inhibitory activity of mTOR from mTORC1 to mTORC2. Finally, immunoblot analysis of GSK3β showed that decreasing MuRF1 expression increased total protein expression of GSK3β in vehicle-treated cardiomyocytes, but neither GSK3β nor p-GSK3β S9 were
different between AdshMuRF1 and its control after IGF-1 stimulation (Figure 5B). Together, our protein expression findings suggest that increasing MuRF1 expression may be inhibiting IGF-1 growth by its effects on total Akt and GSK3β, while MuRF1 knockdown releases this inhibition of IGF-1 signaling by promoting total protein expression of mTOR, and by extension, mTORC2.

**MuRF1 inhibits c-Jun protein expression and phosphorylation in the presence of IGF-1.** Recent studies have implicated that the sirtuin SIRT6 inhibits IGF-1-Akt signaling and the development of cardiac hypertrophy by targeting c-Jun. The authors of this study show that c-Jun promotes the mRNA expression of numerous proteins involved in IGF-1 signaling, including Akt, GSK3β, and mTOR; therefore, they demonstrated that inhibition of c-Jun by SIRT6 inhibited the expression of key components of IGF-1 signaling. We have recently discovered that c-Jun is a MuRF1 substrate. In these previous studies, we identified that MuRF1 inhibited ischemia-reperfusion induced cell death (apoptosis) by preventing JNK signaling. MuRF1 preferentially recognized phospho-c-Jun, it was able to bind activated c-Jun, direct its polyubiquitination, which then was recognized by the 26S proteasome and degraded. This known MuRF1-dependent degradation of phospho-c-Jun led to our investigation the role of c-Jun in IGF-1 signaling in cardiomyocytes in the current study.

We first identified that MuRF1 inhibits total and phosphorylated c-Jun (p-S63-c-Jun and p-S73-c-Jun) expression levels in IGF-1 stimulated HL-1 cells (Figure 8A). These data are consistent with MuRF1’s inhibition Akt, GSK3β, and mTOR expression after IGF-1 stimulation (Figures 5-7) since c-Jun promotes the expression of the genes that code for all three of these proteins. Similarly, increasing MuRF1 in NRVM resulted in a decrease in phosphorylated c-Jun (S63, S73, and T91) in vehicle-treated cardiomyocytes and p-T91-c-Jun specifically in the presence of IGF-1 (Figure 9). Conversely, when MuRF1 was decreased in HL-1 cardiomyocytes total c-Jun expression was significantly increased in the presence of IGF-1 (Figure 8B). This is consistent with MuRF1’s ability to bind and poly-ubiquitinate c-Jun, targeting c-Jun it for proteasome-dependent degradation. Since c-Jun is a transcription factor that drives expression of Akt, GSK3β, and mTOR, our observation...
that Akt, GSK3β and mTOR total protein levels are inhibited by MuRF1 is suggest that MuRF1-dependent limitation of c-Jun in IGF-1-stimulated cardiomyocytes is the mechanism by which MuRF1 inhibits IGF-1-dependent cardiomyocyte growth.

**MuRF1-dependent regulation of IGF-1-dependent cardiomyocyte growth is JNK-dependent.** To further support our the hypothesis that MuRF1 inhibits IGF-1 signaling in a c-Jun-dependent manner, we next determined if inhibiting JNK, a kinase well-known to phosphorylate and activate c-Jun, blocks MuRF1’s ability to inhibit IGF-1-induced cardiomyocyte growth (Figure 10). First, we identified that the JNK inhibitor SP600125 was able to prevent the exaggerated cardiomyocyte hypertrophy stimulated by IGF-1 that is observed when MuRF1 is transiently knocked down (Figure 10). Importantly, JNK inhibitor treatment only acted to limit IGF-1-dependent growth (decreasing area by 27.1%) in cardiomyocytes with decreased MuRF1 (AdshMuRF1), having no effect on the IGF-1-dependent growth on control cells (Adshscrambled) (Figure 10). In addition, decreased MuRF1 expression (AdshMuRF1), JNK inhibitor pre-treatment, and IGF-1 treatment together were required to produce these results (F=11.146, DF=1) (Figure 10). Consistent with these data, MAPK13 expression was inhibited in cardiomyocytes treated with JNK inhibitor only in cells with decreased MuRF1 (AdshMuRF1) (Figure 11). Furthermore, IGF-1-dependent enhancement of RYR1 by knockdown of MuRF1 was lost with JNK inhibition (Figure 11), abrogating AdshMuRF1’s augmentation of RYR1 expression seen in Figure 4. Together, these data illustrate that MuRF1’s regulation of IGF-1-induced growth and Akt-specific gene expression are dependent on JNK signaling. Overall, these data illustrate a role for MuRF1 in regulating IGF-1-induced cardiac hypertrophy through its regulation of JNK/c-Jun activity. In the context with our previous studies that show that MuRF1 inhibits JNK signaling by directly interacting and ubiquitinating cardiac c-Jun to target it for proteasome-dependent degradation, the present data illustrates a role for this mechanism in IGF-1-induced cardiac hypertrophy as well.

**MuRF1 inhibits exercise-induced cardiac hypertrophy in vivo.** Since MuRF1 inhibits IGF-1 signaling in both cardiac-derived and primary cardiomyocytes in vitro, we next determined if
MuRF1 similarly inhibits IGF-1 signaling in vivo. The development of physiological cardiac hypertrophy in response to regular exercise occurs through IGF-1 signaling. So we next challenged MuRF1/-/- and MuRF1 Tg+ mice and their sibling-matched wildtype controls to voluntary unloaded wheel running for 5 weeks to understand the role of MuRF1-dependent inhibition of IGF-1 signaling in vivo. Echocardiographic analysis performed at baseline and 5 weeks of voluntary running revealed that MuRF1 -/- hearts exhibited significant increases in LV anterior and posterior wall thickness and LV mass compared to wildtype control and sham animals (Table 1, Figure 12A-B). Indicators of systolic function (EF% and FS%) did not change over the course of the experiment (Table 1). Consistent with an exaggerated cardiac hypertrophy, MuRF1-/- hearts exhibited a significant increase in LV mass/tibia length and cardiomyocyte cross-sectional area (Figure 12C-D). In contrast, the anterior and posterior wall thickness of MuRF1 Tg+ hearts did not increase in size, by echocardiographic analysis of wall thickness, after 5 weeks of voluntary running compared to matched wildtype control hearts (Table 2, Figure 13A-B). In addition, MuRF1 Tg+ cardiomyocyte cross-sectional areas exhibiting significantly less cardiomyocyte hypertrophy compared to wildtype controls (Figure 13C-D). These echocardiographic and histological analyses illustrate MuRF1’s negative regulation of physiological hypertrophy after 5 weeks of voluntary running at the level of the whole heart and cardiomyocyte.

To further analyze the type of cardiac hypertrophy the MuRF1-/- and MuRF1 Tg+ hearts were undergoing, we quantified the expression of genes up-regulated in cardiac pathological hypertrophy. Brain natriuretic peptide (BNP), atrial natriuretic peptide (ANF), and smooth muscle actin (SM actin) were unchanged by qRT-PCR in cardiac tissue from sham and running mice. Neither MuRF1-/- nor MuRF1 Tg+ hearts demonstrated significant increases in gene expression after running (Figure 12E and 13E, respectively). No inflammatory or fibrotic remodeling was identified by histological analysis of MuRF1-/- or MuRF1 Tg+ hearts (Figure 14A, 14B, respectively). Both the lack of pathological cardiac hypertrophy makers and the absence of any histological pathology
illustrate that both MuRF1-/ and MuRF1 Tg+ hearts respond to voluntary running by undergoing physiological hypertrophy, a process driven by IGF-1 mediated cell signaling in vivo.

Daily analysis of the running time, average speed, and total distance was collected for each of the groups. Interestingly, both the MuRF1-/ and MuRF1 Tg+ mice ran the same amount of time as did their strain-matched controls (Tables 3 and 4, respectively). Interestingly, the MuRF1-/ mice ran significantly farther than their matched control wildtype littermates for the entire 5-week duration of the study (Table 3). Consistent with these findings, MuRF1-/ mice ran farther for the entire 5-week duration of the study (Table 3). In contrast, MuRF1 Tg+ mice ran the same speed and distance compared to their wild type controls. Together, these data suggest that absence of MuRF1 enhances the function of the cardiovascular system due effects on cardiac and/or skeletal muscle (Table 4). Since MuRF1 Tg+ mice, where MuRF1 is specifically expressed in the heart, were not exercise inhibited, these studies suggest that skeletal muscle may be, in part, responsible for our finding that global MuRF1-/ mice have enhanced exercise performance.

Discussion

Increases in cardiac workload induce the mammalian heart to grow by a process called cardiac hypertrophy, that can be induced by either pathological or physiological stimulation. The ability of the muscle-specific ubiquitin ligase MuRF1 to inhibit pathological cardiac hypertrophy has been shown. Growth of primary NRVM with PE, AngII, ET-1, or NE (activators of signaling pathways which drive pathological hypertrophic remodeling), has been reported to be inhibited by MuRF1 by virtue of MuRF1-dependent inhibition of PKCe. In vivo, TAC, a method used to mimic the pressure overload observed during chronic hypertension, of MuRF1-/ mice resulted in exaggerated cardiac hypertrophy. Furthermore, increased activity of the transcription factor SRF, was observed with increased MuRF1 expression in cardiomyocytes, showing that MuRF1 inhibition of SRF contributes to MuRF1’s effect on cardiac growth following TAC. We have recently reported that MuRF1 and MuRF2 are functionally redundant in regulating developmental cardiac hypertrophy by regulating E2F1-mediated gene expression. These reports establish MuRF1’s
widespread inhibitory effect on pathological myocardial growth signaling on the level of both protein kinases (PKCε) and transcription factors (SRF). Due to this broad activity of MuRF1 in limiting these signal transduction cascades, we posited that MuRF1 would also inhibit the signal transduction cascades distinctly activated by physiologic cardiac stimuli.

Cardiomyocyte growth induced by physiologic stimuli, such as repetitive exercise and pregnancy, is beneficial because the changes in size and strength, enhanced vascular perfusion, and metabolism associated with cardiac hypertrophy occur without the adverse long term effects seen in pathologic hypertrophy\(^5\). Physiological stimuli trigger the release of GH that subsequently induces the production of IGF-1 in the liver. At the molecular level, elevated circulating GH and IGF-1 drive physiological cardiac hypertrophy\(^87\). Considerable effort has been made to delineate pro-hypertrophic IGF-1 signaling pathways in the myocardium. Circulating IGF-1 is the main source of IGF-1 for the cardiomyocyte. While it has been shown that cardiomyocytes themselves transiently release IGF-1 in response to exercise, this pool of IGF-1 contributes to the hypertrophy effect at a much lesser extent than circulating IGF-1\(^14-16\). At the cardiomyocyte plasma membrane, IGF-1 binds to and activates the IGF-1R and IR RTKs, which subsequently activate PI3K\(^3\). PI3K catalyze the formation of PIP3, inducing PDK1 translocation to the plasma membrane. The crucial step in IGF-1 signaling transduction is PDK1-dependent phosphorylation of Akt1, the main isoform of Akt in the heart (Akt2 is also present, but to a lesser extent) at T308\(^17,18\). This hypophosphorylated form of Akt is fully activated by phosphorylation at S473 by mTORC2\(^19\). Akt promotes protein translation by phosphorylating and inhibiting GSK3β, an inhibitor of the translation elongation eIF2Be\(^15,18,25\) and phosphorylating mTOR, promoting the formation of mTORC1 and subsequent mTORC1-dependent activation of ribosome biosynthesis and protein translation\(^19\).

In the present study, we detail, for the first time, the ability of MuRF1 to inhibit physiological cardiomyocyte hypertrophy by acting on a novel regulator of IGF-1 signaling, the transcription factor c-Jun. We show that when MuRF1’s expression is knocked-down, cardiomyocyte hypertrophy is enhanced, while increases in MuRF1 inhibited cardiomyocyte hypertrophy (Figure 3).
To date, only a few endogenous molecular inhibitors of IGF-1 signaling in cardiomyocytes have been described: phosphatase and tensin homolog (PTEN), which acts as a phosphatase for PIP3 and Akt\(^88\), and FOXO3a, which activates transcription of genes involved in cardiac atrophy, including MuRF1\(^23\); therefore inhibition of IGF-1 signaling by MuRF1 in the myocardium is a novel finding.

We expanded on MuRF1’s role in inhibiting IGF-1-dependent hypertrophy by showing that MuRF1 inhibits Akt-associated gene expression (Figure 4). If MuRF1 acts at the level of Akt or higher in the signaling pathway, we expected that MuRF1 would limit Akt phosphorylation; however, our findings suggested that Akt phosphorylation was not the main method by which MuRF1 inhibited Akt activity (Figure 5 and 6), despite the observation that phosphorylation of GSK3β, a direct target of Akt, was inhibited by MuRF1 (Figure 5 and 6). These findings are consistent with the findings of Chen et al.\(^85\), who demonstrated that increased MuRF1 expression in adult mouse cardiomyocytes did not alter basal levels of phosphorylated Akt. Instead, we observed that MuRF1 inhibited total Akt, GSK3β (Figure 5 and 6) and mTOR (Figure 7) expression in the presence of IGF-1. In addition, IGF-1-dependent MuRF1 regulation of mTOR suggested that IGF-1 switches MuRF1-dependent inhibition from mTORC1 to mTORC2 (Figure 7). Together, MuRF1’s regulation of three independent proteins involved in the IGF-1 pathway indicated that MuRF1-dependent inhibition of IGF-1-dependent hypertrophy and signaling was independent of Akt activation and likely involved transcriptional regulation.

Since a recently published study identified Akt1, GSK3β, and mTOR in a number of cardiac genes the promoter regions of which are bound and activated by c-Jun\(^22\) we hypothesized that MuRF1 achieves its limitation of Akt, GSK3β, and mTOR protein levels by inhibiting c-Jun in the presence of IGF-1. We were especially interested in c-Jun-dependent regulation of IGF-1 signaling since our laboratory recently identified MuRF1’s ability to inhibit JNK signaling through MuRF1’s direct interaction with, and degradation of, c-Jun\(^74\). In that study, we showed that, in the context of ischemia reperfusion injury, increased c-Jun degradation (by MuRF1) resulted in the inhibition of I/R-induced cell death and cardiomyocyte dysfunction\(^74\). In the present study, we found that increasing MuRF1
expression decreased total c-Jun levels when cardiomyocytes were stimulated with IGF-1; in parallel, MuRF1 reduced the amount of phosphorylated c-Jun present (S73 and T91) (Figure 8 and 9). Conversely, we found that total c-Jun protein levels were increased in the presence of IGF-1 when MuRF1 was knocked down (Figure 8B). Using a c-Jun N-terminal kinase (JNK) inhibitor, we then demonstrated that JNK (an activator of c-Jun) activity is required for the exaggerated IGF-1 mediated cardiomyocyte growth and Akt-associated gene expression observed when MuRF1 is knocked-down (Figure 10 and 11). Taken together, these studies suggest that MuRF1 limits IGF-1 cardiomyocyte hypertrophy and signaling, in part, by its inhibition of JNK signaling. This mechanism is likely through MuRF1-dependent proteasome degradation of phosphor-c-Jun, newly identified as a transcription factor responsible for transcription of multiple genes in the myocardium involved in the IGF-1 pathway. c-Jun’s importance in MuRF1-dependent regulation of IGF-1 hypertrophy is not altogether surprising since IGF-1-induced JNK activation has previously been reported in cardiomyocytes and in the maintenance of cardiac hypertrophy.

There are a growing number of ubiquitin ligases that regulate JNK signal transduction in multiple cell types by recognizing phosphorylated substrates. These ubiquitin ligases include MEKK1 (NIH3T3 cells), FBw7 (neurons), DCXhDET1-hCOP1 (U2Os, HEK293T cells), and itch (T cells). Interestingly, MEKK1, a MAPK3K in the JNK signaling pathways that phosphorylates MAP2K (one kinase that phosphorylates JNK), acts as an ubiquitin ligase that preferentially poly-ubiquitinates phospho-c-Jun and target c-Jun for proteasomal degradation. Showing that poly-ubiquitination of phospho-c-Jun specifically is induced by ubiquitin ligases other than MuRF1. While the mechanism by which MuRF1 regulates c-Jun to inhibit IGF-1-dependent cardiomyocyte growth is novel, given that ubiquitin ligase-dependent inhibition of JNK signaling is being identified in a growing number of cell types and disease processes, it is likely that the mechanism we describe here occurs more commonly than has been reported.

Exercise training induces the release of IGF-1 from the liver, leading to activation of IGF-1 signaling in the myocardium and cardiac growth with limited adverse effects on cardiac function.
an effort to understand MuRF1-dependent regulation of endogenous IGF-1 signaling in the myocardium in vivo, we submitted MuRF1−/− and MuRF1Tg+, and their littermate controls (MuRF1+/+ and Wildtype<sup>MaRF1Tg+</sup>, respectively) to voluntary wheel running exercise to increase circulating IGF-1 in these animals by natural means. We discovered that loss of MuRF1 (MuRF1−/−) caused exercise-induced cardiac hypertrophy to be exaggerated, without provoking pathological remodeling (Figure 12). Conversely, exaggerated cardiac growth in response to exercise was lost in MuRF1 Tg+ animals and blunted on the level of cardiomyocyte-specific growth (Figure 13). These data confirm that MuRF1-dependent regulation of IGF-1 signaling is applicable in whole animal models and provide additional evidence for the importance of the ubiquitin proteasome system (UPS) in the heart.

Our current study showing that MuRF1 regulates IGF-1 signaling in the myocardium in vivo is latest in the growing literature implicating the UPS in maintenance of cardiac function<sup>92</sup>,<sup>103</sup>. Mice lacking the ubiquitin ligase and protein chaperone carboxyl terminus of Hsp70-interacting protein (CHIP) exhibit an exaggerated cardiac hypertrophy in as little as 1 week and as much as 5 weeks of running, which parallels increases in increased phosphorylated Akt after IGF-1 stimulation in vitro<sup>84</sup>. While these studies suggest an inhibitory role of CHIP in Akt activation, Akt was not found to be a direct CHIP substrate<sup>103</sup>. The ubiquitin ligase MAFbx/Atrogin-1 has recently been found to regulate physiological cardiac hypertrophy induced by both IGF-1/GH injections and wheel running exercise. MAFbx/Atrogin-1 achieves this inhibition by ubiquitinating and activating of Forkhead proteins (FOXO1 and FOXO3a)<sup>52</sup>. Interestingly, another recent study identified that aging MuRF1−/− mice exhibit a spontaneous cardiac hypertrophy, lacking observable pathological remodeling, after 6 months of age, with parallel increases in phosphorylated Akt<sup>104</sup>. The mechanism underlying these spontaneous changes in the MuRF1−/− mouse hearts remains unknown, but the mechanistic relationship between MuRF1 and Akt activation was implicated in that model<sup>104</sup>. In contrast, we found that increased phosphorylated Akt was not evident over the transient knock-down periods used in the current study, we did observed increases in cardiac size in MuRF1−/− compared to MuRF1+/+
in our sedentary controls (Figure 12) and incremental, spontaneous increases in cardiomyocyte size with transient MuRF1 knockdown in vitro (Figure 3); the latter indicating how closely the cultured cells parallel in vivo findings in this study.

Regulation of cardiac growth by MuRF1 has recently been identified to contribute to cardiac disease in humans. Patients with hypertrophic cardiomyopathy were found to have 3 MuRF1 variants, including two missense and one deletion, which produce MuRF1 protein with impaired sarcomeric localization and ability to auto-ubiquitinate and ubiquitinate multiple substrates. Mechanistic studies showed that mTOR signaling is specifically altered in cardiomyocytes expressing these MuRF1 variants—confirming that the effect we observed in IGF-1-dependent regulation of mTOR signaling by MuRF1 in this study. Taken together, these emerging studies implicate the UPS and ubiquitin ligases, such as MuRF1, as critical regulators of IGF-1 signaling, with additional components likely to be found. Our current study, as well as recently published reports, illustrate the importance of studying the mechanisms by which ubiquitin ligases limit pro-hypertrophic signaling in the heart, especially for development of therapies for hypertrophic cardiomyopathy and heart failure.

**Figure Legends**

**Figure 3. Knockdown of MuRF1 enhances and increased expression of MuRF1 repression IGF-1-induced cardiomyocyte hypertrophy**

A. MuRF1 was knocked down using AdshMuRF1, with Adshscrambled as control, at MOI 60 for 24 hours in serum-free DMEM followed by treatment with 10nM IGF-1 for 18 hours. Cells were fixed and observed with a fluorescent microscope using a 40X objective lens. Shown are representative images of vehicle or IGF1-treated HL-1 cardiomyocytes transduced with either Adshscrambled or AdshMuRF1. B. Cardiomyocyte area (mm2) measurements of vehicle or IGF1-treated HL-1 cardiomyocytes transduced with either Adshscrambled or AdshMuRF1, averaged over at least 200 cardiomyocytes. Black bars represent vehicle and gray bars represent IGF1-treated cells. C. MuRF1 was increased in expression using AdMuRF1, with AdGFP as a control, at MOI 25 for 24 hours in serum-free DMEM followed by treatment with IGF-1 for 18 hours. Shown are representative fluorescent images of vehicle or IGF1-
treated HL-1 cardiomyocytes transduced with either AdGFP or AdMuRF1. D. Cardiomyocyte area (mm²) measurements of vehicle or IGF1-treated HL-1 cardiomyocytes transduced with either AdGFP or AdMuRF1. Black bars represent vehicle- and gray bars represent IGF-1-treated cells. Cardiomyocyte area measurements are represented as mean area ± SEM. E. MuRF1 was increased in expression in NRVM using AdMuRF1, with AdGFP as a control, at MOI 25 for 24 hours in serum-free M199 followed by treatment with IGF-1 for 18 hours. Shown are representative fluorescent images of vehicle or IGF1-treated cardiomyocytes transduced with either AdGFP or AdMuRF1. F. Cardiomyocyte area (mm²) measurements of vehicle or IGF1-treated NRVM transduced with either AdGFP or AdMuRF1. Black bars represent vehicle- and gray bars represent IGF-1-treated cells. Cardiomyocyte area measurements are represented as mean area ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group, **indicates significance on level of treatment group, %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as #P< 0.05, ##P< 0.001 as determined using a pairwise post-test.

**Figure 4. IGF-1-dependent expression of genes associated with Akt activity is inhibited by MuRF1.** A. AdshMuRF1 and control Adshscrambled were used at MOI 60 for 24 hours in serum-free DMEM to knockdown MuRF1 in HL-1 cardiomyocytes and transduced cells were treated with 10nM IGF-1 for 18 hours. RNA was isolated and cDNA generated for use in measuring expression of MuRF1, MAPK13, RYR1, Igfbp5, and 18S (reference gene). Shown are RTPCR data for each of these genes in vehicle or IGF-1-treated HL-1 cardiomyocytes transduced with either Adshscrambled or AdshMuRF1. B. AdMuRF1 and control AdGFP were used at MOI 25 for 24 hours in serum-free DMEM to increase MuRF1 and transduced cells were treated with 10nM IGF-1 for 18 hours. Shown are RT-PCR data for MuRF1, MAPK13, RYR1, and Igfbp5 in cardiomyocytes transduced with either AdGFP or AdMuRF1 and treated with either vehicle or IGF-1. Raw CT values from three independent experiments were normalized to their respective 18S values, averaged over experimental
group, and subsequently normalized to either to vehicle control (MAPK13, RYR1, and Igfbp5) or adenovirus control (MuRF1). Final data is represented as mean fold change ± SEM. A two-way ANOVA test was used to determine statistical significance. * indicates significance on level of adenovirus group. ** indicates significance on level of treatment group. % indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P< 0.05, ##P< 0.001 as determined using a pairwise post-test. $ indicates significance (p<0.05) between groups within the treatment group, as measured by a Student’s t-test.

**Figure 5. MuRF1 inhibits protein expression of Akt and GSK3β in IGF-1 stimulated HL-1 cardiomyocytes.** A. HL-1 cells were transduced with AdMuRF1 or control AdGFP at MOI 25 for 24 hours in serum-free DMEM to increase MuRF1 expression, followed by treatment with 10nM IGF-1 for 30 minutes. Immunoblots using whole cell lysates from three independent experiments are shown for p-Akt S473, p-Akt T308, Akt, GSK3β, and p-GSK3β S9. Primary antibody against myc was used to assess adenovirus-dependent expression of myc-MuRF1 and immunoblot for MuRF1 was done to determine endogenous protein levels. β-actin was used as a loading control. Densitometry analysis of Akt, p-Akt S473, p-Akt T308, GSK3β, and p-GSK3β S9 are shown for vehicle or IGF-1-treated cardiomyocytes transduced with either AdGFP or AdMuRF1. Total protein levels were normalized to β-actin and phospho-protein levels were normalized first to β-actin and then to total protein levels. B. HL-1 cardiomyocytes were transduced with either AdshMuRF1 or control Adshscrambled at MOI 30 for 48 hours in serum-free DMEM, followed by treatment with 10nM IGF-1 for 30 minutes. Akt and GSK3β expression and phosphorylation in whole cell lysates from three independent experiments was assessed by immunoblot using primary antibodies raised against total Akt or GSK3β and p-Akt S473, p-Akt T308, and p-GSK3β S9, as indicated. Primary antibody against MuRF1 was used to confirm knockdown. β-actin was used as a loading control. Densitometry analysis of Akt, p-Akt S473, p-Akt T308, GSK3β, and p-GSK3β S9 are shown for vehicle or IGF-1-
treated cardiomyocytes transduced with either Adshscrambled or AdshMuRF1. Densitometry analysis is represented as means ± SEM. A two-way ANOVA test was used to determine statistical significance. * indicates significance on level of adenovirus group. ** indicates significance on level of treatment group. % indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P< 0.05, ##P< 0.001 as determined using a pairwise post-test.

**Figure 6. MuRF1 inhibits protein expression of Akt and GSK3β in IGF-1 stimulated NRVM.** NRVM were transduced with AdMuRF1 or control AdGFP at MOI 25 for 24 hours in serum-free M199 to increase MuRF1 expression, followed by treatment with 10nM IGF-1 for 30 minutes. Immunoblots using whole cell lysates from three independent experiments are shown for p-Akt S473, p-Akt T308, Akt, GSK3β, and p-GSK3β S9. Primary antibody against myc was used to assess adenovirus-dependent expression of myc-MuRF1. β-actin was used as a loading control. Densitometry analysis of Akt, p-Akt S473, p-Akt T308, and p-GSK3β S9 are shown for vehicle or IGF-1-treated cardiomyocytes transduced with either AdGFP or AdMuRF1. Total protein levels were normalized to β-actin and phospho-protein levels were normalized first to β-actin and then to total protein levels. Densitometry analysis is represented as means ± SEM. A two-way ANOVA test was used to determine statistical significance. * indicates significance on level of adenovirus group ** indicates significance on level of treatment group. % indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P< 0.05, ##P< 0.001 as determined using a pair-wise post-test.

**Figure 7. MuRF1 knockdown in HL-1 cardiomyocytes induces total mTOR protein levels to increase upon IGF-1 stimulation.** HL-1 cardiomyocytes were transduced with either AdshMuRF1 or control Adshscrambled at MOI 30 for 48 hours in serum-free DMEM, followed by treatment with 10nM IGF-1 for 30 minutes. Total and phosphorylated (S2481) mTOR was measuring
by immunoblot of whole cell lysates from three independent experiments. β-actin was used as a loading control. Densitometry analysis of mTOR and p-mTOR S2481 are shown for vehicle or IGF-1-treated cardiomyocytes transduced with either Adshscrambled or AdshMuRF1. Total protein levels were normalized to β-actin and phosphor-protein levels were normalized first to β-actin and then to total protein levels. Densitometry analysis is represented as means ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group. **indicates significance on level of treatment group. %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P< 0.05, ##P< 0.001 as determined using a pairwise post-test.

Figure 8. MuRF1 inhibits HL-1 cardiomyocyte c-Jun protein levels and phosphorylation in the presence of IGF-1. A. MuRF1 expression was increased in HL-1 cells using AdMuRF1 and control AdGFP at MOI 25 for 24 hours in serum-free DMEM followed by treatment with 10nM IGF-1 for 30 minutes. c-Jun expression and phosphorylation in whole cell lysates from three independent experiments was assessed by immunoblot using primary antibodies raised against total c-Jun, p-c-Jun S63, p-c-Jun S73, and p-c-Jun T91, as indicated. Primary antibody against myc was used to confirm adenovirus-dependent expression of myc-MuRF1. β-actin was used as a loading control. Densitometry analysis of c-Jun, p-c-Jun S63, p-c-Jun S73, and p-c-Jun T91 are shown for vehicle or IGF-1-treated cardiomyocytes transduced with either AdGFP or AdMuRF1. Total protein levels were normalized to β-actin and phospho-protein levels were normalized first to β-actin and then to total protein levels. B. AdshMuRF1 and control Adshscrambled were used at MOI 30 for 48 hours in serum-free DMEM to knockdown MuRF1 in HL-1 cardiomyocytes followed by IGF-1 treatment for 30 minutes. Immunoblot using whole cell lysates from three independent experiments are shown for c-Jun, p-c-Jun S63, p-c-Jun S73, and p-c-Jun T91. MuRF1 primary antibody was used to confirm knockdown. β-actin was used as a loading control. Densitometry analysis of c-Jun, p-c-Jun S63, p-c-Jun S73, and p-c-Jun T91 are shown for vehicle or IGF-1-treated cardiomyocytes transduced.
with either Adshscrambled or AdshMuRF1. Total protein levels were normalized to β-actin and phospho-protein levels were normalized first to β-actin and then to total protein levels. Densitometry analysis is represented as means ± SEM. A two-way ANOVA test was used to determine statistical significance. * indicates significance on level of adenovirus group ** indicates significance on level of treatment group. % indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P< 0.05, ##P< 0.001 as determined using a pair-wise post-test.

**Figure 9. MuRF1 inhibits NRVM c-Jun protein levels and phosphorylation in the presence of IGF-1.** NRVM were transduced with AdMuRF1 or control AdGFP at MOI 25 for 24 hours in serum-free M199 to increase MuRF1 expression, followed by treatment with 10nM IGF-1 for 30 minutes. Immunoblots using whole cell lysates from three independent experiments are shown for c-Jun, p-c-Jun S63, p-c-Jun S73, and p-c-Jun T91. Primary antibody against myc was used to assess adenovirus-dependent expression of myc-MuRF1. β-actin was used as a loading control. Densitometry analysis of c-Jun, p-c-Jun S63, p-c-Jun S73, and p-c-Jun T91 are shown for vehicle or IGF-1-treated cardiomyocytes transduced with either AdGFP or AdMuRF1. Densitometry analysis is represented as means ± SEM. A two-way ANOVA test was used to determine statistical significance. * indicates significance on level of adenovirus group ** indicates significance on level of treatment group. % indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P< 0.05, ##P< 0.001 as determined using a pair-wise post-test.

**Figure 10. Exaggerated IGF-1-dependent cardiomyocyte growth with MuRF1 knockdown requires JNK activity A.** MuRF1 was knocked down using AdshMuRF1, with Adshscrambled as control, at MOI 60 for 24 hours in serum-free DMEM followed by pre-treatment with 10µM SP600125 (JNK inhibitor) for 30 minutes. Cardiomyocytes were subsequently treated
with 10nM IGF-1 for 18 hours. Cells were fixed and observed using with a fluorescent microscope using a 40X objective lens. Shown are representative images and quantification of cardiomyocyte area (mm2) averaged over at least 200 cells per group. Cardiomyocyte area measurements are represented as mean area ± SEM. A three-way ANOVA test was used to determine statistical significance. * indicates significance on level of adenovirus group ** indicates significance on level of pre-treatment group. % denotes significant interactions between either the adenovirus, pre-treatment, or treatment groups, as indicated. %%% indicates a significant interaction between all three groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P < 0.05, ##P < 0.001 as determined using a pairwise post-test.

**Figure 11. Exaggerated IGF-1-dependent Akt-associated gene expression with MuRF1 knockdown requires JNK activity.** AdshMuRF1 and control Adshscrambled were used at MOI 60 for 24 hours in serum-free DMEM to knockdown MuRF1 in HL-1 cardiomyocytes. Transduced cells were pre-treated with 10µM SP600125 (JNK inhibitor) for 30 minutes, followed by treatment with 10nM IGF-1 treatment for 18 hours. RNA was isolated and cDNA generated for use in measuring expression of MuRF1, MAPK13, RYR1, and 18S (reference gene). Raw CT values from three independent experiments were normalized to their respective 18S values, averaged over experimental group, and subsequently normalized to either vehicle control (MAPK13, RYR1, and Igfbp5) or adenovirus control (MuRF1). Final data is represented as mean fold change ± SEM. Shown are RT-PCR data for each of these genes in cardiomyocytes transduced with either Adshscrambled or AdshMuRF1 and treated with vehicle or both JNK inhibitor and IGF-1. A three-way ANOVA test was used to determine statistical significance. * indicates significance on level of adenovirus group ** indicates significance on level of pre-treatment group. % denotes significant interactions between either the adenovirus, pre-treatment, or treatment groups, as indicated. %%% indicates a significant interaction between all three groups. The F statistic and degrees of freedom (DF) were reported when
dependence between groups was found to be a significant source of variation. Significance between groups are represented as \#P < 0.05, \##P < 0.001 as determined using a pairwise post-test.

**Figure 12. MuRF1 regulates physiological cardiac hypertrophy induced by exercise training in MuRF1-/- mice.** MuRF1-/- and sibling-matched wildtype control mice (MuRF1 +/+ ) were randomly assigned to either sedentary or running groups, where running groups were provided with an in-cage exercise wheel for 5 weeks and sedentary groups were not. Cardiac growth and function were measured by echocardiography. **A.** Representative M-mode images, containing at least 10 waveforms, from echocardiography of sedentary and running MuRF1+/+ and MuRF1-/- **B.** LV mass measurements, as measured by echocardiography, normalized to tibia length (TL) for MuRF1+/+ and MuRF1-/- mice assigned to either sedentary or running groups were also analyzed. **C.** Perfused and fixed paraffin-embedded heart sections from sedentary and running MuRF1-/- and wild type control mice were stained with Lectin-TRITC, imaged using fluorescent microscopy to visualize cardiomyocytes, and cross-sectional area analyzed from >180 cardiomyocytes from at least three animals per group. **D.** Histological analysis of hematoxylin and eosin (HE) was also performed. **E.** RNA was isolated from whole heart tissue from sedentary and running MuRF1+/+ and MuRF1-/-, cDNA generated, and BNP, ANF, skeletal muscle actin, TATA box-binding protein (Tbp), and hypoxanthine phosphoribosyltransferase (Hprt) expression determined, where both Tbp and Hprt were used as reference genes. Raw CT values were normalized to the average Ct value over all groups within each genotype pair to calculate ΔCT values. ΔCT values for each animal were then divided by their respective reference gene correction factor (geometric mean of Tbp and Hprt ΔCT values) to obtain final expression data for target genes (BNP, ANF, and skeletal muscle actin). Three animals (n=3) were used per group. A two-way ANOVA test was used to determine statistical significance. A two-way ANOVA test was used to determine statistical significance. * indicates significance on level of genotype group. ** indicates significance on level of exercise (sedentary or running) group. % indicates a significant interaction between genotype and exercise groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to
be a significant source of variation. Significance between groups are represented as \( P < 0.05 \), \( ## P < 0.001 \) as determined using a pair-wise post-test.

**Figure 13. MuRF1 regulates physiological cardiac hypertrophy induced by exercise training in MuRF1Tg+ mice.** MuRF1 Tg+ and sibling-matched wildtype control mice (Wildtype\(^{\text{MuRF1Tg+}}\)) were randomly assigned to either sedentary or running groups, where running groups were provided with an in-cage exercise wheel for 5 weeks and sedentary groups were not. Cardiac growth and function were measured by echocardiography. 

A. Representative M-mode images, containing at least 10 waveforms, from echocardiography of sedentary and running MuRF1 Tg+ and Wildtype\(^{\text{MuRF1Tg+}}\) B. LV mass measurements, as measured by echocardiography, normalized to TL for MuRF1 Tg+ and Wildtype\(^{\text{MuRF1Tg+}}\) mice assigned to either sedentary or running groups were also analyzed. C. Perfused and fix paraffin-embedded heart sections from sedentary and running MuRF1 Tg+ and Wildtype\(^{\text{MuRF1Tg+}}\) control mice were stained with Lectin-TRITC, imaged using fluorescent microscopy to visualize cardiomyocytes, and cross-sectional area analyzed from >180 cardiomyocytes from at least three animals per and cross-sectional area analyzed from >180 cardiomyocytes from at least three animals per group. D. Histological analysis of HE was also performed. E. RNA was isolated from whole heart tissue from sedentary and running Wildtype\(^{\text{MuRF1Tg+}}\) and MuRF1Tg+ mice cDNA generated and BNP, ANF, skeletal muscle actin, Tbp, and Hprt expression determined, where both Tbp and Hprt were used as reference genes (described above).

**Figure 9. High power histological analysis of MuRF1-/-, MuRF1 Tg+, and sibling wildtype control hearts.** Top: HE staining of representative cardiac sections from A. MuRF1-/- and B. MuRF1 Tg+ mice after 5 weeks sham or running challenge. Bottom: Masson's trichrome (MT) staining of representative cardiac sections from C. MuRF1-/- and D. MuRF1 Tg+ mice after 5 weeks sham or running challenge.
CHAPTER 3: MURF1 INHIBITS CARDIAC THYROID HORMONE SIGNALING BY LOCALIZING TRα to CAP350

Introduction

TH plays a critical role in the homeostasis of the heart. Biologically active T3 regulates most of the enzyme systems involved in Ca\textsuperscript{2+} and ion fluxes in the heart. T3 regulates sarcoplasmic Ca\textsuperscript{2+} via by controlling the expression of SERCa2 and PLN\textsuperscript{105, 106}. In addition to regulating cardiomyocyte Ca\textsuperscript{2+}, T3 modulates K\textsuperscript{+} availability by regulating voltage-gated K\textsuperscript{+} channels, affecting cardiomyocyte excitation at the plasma membrane\textsuperscript{107, 108}. At the tissue level, T3 maintains myocyte alignment, ventricular geometry\textsuperscript{109} and function\textsuperscript{110}, the turnover of the extracellular matrix\textsuperscript{111}, and the progression of heart failure\textsuperscript{112, 113}. T3 has also been implicated in restoration of heart function, reducing susceptibility to heart failure after infarction\textsuperscript{114} and converting pathological cardiac hypertrophy to physiological hypertrophy under certain conditions\textsuperscript{115, 116}.

At the molecular level, T3 exerts its activity by binding to TRs, a group of NR transcription factors including TRα and TRβ\textsuperscript{117}. Activation of TRα or TRβ induces their dimerization with another NR, retinoid X receptor (RXR). Together, the NR transcription factor complex binds to thyroid response elements (TREs) and regulate gene expression\textsuperscript{118}. The regulation of TRs by post-translational modifications is just beginning to be elucidated. Recent studies in kidney-derived HEK293T cells have demonstrated that SIRT1 deacetylates TRβ, inhibiting TRβ activity by promoting ubiquitin-dependent turnover of TRβ\textsuperscript{119}. Phosphorylation of TRβ by MAPK in CV-1 cells, inhibits TRβ activity by recruiting transcriptional co-repressors\textsuperscript{120, 121}. The small ubiquitin-like modifier (SUMO) has recently been reported to regulate TH-dependent gene regulation\textsuperscript{122}; using the liver HepG2 cell line, it was shown that the SUMO ligase PIASxβ specifically SUMOylates TRα, while PIAS1, PIAS3 and PIASy all can SUMOylate TRβ\textsuperscript{122}. Poly-ubiquitination of the ligand
binding domain has been reported for TRα in CV-1 cells, where T3 treatment induces TRα poly-ubiquitination and subsequent TRα degradation by the proteasome. Degradation of the TRβ by the proteasome pathway has been reported in QBI-HEK 293A cells; however, no direct measurements of TRβ poly-ubiquitination were made in this study. While these studies show that TRs can be regulated by post-translational modifications, these modifications have not been reported in the heart. Especially absent are investigations of the molecular mechanisms by which cardiac ubiquitin ligases might regulate TRs and downstream TH activity.

In the present study, the cardiac ubiquitin ligase MuRF1 is identified as an inhibitor of T3-induced physiological cardiac hypertrophy in vivo. We identified, for the first time, the role of mono-ubiquitination in regulating TRα activity. Specifically, we show that T3-dependent mono-ubiquitination targets TRα to centosome-associated protein 350 (CAP350), a protein that has recently been identified to interact with other NR transcription factors (including PPARs (α, β/δ, γ) and the liver X receptor (LXR)) in cancer cell lines. The present study confirms the expression of CAP350 in cardiomyocytes, which has only been alluded to by microarray results available on Gene Expression Omnibus (GEO), and establishes CAP350’s involvement in a novel mechanism by which non-canonical ubiquitination regulates TRα.

**Materials and Methods**

**Cell culture, adenovirus transduction, and T3 treatment.** The cardiac-derived HL-1 cell line was maintained as previously described. Briefly, cells were grown in Claycomb media (51800C, Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% norepinephrine, and 1% L-glutamine on dishes coated with 0.02% gelatin (w/v)/0.5% fibronectin (v/v). COS-7 (CRL-1651™, ATCC®, Manassas, VA) cells were cultured according to the manufacturer’s protocols in DMEM-10 and 1% penicillin/streptomycin. Control GFP (AdGFP) or myc-tagged MuRF1/bicistronic GFP (AdMuRF1) were transduced in both HL-1 and COS-7 cells, as previously described. MuRF1 was transiently knocked down using the adenovirus shRNA-MuRF1 or control adenovirus expressing scrambled shRNA (Adshscrambled).
(Vector Biolabs, Inc., Philadelphia, PA) as previously described. Following adenovirus transduction, either HL-1 or COS-7 cells were treated with 3,3’,5-triiodo-L-thyronine (T3) (Cat.#T2877, Sigma-Aldrich, Inc., St. Louis, MO) at a final concentration of 1µM in PBS in serum-free DMEM supplemented with 1% penicillin/streptomycin.

Animals. MuRF1/- mice and α-MHC–MuRF1 (cardiac-specific) transgenic mice and strain-matched wildtype mice (MuRF1+/+ and Wildtype^\text{MuRF1Tg}, respectively) ages 8–16 weeks were used as previously described. Mice were randomly assigned to treatment with triiodothyronine (T3) or vehicle control groups (10–12 mice per group), followed by daily 1mg/kg T3 (dissolved in PBS) or PBS alone intraperitoneal injection for two weeks. Conscious echocardiography analysis was performed at baseline, one week, and two weeks. After two weeks of vehicle- or T3-treatment, mice were anesthetized to collect retro-orbital blood, then euthanized. Hearts were flash frozen and stored at -80°C. Blood was collected in Microtainer SST-Amber tubes (Cat.# 365978, BD Biosciences, San José, CA), allowed to clot at room temperature, centrifuged at 1,300xg; the remaining serum aliquoted and stored at -80°C. Randomly assigned mice were euthanized for cardiac fixation in 4% paraformaldehyde via perfusion for use in histological studies. All animal studies were approved by the institutional care and use committee (IACUC) for animal research at the University of North Carolina at Chapel Hill.

Conscious echocardiographic analysis. A Vevo 770 and 2100 ultrasound biomicroscopy system (FUJIFILM VisualSonics, Toronto, ON Canada) was used to perform echocardiography on conscious mice, at baseline, one week, and two weeks of T3 (or control) treatment, as previously described.

Histological determination of cardiomyocytes cross-sectional area. Fixed cardiac tissue was processed for paraffin-embedding, sectioned, and stained with standard HE or MT. Slides were scanned using an Aperio Scanscope (Aperio Technologies, Vista, CA) analyzed for fibrosis (MT stained), and images exported in Aperio Imagescope (Aperio Technologies). Cardiomyocyte cross-
sectional area was determined on MT-stained slides using ImageJ software on a minimum of 6 random sections per image (100 X magnification) from three different hearts per group.

**Analysis of T3 and T4 in serum.** T3 and T4 measurements from serum was completed using the Luminex MAGPIX® system (EMD Millipore, Billerica MA) utilizing the MILLIPLEX® MAP magnetic bead-based Multi-Species Steroid/Thyroid Hormone assay (Cat# STTHMAG-21K) analyzed on the MAGPIX® Multiplexing instrument. Serum from at least three animals per experimental group was used for the final analyte analysis.

**Plasmid constructs and transfection protocols.** The pGL4 luciferase mammalian expression vector with the growth hormone (GH) thyroid response element (TRE) driving luciferase expression was used as described previously. The pcDNA™3.1 (V790-20, Life Technologies Invitrogen™, Carlsbad, CA). The pcDNA™3.1 β-galactosidase vector was described previously. Mouse TRα cDNA sequence was subcloned into the p3XFLAG®-CMV-14 expression vector (Cat.#E4901, Sigma-Aldrich, Inc.) at EcoRI and BamHI. A pcDNA™3.1 (V790-20, Life Technologies Invitrogen™, Carlsbad, CA) expression vector containing DNA coding for hemagglutinin (HA)-ubiquitin, as described previously. Mutation strategies for the lysine to arginine (K to R) residue changes within specific TRα domains were devised by identifying all lysines within each domain (A/B, C, D, and E/F) and synthesizing DNA sequences replacing all lysine codons with arginine codons within a single domain with the remaining domains left intact (as shown in Figure 20). The four DNA sequences (A/B K to R, C K to R, D K to R, and E/F K to R) were then subcloned into the p3xFLAG®-CMV-14 expression vector at its EcoRI and BamHI sites.

HL-1 cells were transfected with plasmids using Lipofectamine® LTX with PLUS™ (Cat.#15338030, Life Technologies, Inc., Grand Island, NY ) and transfection experiments in Cos-7 cells were carried using the Lipofectamine®2000 reagent (Cat.#11668019, Life Technologies, Inc.), according to the manufacturer’s protocols. Plasmid DNA and Lipofectamine® reagents were diluted in OPTI-MEM® I Reduced Serum Medium (Cat.#31985070, Life Technologies, Inc.). DNA complexes were applied to cells (4 hours) and cells recovered overnight Claycomb Media
supplemented with 10% FBS, 1% pen/strep, 1% norepinephrine, and 1% L-glutamine (HL-1 cells) or DMEM-10 with 10% FBS and 1% pen/strep (Cos-7 cells). When adenoviruses were used in conjunction with transfection, cells were then transduced with adenovirus and treated with T3 (described above).

**Isolation and culturing of NRVM.** NRVMs were isolated as previously described using a commercial kit according to the manufacturer’s protocols (Cat.#LK003300, Worthington Biochemical Corporation, Lakewood, NJ)\(^1\). NRVMs were cultured in 6-well plates coated with 40µg/mL fibronectin, at a concentration of 5x10^5 cells per well in Medium 199 (M199) supplemented with 15% FBS and 0.5% penicillin/streptomycin. After 48 hours, cells were placed in M199 media. Twenty four hours later, AdGFP or AdMuRF1 (described above) were added in M199 media. Following adenovirus transduction, cells were treated with T3 in PBS in serum-free M199 (described above).

**Fluorescent microscopy and cardiomyocyte size analysis.** Optically active flexible membrane 6-well culture plates (Flexcell International, Hillsborough, NC) were used to culture either HL-1 cells or NRVM, as described previously\(^1\). Cells were transduced with AdGFP and AdMuRF1, treated with T3, fixed membranes were prepared to be mounted onto glass slides, as described previously\(^1\). A Leica DMIRB inverted fluorescence microscope (Leica Microsystems, Buffalo Grove, IL) at 400X with a Hamamatsu Orca ER camera (Bridgewater, NJ) was used. As previously described\(^1\), cell area was measured using ImageJ software. At least 200 cells in replicate wells were analyzed in each experimental group.

**Total RNA isolation from tissue and qPCR analysis of mRNA.** Ventricular tissues was homogenized using the TissueLyser LT (Cat.#85600, Qiagen, Valencia, CA), according to the manufacturer’s protocols. Briefly, two 5mm stainless steel beads (69989, Qiagen, Inc.) were used to ~5mg of apical ventricles were homogenized in 1mL of TRIzol® (Cat.#15596-026, Life Technologies Invitrogen™, Grand Island, NY). Total RNA was isolated and purified from the homogenized tissue according to the manufacturer’s protocols. cDNA was generated from 1µg RNA
using a High Capacity cDNA Archive kit (Cat.#4374967, Life Technologies Invitrogen™). One µl of
cDNA product was then amplified on a Lightcycler® 480 Sequence Detection System (Roche,
Indianapolis, IN) in 10 µl final volume using the Lightcycler® 480 Probes Master Mix
(Cat.#04707494001, Roche). The PCR reaction mix included 0.5 µl of mouse-specific 20x Taqman®
probes (Life Technologies Applied Biosystems®) for either BNP (Mm00435304_g1), ANF
(Mm01255747_g1), skeletal muscle actin (Mm00808218_g1), monocarboxylate transporter 8
(MCT8) (Mm01232724_m1), or monocarboxylate transporter 10 (MCT10) (Mm00661045_m1). Tbp
(Mm00446971_m1) was assayed as a reference gene. The ΔΔCt method was used to analyze raw CT
values. Final expression data for each target gene was calculated normalizing all groups to the sham
wildtype group. Expression data for BNP, ANF, skeletal muscle actin, MCT8, and MCT10 were
averaged over three animals per experimental group.

**TRE luciferase assay.** Cos-7 cells were co-transfected with plasmids expressing β-
galactosidase, growth hormone (GH) thyroid response element (TRE) luciferase reporter, and either
empty p3XFLAG®-CMV-14 or FLAG-TRα expression vectors. Following transfection, cells were
transduced with AdGFP or AdMuRF1, treated with either vehicle or T3 and luciferase activity was
detected using the Dual-Light® Luciferase & β-galactosidase Reporter Gene Assay Kit (T1003,
Applied Biosystems), as described previously. Assays were run in quadruplicate normalized to β-
galactosidase expression.

**Immunoblot and antibodies.** Cells were lysed in Cell Lysis Buffer (Cat.#9803S, Cell
Signaling, Danvers, MA), supplemented with protease inhibitor cocktail (Cat.#11697498001, Roche),
phosphatase inhibitor cocktail (Cat.#04906845001, Roche), and 0.2M glycerol-2-phosphate (Sigma-
Aldrich) and resolved on NuPAGE® gels (Cat.#s NP0321 and NP0301, Life Technologies Novex®).
Protein was transferred to PVDF (Cat.#IPVH00010, EMD Millipore), blocked in 2% Amersham™
ECL™ Prime Blocking Reagent (Cat.#RPN418, GE Healthcare Life Sciences, Pittsburgh, PA), and
incubated with primary antibody overnight at 4ºC. Membranes were washed, in HRP-linked
secondary antibody for one hour at RT. Amersham™ ECL™ Prime or ECL™ Select (Cat.#s
RPN2232 and RPN2235, respectively, GE Healthcare Life Sciences) was used to detect signal and final immunoblot results were acquired using Amersham™ Hyperfilm ECL™ (Cat.# 28-9068-38, GE Healthcare Life Sciences). Densitometry analysis was performed using Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA).

Primary antibodies for TRα (PA1-211A, Thermo Scientific, Inc., Rockford, IL, 1:500 dilution), FLAG-TRα (anti-FLAG Cat.# F7425, Sigma-Aldrich, 1:1000), MuRF1 (Cat.#AF5366, RD Systems, Minneapolis, MN, 1:250), β-actin and GAPDH (Cat.#s A5441 and G8795, Sigma-Aldrich, Inc., 1:4000 dilution), p-Rb (Cat.#sc-50, Santa Cruz Biotechnology, Dallas, TX), myc-tagged MuRF1 (anti-myc [Cat.#C4439, Sigma Aldrich, 1:4000] or [anti-myc-HRP-linked primary [Cat.#A5598, 1:10,000]), glutathione-S-transferase (GST)-tagged TRα (anti-GST [Cat.#G1160], Sigma Aldrich, Inc., 1:10,000), HA-ubiquitin (anti-HA [Cat.#sc-57592], 1:1000) or anti-HA-HRP-linked primary [Cat.#12013819001], Roche, 1:500) were used. HRP-linked secondary antibodies against mouse (Cat.# NA931V, GE Healthcare Life Sciences, 1:10,000), rabbit (Cat.#A9169, Sigma Aldrich, 1:20,000) and goat (Cat.#A5420, Sigma Aldrich, 1:10,000) were used.

Cytoplasmic and nuclear fractionation. Cellular fractionation was carried out using the Nuclear Extraction Kit (Cat.#AY2002) purchased from Panomics (Fremont, CA) according to the manufacturer’s protocol.

Confocal immunofluorescence and quantitative colocalization analysis. HL-1 cells grown on Flexcell plates (Cat.# BF-3001U, Flexcell International, Hillsborough, NC) were transfected with the plasmids indicated, transduced with 25 MOI AdGFP or AdMuRF1, treated with vehicle or 1µM T3 for two hours, then fixed in 4% formaldehyde in PBS, cut into 1 x 1 cm sections and blocked in 0.4% TritonX100/5% FBS. Membranes were exposed to primary antibody overnight and signal was detected using the appropriate AlexaFluor® secondary antibodies. After washing, membranes were mounted onto glass slides using a Fluoro-Gel II Mounting Media containing DAPI (17985-50, Electron Microscopy Services, Hatfield, PA). Samples were observed via confocal microscopy using the FV1000 MPE SIM Laser Scanning Confocal Microscope (Olympus, Center
Valley, PA) and the 60X objective lens. Final images were taken at 2X magnification. FLAG-TRα was detected using either a rabbit anti-FLAG antibody (F7425) or mouse anti-FLAG antibody (F1804), both purchased from Sigma-Aldrich, at 1:100 dilution. myc-MuRF1 was detected using either a rabbit anti-myc (C3956) or mouse anti-myc (C4439), both purchased from Sigma-Aldrich, at 1:100 dilution. Endogenous CAP350 was detected using an anti-CAP350 antibody (sc-161481) purchased from Santa Cruz Biotechnology at 1:50 dilution. Secondary AlexaFluor® anti-goat 488 (Cat.#11055), anti-rabbit 568 (Cat.#11011), and anti-mouse 568 (Cat.#110040) antibodies from Life Technologies Molecular Probes® were used as were anti-rabbit 647 (Cat.#A21443) and anti-mouse 647 (Cat.#A21463) from Life Technologies Invitrogen™. Colocalization analysis was performed by determining the coefficient values representing the degree in which two different fluorescence channels (450, 488, 568, or 647) overlap in isolated regions of the cardiomyocyte using Volocity® 3D Images Software Quantitation (PerkinElmer, Waltham, MA), as described previously by other groups. Measurements were made using the Costes’ Pearson’s Coefficient correlation statistics function provided within the software which uses the threshold values for each channel to adjust the final coefficient data.

Coimmunoprecipitation. Cos-7 cells were transduced with either AdGFP or AdMuRF1 and subsequently treated with either vehicle or T3, lysed (50 mM Tris, pH 7.5, 30mM NaCl, 1mM EDTA, 0.5% (v/v) Triton X100) supplemented with 2x protease inhibitor cocktail (Roche) and 1x phosphatase inhibitor cocktail (Roche). Lysates were precleared with 5 µg rabbit IgG (I5006, Sigma-Aldrich) and 2% (v/v) Protein A/G PLUS Agarose beads (sc-2003, Santa Cruz Biotechnology) per 1 mg of protein lysate for one hour at 4ºC with rotation. Myc-MuRF1 was immunoprecipitated using 5% (v/v) EZ View™ Red Anti-c-Myc Affinity Gel (E6654, Sigma-Aldrich) by incubating precleared lysates with equilibrated anti-c-myc beads overnight at 4ºC with rotation. Beads were then washed seven times with the lysis buffer (12.5x bead volume) and eluted with 20 µg myc peptide (M2435, Sigma-Aldrich). Immunoprecipitated proteins were analyzed by immunoblot.
**In vivo ubiquitination assay.** Cos-7 cells were transfected with either empty p3XFLAG®-CMV-14 or FLAG-TRα and subsequently transduced with either AdGFP or AdMuRF1. Cells were lysed using 50mM Tris, pH 7.5, 30mM NaCl, 1mM EDTA, 0.5% (v/v) Triton X100 supplemented with 2x protease inhibitor cocktail (Roche) and 1x phosphatase inhibitor cocktail (Roche). Prior to immunoprecipitation, 1mg of protein lysates was precleared using 5µg mouse IgG (Cat.#I5381, Sigma-Aldrich) and 2% (v/v) Protein A/G PLUS Agarose beads (Cat.#sc-2003, Santa Cruz Biotechnology) for one hour at 4ºC with rotation. FLAG-TRα was immunoprecipitated using 5% (v/v) EZ View™ Red Anti-FLAG Affinity Gel (Cat.#F2426, Sigma-Aldrich). Precleared lysates were then incubated with equilibrated anti-c-myc beads overnight at 4ºC with rotation. Beads were then washed seven times with the lysis buffer (12.5x bead volume) and eluted using 20µg FLAG peptide (Cat.#F3290, Sigma-Aldrich). Immunoprecipitated proteins were analyzed by immunoblot.

**Statistics.** SigmaPlot software (Ver. 11, Systat Software, Inc., Chicago, IL) was used for all statistical analyses. When data included two independent variables, a two-way ANOVA test was used to determine the source of variation. Interactions between the two variables were reported when significant (p<0.05) and the F-statistic (regression variable from the dependent variable mean/residual regression variation) and degrees of freedom (DF) were reported. A Holm-Sidak post-test method using the all-pairwise procedure was used to determine differences between specific groups. For *in vivo* studies, the two variables were defined as genotype and treatment assignment group (vehicle or T3). For *in vitro* studies, the two variables were defined as adenovirus/plasmid expression and treatment. Since only one independent variable was present in the quantitative coimmunofluorescence experiments using TRα K to R domain mutants, a one-way ANOVA test was used to determine significance with an all pairwise post-test to determine differences between specific groups.

For *in vitro* cardiomyocyte area experiments, n-values were defined as the total number of measured cells from at least two separate slides from three independent wells where at least 200 cardiomyocytes in total were measured per treatment group. For *in vivo* cardiomyocyte area experiments, n-values were defined as the total number of measured cells in three random fields from
four separate 100X images, at least four image per animal, where there were three animals per experimental group. A minimum of 500 cardiomyocytes in total over all three animals per experimental group were measured. For luciferase assays, n-values were defined as a single well in three independent experiments. For quantitative coimmunofluorescence experiments, n-values were defined as the total number of measured cells from at least two separate slides from three independent wells where at least 15 cardiomyocytes in total were measured per treatment group. For *in vitro* immunoblot experiments, each treatment group is represented in three lanes, each lane from one independent experiment and n=1 was defined as one lane. Significance level for all statistical analysis was set at *p* < 0.05.

**Results**

**MuRF1 inhibits T3-dependent cardiac hypertrophy.** Previous studies have indicated that MuRF1 regulates transcription factors in the cardiomyocytes, including SRF and the nuclear receptor PPARα⁷⁰-⁷³, critical for the adaptions needed for cardiac hypertrophy/pathological heart failure and fatty acid metabolism, respectively. Moreover, we have identified that MuRF1 inhibits processes in physiological hypertrophy¹²⁶, thereby illustrating MuRF1’s regulation of both pathological and physiological hypertrophy *in vivo*. In the present study, we hypothesized that MuRF1 regulates T3-induced physiological hypertrophy *in vivo* by acting on the primary TR in cardiomyocytes, the TRα³⁴. To determine MuRF1’s role in regulating cardiomyocytes, we stimulated the HL-1 cardiac-derived cell line and NRVMs with 1μM triiodothyronine (T3). T3 uniformly increased the size of both cell types (**Figure 15 A-C**); knock-down of MuRF1 by 50% resulted in an exaggerated increased in cardiomyocyte area in HL-1 cells (**Figure 15A**). Increasing MuRF1 expression in HL-1 and NRCM inhibited T3-induced cardiomyocyte hypertrophy, preventing the growth response (**Figure 15B and C**).

With experimental evidence for MuRF1’s regulation of cardiomyocyte hypertrophy *in vitro*, we next tested the hypothesis that MuRF1 regulates cardiomyocyte hypertrophy *in vivo*. We
challenged the recently described MuRF1−/−, 73, 75, 81 and cardiomyocyte-specific MuRF1 Tg+ mice68, 74 with daily intraperitoneal injections of 1 mg/kg T3 (or PBS vehicle alone) for 14 days and followed the cardiac phenotype by conscious echocardiography and histology. After 2 weeks, T3 treatment induced significant growth in MuRF1+/+ hearts compared to sham controls (Figure 16A). MuRF1−/− hearts were found to exhibit an exaggerated heart weight, significantly larger than that of T3-treated MuRF1+/+ hearts (Figure 16A). In contrast, the MuRF1 Tg+ mice had significantly attenuated T3-dependent increases in heart weight (36.0%) compared to sibling-matched wildtype controls (47.9%) (Figure 16B). Consistent with these findings, T3 treatment of wildtype mice significantly increased both anterior and posterior wall thickness, as measured by echocardiography, versus sham-treated mice (detailed echocardiographic analysis in Tables 5-8). Histological analysis of both MuRF1−/− and MuRF1 Tg+ perfused hearts confirmed these differences in size (Figure 16C), while detailed analysis of cross-sectional areas demonstrated that MuRF1−/− cardiomyocytes exhibited an exaggerated hypertrophy (Figure 16D) while parallel studies of MuRF1 Tg+ cardiomyocytes demonstrated inhibition of cardiomyocyte hypertrophy in response to T3 treatment (Figure 16E). QPCR analysis of genes associated with pathological cardiac hypertrophy (BNP, ANF, and skeletal muscle actin) illustrated that challenging MuRF1−/− and MuRF1 Tg+ mice with T3 did not result in pathological hypertrophy (Figure 17A and B).

Since the T3-induced hypertrophy response is dose dependent, we measured circulating T3 and T4 levels in serum at the time of harvest to determine if the significant changes in cardiac hypertrophy seen in our mouse models were due to differences in T3 and T4. Circulating levels of T3 was significantly increased in T3-treated MuRF1 Tg+, but were unchanged in MuRF1−/− (Figure 18A and B). Consistent with these finding, T4 was also increased in MuRF1 Tg+ serum, while being decreased in MuRF1−/− (sham-treated animals) (Figure 18A and B). These data show that the exaggerated hypertrophic response to T3 in MuRF1−/− mice was not merely a result of increased circulating T3/T4. In addition, these data also suggested to us that T3/T4 tissue uptake is impaired in
MuRF1 Tg+ animals. While it was previously believed that T3/T4 freely diffuse across the plasma membrane, more recent reports have shown that plasma membrane transporters are required for the movement of T3/T4 into the cell from the circulation\textsuperscript{36, 130}. The most well-studied transporters responsible for the passage of T3/T4 across the plasma membrane include two members of the monocarboxylate transporter (MCT) family, MCT8 and MCT10\textsuperscript{36}. Recent studies have shown that MCT8 and 10 expression in the heart are transcriptionally regulated by T3\textsuperscript{38}. Consequently, we measured mRNA expression of MCT8 and MCT10 by qPCR in the hearts of sham- and T3-treated MuRF1-/− and MuRF1 Tg+ mice and their respective wildtype controls. Significant differences in expression were only observed for MCT10; however, this result was not altogether surprising since T3-dependent regulation of MCT10 is more robust than MCT8 in the heart\textsuperscript{38}. MuRF1-/− animals had increased MCT10 expression approaching significance (p=0.052) (Figure 19A) and T3-dependent expression of MCT10 was inhibited in MuRF1 Tg+ animals (Figure 19B). These data provide an explanation for the differences observed for serum T3/T4 (Figure 18A and B) in these animals: decreased T4 in MuRF1-/− is a result of increased movement of TH into cardiomyocytes via MCT10 and, conversely, increased T3/T4 in MuRF1 Tg+ is a result of inhibition of TH movement into cardiomyocytes because of decreased expression MCT10. Since MCT10 is a T3-dependent gene in the cardiomyocyte\textsuperscript{38}, these data illustrate that MuRF1’s inhibition of cardiomyocyte hypertrophy in vivo is a result of MuRF1’s regulation of T3/T4 molecular signaling, most likely via transcriptional pathways.

\textbf{MuRF1 inhibits TRα transcriptional activity without affecting TRα total protein levels.}

While thyroid hormone signals through TRα and TRβ receptors, recent studies have demonstrated that TRα is specifically responsible for the effects of TH in the heart. Mice lacking TRα exhibit the contractile abnormalities associated with thyroid hormone depletion, while mice lacking TRβ do not\textsuperscript{131, 132}. These in vivo studies reflect the contrary functions of TRα and TRβ at the molecular level: TRβ inhibits TH signaling via recruitment of transcriptional co-repressors\textsuperscript{133}, while TRα, the predominant cardiac TR representing 70% of TR at the mRNA and protein level, stimulates TH
We therefore focused our studies on how MuRF1 regulates TRα. First, we measured MuRF1’s effect on TRα transcriptional activity using luciferase reporter assays. In the presence of 1μM T3, increasing TRα expression in the presence of T3 increased TRE-driven luciferase activity >6 fold (Figure 20A). Increasing MuRF1 expression significantly inhibited TRα activity (Figure 20A), indicating that MuRF1’s inhibition of thyroid hormone activity occurs at the level of TRα’s transcriptional activity. As a ubiquitin ligase, MuRF1’s regulation of transcriptional activity has been associated with both proteasome-mediated degradation (e.g. c-Jun)\textsuperscript{74} and non-degradation-associated inhibition (e.g. SRF, PPARα)\textsuperscript{70-73}. We next determined how MuRF1 expression affects steady state levels of TRα in cardiomyocytes. Knocking down endogenous MuRF1 expression by shMuRF1 did not affect endogenous TRα levels (Figure 20B); similarly, increasing MuRF1 in cardiomyocytes also did not affect endogenous TRα levels (Figure 20C). These studies suggest that MuRF1 inhibits T3-dependent cardiac hypertrophy by inhibiting TRα transcriptional activity but without affecting the TRα protein levels; indicating that MuRF1’s inhibition of T3-mediated signaling in the cardiomyocyte occurs through mechanisms independent of proteasome-mediated degradation of TRα.

**MuRF1 causes TRα to accumulate in the nucleus.** To provide more detail on MuRF1-dependent regulation of TRα activity, we performed immunofluorescence confocal analysis of TRα to determine how MuRF1 affects TRα localization in the cardiomyocyte. HL-1 cardiomyocytes were transfected with a plasmid expressing TRα (for empty vector see Figure 22) and transduced with AdMuRF1 or empty control adenovirus, with and without T3. Cells expressing TRα (transduced with control adenovirus) had significantly more TRα localized to the nucleus in the presence of T3 (Figure 21A, column 2 vs. column 1). These data are consistent with previously published reports in *Xenopus* oocytes showing that T3 induces TRα nuclear retention\textsuperscript{134}. Unexpectedly, increasing MuRF1 expression increased TRα localization to the nucleus (Figure 21A, column 3 vs column 1), despite our data showing MuRF1’s inhibitory effect on TRα (Figure 20A). MuRF1 co-localized with TRα in the nucleus (Figure 21B), which was significantly enhanced in the presence of T3 (Figure 21B, column 4 vs column 3). Compared to secondary antibody controls (Figure 21C), these studies
identified that MuRF1 both increased TRα levels in the nucleus and co-localized with TRα in a distribution around the periphery of the nucleus.

Since it was unexpected that MuRF1 increases nuclear localization of TRα, while decreasing TRα activity, we sought to quantify this relationship using a second method. Immunoblot analysis was performed to determine if MuRF1 affected the localization of endogenous TRα in nuclear- and cytoplasmic-enriched fractions of cardiomyocytes transduced with adenovirus driving MuRF1 expression (AdMuRF1). In the presence of T3, we found that MuRF1 significantly increases the levels of endogenous TRα in the nucleus (Figure 23). Increasing MuRF1 expression in the presence of T3 does not significantly alter the cytoplasmic localization for the cytoplasm compared to vehicle controls (Figure 23). These studies confirm our unanticipated finding that increasing cardiomyocyte MuRF1 expression enhances endogenous TRα localization to the nucleus, despite simultaneous MuRF1-dependent inhibition of T3-driven TRα activity.

**MuRF1 interacts with and mono-ubiquitinates TRα.** To determine if MuRF1 regulates TRα activity by interacting with TRα, we performed immunoprecipitation assays. In cells transduced with AdMuRF1, we immunoprecipitated MuRF1 and found that it bound to endogenous TRα specifically in the presence of T3 (Figure 24). To identify how MuRF1 posttranslationally modifies TRα, we performed in vivo ubiquitination assays in COS-7 cells. Using exogenously transfected HA-tagged ubiquitin, we identified that immunoprecipitated TRα exhibited both poly-ubiquitination and mono-ubiquitination (Figure 25). A mono-ubiquitinated species (~52kDa, see asterisk) of TRα was present with increased MuRF1 (which was absent in adenovirus control), while poly-ubiquitinated TRα appeared to be unaffected by MuRF1 (Figure 25A). Additional studies revealed that the background poly-ubiquitination of immunoprecipitated TRα was actually inhibited in the presence of increased MuRF1 expression (Figure 25B). Together, these data show that MuRF1 increases TRα mono-ubiquitination while concomitantly decreasing TRα poly-ubiquitination, suggesting that mono-ubiquitination of TRα by MuRF1 competes with poly-ubiquitination of TRα by another, yet to be identified, ubiquitin ligase(s) and possibly for the same residue (lysine) to modify with ubiquitin.
MuRF1 promotes the interaction between TRα and CAP350. We next searched for further mechanisms that have been implicated in inhibiting NR activities and share the characteristics of enhancing nuclear localization. To our surprise, we found a study from 2005 that identified that centrosome-associated protein 350 (CAP350) is a negative regulator of PPARα in NIH3T3 cancer cells\textsuperscript{125}. This study found that increasing CAP350 expression caused PPARα to localize to a discrete peri-nuclear region, where CAP350 also co-localized with PPARα\textsuperscript{125}. The authors went on to show that PPARα’s capture within the nucleus by CAP350 prevents PPARα transcriptional activity\textsuperscript{125}. CAP350 has neither been identified nor implicated in the regulation of nuclear receptors in cardiomyocytes, but based on our data showing that MuRF1 co-localizes with TRα in the peri-nuclear region of the cardiomyocyte, we hypothesized that CAP350 is involved in TRα’s accumulation to this region. To test this hypothesis, we performed immunofluorescence confocal analysis of endogenous CAP350 and TRα in cardiomyocytes (Figure 26). We found the increasing MuRF1 expression significantly increased the co-localization of CAP350 and TRα in cardiomyocytes (Figure 26A, right two columns vs. left two columns). In addition, the co-localization of CAP350 and TRα (Figure 26A, yellow, right-most column) had a distinctly perinuclear distribution and appeared to have extranuclear connections just adjacent to the nucleus.

CAP350 is known to interact with intermediate filaments\textsuperscript{125, 135-137} and has a role in microtubule stability in association with the Golgi complex\textsuperscript{135-137}. Therefore, we were interested to see if co-localization of TRα and CAP350 at the nuclear membrane involved any cytoskeletal proteins in that region of the cardiomyocyte. Two intermediate filament proteins localized to the nuclear membrane in cardiomyocytes are desmin and laminβ1, which are both imperative for contractile function\textsuperscript{138}. To determine if MuRF1 promotes the interaction of TRα and either of these intermediate filament proteins, we performed immunofluorescence confocal analysis of TRα and endogenous desmin or laminβ1. Indeed, we found that MuRF1 promotes the interaction between TRα and desmin at the nuclear membrane, specifically in the presence of T3 (Figure 27A, column 4 vs. column 2 and 3). Compared to secondary antibody controls (Figure 27B), these data suggest both that the MuRF1-
dependent complex of TRα and CAP350 includes desmin and that this complex is localized on the outer face of the nuclear membrane of cardiomyocytes (where TRα does not have access to DNA) since desmin is a cytoplasmic intermediate filament protein. Conversely, MuRF1 inhibits the T3-dependent interaction of TRα and laminβ1 at the nuclear membrane (Figure 28A, column 2 vs. column 4), compared to secondary antibody controls (Figure 28B). A recent study has shown that lamins, by virtue of their presence directly within the nuclear membrane bilayer and interaction with chromatin, promote the activity of the mechanosensitive transcription factor megakaryoblastic leukemia 1 (MLK1) (a transcriptional co-activator for SRF) in the heart. Interestingly, interaction between TRα and laminβ1 was increased in T3-treated cells transduced with AdGFP (Figure 28A, column 1 vs. column)—suggesting that this newfound lamin-dependent mechanism of promoting transcriptional activity could apply to TRα’s interaction with laminβ1. Since increasing MuRF1 expression interrupts the interaction between TRα and laminβ1, these data could explain how MuRF1 inhibits TRα-dependent transcription. Taken together, these studies identify for the first time that CAP350 is expressed in the heart and is localized adjacent to the connecting intermediate filaments (desmin) peripheral to the nucleus and suggest that CAP350 interacts specifically with mono-ubiquitinated TRα.

As an ubiquitin ligase, MuRF1’s activity has been found to depend on its post-translational modification of protein substrates. We identified that MuRF1 mono-ubiquitinates endogenous TRα in vivo (Figure 26A), while promoting the co-localization of TRα and CAP350 in the nucleus (Figure 26) and inhibiting TRα activity (Figure 20). We next sought to determine the specific regions of TRα that are important for MuRF1’s regulation of TRα nuclear activity in cardiomyocytes. Since MuRF1 places ubiquitin on lysine residues of substrates, as do all ubiquitin ligases, full-length TRα constructs with lysines mutated to arginine (K to R) in each of the four functional domains (A/B, C, D, E/F) were created to prevent ubiquitination of TRα in those individual regions (amino acid sequences of these mutants shown in Figure 29). We transfected cardiomyocytes with these each of these mutants, increased MuRF1, treated all groups with T3 (since these are the experimental
conditions under which TRα nuclear accumulation and interaction with CAP350 was most robust (Figure 21 and Figure 25) and measured TRα nuclear localization using immunofluorescence confocal analysis (Figure 30). Like cells transfected with the full-length wild type TRα, cardiomyocytes expressing A/B (N-terminal), C (DNA Binding domain), and D (Hinge region) domain lysine mutants did not differ in their nuclear localization (Figure 30A). In contrast, the nuclear localization of the TRα E/F domain lysine mutant was significantly decreased (Figure 30A). This result was not merely due to MuRF1’s lack of binding to the E/F mutant, since the mutant co-localized with MuRF1 to the same degree as wildtype TRα, despite the dispersion of MuRF1 and the TRα E/F mutant throughout the nucleus (Figure 30B). These data indicate that MuRF1’s activity in relation to TRα is dependent upon ubiquitination of lysines in the E/F (ligand binding) domain of TRα (Figure 30A). Our finding was not altogether surprising since the E/F region/ligand binding domain of TRα has previously been shown to be poly-ubiquitinated123 and SUMOylated122. These data are also consistent with our data showing that MuRF1 inhibits the poly-ubiquitination of TRα (Figure 25B)—since the lysine responsible for MuRF1’s regulation of TRα is within the same domain that is known to be poly-ubiquitinated123. Co-localization of CAP350 with the E/F mutant in the nucleus was significantly decreased compared with wildtype TRα (Figure 30C). Furthermore, CAP350 staining was diffuse throughout the nucleus, instead of localized to the nuclear membrane, in the presence of the TRα E/F domain lysine mutant (Figure 30C). These data suggest the lysines within the E/F region of TRα are important for the interaction between TRα and CAP350; by extension, these results also suggest that mono-ubiquitination of TRα at a lysine residue within its E/F region by MuRF1 promotes the binding of TRα and CAP350. Furthermore, intra-nuclear localization of both CAP350 and MuRF1 is impacted by the expression of the TRα E/F domain lysine mutant, in that they were dispersed throughout the nucleus rather than being localized to the nuclear membrane; therefore these data suggest that intra-nuclear trafficking of CAP350 and MuRF1 is dependent upon ubiquitination of TRα by MuRF1.
Discussion

Increasing attention has recently been given to the role of TH in cardiac disease—focusing specifically on the beneficial effects of TH on patients with heart failure. Specifically, TH is known to enhance contractile and relaxation properties of the heart, while preserving cardiac structure and performance under basal conditions and in response to injury. Consequently, negative regulation of TH signaling, even at low levels, is associated with worsening prognosis for heart failure patients\textsuperscript{112}. While the study of the systemic importance of TH has been ongoing, work on the specific regulation of TH signaling at the molecular level is just beginning. Importantly, these new mechanistic studies may offer us a more precise picture of how to regulate the beneficial effects of TH on the myocardium, especially in heart failure. For example, recent studies have identified that TR\textalpha{} is down-regulated in the heart post-ischemia—potentially causing cardiac-specific decreases in the efficacy of TH, termed “tissue hypothyroidism”\textsuperscript{142,143}. Like TH deficiency at the systemic level, cardiac “tissue hypothyroidism” can potentially result in decreased contractility (by decreasing the expression levels of SERCa2 and PLN) and alterations in MHC isoform expression\textsuperscript{34}.

In the current study we show that the striated muscle-specific ubiquitin ligase MuRF1 inhibits T3-dependent cardiac hypertrophy \textit{in vivo} (Figure 16)—implicating MuRF1 as a master regulator of TH signaling in the cardiomyocyte. We also found that MuRF1 causes TH accumulate in serum (Figure 18A and B) limiting the access of the heart to TH. Indicating that MuRF1, despite being expressed in skeletal and cardiac muscles (and at low levels in smooth muscle), can affect systemic circulation. Furthermore, we found that MuRF1 inhibits the expression of MCT10, a well-established plasma membrane TH transporter, in cardiac tissue (Figure 19A and B). Showing that MuRF1 limits the ability of TH to enter the cardiomyocyte and consequently, TH-dependent signaling in the myocardium. Recent studies have shown that MCT10 gene expression is regulated by T3 in the myocardium\textsuperscript{38}, therefore we hypothesized that MuRF1 enacts it limitation of T3-dependent physiologic cardiac hypertrophy by limiting TH-dependent transcription.
It has been recognized for some time that regulation of cardiac function by TH occurs predominantly through transcriptional activation via TRs. The activity of TRα, especially, is most frequently studied in the heart since it is the main form of TR that is positively regulated by T3 in cardiomyocytes. In the presence of hormone, TRα binds to TRE DNA sequences in gene promoter sequences (as a monomer, homodimers, or heterodimers with RXR or TRβ) and acts to enhance gene expression. In the absence of T3, TRα forms a complex with co-repressor proteins.

How post-translational modification regulates TRα, especially via the ubiquitin proteasome system (UPS), is just beginning to be understood. Poly-ubiquitination of TRα at its ligand binding domain has been shown in kidney fibroblasts. Indirect regulation of TRα activity by the UPS has also been reported. Recent studies have shown that the ubiquitin ligase mSiah2 positively regulates the process of T3-driven adipogenesis in PV cells. This study found that mSiah2 enhanced T3 activity by targeting the transcriptional co-repressor NCoR for degradation, releasing NCoR’s inhibition of TRα and resulting in enhanced TRα transcriptional activity. TRα is SUMOylated (closely associated with ubiquitination) at lysines 283 and 389, both of which are found in the ligand binding/E/F domain, by PIASxβ in HepG2 (liver) cells. These investigators did show that SUMOylated TRα is present in cardiac tissue, but the impact of SUMOylation on TRα (inhibition) was only shown in HepG2 cells. Together, these studies show that ubiquitination and SUMOylation regulate TRα activity, but the impact of these post-translational mechanisms has yet to be understood in the heart. By understanding the role of post-translational regulation of TRα, especially in beneficial TH-induced physiological cardiac hypertrophy, novel ways to potentiate cardiac function at the molecular level may be identified.

In the present study, we show that TRα is mono-ubiquitinated by MuRF1, a striated muscle specific ubiquitin ligase (Figure 25A). We also show that mono-ubiquitination of TRα by MuRF1 inhibits T3-dependent TRα transcriptional activity (Figure 20A), suggesting that T3-induced conformational changes in TRα may be necessary for MuRF1 to gain access to and ubiquitinate TRα. MuRF1 also causes TRα to accumulate in the nucleus of cardiomyocytes (Figure 23A and Figure
Specifically, MuRF1-dependent localization of TRα to the nucleus in the presence of T3 relies on the lysines located in the TRα E/F domain (Figure 30A). Interestingly, the TRα lysines previously shown to be ubiquitinated and SUMOylated are also located to the E/F domain\textsuperscript{122,123}, therefore it is possible that other ligases compete with MuRF1 for the specific lysines in this region. This competition may explain our findings that increasing MuRF1 expression enhances TRα mono-
ubiquitination while at the same time decreases TRα poly-
ubiquitination (Figure 25A and B).

We also extend our knowledge of TH signaling in the cardiomyocyte by illustrating, for the first time, that TRα is targeted to CAP350 in a T3-dependment manner (Figure 25 and Figure 29C). CAP350 has been identified to be expressed in cardiomyocytes in GEO. Our query made in January 2014 for “CEP350 cardiomyocytes” yielded 24 results. The studies found in the show that octanoic acid (GEO Accession #GDS3648) and mechanical stretch (GEO Accession #GDS3117) increase expression of CAP350 in cardiomyocytes. Conversely, polyunsaturated fatty acids (GEO Accession #GDS2607) decrease expression of CAP350 in cardiomyocytes. To our knowledge our current study is the first to determine the effect of CAP350 specifically on cardiomyocyte function. CAP350’s link to NRs, however, has already been established. A previous study identified that CAP350 interacts with and inhibits PPARα activity by localizing it to the perinuclear region of NIH3T3 cells\textsuperscript{125}. Parallel studies by this group showed that TRα localization was not altered by co-expression of CAP350, but the investigators did not assay the effect of T3 on this interaction\textsuperscript{125}. The latter is a critical point, because in our current study the enhanced TRα-CAP350 perinuclear localization was observed only in the presence of T3 (Figure 25).

MuRF1-dependent regulation of has cardiomyocyte transcription factors in cardiomyocytes has become a specific mechanistic pattern by which MuRF1 effect cardiac function. MuRF1 inhibits the SRF to attenuate pressure overload-induced cardiac hypertrophy \textsuperscript{73}. Like the current study, MuRF1 inhibits SRF activity without degrading SRF \textsuperscript{73}. In contrast, MuRF1 poly-ubiquitinates and degrades phospho-c-Jun following ischemia reperfusion injury, thereby inhibiting AP-1-dependent transcription downstream apoptotic cell death\textsuperscript{74}. Recent studies from our laboratory have identified
that MuRF1 regulates other NRs transcription factors in addition to TRα. MuRF1 mono-ubiquitinates PPARα, targeting PPARα nuclear export by altering PPARα’s ability to interact with CRM1 (main mediator of nuclear export machinery)70-72. While these studies and the current study were carried out under dynamically different conditions, MuRF1 appears to interact and regulate multiple transcription factors via ubiquitination, albeit by different mechanisms. Taken together, these investigations provide evidence for the universality of MuRF1-dependent ubiquitination as a regulator of transcription factor complexes in the cardiomyocyte. In the current context, the present studies may elucidate the first steps in determining how the UPS (via MuRF1) can be harnessed to enhance TH’s beneficial effect on the heart during health and disease states.

**Figure Legends**

**Figure 15.** MuRF1 inhibits T3-induced cardiomyocyte hypertrophy *in vitro and in vivo.*

A. MuRF1 was knocked down in HL-1 cardiomyocytes using AdshMuRF1, with Adshscrambled as control, at MOI 60 for 24 hours in serum-free DMEM followed by treatment with 1µM T3 for 18 hours. Shown are representative fluorescent images and area analysis for vehicle- and T3-treated cardiomyocytes transduced with either Adshscrambled or AdshMuRF1. B. MuRF1 was increased in expression in HL-1 cardiomyocytes using AdMuRF1, with AdGFP as a control, at MOI 25 for 24 hours in serum-free DMEM followed by treatment with 1µM T3 for 18 hours. Shown are representative fluorescent images and area analysis of vehicle- or T3-treated HL-1 cardiomyocytes transduced with either AdGFP or AdMuRF1. C. MuRF1 was increased in expression in NRVM using AdMuRF1, with AdGFP as a control, at MOI 25 for 24 hours in serum-free M199 followed by treatment with 1µM T3 for 18 hours. Shown are representative fluorescent images and area analysis of vehicle- or T3-treated NRVM transduced with either AdGFP or AdMuRF1. Data are represented as mean ± SEM. Black bars represent vehicle and gray bars represent T3-treated cells or animals. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus or genotype group, **indicates significance on level of treatment group. %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of
freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as \#P< 0.05, \##P< 0.001, as determined using a pairwise post-test.

**Figure 16. MuRF1 inhibits T3-induced cardiomyocyte hypertrophy in vivo.** MuRF1-/-, MuRF1 Tg+, and sibling-matched wildtype control mice (MuRF1 +/-, Wildtype$^{\text{MuRF1Tg+}}$) were randomly assigned to either vehicle or T3 groups, where T3 groups were injected with 1mg/kg T3 and vehicle groups were injected with PBS intraperitoneally each day for 14 days. **A.** Heart weight (HW) measurements normalized to body weight (BW) for MuRF1+/+ and MuRF1-/- vehicle and T3 groups. **B.** Heart weight (HW) measurements normalized to body weight (BW) for Wildtype$^{\text{MuRF1Tg+}}$ and MuRF1 Tg+ vehicle and T3 groups. **C.** Representative images of histological analysis of perfused and fixed heart sections stained with hemotoxylin and eosin (HE) from T3-treated MuRF1+/+, MuRF1 -/-, Wildtype$^{\text{MuRF1Tg+}}$, and MuRF1 Tg+. **D.** Representative 100x images of histological analysis of perfused and fixed heart sections stained with Masson’s Trichrome (MT) from vehicle- and T3-treated MuRF1+/+ and MuRF1-/- used for cardiomyocyte area measurements. Analysis of cardiomyocyte area of vehicle- and T3-treated MuRF1+/+ and MuRF1-/-. **E.** Representative 100x images of histological analysis of perfused and fixed heart sections stained with MT from vehicle- and T3-treated Wildtype$^{\text{MuRF1Tg+}}$ and MuRF1 Tg+ used for cardiomyocyte area measurements. Analysis of cardiomyocyte area of vehicle- and T3-treated Wildtype$^{\text{MuRF1Tg+}}$ and MuRF1 Tg+. Data are represented as mean ± SEM. Black bars represent vehicle and gray bars represent T3-treated cells or animals. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus or genotype group, **indicates significance on level of treatment group. %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as \#P< 0.05, \##P< 0.001, as determined using a pairwise post-test.
Figure 17. Gene expression associated with pathological hypertrophy is not altered in MuRF1-/- and MuRF1Tg+ mice treated with T3. A. RNA was isolated from whole heart tissue from vehicle- and T3-treated MuRF1+/+ and MuRF1-/-, cDNA generated, and BNP, skeletal muscle actin, ANF, and TATA box-binding protein (Tbp) expression determined, where Tbp was used as a reference gene. B. RNA was isolated from whole heart tissue from vehicle- and T3-treated Wildtype$^{\text{MuRF1Tg+}}$ and MuRF1 Tg+, cDNA generated, and BNP, skeletal muscle actin, ANF, and TATA box-binding protein (Tbp) expression determined, where Tbp was used as a reference gene. In vitro cardiomyocyte area measurements were averaged over at least 200 cardiomyocytes. In vivo cardiomyocyte area measurements were averaged over at least 400 cardiomyocytes from three different animals per group. RT-PCR analysis was carried out using the $\Delta \Delta CT$ method, where raw CT values were first normalized to Tbp (CT values) and then to vehicle-treated wildtype groups (Tbp values). RT-PCR data were averaged over 3 animals per group. Data are represented as mean ± SEM. Black bars represent vehicle and gray bars represent T3-treated cells or animals.

Figure 18. MuRF1 inhibits TH cardiac uptake. A. Serum T3 and T4 analysis of MuRF1-/- and sibling-matched wildtype control mice (MuRF1 +/-) injected with either 1mg/kg T3 or vehicle (PBS) intraperitoneally each day for 14 days. Black bars represent MuRF1+/+ and gray bars represent MuRF1-/-.

B. Serum T3 and T4 analysis of MuRF1 Tg+ and sibling-matched wildtype control mice (Wildtype$^{\text{MuRF1Tg+}}$) injected with either 1mg/kg T3 or vehicle (PBS) intraperitoneally each day for 14 days. Black bars represent Wildtype$^{\text{MuRF1Tg+}}$ and gray bars represent MuRF1 Tg+.

Data are represented as mean ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group, **indicates significance on level of treatment group %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as $P<0.05$, $$$P<0.001$ as determined using a pairwise post-test.
Figure 19. MuRF1 inhibits cardiac MCT10 gene expression. A. RNA was isolated from whole heart tissue from vehicle- and T3-treated MuRF1+/+ and MuRF1−/−, cDNA generated, and monocarboxylate transporter 8 (MCT8), MCT10, and Tbp expression determined, where Tbp was used as a reference gene. Black bars represent MuRF1+/+ and gray bars represent MuRF1−/−. B. RNA was isolated from whole heart tissue from vehicle- and T3-treated WildtypeμRF1Tg+ and MuRF1 Tg+, cDNA generated, and monocarboxylate transporter 8 (MCT8), MCT10, and Tbp expression determined, where Tbp was used as a reference gene. Black bars represent WildtypeμRF1Tg+ and gray bars represent MuRF1 Tg+. RT-PCR analysis was carried out using the ΔΔCT method, where raw CT values were first normalized to Tbp (ΔCT values) and then to vehicle-treated wildtype groups (ΔΔCT values). RT-PCR data were averaged over 3 animals per group. Data are represented as mean ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group, **indicates significance on level of treatment group %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as #P < 0.05, ##P < 0.001 as determined using a pairwise post-test.

Figure 20. MuRF1 inhibits TRα transcriptional activity, but does not target TRα for proteasomal degradation. A. To assess the role of MuRF1 on TRα transcriptional activity, Cos-7 cells were co-transfected with luciferase plasmid driven by the thyroid response element (TRE) of the GH gene, as described previously, β-galactosidase plasmid (for transfection control), and FLAG-TRα plasmid, with empty FLAG as a control, transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 4 hours, and treated with 1µM T3 for 24 hours. Parallel plates were used to confirm expression of FLAG-TRα and myc-MuRF1 by immunoblot using FLAG and myc antibodies, respectively. Black bars represent AdGFP- and gray bars represent AdMuRF1-transduced cells. B. MuRF1 was knocked down in HL-1 cardiomyocytes using AdshMuRF1, with Adshscrambled as control, at MOI 60 for 24 hours. Immunoblot using whole cell lysates was used to access protein
expression of endogenous TRα. MuRF1 antibody was used to confirm knockdown. β-actin was used as a loading control. C. MuRF1 was increased in expression in HL-1 cardiomyocytes using AdMuRF1, with AdGFP as a control, at MOI 25 for 24 hours. Immunoblot using whole cell lysates was used to access protein expression of endogenous TRα. Myc antibody was used to confirm expression of myc-MuRF1. β-actin was used as a loading control. Luciferase data is representative of three independent experiments. Data are represented as mean ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of plasmid group. %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as #P< 0.05 as determined using a pairwise post-test.

**Figure 21. Localization of TRα in the nucleus of cardiomyocytes is increased by MuRF1.** HL-1 cardiomyocytes were transfected with FLAG-TRα plasmid, or with empty FLAG as a control (see Figure 14), transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 4 hours, and treated with 1µM T3 for 2 hours. A. Nuclear localization of TRα was assessed using confocal microscopy, immunofluorescent staining of TRα (anti-FLAG, red) and nuclei (DAPI, blue), and quantitatively evaluated by determining the degree of TRα (red) and DAPI (blue) overlap via correlation coefficient measurements. B. The co-localization of MuRF1 and TRα in the nucleus was assessed using confocal microscopy, immunofluorescent staining of MuRF1 (anti-myc, green) and TRα (anti-FLAG, red), and quantitatively evaluated by determining the degree of MuRF1 (green) and TRα (red) overlap, isolated to the nucleus, via correlation coefficient measurements. C. Control confocal analysis of immunofluorescent staining with secondary antibodies (568 for TRα and 647 for MuRF1) alone. Quantitative immunofluorescence measurements were made using Volocity imaging software. For correlation coefficients, numeric range is from 0 to 1, where 0 indicates no correlation and 1 indicated perfect correlation. Data are represented as mean ± SEM. For TRα/DAPI co-immunofluorescence, a two-way ANOVA test was used to determine statistical significance.
*indicates significance on level of adenovirus group, **indicates significance on level of treatment group. Significance between groups is represented as ##P < 0.001 as determined using a pairwise post-test. For TRα/MuRF1 coimmunofluorescence, a Student’s t-test was used to measure statistical significance where $ indicates P < 0.001 between groups.

**Figure 22. Empty FLAG vector controls for FLAG-TRα immunofluorescence.** HL-1 cardiomyocytes were transfected with empty FLAG as a control, transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 4 hours, and treated with 1µM T3 for 2 hours. Control confocal analysis of immunofluorescent staining with anti-FLAG (red) and nuclei (DAPI, blue).

**Figure 23. Endogenous TRα accumulates in the nucleus in a MuRF1- and T3-dependent manner.** MuRF1 was increased in expression in HL-1 cardiomyocytes using AdMuRF1, with AdGFP as a control, at MOI 25 for 24 hours in serum-free DMEM followed by treatment with 1µM T3 for 2 hours. Immunoblot using nuclear- and cytoplasmic-enriched fractions was used to access nuclear localization of endogenous TRα. p-Rb was used as a nuclear marker and GAPDH was used as a cytoplasmic marker. Densitometry analysis of TRα is shown for AdGFP- and AdMuRF1-transduced cardiomyocytes treated with vehicle (PBS) or T3. Data are represented as mean ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group, **indicates significance on level of treatment group % indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as #P < 0.05 as determined using a pairwise post-test.

**Figure 24. MuRF1 interacts with TRα specifically in the presence of T3.** Cos-7 cells were transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 24 hours, and treated with 1µM T3 for 2 hours. To determine if MuRF1 physically interacts with TRα, whole cell lysates were submitted to specific immunoprecipitation of myc-MuRF1 followed by immunoblot of endogenous TRα.
Figure 25. MuRF1 mono-ubiquitinates TRα, inhibiting TRα’s poly-ubiquitination. Cos-7 cells were co-transfected with HA-Ub and FLAG-TRα plasmids, with empty HA and FLAG as controls, and transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 4 hours. To assess the effect of MuRF1 expression on TRα mono-ubiquitination (B), whole cell lysates were submitted to specific immunoprecipitation of FLAG-TRα followed by immunoblot of HA using anti-HA-horseradish peroxidase (HRP)-linked primary antibody (to prevent IgG heavy chain signal). To assess the effect of MuRF1 expression on TRα poly-ubiquitination (C), whole cell lysates were submitted to specific immunoprecipitation of FLAG-TRα followed by immunoblot of HA using primary anti-HA and HRP-linked secondary antibody.

Figure 26. MuRF1 promotes the interaction of TRα and CAP350 in the nucleus. HL-1 cardiomyocytes were transfected with FLAG-TRα plasmid, or with empty FLAG as a control (see Figure 22), transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 4 hours, and treated with 1µM T3 for 2 hours. A. The co-localization of TRα and endogenous centrosome-associated protein 350 (CAP350) in the nucleus was assessed using confocal microscopy, immunofluorescent staining of TRα (anti-FLAG, red) and CAP350 (green), and quantitatively evaluated by determining the degree of TRα (red) and CAP350 (green) overlap, isolated to the nucleus, via correlation coefficient measurements. B. Control confocal analysis of immunofluorescent staining with secondary antibodies (568 for TRα and 488 for CAP350) alone. Quantitative immunofluorescence measurements were made using Volocity imaging software. For correlation coefficients, numeric range is from 0 to 1, where 0 indicates no correlation and 1 indicated perfect correlation. Data are represented as mean ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group, **indicates significance on level of treatment group. Significance between groups is represented as ##P< 0.001 as determined using a pairwise post-test.

Figure 27. Interaction between desmin and TRα at the cardiomyocyte nuclear membrane is MuRF1- and T3-dependent. HL-1 cardiomyocytes were transfected with FLAG-TRα
plasmid, or with empty FLAG as a control (see Figure 14), transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 4 hours, and treated with 1µM T3 for 2 hours. A. The co-localization of TRα and endogenous desmin at the nuclear membrane was assessed using confocal microscopy, immunofluorescent staining of TRα (anti-FLAG, red) and desmin (green), and quantitatively evaluated by determining the degree of TRα (red) and desmin (green) overlap, isolated to the nuclear membrane, via correlation coefficient measurements. B. Control confocal analysis of immunofluorescent staining with secondary antibodies (568 for TRα and 488 for desmin) alone. Quantitative immunofluorescence measurements were made using Volocity imaging software. For correlation coefficients, numeric range is from 0 to 1, where 0 indicates no correlation and 1 indicated perfect correlation. Data are represented as mean ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group, **indicates significance on level of treatment group %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as #P< 0.05 as determined using a pairwise post-test.

Figure 28. T3 promotes the interaction between laminβ1 and TRα, which is inhibited by MuRF1. HL-1 cardiomyocytes were transfected with FLAG-TRα plasmid, or with empty FLAG as a control (see Figure 14), transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 4 hours, and treated with 1µM T3 for 2 hours. A. The co-localization of TRα and endogenous laminβ1 at the nuclear membrane was assessed using confocal microscopy, immunofluorescent staining of TRα (anti-FLAG, red) and laminβ1 (green), and quantitatively evaluated by determining the degree of TRα (red) and laminβ1 (green) overlap, isolated to the nuclear membrane, via correlation coefficient measurements. B. Control confocal analysis of immunofluorescent staining with secondary antibodies (568 for TRα and 488 for laminβ1) alone. Quantitative immunofluorescence measurements were made using Volocity imaging software. For correlation coefficients, numeric range is from 0 to 1, where 0 indicates no correlation and 1 indicated perfect correlation. Data are represented as mean ±
A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group, **indicates significance on level of treatment group %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as #P < 0.05, ##P < 0.001 as determined using a pairwise post-test.

**Figure 29. Amino acid sequences of mouse TRα domain lysine mutants.** Final amino acid sequences of TRα WT, TRα A/B KtoR, TRα C KtoR, TRα D KtoR, and TRα E/F KtoR, as expressed via the p3XFLAG®-CMV-14 vector. Plasmid cloning described in data supplement.

**Figure 30. Mutation of the lysine residues within the E/F region of TRα inhibits MuRF1-dependent TRα nuclear accumulation.** HL-1 cardiomyocytes were transfected with indicated FLAG-TRα domain lysine to arginine (KtoR) mutant plasmids, or with empty FLAG as a control (see Figure 22), transduced with AdMuRF1 at MOI 25 for 4 hours, and treated with 1µM T3 for 2 hours. A. Nuclear localization of TRα was assessed using confocal microscopy, immunofluorescent staining of TRα (anti-FLAG, red) and nuclei (DAPI, blue), and quantitatively evaluated by determining the degree of TRα (red) and DAPI (blue) overlap via correlation coefficient measurements. B. The co-localization of MuRF1 and TRα in the nucleus was assessed using confocal microscopy, immunofluorescent staining of MuRF1 (anti-myc, green) and TRα (anti-FLAG, red), and quantitatively evaluated by determining the degree of MuRF1 (green) and TRα (red) overlap, isolated to the nucleus, via correlation coefficient measurements. C. The co-localization of TRα and endogenous CAP350 in the nucleus was assessed using confocal microscopy, immunofluorescent staining of TRα (anti-FLAG, red) and CAP350 (green), and quantitatively evaluated by determining the degree of TRα (red) and CAP350 (green) overlap, isolated to the nucleus, via correlation coefficient measurements. Quantitative immunofluorescence measurements were made using Volocity imaging software. For correlation coefficients, numeric range is from 0 to
1, where 0 indicates no correlation and 1 indicated perfect correlation. Data are represented as mean ± SEM. A one-way ANOVA test was used to determine statistical significance. Significance between groups is represented as ##P< 0.001 as determined using a pairwise post-test.
CHAPTER 4: DISCUSSION AND PERSPECTIVES

The ubiquitin proteasome system (UPS) and muscle atrophy

Diseases of skeletal muscle atrophy involve the UPS. The involvement of the UPS in the regulation of muscle atrophy was first discovered by David Glass and colleagues in 2001 (Bodine et al.)\textsuperscript{57}. In this study, the authors induced muscle atrophy in the hindlimb of rats by hindlimb suspension, immobilization or denervation, and analyzed gene expression levels. The expression of two E3 ubiquitin ligases, MuRF1 and muscle atrophy F box (MAFbx), were found to be most highly upregulated in all the models of muscle atrophy\textsuperscript{57}. Knocking out either MuRF1 or MAFbx partially maintained muscle mass in response to denervation, suggesting that these ubiquitin ligases play a role in the induction of muscle atrophy\textsuperscript{57}. Consequently, ubiquitin ligases were presumed to be involved in muscle wasting diseases—leading to the study of the activity of the UPS and ubiquitin ligases in these diseases in hope of developing therapies effective in restoring muscle mass and function\textsuperscript{145}. The importance of ubiquitin ligases, especially MuRF1 and MAFbx, continues to be shown in immobilization- and starvation-induced atrophy, cachexia, and sarcopenia\textsuperscript{146-149}. The identification of new ubiquitin ligases involved in atrophy is ongoing: for instance, the expression and activity of tumor necrosis factor (TNF) receptor activated factor 6 (TRAF6), an ubiquitin ligase activated downstream of inflammatory cytokines, is increased following atrophic stimuli has recently been established\textsuperscript{150}. Together these studies demonstrate the significance of the UPS, especially ubiquitin ligases, in skeletal muscle atrophy.

The UPS in DMD. One of the most devastating diseases defined by loss of muscle mass is Duchenne muscular dystrophy (DMD)\textsuperscript{151}. Proteasome inhibition has been considered as possible new therapy for the treatment DMD\textsuperscript{152} because protein degradation has been shown to be increased overall in the \textit{mdx} mouse model of DMD\textsuperscript{153} and proteasome expression is increased in human DMD
patients (Kumamoto et al.\textsuperscript{154}, described in Table 7). As shown in Table 7, multiple studies have tested the effectiveness of proteasome inhibitors to improve the pathophysiology of muscular dystrophy using the \textit{mdx} mouse model of muscular dystrophy and human muscle explants from DMD patients. Overall, proteasome inhibition improves function and diminishes histological markers of dystrophy, while reducing molecular markers of inflammation and increasing expression of proteins within the dystrophin-glycoprotein complex (DGC) (Table 7). While these reports provided evidence for improvement of dystrophic muscle disease by proteasome inhibitors, at that time, they did so only \textit{in the mouse}, an animal for which loss of function mutations in dystrophin gene produces only mild myopathy\textsuperscript{155, 156}, in only three types of skeletal muscle (Table 7), and not in cardiac muscle (likely because cardiomyopathy in the \textit{mdx} mouse is delayed\textsuperscript{157}). (It is important to note that Gloria Bonuccelli and colleagues, authors of most of the studies described in Table 7, have since investigated the role of proteasome inhibition in Golden retriever muscular dystrophy (GRMD)\textsuperscript{158}, the model of DMD used in our laboratory’s study\textsuperscript{159} described below).

To provide a more complete understanding of the regulation of the UPS in DMD, our laboratory undertook a comprehensive study of the activity of the proteasome and calpain 1 and 2 (Ca\textsuperscript{2+}-dependent cysteine proteases also involved in the pathogenesis of DMD\textsuperscript{160}) and expression of multiple members of the UPS (including multiple E2 enzymes and ubiquitin ligases), proteasomal subunits, and calpain 1 and 2 in five different skeletal muscles and heart from dogs with GRMD\textsuperscript{159}. We chose the GRMD model of DMD specifically because the severity of myopathy in these animals compares to that observed in humans\textsuperscript{155, 156}. The resulting study is reprinted in its entirety as the Appendix of this dissertation. Overall, we did find significant differences the expression of E2 enzymes, ubiquitin ligases, proteasomal subunits, and calpain 1 and 2 and the activity of the proteasome and calpain 1 and 2, but, unexpectedly, these metrics were not all increased, as would be expected based on the studies described in Table 7. Instead, certain muscles displayed explicit patterns. While expression of E2 enzymes, ubiquitin ligases, proteasomal subunits, and calpain 1 and calpain 2 were all increased overall in skeletal muscle (Figures 33-37 and Tables 9 and 10), these
increases did not translate to differences in protease activity in all these muscles—most notably protease activity was increased across the board in the long digital extensor, but remained unchanged in the cranial sartorius (Table 10). Interestingly, protease activity correlated with the known phenotype for each of these muscles: the long digital extensor undergoes severe atrophy and cranial sartorius undergoes hypertrophy (Table 10). These data suggest that the UPS plays a role in the pathophysiology of DMD, but that the effects are specific to each muscle type and, possibly, use (the long digital extensor is an extensor muscle and cranial sartorius is a flexor muscle (Table 10). Most importantly, these findings show that proteasome inhibition in GRMD may improve function for certain muscles for which proteasome activity is increased (e.g. long digital extensor), but worsen function for certain muscles for which proteasome activity is unchanged (e.g. cranial sartorius).

Following the publication of Wadosky et al., Gloria Bonuccelli and colleagues showed that treatment of GRMD dogs with the proteasome inhibitor bortezomib did improve disease pathology via histology (increased fiber diameter, decreased lymphocytic invasion, and decreased collagen deposition) and immunohistochemistry (increased plasma membrane localization of DGC proteins), but these improvements did not translate to clinical progress since neither physical nor hematological signs of disease were improved in the two treated dogs. In addition, the particular skeletal muscle biopsied was never indicated in this study—so it is still unclear whether certain skeletal muscles (e.g. cranial sartorius) are adversely affected by proteasome inhibitor treatment in GRMD. Neither the studies in the mdx mouse (Table 7) nor the recent study in GRMD dogs assessed how proteasome inhibition treatment affects the heart of diseased animals. Since dilated cardiomyopathy is a severe complication of DMD, this omission is considerable; therefore, we also measured the expression of E2 enzymes, ubiquitin ligases, proteasomal subunits, and calpain 1 and calpain and activity of the proteasome and calpain 1 and 2 in the hearts of GRMD and control animals (Figures 33-37 and Tables 9 and 10). In contrast to skeletal muscle, expression of one E2 enzyme (UNC4/5), two ubiquitin ligases (CHIP and MDM2), all three proteasomal subunits, and both calpain 1 and 2 where decreased in GRMD cardiac muscle (Figures 33-37 and Tables 9 and 10).
These data provide substantial evidence that proteasome inhibitor treatment would be detrimental to the DMD heart, which is already undergoing pathological remodeling. Use of bortezomib is approved for treatment of multiple myeloma\textsuperscript{165} and has been linked to adverse cardiac outcomes\textsuperscript{166}—for instance, one patient with no previous history of cardiac disease underwent myocardial infarction only 5 days starting treatment with bortezomib and dexamethasone\textsuperscript{167}. In addition, studies have shown that the progression of cardiac dysfunction in general (not specific to DMD) decreases proteasome activity\textsuperscript{168-172}. Altogether these studies suggest that use of proteasome inhibitors in DMD patients could worsen their existing cardiomyopathy; therefore, identification of another way to target the UPS, perhaps one that can be injected in certain skeletal muscle rather than be administered systemically, would be a superior treatment option.

**The UPS in cardiac disease**

The adverse effects of proteasome inhibition on the heart\textsuperscript{166, 167} and the endogenous decreases in proteasome activity in the myocardium during pathological remodeling\textsuperscript{168-172} illustrate the importance of the UPS to cardiac biology\textsuperscript{92, 173-175}. Since cardiomyocytes, the cell type that makes up 70-80\% of the adult heart\textsuperscript{2}, are post-mitotic, their post-damage repair relies on protein quality control mechanisms of which the UPS is one (in addition to molecular chaperones and autophagy)\textsuperscript{175}. Continuous rounds of sarcomeric contraction and relaxation provokes protein wear and tear requires that the UPS is functioning properly. Not surprisingly, cardiac disease states, such ischemic heart disease, certain cardiomyopathies, and pathological hypertrophy, are associated with altered UPS function and imbalance between protein synthesis and protein degradation\textsuperscript{92, 173-175}.

**Cardiac hypertrophy and ubiquitin ligases.** In cardiac hypertrophy, the balance between protein synthesis and protein degradation is skewed toward protein synthesis (Figure 31)\textsuperscript{175}. Since the purpose of hypertrophic remodeling is to increase the number of sarcomeres, making new and limiting the breakdown of sarcomeric proteins is required for the maintenance of heart function in the face of increased load\textsuperscript{2, 175}. Biasing this overall balance toward synthesis can involve increases in growth signaling or decreases degradation signaling, or both (Figure 31). Signaling cascades can
Even affect both protein synthesis and degradation. Depending on which pathway is activated, shifting the balance toward protein synthesis can be good or bad for the myocardium. Pathological cardiac hypertrophy, induced by hypertension, aortic stenosis, and myocardial infarction, activates GPCRs and downstream MAPK signaling while physiological hypertrophy, induced by developmental growth, exercise, and pregnancy, activates RTKs and downstream PI-3K/Akt signaling. While distinct signaling cascades have been linked to each type of hypertrophy, why continued activation of MAPKs causes heart failure and RTKs do not, has yet to be established. Interestingly, decreased proteasome function and accumulation of ubiquitinated proteins has been shown to occur in cardiomyocytes during the development of pathological hypertrophy, implicating the inhibition of the UPS in this maladaptive cardiac growth. IGF-1/Akt signaling, activated downstream of physiologic growth activation, activates protein translation while inhibiting FOXO proteins, transcription factors that drive the expression of cardiac ubiquitin ligases (Figure 1); the latter of which again implicates inhibition of the UPS and ubiquitin ligases in this form of cardiac hypertrophy that occurs without pathological remodeling. While these findings are in opposition— inhibition of the UPS is involved in both pathological and physiological growth—they show that the study of the UPS and ubiquitin ligases is imperative to understanding what makes pathological hypertrophy “bad” and physiological hypertrophy “good” for the myocardium, and perhaps the key to converting improving function in heart failure patients by stimulate “physiologic” type growth.

Since the discovery that ubiquitin ligases regulate skeletal muscle atrophy, these enzymes have been extensively studied in the context of cardiac hypertrophy. MAFBx/Atrogin-1 inhibits pressure overload-induced pathological cardiac hypertrophy by degrading calcineurin, a phosphatase that de-phosphorylates and activates the growth-promoting transcription factor NFAT. IGF-1- and exercise-induced physiological hypertrophy is also inhibited by MAFBx/Atrogin-1 via MAFBx/Atrogin-1-dependent ubiquitination of FOXOs, protecting FOXOs from Akt-dependent phosphorylation and inhibition. Another ubiquitin ligase, CHIP has been found to inhibit both pathological and physiological cardiac hypertrophy. CHIP inhibits pathological hypertrophy by
promoting the activity of AMP activated kinase (AMPK), a metabolic sensor that orchestrates the cardiac stress response to increased ATP requirements\textsuperscript{178}, and thereby retaining the availability of ATP to the myocardium\textsuperscript{179}. Our laboratory has shown that CHIP also inhibits physiological hypertrophy—where CHIP was found to inhibit beneficial autophagy in response to exercise\textsuperscript{84}. It has also been shown by our laboratory that MuRF1, but not MuRF2, inhibits pressure overload-induced cardiac hypertrophy by inhibiting the activity of the growth promoting transcription factor SRF\textsuperscript{73}. In contrast, recent studies by our laboratory have shown that both MuRF1 and MuRF2 inhibit developmental cardiac growth by regulating E2F1 promoter occupancy\textsuperscript{75}. Indicating that MuRF1 and MuRF2 inhibit physiological hypertrophy, at least during post-natal development. A recent study has shown that MuRF1-/- animals undergo spontaneous age-dependent cardiac hypertrophy and have decreased phosphorylation of Akt and mTOR\textsuperscript{104}, providing additional evidence for MuRF1’s inhibition of physiological hypertrophy. A common theme which reveals itself in the studies described above is that ubiquitin ligases can inhibit both pathological and physiological hypertrophy. After the reviewing this body of work the paucity of investigations studying MuRF1’s regulation of physiological hypertrophy is also significant. Therefore, the purpose of this dissertation is to fill that void by providing rigorous study of MuRF1 in the context of physiological cardiac hypertrophy induced by growth hormones and exercise.

**MuRF1 inhibits IGF-1- and exercise-dependent cardiac hypertrophy**

**Regulation of IGF-1/Akt pathway by ubiquitination and SUMOylation.** Ubiquitination regulates IGF-1/Akt signal transduction at multiple levels of the kinase cascade and Akt ubiquitination, specifically, has been the most well-studied. The initial recruitment of Akt to the plasma membrane, where it is phosphorylated by PDK1 (Figure 1), requires poly-ubiquitination. The ubiquitin ligase TRAF6, which interacts with IGF-1R, is activated by IGF-1 engagement of IGF1R\textsuperscript{180}. TRAF6 then specifically polyubiquitinates unphosphorylated Akt via a K63 linkage (an atypical chain type (see Figure 2), which induces Akt membrane localization, promotes the interaction of Akt and PDK1, and subsequent phosphorylation of Akt at T308 by PDK1\textsuperscript{180}. This ubiquitin-dependent
mechanism of Akt by TRAF6 has been shown to be present in skeletal and cardiac muscle\textsuperscript{180}. In addition, new evidence has shown that TRAF4 can also ubiquitinate and activate Akt in the context of lung cancer\textsuperscript{181}. Akt nuclear localization, and thereby Akt-dependent phosphorylation and inhibition of FOXO transcription factors (\textbf{Figure 1}), is also dependent on ubiquitination. Fully phosphorylated Akt (S473 and T308) is recognized by the ubiquitin ligase tetratricopeptide repeat domain 3 (TTC3), that polyubiquitinates Akt via a K48 linkage and promotes the nuclear localization of Akt\textsuperscript{182}. While nuclear-specific activity of Akt has been shown to be important for cardiomyocyte survival\textsuperscript{183} and Akt-dependent inhibition of pathological hypertrophic cardiac remodeling\textsuperscript{184}, the role of TTC3-dependent ubiquitination of Akt have yet to be studied in the heart. Activation of Akt has also been shown to be dependent on SUMOylation by PIAS, where Akt kinase activity, cell proliferation, and tumorigenesis are inhibited when an Akt lysine mutant unable to be SUMOylated is expressed\textsuperscript{185}.

Ubiquitination also plays a role in inhibiting Akt. After passage to the nucleus Akt also recognized for degradation by the nuclear proteasome\textsuperscript{182}, a negative feedback mechanism postulated to keep growth signaling in check by ensuring that activation of IGF-1 signaling originates via extracellular ligand and not by unrestrained activated Akt\textsuperscript{186}. Negative regulation of IGF-1 signaling by ubiquitination of Akt also occurs in the cytoplasm, where mTORC2-dependent phosphorylation of Akt at S473 has been shown to promote the degradation of Akt\textsuperscript{187} and at the outer mitochondrial membrane, where mitochondria-specific ubiquitin ligase mitochondrial ubiquitin ligase activator of NF-\kappa B (MULAN)\textsuperscript{188} polyubiquitinates phosphorylated Akt via a K48 linkage, promoting the degradation of Akt\textsuperscript{189}. Together, these studies show that negative feedback control of IGF-1 signaling via proteasome-dependent degradation of Akt occurs by multiple mechanisms.

\textbf{MuRF1 inhibits IGF-1 signaling by ubiquitin-mediated degradation of c-Jun.} The studies described above show that a ubiquitin/SUMO ligase network, in addition to the kinase network, regulates IGF-1/Akt signaling. However, the majority of the studies investigating the regulation of IGF-1/Akt signaling by ubiquitination have been carried out in the context of cancer, not in cardiac hypertrophy. The work shown in \textbf{Chapter 2}\textsuperscript{126} of this dissertation reveals that ubiquitin-
dependent regulation of IGF-1/Akt signaling occurs in the myocardium. In addition, the work presented also shows that this regulation occurs at a level that had yet to be discovered for IGF-1/Akt signaling in the heart—that of ubiquitination-dependent transcriptional regulation of the IGF-1 pathway by the action of the ubiquitin ligase MuRF1. MuRF1 limits the total protein expression of Akt, GSK3β, and mTOR (Figure 5-7) by reducing total and phosphorylated c-Jun (Figure 8 and 9), a transcription factor recently shown to regulate the transcription of multiple genes that code for proteins that make up the IGF-1 signaling cascade²², via proteasome-dependent degradation upon poly-ubiquitination by MuRF1⁷⁴. Inhibition of IGF-1-dependent cardiomyocyte hypertrophy (Figure 1) was consequent upon MuRF1-dependent inhibition of IGF-1 pathway transcription via c-Jun (Figure 10 and 11). Showing that MuRF1’s ubiquitination of c-Jun in the context of IGF-1 signaling has significance for growth on the cardiomyocyte level.

The next goal was to determine if MuRF1’s regulation of IGF-1 signaling applied systemically. Indeed, we found that MuRF1 inhibits cardiac hypertrophy in response to running exercise in the mouse (Figure 12 and 13). Importantly, the hypertrophy observed in these studies was without cardiac dysfunction (Tables 1 and 2) or markers of pathological remodeling (Figures 12E, 13E, and 14), as we expected to be the case for cardiac hypertrophy induced by running exercise 21487028. These data link MuRF1 activity to the inhibition of exercise-induced physiological hypertrophy for the first time. MuRF1’s influence even has an effect on a mouse’s ability to exercise—in that, global knockout of MuRF1 increases the running speed and distance that a mouse can run (Tables 3 and 4). One caveat of these findings, however, is that it cannot be determined from these data whether increased exercise performance is associated with MuRF1 activity in the heart or the skeletal muscle, since is not expressed in either in this animal model⁵⁷.

Altogether, Chapter 2¹²⁶ has established, for the first time, that MuRF1 inhibits physiological cardiac hypertrophy stimulated by aerobic exercise. In addition, the work in that chapter also established that MuRF1 can be listed among the ubiquitin/SUMO ligases, including TRAF6, TRAF4, TTC3, MULAN, and PIAS, that are part of a network that regulates IGF-1/Akt
growth signaling. Interestingly, in regulating c-Jun, MuRF1 acts upstream of all of the other ligases within this network by regulating the transcription of gene which code for the proteins, namely Akt, that these ligases call substrates. Since MuRF1’s inhibition of IGF-1/Akt signaling has a substantial effect on cardiac physiology in response to exercise, the novel mechanism detailed in Chapter 2 may prove to be useful in the development of therapies that interrupt MuRF1’s inhibition of IGF-1 signaling for use in promoting beneficial cardiac remodeling in heart failure patients.

**MuRF1 inhibits TH-dependent cardiac hypertrophy**

**Ubiquitination of NRs.** Regulation of NRs by ubiquitination has been the subject of emerging research in the last several years in the study of NR regulation in multiple diseases. The ubiquitin ligase RING finger protein 4 (RNF4) promotes the nuclear localization of androgen receptor (AR), thereby increasing testosterone-mediated transcription. Another RING finger protein, RNF6, also positively regulates AR transcriptional activity by poly-ubiquitinating AR. Canonical poly-ubiquitination and subsequent proteasomal degradation also plays a role in AR regulation: the ubiquitin ligases MDM2 and CHIP have both been shown to poly-ubiquitinate AR and promote AR degradation. The UPS is also important for the regulation of ERα, in that proteasome-dependent degradation of ERα is implicated in maintaining estrogen-dependent transcription by promoting ERα turnover. In addition to targeting AR, MDM2 and CHIP also target ERα for poly-ubiquitination and subsequent proteasomal degradation. ERα is targeted by a number of other ubiquitin ligases, including estrogen-responsive finger protein (EFP) and E6 associated protein (E6AP). Most notably, Breast cancer early onset 1 (BRCA1), a tumor suppressor coded for by a gene that commonly mutated in breast tumors, complexed with BRCA1-associated RING domain 1 (BARD1) monoubiquitinates ERα, inhibiting ERα-dependent transcription. Not surprisingly, BRCA1/BARD1 also poly-ubiquitinates and targets progesterone receptor (PR) for proteasome-dependent degradation, inhibiting the action of progesterone (the dysregulation of which is also found in breast cancer). Liver X receptor α (LXRα), that regulates lipid homeostasis and inflammation in multiple tissues, is also poly-ubiquitinated by
BRCA1/BARD1, promoting the LXRα degradation by the proteasome; illustrating that the activity of this ubiquitin ligase complex is important in tissues other than estrogen/progesterone target tissues. Finally, the UPS also regulates glucocorticoid receptor (GR)—where CHIP-dependent polyubiquitination of GR promoting GR’s degradation by the proteasome. Like ERα, the regular turnover of GR via the UPS maintains GR function; therefore CHIP-mediated degradation of GR promoted, rather than inhibited, GR transcriptional activity. Altogether, these studies show that ubiquitination of NRs has a wide effect on numerous organ systems and thereby is involved in the pathogenesis of multiple disease, including prostate cancer (AR), breast cancer (ERα and PR), inflammatory/auto-immune diseases (LXR and GR), type II diabetes (LXR, PPAR), obesity (LXR, PPAR), atherosclerosis (LXR, PPAR), and liver disease (LXR, PPAR). Except for two recent studies linking AR and ERα to striated muscle function and growth, PPARs and TRs (and the co-receptor for both of these NRs, RXRα) are the NRs which have most well-studied in cardiac diseases; therefore the ubiquitin-dependent regulation of these NRs will be discussed in more detail below.

**Regulation of PPARα by the UPS.** Over the past 10 years, several lines of evidence have implicated the ubiquitin proteasome system (UPS) in the regulation of PPARα activity. In early studies, GFP- or HA-tagged PPARα expression vectors were transfected into HeLa cells and analyzed by Western blot analysis for PPARα. These studies revealed that PPARα protein stability is greatly affected by the presence or absence of a selective PPARα ligand. Similar observations have been reported in HepG2 cells transfected with PPARα. In these cells, cotransfection with PPARα and HA-tagged ubiquitin reveals that PPARα can be polyubiquitinated and that this post-translational modification is lost in the presence of multiple PPARα ligands. Likewise, increasing the expression of the RXRα and the CREB binding protein coactivator of PPARα (both PPARα coactivators) in HepG2 cells leads to a decrease in PPARα ubiquitination. Finally, when these cells are treated with MG132, a

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3 This section has previously appeared in *American Journal of Physiology Heart and Circulatory Physiology*. The original citation for the publication which includes this excerpt is as follows: *Am J Physiol Heart Circ Physiol* 2012 Feb 1;302(3):H515-26.
proteasome inhibitor, PPARα activity is increased, presumably because of the decrease in ubiquitin-mediated proteasomal degradation of PPARα\textsuperscript{211}.

Recently, additional molecular details surrounding PPARα ubiquitination and its effect on PPARα activity have been reported by Gopinathan et al.\textsuperscript{212}. In this study, an interesting relationship between PPARα activity and the ratio of PPARα to MDM2, was revealed. MDM2 associates with PPARα through the A/B domain of PPARα and the coexpression of MDM2 increases PPARα ubiquitination\textsuperscript{212}. Increasing MDM2 levels relative to PPARα and PPARβ/δ, but not PPARγ, leads to a decrease in PPAR activity\textsuperscript{212}, whereas knocking down MDM2 expression levels with small interfering RNA in rat hepatoma cells inhibits mRNA expression of several PPARα targets\textsuperscript{212}. Increasing MDM2 levels in the presence of Wy-14643 (a PPARα selective agonist) leads to enhanced PPARα activity up to a ratio of MDM2 to PPARα <0.5 to 1; however, ratios of MDM2 to PPARα ≥ 1 inhibits PPARα activity\textsuperscript{212}. In summary, these studies demonstrate the ubiquitination of PPARα in a ligand-dependent manner and that effect of ubiquitination on PPARα activity depends on the systems studied.

**Regulation of PPARβ/δ by the UPS\textsuperscript{4}**. Like PPARα, the mechanism by which ubiquitination affects PPARβ/δ activity and protein levels has been studied in different cell types. In U2OS human osteosarcoma cells expressing recombinant PPARβ/δ, PPARβ/δ is ubiquitinated and rapidly degraded in the absence of a ligand. However, the addition of PPARβ/δ-specific agonists such as L-165041, GW-501516, and the stable prostaglandin analog carbaprostacyclin PGI\textsubscript{2} completely inhibit PPARβ/δ degradation, a process that is reliant on the DNA binding domain of PPARβ/δ\textsuperscript{213}. When U2OS cells are treated with puromycin (an inhibitor of protein synthesis) and the proteasome inhibitor PS341, PPARβ/δ protein levels are stabilized to levels similar to what is seen with puromycin and L-165041 (a PPARβ/δ agonist) treatment, suggesting that L-165041 blocks

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\textsuperscript{4} This section has previously appeared in *American Journal of Physiology Heart and Circulatory Physiology*. The original citation for the publication which includes this excerpt is as follows: *Am J Physiol Heart Circ Physiol* 2012 Feb 1;302(3):H515-26.
ubiquitination and degradation of PPARβ/δ in U2OS cells\textsuperscript{213}. Independent of basal levels of PPARβ/δ, ligand binding of PPARβ/δ prevents its ubiquitination and subsequent degradation\textsuperscript{213}.

In contrast to the study described above, studies using mouse fibroblasts demonstrate that the ligand dependency of ubiquitination and degradation of PPARβ/δ is determined by PPARβ/δ protein levels. At low PPARβ/δ protein concentration, PPARβ/δ ubiquitination and degradation is not influenced by the synthetic agonist GW-501516\textsuperscript{214}. However, at high PPARβ/δ levels, GW-501516 strongly inhibits the ubiquitination and degradation of PPARβ/δ\textsuperscript{214}. These findings have implications not only in the biological regulation of PPARβ/δ but also in the experimental design of overexpression systems used to determine the function and regulation of PPARβ/δ.

**Regulation of PPARγ by the UPS\textsuperscript{5}**. There are two forms of PPARγ that are generated from the same gene by alternative promoter usage, PPARγ1 and PPARγ2. PPARγ1 is found in most cell types, whereas PPARγ2 is found exclusively in adipose tissues\textsuperscript{215}. Ubiquitination of PPARγ has only been studied in adipocytes\textsuperscript{216-218}, and therefore the knowledge of the regulation of PPARγ by the UPS is limited to the PPARγ2 moiety. Like other PPAR family members, the rate at which PPARγ2 is degraded is dependent on its interaction with ligands and appears to be mediated by an ubiquitin-dependent mechanism. However, unlike PPARα and PPARβ/δ, ubiquitin-mediated regulation of PPARγ2 is enhanced rather than inhibited in the presence of PPARγ2-specific ligands. When differentiated adipocyte cells (3T3-F442A) are treated with the PPARγ2 ligand pioglitazone (or other thiazolidinediones), a dose-dependent increase in ubiquitination and a subsequent decrease in the PPARγ2 protein expression are observed\textsuperscript{217}. When the proteasome is inhibited in this adipocyte cell line, the degradation of PPARγ2 is also inhibited, indicating that the degradation of PPARγ2 in the presence of ligand occurs via proteasomal degradation. Interestingly, PPARγ2 constructs containing mutations in the activation function-1 domain, part of the ligand binding domain, are able to attenuate this ligand-dependent degradation of PPARγ2, supporting the theory that ligand binding to PPARγ2 is

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necessary for its ubiquitin-mediated proteasomal degradation\textsuperscript{217}. In the context of the previous two sections, these findings demonstrate that all three members of the PPAR family are degraded by the UPS, albeit by different mechanisms.

**Regulation of RXRα by the UPS**\textsuperscript{6}. Ubiquitination of the PPAR/TR coreceptor RXRα has been reported, although there are a limited number of studies that address this particular post-translational modification. Since PPAR transcription factors work by dimerizing with RXRα, these studies likely have relevance to the post-translational regulation of PPARs. Recent studies have demonstrated that RXR homologs are ubiquitinated by ubiquitin ligases. However, it has not been determined whether this leads to degradation of RXR, which would be expected to inhibit PPAR activity overall. A yeast two-hybrid screen of the *Schistosoma mansoni* (Sm) cDNA library using SmRXR1 and SmRXR2 as bait identified the RING finger protein Sm seven in absentia (SmSINA) as a potential ubiquitin ligase specific for SmRXRs\textsuperscript{219}. In vitro ubiquitination assays demonstrated that SmSINA has ubiquitin ligase activity and can polyubiquitinate both SmRXR1 and SmRXR2, targeting them for proteasomal degradation\textsuperscript{219}. The DNA binding domain of SmRXRs shares 80\% homology with mammalian RXRα, but the ligand-binding E domain of SmRXRs shares only 22–25\% homology with mammalian RXRα\textsuperscript{220}. Since SmSINA interacts with the E domains of SmRXR1 and SmRXR2, where *S. mansoni* and mammals share the least homology in RXR sequence, it is unclear whether SINA-dependent degradation of RXRα would occur in mammals. Another RING finger protein, RNF8, has also been identified as an RXRα-interacting protein using a yeast two-hybrid screen of a human liver cDNA library\textsuperscript{221}. Interestingly, increasing expression of RNF8 in COS7 cells has no effect on RXRα ubiquitination but increases its transactivation ability, a phenomenon that is enhanced by retinoic acid treatment\textsuperscript{221}. Although this pathway does not lead to RXRα ubiquitination, it is an interesting example of how interaction with an ubiquitin ligase can affect a protein's function in an ubiquitin-independent manner.

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Further studies aimed at determining the physiological relevance of RXRα ubiquitination have revealed that RXRα is polyubiquitinated in smooth muscle cells (SMCs) derived from the myometrium and that this ubiquitination is significantly inhibited in cells isolated from leiomyomas, a benign smooth muscle neoplasm of the uterus. Protein lysates from tissue samples from leiomyomas and healthy myometrium contain three RXRα immunoreactive bands: one at 54 kDa, representing the full-length RXRα, and two bands at lower molecular mass at 45 and 42 kDa. Interestingly, the lower molecular mass bands (presumably degradation products) are predominantly in protein lysates from healthy myometrium, whereas the single higher molecular mass band is predominant in protein lysates isolated from leiomyomas, suggesting that RXRα degradation is inhibited in leiomyomas. Interestingly, although degradation of RXRα is inhibited in SMCs derived from leiomyomas, it can be rescued by the addition of the RXR ligand 9-cis-retinoic acid, indicating that 9-cis-retinoic acid promotes the degradation of RXRα in leiomyomas. These results suggest that RXRα is poly-ubiquitinated and degraded by the proteasome in SMCs derived from the myometrium and that this process is inhibited during the pathogenesis of leiomyoma, leading to the accumulation of RXRα. Furthermore, 9-cis-retinoic acid can attenuate RXRα protein accumulation by restoring RXRα's polyubiquitination and degradation to basal levels, showing the potential of 9-cis-retinoic acid as an efficient treatment of leiomyomas. Since RXRα is the protein PPAR dimerizes with to enhance PPAR activity, it would not be surprising if PPAR activity was increased in leiomyomas, because of increased RXRα protein and activity levels.

**Regulation of TRα by ubiquitination and SUMOylation.** Being that the UPS regulates a variety of other NRs, including its co-receptor RXRα, it is anticipated that TRs is also regulated by the UPS; especially because of its widespread effect on cardiomyocytes (Figure 1). Surprisingly, there has been little work done to identify the role of the UPS in controlling TRs. Both TRα and TRβ have been shown to be polyubiquitinated and degraded by the proteasome in a T3-dependent manner in fibroblasts and lymphocytes. These authors went on to show that the ligand binding domain (LBD) was required for the poly-ubiquitination of TRs (Figure 32), not unsurprising since T3 is
required for poly-ubiquitination of TRs\textsuperscript{123}. Unfortunately, this study did not identify the ubiquitin ligase that modifies TRα and TRβ, which still remains unknown. The only other study that has addressed this type of post-translational modification is that of Liu et al.\textsuperscript{122} where the authors showed that both TRα and TRβ are mono-SUMOylated. In this study, it was shown that TRs are SUMOylated by PIAS proteins, established SUMO ligases\textsuperscript{223}; specifically, TRα is SUMOylated by PIASxβ and TRβ is SUMOylated by PIAS1, PIAS3, and PIASγ\textsuperscript{122}. Interestingly, dependence of TR SUMOylation on T3 differed for TRα and TRβ: TRα SUMOylation occurs independent of T3, while TRβ SUMOylation depends on T3\textsuperscript{122}. The effect of SUMOylation, however, was similar for both TRα and TRβ, in that SUMOylation inhibits TR transcriptional activity\textsuperscript{122}. While the lysines important for SUMOylation were both located in the LBD of TRα (K283 and K389) (Figure 32), lysines important for SUMOylation of TRβ we located in the A/B (K50), DNA-binding domain (DBD) (K146), and LBD (K443)\textsuperscript{122}. Importantly, the authors identified that mono-SUMOylated species of both TRα and TRβ were present in liver, white adipose, and cardiac tissue\textsuperscript{122}, showing that this SUMO-dependent mechanism of TR regulation is relevant \textit{in vivo}. An essential detail in these findings is that SUMOylation of TRα was most robust in cardiac tissue, compared to liver and white adipose and compared to SUMOylation of TRβ in all tissues evaluated\textsuperscript{122}. These studies indicate that SUMOylation of TRα in the cardiomyocyte may be regulating the numerous activities of TRα that regulate myocardial contractility (Figure 1).

**MuRF1-dependent inhibition of cardiac hypertrophy by TRα monoubiquitination.**

Other than SUMO-TRα expression in cardiac tissue\textsuperscript{122}, studies of ubiquitin/SUMO modifications have not been studied in the myocardium. Since T3-dependent TRα activity throughout the cardiomyocyte promotes cardiac growth (Figure 1) we were interested in determining how ubiquitination affects this mechanism. The effect of TRα ubiquitination of TH-dependent cardiac hypertrophy, an adaptive form of growth, was especially interesting to us because we already found that ubiquitin-dependent inhibition of physiological hypertrophy induced by IGF-1 and exercise (Chapter 2\textsuperscript{126}). Specifically, we found that MuRF1 inhibits IGF-1/exercise induced cardiac
hypertrophy by its poly-ubiquitination and degradation of the transcription factor c-Jun (Chapter 2 126); therefore we set out to determine the effect of MuRF1 on TH-dependent cardiac hypertrophy, hypothesizing that MuRF1 ubiquitinates of TRα. The latter was the focus of our study because MuRF1 has also been shown to regulate PPARα by mono-ubiquitination70-72. As described in Chapter 3, MuRF1 inhibits cell growth following T3 treatment in cardiac-derived HL-1 cells and primary NRVM (Figure 15A, B, and C). MuRF1 also limited T3-dependent cardiac growth in vivo, where MuRF1-/ mice had exaggerated and MuRF1 Tg+ has blunted cardiac hypertrophy in response to T3 treatment (Figure 16). Interestingly, MuRF1 also limited the expression of MCT10 (Figure 19), a plasma membrane TH transporter (Figure 1). Consequently, T3/T4 was found to accumulate in the serum in MuRF1 Tg+ animals (Figure 18B), suggesting that decreased expression of MCT10 in the cardiac tissue of these animals is inhibiting the passage of TH into the cardiomyocyte (Figure 1).

Lack of available T3 in the cardiomyocyte, in addition to inhibiting the activity of TRs (Figure 1), can have other adverse circumstances because T3/T4 itself activates pathways important for cardiomyocyte growth, contractility, and metabolism6,40. T3 can activate Akt signaling44,224,225, promoting transcription of growth genes and protein transcription (Figure 1). T3 also increases phosphorylation of PLN226, inactivating PLN, releasing SERCa2 from PLN’s inhibition, and allowing for increased Ca2+ uptake into the sarcoplasmic reticulum (Figure 1). These studies show that MuRF1’s systemic effect on TH tissue uptake, in addition to MuRF1’s inhibition of TRα (described below), is likely to adversely affect the heart based on TH’s stand-alone effect on the cardiomyocyte.

We chose to focus our attention on TRα in investigating the mechanism of MuRF1-dependent inhibition of TH-dependent cardiac hypertrophy because 1) MuRF1 is already known to regulate PPARα, and 2) TRα activity in relation to cardiac hypertrophy is the most established mechanism of TH-dependent hypertrophy. As shown in Chapter 3, MuRF1’s inhibits TRα transcriptional activity (Figure 20A) via mono-ubiquitination (Figure 25A) and not by poly-ubiquitination-dependent degradation (Figure 25B). Mono-ubiquitinated TRα accumulates in the
nucleus (Figures 21 and 23) and interacts with CAP350 (Figure 26) and intermediate filaments (desmin) (Figure 27) at the nuclear membrane specifically in the presence of T3. While this was the first direct evidence of CAP350 in the cardiomyocyte, it was not altogether surprising that interaction with CAP350 inhibited TRα activity since this mechanism has already been recognized for PPARα.\(^{125}\)

We went on to show that MuRF1-dependent targeting of TRα to CAP350 was dependent on the lysines located to the E/F/LBD domain of TRα (Figures 30). These data were exciting to us because that region of TRα contains the lysines which are ubiquitinated and SUMOylated (Figure 32), indicating that mono-ubiquitination of TRα’s E/F/LBD domain could compete with these other post-translational modifications. Indeed, we found that MuRF1 inhibits poly-ubiquitination of TRα (Figure 25B). Regular turnover of some NRs, including ERα and GR (described above), is imperative for their transcriptional activity\(^{190,208}\), explaining how MuRF1’s inhibition of TR poly-ubiquitination (Figure 25B) inhibits rather than activates TRα transcriptional activity (Figure 20A).

Altogether, data provided in Chapter 3 establish that MuRF1 impairs TH-signaling in the cardiomyocyte via its mono-ubiquitination of TRα. Moreover, the effect that mono-ubiquitination has on TRα in this context revealed a newfound mechanism by which TRα is inhibited—via interaction with CAP350 and inhibition of poly-ubiquitination. TH supplementation therapy has recently been shown to improve cardiac function for patients in the clinic\(^{112,227}\). The new information this dissertation provides on the inhibitor function of MuRF1 on TH-dependent hypertrophy could be important in the treatment of these patients, since they might fare better on TH supplementation therapy if MuRF1 could be inhibited.

**Targeting MuRF1 in the clinic: druggable opportunities for heart failure?**

Restoring the damaged myocardium by promoting a “physiologic” cardiac phenotype has been a dream of cardiac researchers and clinicians for some time and TH supplementation has so far been the avenue by which activation of adaptive cardiac remodeling hopes to be achieved in the clinic\(^{228,229}\). Serum levels of T3 are reduced in patients with congestive heart failure, undergo rapid decline in the week following myocardial infarction, and fall in infants undergoing cardiopulmonary
bypass surgery. Consequently, several clinical studies have shown that TH repletion therapy improves symptoms of heart failure\textsuperscript{112,227}. TH supplementation improves cardiac performance with minimal side effects in patients with ischemic and nonischemic heart failure and decreases postoperative time to extubation in infants who received surgery to correct cardiac defects\textsuperscript{112,227}.

Furthermore, recent studies have shown that a TH-dependent mechanism is responsible for the improvement of patients with end-stage heart failure who underwent regimented moderate intensity exercise training\textsuperscript{230}. Patients bridged to heart transplantation using a ventricular assist device (VAD) undertook an exercise regimen, where cardiac tissue was collected pre-VAD and at the time of heart transplantation. The authors found that TR\textalpha{}1 and phospho-Akt expression were both increased in cardiac tissue over pre-VAD levels only in patients who received exercise training\textsuperscript{230}. It was also found that patients who exercised has increased serum concentrations of T3—suggesting that exercise-dependent increases in T3 may have causative effect on the positive regulation of TR\alpha{} and phospho-Akt\textsuperscript{230} (especially considering what we know about the T3-dependent activation of growth signaling\textsuperscript{6,40}). This study is especially noteworthy and exciting in the context of this dissertation work, since it shows that both exercise and TH signaling, both of which are shown here to be inhibited by MuRF1 (\textit{Chapters 2 and 3}), can promote a molecular “physiologic” phenotype in the damaged myocardium of humans. One can only imagine how much more beneficial exercise training and TH supplementation could be for heart failure patients if MuRF1 were selectively inhibited in the myocardium. Hopefully, the detailed mechanisms by which cardiac MuRF1 inhibits IGF-1-, exercise-, and TH-induced hypertrophic remodeling provided in this dissertation work will uncover at least one druggable interaction that will make an effectual treatment regimen possible that reverses the symptoms of heart failure.
APPENDIX: REGULATION OF THE CALPAIN AND UBIQUITIN PROTEASOME SYSTEMS IN A CANINE MODEL OF MUSCULAR DYSTROPHY

Introduction

The muscle damage sustained in Duchenne muscular dystrophy (DMD) is cleared by two major proteolytic systems: the calpain and ubiquitin proteasome systems\(^1\)\(^2\). The involvement of these proteolytic systems has prompted investigators to explore their role in the pathogenesis of DMD mainly using the \textit{mdx} mouse model\(^3\)\(^4\). At least eight studies have tested the hypothesis that calpain and/or proteasome inhibition is beneficial in disease, and most have shown significant improvement in histology and function in short-term studies (as summarized in Tables 11 and 12)\(^5\)\(^6\).\(^7\)\(^8\). Paralleling the therapeutic utility of calpain and proteasome inhibition on the \textit{mdx} phenotype, there has been the largely uniform evidence that calpain and proteasome activities are enhanced in DMD (Tables 11 and 12).

These studies have been limited by their sampling of only one or two skeletal muscle types and their use of the \textit{mdx} mouse model, which has a relatively mild phenotype compared with DMD in humans. We hypothesized that different muscle groups, including the heart, might have a more diverse expression and activity of calpain and the ubiquitin-proteasome systems. If so, it could result in unexpected and possibly unintended consequences in response to calpain and proteasome inhibition. In this study, we investigated the diversity of proteasome and calpain activities in five representative skeletal muscles and the heart in the GRMD model at 6 months of age. Despite the more severe disease phenotype in these animals, we found that only one (of six) muscle had increased trypsin-like (proteasome) activity. In all six muscles tested, including the left ventricle, no differences in caspase-like and chymotrypsin-like (proteasome) activities were seen. Similarly, none of the five skeletal muscles or the left ventricle had significant increases in calpain 1 and 2 activities. In addition, transcriptional regulation of the ubiquitin-proteasome system was most pronounced in the heart, where numerous components were significantly decreased, including the ubiquitin ligases CHIP

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and MDM2, and the E2 enzyme uncoordinated phenotype 4/5 (UNC4/5). The left ventricle also had decreased calpain 1 and 2 expression, without affecting the overall calpain 1 and 2 activities. These findings illustrate the muscle-specific differences in calpain and ubiquitin-proteasome system expression and activity in GRMD, a DMD model that parallels human disease in many ways. These findings illustrate how proteasome and calpain inhibitors used to treat DMD might have unexpected consequences that are muscle-specific, particularly in the heart.

**Materials and Methods**

**Animals.** Dogs were cared for and utilized according to principles outlined in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health). Newborn GRMD dogs were identified based on elevation of serum creatine kinase (CK). Genotype was confirmed by polymerase chain reaction (PCR) when CK results were ambiguous. Dogs subsequently developed characteristic clinical signs. Long digital extensor, lateral head of gastrocnemius, vastus lateralis, biceps femoris, cranial sartorius, and left ventricle of the heart were harvested from 7 GRMD and 8 control dogs at 6 months of age (Table 1). Dogs were anesthetized using conventional pre-anesthetic drugs, propofol (normal dogs only) and sevoflurane. Of the 15 dogs, 12 were harvested after being euthanized, and 3 underwent biopsy before recovery and subsequent adoption (see Table 1). The muscle(s) were exposed sharply at surgery to allow removal of a sample of approximately 1 x 0.5 x 0.5 cm, snap frozen in liquid nitrogen, and stored at -80°C for further processing. These studies have been approved by the institutional animal care and use committee at the University of North Carolina.

**Real-Time PCR Analysis of Ubiquitin-Proteasome System Components.** Total RNA was isolated, cDNA was generated, and PCR products were amplified as described previously. TaqMan probes were from Applied Biosystems, Inc. (Carlsbad, California) and included dog-specific probes for calpain 1 (Cf02704115_m1), calpain 2 (Cf02645870_m1), CHIP (Cf02644017_m1), muscle atrophy F-box (MAFbx; Cf02667148_mi), MDM2 (Cf026759237_m1), muscle ring finger protein 1 (MuRF1; Cf02649993_mi), proteasome subunit alpha type 6 (PSMA6; Cf02666165_g1), proteasome subunit beta type 4 (PSMB4; Cf01123846_m1), proteasome subunit activator type 1 (PSME1;
Cf02646187_g1), ubiquitin (Mm01622233_g1), UBC9 (Cf02655738_g1), and UNC4/5 (Cf02657121_m1). Relative mRNA expression was normalized to 18S (Hs99999901_s1).

**Measuring 26S Proteasome Activity Using Fluorogenic Substrates.** Assaying specific 26S proteasome activities was performed as previously described\(^{247-249}\). Briefly, tissue was homogenized in lysis buffer [250 mM sucrose, 50 mM Tris (pH 7.5), 5 mM MgCl\(_2\), 0.5 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol, 2 mM adenosine triphosphate (ATP), and 0.025% digitonin. Approximately 25 μg of protein was added to the proteasome reaction buffer [50 mM Tris (pH 7.5), 40 mM KCl, 1 mM MgCl\(_2\), 1 mM dithiothreitol, 0.5 mM ATP, and 0.05 mg/ml bovine serum albumin] that contained fluorogenic substrates (75 μM of Suc-LLVY-AMC for chymotrypsin-like activity, 150 μM of Boc-LRR-AMC for trypsin-like activity, 75 μM of Ac-nL-PnLD-AMC for caspase-like activity; Enzo Life Sciences International, Inc., Farmingdale, New York). Fluorescence was measured using a spectrophotometer (excitation 355 nm, emission 460 nm; Wallace Victor 2) 40 times every 2 minutes at 37°C. Parallel samples were preincubated with the proteasome inhibitor epoxomicin (20 μM) for 30 min at 37°C to determine the non-specific substrate hydrolysis. These fluorescence units were then subtracted from each measurement.

**Measuring Calpain 1 and 2 Activity Using Fluorogenic Substrates.** Calpain 1 and 2 activity assays were performed using the Sensolyte AMC Calpain Activity Flurometric Assay Kit, according to the manufacturer’s protocol (Anaspec, Inc., Fremont, California). Briefly, calpain 1 and 2 activity in tissue extracts was assessed by hydrolysis of the fluorogenic peptide substrate succinyl-leucine–leucine–valine–tyrosine–4-methyl-7-coumarylamide (Suc-LLVY-AMC). Fifty micrograms per well of tissue extract was placed into a 96-well black opaque plate followed by addition of the substrate solution. Fluorescence was measured using a GENios microplate reader (excitation 355 nm and emission 442 nm; Tecan Group, Ltd., Durham, North Carolina).

**Summaries of Previous Studies of Muscular Dystrophy, Proteasome, and Calpains.** Summaries of previous studies are shown in Tables 11 and 12. The studies were identified by searching PubMed using the search criteria indicated in the tables.
**Statistical Analysis.** A rank-sum test was performed because most of the data were determined to be non-parametric using a normality test and (when appropriate) an equal variance test in SigmaStat 2.03 (Systat Software, Inc., San Jose, California). Statistical significance was set at $P < 0.05$.

**Results**

**Differential Expression of Ubiquitin-Proteasome Components Is Muscle-Specific.** The ubiquitin ligases MuRF1 and MAFbx/atrogen-1 have been prominently studied in skeletal muscle atrophy and are linked mechanistically to degradation of the sarcomere$^{57}$. Their role in cardiac hypertrophy, atrophy, and metabolism has also recently been reported$^{52, 68, 73, 81}$. Therefore, we investigated their expression in the GRMD model. MuRF1 and MAFbx/atrogen-1 mRNA levels in GRMD did not differ from age-matched controls in the muscles investigated (Figure 33A and B). The five GRMD skeletal muscles (long digital extensor, lateral head of the gastrocnemius, vastus lateralis, biceps femoris, and cranial sartorius) generally had increased MuRF1 and MAFBx expression compared with controls, but these increases were not uniform.

Apoptosis has also been linked to the pathophysiology of DMD$^{250-252}$. The ubiquitin ligases CHIP and MDM2 have been reported to inhibit apoptosis by targeting p53 for degradation by the proteasome$^{253-256}$. Furthermore, CHIP has been implicated in the pathogenesis of a *Caenorhabditis elegans* form of muscular dystrophy$^{257}$. We therefore measured CHIP and MDM2 expression in GRMD. Both CHIP and MDM2 were significantly decreased in the left ventricle (Figure 33C and D); MDM2 was increased only in the vastus lateralis (Figure 33D). Expression of CHIP and MDM2 did not differ significantly from that in control animals in the long digital extensor, lateral head of the gastrocnemius, biceps femoris, and cranial sartorius (Figure 33C and D). However, individual animals had increased CHIP and MDM2 in the biceps femoris and cranial sartorius. Expression of ubiquitin ligases in GRMD was largely unaffected in the skeletal muscles, whereas downregulation of two of the four investigated in this study was seen in the heart, as summarized in Table 9.
To determine whether expression of other UPS components are altered in GRMD, we measured mRNA expression levels of the ubiquitin UNC4/5, a protein chaperone involved in integrating myosin into the sarcomere\(^{258}\), and ubiquitin-like protein SUMO-1–conjugating enzyme 9 (UBC9), the sole conjugating (E2) enzyme for the small ubiquitin like modifier protein (SUMO)\(^{259}\). There were no differences in mRNA expression levels of ubiquitin between GRMD and control muscles (Figure 34A), consistent with a previous study in DMD patients\(^{237}\). UNC4/5 expression was decreased in GRMD left ventricle compared with controls, but it was unaffected in the five skeletal muscles (Figure 34B). UBC9 expression was significantly increased only in the cranial sartorius compared with controls (Figure 34C).

**Expression of Proteasome Subunits Is Significantly Decreased in GRMD Heart.** We next determined the expression of three different proteasome subunits: PSMA6, PSMB4, and PSME1 (Figure 35). PSMA6 and PSMB4 are non-catalytic subunits of the 20S proteasome\(^{59}\), and PSME1 is the alpha subunit in the activator heteroheptamer ring, which binds to one or both ends of the 20S proteasome and allows peptides to enter the 20S subunit\(^{59}\). GRMD left ventricle showed a significant decrease in the expression of all three proteasome subunits (Figure 35A–C). PSMB4 was increased in four of the five GRMD skeletal muscles (Figure 35B). Expression of PSMA6 and PSME1 was unchanged in all GRMD skeletal muscles.

**Proteasome Activity Is Generally Unchanged in GRMD Skeletal Muscle and Heart.** Using a fluorometric assay, we assayed proteasome trypsin-like, caspase-like, and chymotrypsin-like activities from GRMD and control animals in skeletal muscles and heart (Figure 36). The only significantly different proteasome activity in GRMD muscles was found in the lateral head of the gastrocnemius, with increased trypsin-like activity. Individual animals had increases in caspase-like and chymotrypsin-like activity in the gastrocnemius as well, but these increases were not uniform. Similarly, all three activities were increased in the long-digital extensor, but this was not uniform among all the animals (i.e., not significant). The disconnect between the decreased proteasome
expression shown in Figure 36 and the unaffected proteasome activities is pronounced in the GRMD left ventricle (Table 10).

**Calpain 1 and 2 Expression Is Decreased in GRMD Heart.** Calpains are Ca\(^{2+}\)-dependent cysteine proteases that initiate the release of proteins from the myofibril, making myofibrillar proteins available for degradation by the UPS\(^{260}\). Calpain 1 (µ-calpain) and calpain 2 (m-calpain), so named because they are activated by micromolar and millimolar Ca\(^{2+}\) concentrations, respectively, are the most widely studied members of the calpain family and have been shown to be involved in the pathogenesis of DMD\(^{232, 237, 238, 242, 261}\). Because we found that the UPS is altered in GRMD muscle, we next measured calpain 1 and 2 expression in GRMD. Both calpain 1 and 2 expression were significantly decreased in the GRMD left ventricle (Figure 37A and B). Calpain 2 expression was increased in many of the animals in all five GRMD skeletal muscles, although only three muscle groups reach significance (gastrocnemius, vastus lateralis, and cranial sartorius) (Figure 37B).

**Calpain 1 and 2 Activity Is Not Uniformly Increased in GRMD Skeletal Muscles and Heart.** Calpain 1 and 2 activities were not significantly different from that of age-matched controls in any of the skeletal muscles or left ventricle (Figure 37C). However, four of the five skeletal muscles and the left ventricle had individuals with increased calpain 1 and 2 activity, which did not reach significance.

**Discussion**

For nearly two decades, the role of intracellular proteolysis has been appreciated in DMD\(^{153}\). At least eight studies have reported that calpain and/or proteasome inhibition is beneficial in disease (summarized in blue in Tables 11 and 12)\(^{232-236, 239-241}\). Paralleling the therapeutic utility of calpain and proteasome inhibition with the mdx mouse phenotype, there has been largely uniform evidence in four studies that calpain and proteasome activities are enhanced in DMD (summarized in white in Tables 11 and 12). What is striking about these studies is that they largely investigated short-term proteasome and calpain inhibition on mainly the milder mdx muscular dystrophy in a very limited number of skeletal muscles, generally three or less. In this study, we investigated more broadly how
muscular dystrophy in the GRMD model affected components of the ubiquitin-proteasome system and the proteasome and calpain activities (summarized in Tables 9 and 10 and the Supplemental Results Section). Although we detected increased proteasome and calpain activities in a minority of the muscles tested, we found that the heart had dramatically decreased expression of ubiquitin ligases (CHIP, MDM2), the proteasome (PSMA6, PSMB4, PSME1), and calpains (calpain 1 and calpain 2). This raises the concern that proteasome inhibition may inhibit proteasome and calpain activities in muscles that do not have increased proteolysis and may potentially further inhibit muscles that have decreased expression of ubiquitin proteasome components and calpains, particularly in the heart.

Inhibiting proteasome and calpain activities in muscles without increased activity is worrisome considering their diverse roles in the maintenance of the cell. This is a point that may have become obscured by the large number of studies implicating these proteolytic systems in the degradation of specific substrate targets in the sarcomere. Activity of the proteasome has been implicated at multiple points in the regulation of gene expression. The 26S proteasome specifically plays a role in modifying activators, co-activators, and co-repressors of transcription necessary for gene transcription. Proteasome activity is also essential in numerous signaling pathways, including calcineurin, β-catenin, NF-κB, and caspases, which are responsible for the regulation of cell death. Similar to the proteasome, the calpain system plays diverse roles in cell biology. For example, calpains have been implicated in proliferation, differentiation, cell cycle progression, apoptosis, and cell signaling. Calpain activity has been implicated in signal transduction through PKC, GSK3β, calcium/calmodulin-dependent protein kinase (CaMK) II and IV, MLK kinase, and calcineurin (recently reviewed by Bukowska et al.). Calpains have also been implicated in the regulation of transcription factor regulation, including NF-κB, AP-1/c-Jun/c-Fos, and c-Myc, among others. Given the diverse roles both the proteasome and calpains play in cell biology, inhibiting their activity broadly throughout all cells could potentially uncover many side effects, at the very least, and potentially worsen outcomes.
Examples of potential side effects come from experimental studies of proteasome inhibitors. Proteasome inhibitors are useful for the treatment of multiple myeloma, because they enhance cell death in myeloma cells\textsuperscript{267}. How the heart may be affected by these therapies is just now starting to be appreciated. For example, inhibiting the proteasome in cardiomyocytes can induce cell death in cultured cardiomyocytes\textsuperscript{268, 269} and can adversely affect cardiac function in hearts challenged by pressure overload\textsuperscript{168, 270}. In humans treated with proteasome inhibitors for multiple myeloma, ischemic heart disease complications have been reported\textsuperscript{167}, raising the concern of systemic proteasome inhibition. The activity of calpains in cardiomyocytes has also been implicated in cardiac pathology\textsuperscript{266}. Although inhibiting calpains experimentally has been shown to reduce myocardial stunning, contractile dysfunction due to tachypacing, and damage due to atrial fibrillation, long-term therapeutic benefits have not been studied\textsuperscript{266}. For example, although calpain inhibition may inhibit apoptosis in some cardiac conditions, it is not clear if this is protective or whether it induces alternative forms of cell death, namely necrosis \textsuperscript{19813082}. Last, the complexity of interpreting all these studies is that DMD patients largely have an underlying cardiac defect caused by DMD itself. Specifically, most DMD patients exhibit dilated cardiomyopathy and ventricular arrhythmias due to their disease\textsuperscript{271-277}, thus the heart has increases susceptibility to further insult. This should raise concerns for unexpected side effects from inhibiting proteasome or calpain activity in a more complex milieu of DMD-related cardiac disease and should highlight the need for testing these therapies in DMD patients.

Limiting factors in this investigation include the number of animals studied and the variability seen in the GRMD disease model. Although statistical differences were seen in 19 of the parameters tested, an additional 19 parameters had trends that were close, but not statistically different (see Tables 9 and 10). The trends that did not reach statistical significance were likely due to the fact that our study utilized a limited number of highly variable samples. This variation makes it more difficult to delineate differences because of the heterogeneity of the population being investigated. Despite these weaknesses, our major conclusions are largely not affected by these
issues and allow us to show that both the calpain and ubiquitin-proteasome systems are differentially regulated by different muscle types and are for the most part not increased.

Although we have emphasized the expanding role of the ubiquitin-proteasome and calpain systems in diverse biological processes, they are best known for their roles in protein quality control in muscle\textsuperscript{262, 263}. Because widespread destruction of muscle and the sarcomere is involved in the pathophysiology of muscular dystrophy, inhibiting the destruction of damaged proteins may be one reason that proteasome and calpain inhibition have worked. By slowing the destruction of damaged sarcomere, more sarcomere is kept and used, despite its imperfect quality. The build-up of posttranslational modifications and reduced quality control could be the compromise needed to maintain function. However, the concern is not so much for the few skeletal muscles improved with proteasome and/or calpain inhibition. It is more for the heart, which in DMD patients is compromised to begin with and insulted further by inhibition of two of the three proteolytic systems necessary for maintaining the cardiomyocytes.

Figure Legends

**Figure 33. Ubiquitin ligase (E3) expression in golden retriever muscular dystrophy (GRMD).** Quantitative mRNA expression analysis of the ubiquitin ligases (A) muscle ring finger 1 (MuRF1), (B) muscle F-box protein (MAFbx), (C) carboxyl terminus of Hsp70-interacting protein (CHIP), and (D) mouse double minute-2 (MDM2) in GRMD lateral digital extensor, the lateral head of the gastrocnemius, vastus lateralis, biceps femoris, left ventricle, and cranial sartorius. mRNA expression is shown as the fold change compared with age-matched control animals. Data represent 3–5 dogs per group (outlined in Table 13), as indicated above each vertical boxplot. The vertical boxplot presents the median (50% percentile), indicated by the middle line inside the box, the 75th percentile indicated by the top of the box, and the 25th percentile indicated by the bottom of the box. A rank-sum test was used to determine the differences in mRNA expression. *$P< 0.05$.

**Figure 34. Expression of ubiquitin, UNC4/5, and the E2 ubiquitin-conjugating enzyme UBC9 in GRMD skeletal muscle and heart.** Quantitative mRNA expression analysis of (A)
ubiquitin, (B) the protein chaperone UNC4/5, and (C) E2 UBC9 in the GRMD lateral digital extensor, 
the lateral head of the gastrocnemius, vastus lateralis, biceps femoris, left ventricle, and cranial 
sartorius. mRNA expression is shown as the fold change compared with age-matched control animals. 
Data represent 3–5 dogs per group (outlined in Table 13), as indicated above each vertical boxplot. 
The vertical boxplot presents the median (50th percentile), indicated by the middle line inside the 
box, the 75th percentile indicated by the top of the box, and the 25th percentile indicated by the 
bottom of the box. A rank-sum test was used to determine the differences in mRNA expression. *P < 0.05.

Figure 35. Proteasome subunit expression in GRMD skeletal muscle and heart.
Quantitative mRNA expression analysis of the proteasome subunits (A) PSMA6, (B) PSMB4, 
and (C) PSME1 in GRMD long digital extensor, lateral head of the gastrocnemius, vastus lateralis, 
biceps femoris, left ventricle, and cranial sartorius. mRNA expression is shown as the fold change 
compared with age-matched control animals. Data represent 3–5 dogs per group (outlined in Table 
13), as indicated above each vertical boxplot. The vertical boxplot presents the median (50th 
percentile), indicated by the middle line inside the box, the 75th percentile indicated by the top of the 
box, and the 25th percentile indicated by the bottom of the box. A rank-sum test was used to 
determine the differences in mRNA expression. *P < 0.05.

Figure 36. Muscle-specific proteasome activities in GRMD. (A) Trypsin-like, (B) caspase- 
like, and (C) chymotrypsin-like activities in GRMD long digital extensor, lateral head of the 
gastrocnemius, vastus lateralis, biceps femoris, left ventricle, and cranial sartorius. Proteasome 
activities are presented as arbitrary fluorescent units (AFU). Data represent 3–5 dogs per group 
(outlined in Table 13), as indicated above each vertical boxplot. The vertical boxplot presents the 
median (50th percentile), indicated by the middle line inside the box, the 75th percentile indicated by 
the top of the box, and the 25th percentile indicated by the bottom of the box. A rank-sum test was 
used to determine the differences in proteasome activity. *P < 0.05.
Figure 37. Muscle-specific calpain 1 and 2 activities in GRMD. Quantitative mRNA expression analysis of (A) calpain 1, and (B) calpain 2 in GRMD long digital extensor, lateral head of the gastrocnemius, vastus lateralis, biceps femoris, left ventricle, and cranial sartorius. Expression levels are presented as a percentage of age-matched controls. (C) Calpain 1 and 2 activity determined by a fluorometric assay based on GRMD long digital extensor, lateral head of the gastrocnemius, vastus lateralis, biceps femoris, left ventricle, and cranial sartorius. mRNA expression is shown as the fold change compared with age-matched control animals. Calpain 1 and 2 activity presented as relative fluorometric units. Data represent 3–5 dogs per group (outlined in Table 13), as indicated above each vertical boxplot. The vertical boxplot presents the median (50th percentile), indicated by the middle line inside the box, the 75th percentile indicated by the top of the box, and the 25th percentile indicated by the bottom of the box. A rank-sum test was used to determine the differences in mRNA expression and calpain 1 and 2 activity. *P < 0.05.

Supplemental Results

Previous studies of the proteasome and calpain systems in muscular dystrophy. The effects of proteasome inhibition on the mdx mouse phenotype has been reported at least 3 times on mice 7 weeks, 6 months, and 8 months old (Table 11). These studies have shown improvement in terms of the dystrophin-glycoprotein-complex stability histologically in the gastrocnemius and diaphragm using mdx mice 7 weeks to 8 months old as determined by muscle architecture after 24 hours to 14 days. In humans, the proteasome inhibitor Velcade was just reported to stabilize the dystrophin-glycoprotein-complex in quadricep explants from 8 DMD patients after 16 hours at 2 doses. While these studies are impressive, they do not address a number of issues raised in the present study. First, they have not looked at the effect of proteasome therapy in a diversity of muscles, particularly the heart, where the ubiquitin proteasome system has been shown to regulate many signaling processes associated with cardiac disease (as recently reviewed). The present study demonstrates that many skeletal muscles and the heart do not have enhanced proteasome activities (Table 9), raising the question of how therapies which inhibit the proteasome
may complicate DMD disease by altering the protein quality control mechanisms largely attributed to the ubiquitin proteasome system\(^{103, 265, 278, 279}\).

Five previous studies have reported the efficacy of calpain inhibition in the mdx model of DMD (Table 12). \textit{Mdx} mice ages 2-5 weeks old were treated with pharmacologic calpain inhibitors or had transgenic expression of the calpain inhibitor calpastatin\(^{232, 235, 236, 240, 241}\). These studies found histological and functional improvements in \(~6\) muscles after 4 weeks of treatment. However, studies investigating the effects of calpain inhibition for 6 months did not find durable improvement in diaphragm function\(^{240}\). Since only the diaphragm was tested in this study, it is difficult to extrapolate these findings to other critical muscles. Similarly, it is difficult to extrapolate the uniform improvement of muscle histologically over short term treatments in a model of DMD that is so mild (mdx). In more severe disease, such as GRMD, it is not clear how calpain inhibition would affect the muscles which do not have enhanced calpain activities, such as the gastrocnemius, vastus lateralis, and cranial sartorius (Table 9).

**Supplemental Discussion**

In the current study, we identified that the expression of ubiquitin is not significantly affected in GRMD (Figure 26A). Only 1 previous study has investigated how the expression of ubiquitin is regulated in DMD patients. Like the current study, 5 patients ages 7-21 did not have changes in ubiquitin expression\(^{290}\). The changes in UNC4/5 and UBC9 found in the current study have previously not been investigated.

The findings in the current study suggest that the proteasome number is decreased in the heart. The expression of the \(\beta\)-subunit of the proteasome (e.g. PSMB4) is generally indicative of the number of assembled proteasomes in the cell\(^{280, 281}\). In Figure 27B, we identified decreased PSMB4, which may indicate a decrease in proteasome number in the GRMD heart. Yet PSMB4 is increased in four of the five GRMD skeletal muscles tested (Figure 27B). A study of 2 DMD patients reported that biceps and rectus femoris biopsies had significantly increased 26S proteasome-positive fibers by IHC\(^{154}\). Another study of 5 DMD patients (ages 7-21) identified no changes in the RC2 and HC2
proteasome subunits in the deltoid, triceps, hip adductor, and tensor fasciae\textsuperscript{237}. The findings in the current study on skeletal muscle are consistent with both of these studies with either increases of or no effect on proteasome numbers. Our identification of decreased expression of all three proteasome subunits in in the heart is the first known report of the cardiac proteasome makeup in DMD and/or DMD models and may reflect its unique biology.

Despite either increases in PSMB4 subunits in 4 of the 5 skeletal muscles, we only identified increases in proteasome activity in the lateral head of the gastrocnemius. Here we only saw increases in the trypsin like activity (\textbf{Figure 28A}). This disconnect between expression and activity may be due to the influence of post-translational modifications on proteasome activity, such as phosphorylation. For example, recent studies have shown that PKA phosphorylation of the catalytic subunits in the core 20S proteasome in the heart stimulates all three types of proteasome activities\textsuperscript{282}. Since abnormal changes in PKA localization and activity have been reported in dystrophic muscle\textsuperscript{283},\textsuperscript{284}, it is possible that dystrophic regulation of PKA may alter proteasome activities, and these alterations are muscle/heart specific.

A similar disconnect between increased calpain 2 mRNA expression and activity was also identified in \textbf{Figure 29}. The increased calpain 2 expression in the 3 skeletal muscles did not affect the calpain 1/2 activity (gastrocnemius, vastus lateralis, cranial sartorius) (\textbf{Table 9}). Similarly, the decreased calpain 1 and 2 expression did not affect calpain 1/2 activity in the left ventricle (\textbf{Table 9}). One possible explanation for this disconnect may be the muscle-specific levels of Ca\textsuperscript{2+} in GRMD muscles. Increased intracellular Ca\textsuperscript{2+} has been reported in the necrotic fibers of several GRMD muscles\textsuperscript{285}. In addition, the flexor digitorum brevis, lumbrical muscles of the hind limb, isolated myotubes, and cardiomyocytes of \textit{mdx} mice have been shown to have increased intracellular Ca\textsuperscript{2+} concentration\textsuperscript{286-290}. Millay and colleagues\textsuperscript{291} have shown that Ca\textsuperscript{2+} influx alone, through the overexpression of a nonselective cation channel called transient receptor potential canonical 3 (TRPC3), can induce a muscular dystrophy-like phenotype in mouse quadriceps. The increased intracellular Ca\textsuperscript{2+} in dystrophic myocytes is believed to occur by several mechanisms, including the
dysregulation of sarcolemmal and sarcoplasmic reticulum Ca\(^{2+}\) channels, microruptures in the sarcolemma and the sarcoplasmic reticulum, and involvement of Ca\(^{2+}\)-binding proteins\(^{292}\). Therefore, muscle-specific increases in Ca\(^{2+}\) may therefore drive calpain 1/2 activity in GRMD muscles, independent of the expression of calpain 1 and 2.

The X-linked GRMD model of muscular dystrophy is caused by a mutation at the consensus splice acceptor site in intron 6 of the canine dystrophin gene\(^{293}\). Previous studies on the GRMD cohort used in these studies have determined that specific skeletal muscle types atrophy (long digital extensor) while others demonstrate true hypertrophy (cranial sartorius), as summarized in Table 9, right columns\(^{161}\). In the present study we found that the muscle with severe atrophy (long digital extensor) unique had the most upregulation of the ubiquitin proteasome and calpain systems, although many of these are near significance (Table 9, top row). However, muscles undergoing atrophy (gastrocnemius and biceps formalis) or hypertrophy (cranial sartorius) do not have unique UPS/calpain system expression or activity signatures (Table 9), indicating that the 2 may not necessarily be related and/or the complex pathophysiology of DMD may be complicating the relationship between the two.
TABLE 1 – Echocardiographic analysis of conscious MuRF1 +/- and sibling-matched wildtype control mice at baseline and 5 weeks after voluntary running (or sham conditions).

<table>
<thead>
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<th>M mode Measure</th>
<th>MuRF1 +/- Baseline N=7</th>
<th>MuRF1 +/- Sham N=10</th>
<th>MuRF1 +/- Baseline Running N=7</th>
<th>MuRF1 +/- 5 Weeks Sham N=8</th>
<th>MuRF1 +/- 5 Weeks Running N=10</th>
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<td>1.07±0.01</td>
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<td>LV Anterior Wall v</td>
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<td>Heart Rate</td>
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Rarely, mice stopped running and were excluded from further study (preventing a repeated measures statistical analysis). Data is represented as mean±standard error. A one-way ANOVA was performed to compare among all 8 groups. *p<0.01 vs. all other groups. **p<0.05 vs. all baseline

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http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013 DOI:10.1152/ajpendo.00326.2013
TABLE 2 – Echocardiographic analysis of conscious MuRF1 Tg+ and sibling-matched wildtype control mice at baseline and 5 weeks after voluntary running (or sham conditions).

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<th>MuRF1 Wildtype Baseline Running N=10</th>
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<th>MuRF1 Wildtype 5 Weeks Sham N=9</th>
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<td>LV Posterior Wall d</td>
<td>1.04±0.01</td>
<td>0.85±0.02</td>
<td>0.99±0.03</td>
<td>0.81±0.02</td>
<td>1.07±0.02</td>
<td>0.84±0.02</td>
<td>1.22±0.03</td>
<td>1.02±0.04</td>
</tr>
<tr>
<td>LV Anterior Walls s</td>
<td>1.72±0.04</td>
<td>1.46±0.02</td>
<td>1.67±0.04</td>
<td>1.48±0.03</td>
<td>1.73±0.03</td>
<td>1.51±0.03</td>
<td>1.89±0.04</td>
<td>1.68±0.04</td>
</tr>
<tr>
<td>LV LVIDs s</td>
<td>1.09±0.05</td>
<td>1.68±0.06</td>
<td>1.28±0.09</td>
<td>1.76±0.07</td>
<td>1.17±0.05</td>
<td>1.75±0.05</td>
<td>1.23±0.09</td>
<td>1.65±0.11</td>
</tr>
<tr>
<td>LV Posterior Walls s</td>
<td>1.56±0.05</td>
<td>1.26±0.02</td>
<td>1.51±0.05</td>
<td>1.25±0.03</td>
<td>1.63±0.04</td>
<td>1.33±0.02</td>
<td>1.77±0.07</td>
<td>1.41±0.04</td>
</tr>
<tr>
<td>LV V0,d</td>
<td>23.0±1.8</td>
<td>32.9±3.1</td>
<td>28.0±2.1</td>
<td>36.2±2.0</td>
<td>28.2±2.3</td>
<td>36.3±1.6</td>
<td>26.5±2.1</td>
<td>31.9±2.8</td>
</tr>
<tr>
<td>LV V0,s</td>
<td>2.7±1.0</td>
<td>8.4±0.8</td>
<td>4.4±0.8</td>
<td>9.5±0.9</td>
<td>3.3±0.3</td>
<td>9.3±0.7</td>
<td>8.4±1.5</td>
<td>8.4±1.5</td>
</tr>
<tr>
<td>%EF</td>
<td>88.3±0.6</td>
<td>74.3±1.1</td>
<td>84.9±2.0</td>
<td>74.1±1.6</td>
<td>88.4±0.5</td>
<td>74.2±1.2</td>
<td>85.6±2.0</td>
<td>75.0±2.7</td>
</tr>
<tr>
<td>% FS</td>
<td>56.5±0.6</td>
<td>41.9±1.0</td>
<td>53.1±2.1</td>
<td>42.0±1.4</td>
<td>57.0±0.7</td>
<td>42.2±1.1</td>
<td>53.9±2.3</td>
<td>42.9±1.8</td>
</tr>
<tr>
<td>Heart Rate</td>
<td>704±9</td>
<td>705±9</td>
<td>731±8</td>
<td>718±14</td>
<td>750±8</td>
<td>737±13</td>
<td>754±10</td>
<td>739±7</td>
</tr>
</tbody>
</table>
Rarely, mice stopped running and were excluded from further study (preventing a repeated measures statistical analysis). Data is represented as mean±standard error. A one-way ANOVA was performed to compare among all 8 groups. *p<0.01 vs. all other groups. **p<0.05 vs. all other groups (except MuRF1 Wildtype Baseline Sham). &p<0.01 vs. MuRF1 Wildtype Baseline groups. &&p<0.01 vs. all wildtype groups.

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9 As of this writing, this table appears in an Article in Press (published January 14, 2014) in *American Journal of Physiology Endocrinology and Metabolism* at the following website: http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013 DOI: 10.1152/ajpendo.00326.2013
TABLE 3 – Voluntary wheel running exercise performance of MuRF1+/+ and MuRF1/-/ mice.

<table>
<thead>
<tr>
<th>Running Time (min/day)</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Overall (Weeks 1-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MuRF1+/+</td>
<td>329.8±28.8</td>
<td>348.4±18.5</td>
<td>323.0±25.4</td>
<td>359.8±19.3</td>
<td>341.3±16.3</td>
<td>343.1±14.8</td>
</tr>
<tr>
<td>MuRF1/-/</td>
<td>349.9±28.8</td>
<td>375.5±24.6</td>
<td>325.1±24.3</td>
<td>384.5±17.0</td>
<td>382.3±31.1</td>
<td>363.7±18.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Speed (mph)</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Overall (Weeks 1-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MuRF1+/+</td>
<td>2.80±0.199</td>
<td>3.30±0.161</td>
<td>3.31±0.171</td>
<td>3.59±0.146</td>
<td>3.64±0.169</td>
<td>3.31±0.142</td>
</tr>
<tr>
<td>MuRF1/-/</td>
<td>3.46±0.224*</td>
<td>4.84±1.71</td>
<td>4.52±0.303*</td>
<td>4.92±0.256#</td>
<td>4.92±0.407*</td>
<td>4.47±0.279*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Distance (km)</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Overall (Weeks 1-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MuRF1+/+</td>
<td>10.1±1.2</td>
<td>12.2±0.93</td>
<td>11.2±0.96</td>
<td>13.9±0.87</td>
<td>13.1±0.91</td>
<td>12.1±0.73</td>
</tr>
<tr>
<td>MuRF1/-/</td>
<td>13.0±1.7</td>
<td>19.0±2.1*</td>
<td>15.6±1.83*</td>
<td>19.5±1.32*</td>
<td>19.9±2.5*</td>
<td>17.2±1.5*</td>
</tr>
</tbody>
</table>
MuRF1+/+ n=8-9 per group per week; MuRF1-/− n=8 per week. Rarely, mice stopped running and were excluded from further study (preventing a repeated measures statistical analysis). Data is represented as mean±standard error. A Student’s t-test was performed to compare MuRF1+/+ and MuRF1-/− for each week. *p<0.05, #<0.00110

10 As of this writing, this table appears in an Article in Press (published January 14, 2014) in American Journal of Physiology Endocrinology and Metabolism at the following website: http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013 DOI:10.1152/ajpendo.00326.2013
### TABLE 4 – Voluntary wheel running exercise performance of Wildtype$^{\text{MuRF1Tg+}}$ and MuRF1Tg+ mice

<table>
<thead>
<tr>
<th>Running Time (min/day)</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Overall (Weeks 1-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype$^{\text{MuRF1Tg+}}$</td>
<td>419.4±19.0</td>
<td>383.0±31.2</td>
<td>332.5±26.1</td>
<td>309.4±38.4</td>
<td>324.4±37.5</td>
<td>357.0±20.0</td>
</tr>
<tr>
<td>MuRF1Tg+</td>
<td>387.9±35.1</td>
<td>361.9±35.8</td>
<td>351.7±31.7</td>
<td>342.2±38.8</td>
<td>378.7±51.8</td>
<td>366.2±32.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Speed (mph)</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Overall (Weeks 1-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype$^{\text{MuRF1Tg+}}$</td>
<td>3.20±0.179</td>
<td>4.29±0.188</td>
<td>3.92±0.197</td>
<td>3.83±0.146</td>
<td>4.29±0.216</td>
<td>3.87±0.141</td>
</tr>
<tr>
<td>MuRF1Tg+</td>
<td>3.38±0.177</td>
<td>4.14±0.281</td>
<td>4.36±0.373</td>
<td>4.13±0.417</td>
<td>4.50±0.298</td>
<td>4.09±0.286</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Distance (km)</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Overall (Weeks 1-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype$^{\text{MuRF1Tg+}}$</td>
<td>14.0±0.82</td>
<td>17.6±1.6</td>
<td>14.1±1.6</td>
<td>12.5±1.8</td>
<td>14.9±2.3</td>
<td>14.5±1.1</td>
</tr>
<tr>
<td>MuRF1Tg+</td>
<td>14.0±1.2</td>
<td>15.8±1.1</td>
<td>15.8±1.6</td>
<td>15.6±2.7</td>
<td>17.2±2.1</td>
<td>15.7±1.4</td>
</tr>
</tbody>
</table>
Wildtype\textsuperscript{MuRF1Tg+} n=6-8 per week; MuRF1Tg+ n=7 per week. Rarely, mice stopped running and were excluded from further study (preventing a repeated measures statistical analysis). Data is represented as mean±standard error. A Student’s t-test was performed to compare Wildtype\textsuperscript{MuRF1Tg+} and MuRF1Tg+ for each week. No differences between groups was identified (p>0.05)\textsuperscript{11}

\textsuperscript{11} As of this writing, this table appears in an Article in Press (published January 14, 2014) in \textit{American Journal of Physiology Endocrinology and Metabolism} at the following website: http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013 DOI:10.1152/ajpendo.00326.2013
TABLE 5 – High resolution transthoracic echocardiography performed on conscious MuRF1/- and age-matched wild type mice at baseline, 1 week, and 2 weeks after daily i.p. T3 treatment.

<table>
<thead>
<tr>
<th></th>
<th>MuRF1 +/+ Baseline N=10</th>
<th>MuRF1 +/- Baseline N=12</th>
<th>MuRF1 +/- Thyroid Hormone 1 Week N=10</th>
<th>MuRF1 +/- Thyroid Hormone 1 Week N=12</th>
<th>MuRF1 +/- Thyroid Hormone 2 Weeks N=10</th>
<th>MuRF1 +/- Thyroid Hormone 2 Weeks N=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>AWTD (mm)</td>
<td>1.05±0.02</td>
<td>1.13±0.04</td>
<td>1.20±0.03</td>
<td>1.60±0.09</td>
<td>1.38±0.04</td>
<td>1.70±0.07</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.03±0.11</td>
<td>3.08±0.12</td>
<td>3.13±0.13</td>
<td>3.87±0.15</td>
<td>3.11±0.11</td>
<td>3.00±0.14</td>
</tr>
<tr>
<td>PWTD (mm)</td>
<td>0.97±0.02</td>
<td>1.04±0.04</td>
<td>1.12±0.02</td>
<td>1.37±0.07</td>
<td>1.28±0.05</td>
<td>1.58±0.05</td>
</tr>
<tr>
<td>AWTS (mm)</td>
<td>1.77±0.04</td>
<td>1.82±0.06</td>
<td>1.97±0.02</td>
<td>2.23±0.08</td>
<td>2.23±0.07</td>
<td>2.44±0.06</td>
</tr>
<tr>
<td>LVEDS (mm)</td>
<td>1.36±0.05</td>
<td>1.42±0.07</td>
<td>1.44±0.08</td>
<td>1.11±0.07</td>
<td>1.42±0.08</td>
<td>1.25±0.08</td>
</tr>
<tr>
<td>PWTS (mm)</td>
<td>1.58±0.01</td>
<td>1.65±0.07</td>
<td>1.81±0.06</td>
<td>2.10±0.08</td>
<td>1.93±0.08</td>
<td>2.18±0.07</td>
</tr>
<tr>
<td>LV Vol;d (µl)</td>
<td>36.5±3.3</td>
<td>38.2±3.8</td>
<td>39.8±3.7</td>
<td>32.6±3.8</td>
<td>39.0±3.4</td>
<td>36.3±4.1</td>
</tr>
<tr>
<td>LV Vol;s (µl)</td>
<td>4.8±0.5</td>
<td>5.5±0.8</td>
<td>5.8±0.8</td>
<td>3.0±0.5</td>
<td>5.7±1.0</td>
<td>4.0±0.7</td>
</tr>
<tr>
<td>EF%</td>
<td>86.8±0.5</td>
<td>86.0±0.6</td>
<td>86.0±1.0</td>
<td>90.9±0.8</td>
<td>86.0±1.3</td>
<td>89.4±0.7</td>
</tr>
<tr>
<td>FS%</td>
<td>55.0±0.6</td>
<td>54.1±0.7</td>
<td>54.3±1.2</td>
<td>61.2±1.3</td>
<td>54.5±1.5</td>
<td>58.8±1.1</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>107.3±6.1</td>
<td>124.8±11.1</td>
<td>139.4±7.6</td>
<td>187.8±17.6</td>
<td>170.3±8.8</td>
<td>237.4±19.8</td>
</tr>
<tr>
<td>LV Mass/BW (mg/g)</td>
<td>4.1±0.3</td>
<td>4.7±0.2</td>
<td>5.2±0.3</td>
<td>6.4±0.2</td>
<td>5.9±0.3</td>
<td>8.0±0.2</td>
</tr>
<tr>
<td>BW (g)</td>
<td>26.1±0.8</td>
<td>26.7±2.5</td>
<td>27.3±0.7</td>
<td>29.0±1.9</td>
<td>28.9±1.1</td>
<td>29.5±1.8</td>
</tr>
<tr>
<td>LV Mass/TL (mm)</td>
<td>6.3±0.3</td>
<td>7.5±0.7</td>
<td>8.2±0.4</td>
<td>11.4±0.9</td>
<td>10.0±0.5</td>
<td>14.3±1.0</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>665±9</td>
<td>670±13</td>
<td>708±21</td>
<td>727±11</td>
<td>730±14</td>
<td>729±10</td>
</tr>
</tbody>
</table>

111
Data represent means ± SEM. HR, heart rate; ExLVD, external left ventricular diameter; bpm, heart beats per minute; AWTD, anterior wall thickness in diastole; AWTS, anterior wall thickness in systole; PWTD, posterior wall thickness in diastole; PWTS, posterior wall thickness in systole; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; FS, fractional shortening, calculated as (LVEDD-LVESD)/LVEDD x 100; EF%, ejection fraction calculated as (end Simpson’s diastolic volume – end Simpson’s systolic volume)/end Simpson’s diastolic volume * 100
TABLE 6 – High resolution transthoracic echocardiography performed on conscious on MuRF1 Tg+ and age-matched wild type mice at baseline, 1 week, and 2 weeks after daily i.p. T3 treatment.

<table>
<thead>
<tr>
<th></th>
<th>MuRF1 wildtype Tg+ Baseline N=16</th>
<th>MuRF1 Tg+ Baseline N=11</th>
<th>MuRF1 wildtype Tg+ Thyroid Hormone 1 Week N=16</th>
<th>MuRF1 Tg+ Thyroid Hormone 1 Week N=10</th>
<th>MuRF1 wildtype Tg+ Thyroid Hormone 2 Weeks N=16</th>
<th>MuRF1 Tg+ Thyroid Hormone 2 Weeks N=11</th>
</tr>
</thead>
<tbody>
<tr>
<td>AWT D (mm)</td>
<td>1.05±0.02</td>
<td>0.96±0.03</td>
<td>1.35±0.03</td>
<td>1.08±0.03</td>
<td>1.44±0.04</td>
<td>1.10±0.04</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.06±0.08</td>
<td>3.17±0.16</td>
<td>2.12±0.04</td>
<td>1.78±0.04</td>
<td>2.12±0.04</td>
<td>1.70±0.05</td>
</tr>
<tr>
<td>PWTD (mm)</td>
<td>1.02±0.02</td>
<td>0.96±0.02</td>
<td>3.15±0.10</td>
<td>3.27±0.11</td>
<td>3.20±0.11</td>
<td>3.54±0.13</td>
</tr>
<tr>
<td>AWTS (mm)</td>
<td>1.70±0.05</td>
<td>1.54±0.06</td>
<td>1.48±0.07</td>
<td>1.80±0.09</td>
<td>1.64±0.10</td>
<td>2.23±0.14</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.53±0.07</td>
<td>1.88±0.14</td>
<td>1.36±0.06</td>
<td>1.18±0.12</td>
<td>1.41±0.04</td>
<td>1.11±0.03</td>
</tr>
<tr>
<td>PWTS (mm)</td>
<td>1.58±0.04</td>
<td>1.33±0.06</td>
<td>1.86±0.05</td>
<td>1.60±0.03</td>
<td>1.92±0.05</td>
<td>1.50±0.06</td>
</tr>
<tr>
<td>LV Vol:d (µl)</td>
<td>37.4±2.4</td>
<td>41.6±4.6</td>
<td>40.3±3.0</td>
<td>44.1±3.7</td>
<td>42.1±3.3</td>
<td>53.4±4.6</td>
</tr>
<tr>
<td>LV Vol:s (µl)</td>
<td>6.8±0.8</td>
<td>12.0±2.1</td>
<td>6.3±0.9</td>
<td>10.4±1.1</td>
<td>8.5±1.4</td>
<td>17.9±2.4</td>
</tr>
<tr>
<td>EF%</td>
<td>82.5±1.3</td>
<td>72.9±2.4</td>
<td>84.9±1.1</td>
<td>74.6±4.1</td>
<td>81.3±1.8</td>
<td>67.4±3.1</td>
</tr>
<tr>
<td>FS%</td>
<td>50.4±1.4</td>
<td>41.2±1.9</td>
<td>53.1±1.3</td>
<td>43.6±3.4</td>
<td>49.4±1.8</td>
<td>37.3±2.5</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>116.6±7.3</td>
<td>107.4±9.3</td>
<td>178.5±8.3</td>
<td>137.7±7.4</td>
<td>201.8±110.8</td>
<td>155.0±10.2</td>
</tr>
<tr>
<td>LV Mass/BW (mg/g)</td>
<td>4.1±0.3</td>
<td>5.1±0.7</td>
<td>6.2±0.2</td>
<td>5.2±0.3</td>
<td>6.7±0.2</td>
<td>5.5±0.3</td>
</tr>
<tr>
<td>BW (g)</td>
<td>29.3±1.3</td>
<td>27.7±1.8</td>
<td>29.9±1.1</td>
<td>26.9±1.5</td>
<td>29.8±0.9</td>
<td>28.1±1.1</td>
</tr>
<tr>
<td>LV Mass/TL (mm)</td>
<td>6.8±0.4</td>
<td>5.1±0.5</td>
<td>10.3±0.5</td>
<td>7.9±0.4</td>
<td>11.8±0.6</td>
<td>9.2±0.7</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>711±13</td>
<td>679±10</td>
<td>802±9</td>
<td>802±7</td>
<td>798±13</td>
<td>768±6</td>
</tr>
</tbody>
</table>
Data represent means ± SEM. HR, heart rate; ExLVD, external left ventricular diameter; bpm, heart beats per minute; AWTD, anterior wall thickness in diastole; AWTS, anterior wall thickness in systole; PWTD, posterior wall thickness in diastole; PWTS, posterior wall thickness in systole; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; FS, fractional shortening, calculated as (LVEDD-LVESD)/LVEDD x 100; EF%, ejection fraction calculated as (end Simpson’s diastolic volume – end Simpson’s systolic volume)/end Simpson’s diastolic volume * 100
TABLE 7 – High resolution transthoracic echocardiography performed on conscious MuRF1 +/- and age-matched wild type mice at baseline, 1 week, and 2 weeks after sham daily i.p. treatment

<table>
<thead>
<tr>
<th></th>
<th>MuRF1 +/- Baseline N=4</th>
<th>MuRF1 +/- Baseline N=5</th>
<th>MuRF1 +/- Vehicle 1 Week N=8</th>
<th>MuRF1 +/- Vehicle 1 Week N=8</th>
<th>MuRF1 +/- Vehicle 2 Weeks N=7</th>
<th>MuRF1 +/- Vehicle 2 Weeks N=8</th>
</tr>
</thead>
<tbody>
<tr>
<td>AWTD (mm)</td>
<td>1.00±0.02</td>
<td>1.12±0.07</td>
<td>1.05±0.02</td>
<td>1.17±0.04</td>
<td>1.05±0.03</td>
<td>1.15±0.04</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.13±0.30</td>
<td>3.38±0.37</td>
<td>2.84±0.08</td>
<td>3.15±0.08</td>
<td>3.05±0.11</td>
<td>3.07±0.19</td>
</tr>
<tr>
<td>PWTD (mm)</td>
<td>1.06±0.05</td>
<td>1.13±0.05</td>
<td>1.04±0.03</td>
<td>1.19±0.03</td>
<td>1.03±0.03</td>
<td>1.19±0.03</td>
</tr>
<tr>
<td>AWTS (mm)</td>
<td>1.69±0.02</td>
<td>1.93±0.13</td>
<td>1.77±0.04</td>
<td>2.13±0.04</td>
<td>1.72±0.03</td>
<td>2.07±0.06</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.48±0.15</td>
<td>1.51±0.21</td>
<td>1.25±0.06</td>
<td>1.39±0.05</td>
<td>1.38±0.08</td>
<td>1.39±0.11</td>
</tr>
<tr>
<td>PWTS (mm)</td>
<td>1.69±0.03</td>
<td>1.91±0.04</td>
<td>1.69±0.05</td>
<td>2.08±0.07</td>
<td>1.64±0.04</td>
<td>2.00±0.06</td>
</tr>
<tr>
<td>LVEDS (µl)</td>
<td>39.8±6.4</td>
<td>50.2±12.0</td>
<td>30.9±2.1</td>
<td>39.7±2.3</td>
<td>37.0±3.1</td>
<td>38.8±5.8</td>
</tr>
<tr>
<td>LV Vol;d (µl)</td>
<td>6.3±1.7</td>
<td>7.1±2.5</td>
<td>3.9±0.5</td>
<td>5.0±0.4</td>
<td>5.1±0.72</td>
<td>5.4±1.1</td>
</tr>
<tr>
<td>EF%</td>
<td>85.0±1.8</td>
<td>87.0±1.4</td>
<td>87.7±0.80</td>
<td>87.5±0.5</td>
<td>86.6±1.1</td>
<td>86.8±0.9</td>
</tr>
<tr>
<td>FS%</td>
<td>53.0±2.0</td>
<td>55.7±1.4</td>
<td>56.0±1.0</td>
<td>56.1±0.7</td>
<td>55.0±1.5</td>
<td>55.2±1.0</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>117.6±17.0</td>
<td>153.0±26.5</td>
<td>102.9±3.0</td>
<td>153.7±7.0</td>
<td>91.5±5.8</td>
<td>143.2±13.0</td>
</tr>
<tr>
<td>LV Mass/BW (mg/g)</td>
<td>4.59±0.68</td>
<td>4.83±0.65</td>
<td>4.40±0.30</td>
<td>5.11±0.27</td>
<td>5.2±0.4</td>
<td>5.4±1.1</td>
</tr>
<tr>
<td>BW (g)</td>
<td>25.7±0.8</td>
<td>31.1±2.0</td>
<td>24.9±0.40</td>
<td>30.4±1.4</td>
<td>24.6±0.6</td>
<td>30.0±1.3</td>
</tr>
<tr>
<td>LV Mass/TL (mg/mm)</td>
<td>6.7±0.9</td>
<td>9.0±1.7</td>
<td>5.8±0.2</td>
<td>8.3±0.5</td>
<td>6.5±0.4</td>
<td>6.5±0.8</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>618±41</td>
<td>616±25</td>
<td>717±8</td>
<td>632±21</td>
<td>683±18</td>
<td>631±27</td>
</tr>
</tbody>
</table>
Data represent means ± SEM. HR, heart rate; ExLVD, external left ventricular diameter; bpm, heart beats per minute; AWTD, anterior wall thickness in diastole; AWTS, anterior wall thickness in systole; PWTD, posterior wall thickness in diastole; PWTS, posterior wall thickness in systole; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; FS, fractional shortening, calculated as (LVEDD-LVESD)/LVEDD x 100; EF%, ejection fraction calculated as (end Simpson’s diastolic volume – end Simpson’s systolic volume)/end Simpson’s diastolic volume * 100
TABLE 8 – High resolution transthoracic echocardiography performed on conscious on MuRF1 Tg+ and age-matched wild type mice at baseline, 1 week, and 2 weeks after sham daily i.p. treatment.

<table>
<thead>
<tr>
<th></th>
<th>MuRF1 wildtype&lt;sup&gt;Tg+&lt;/sup&gt; Baseline</th>
<th>MuRF1 Tg+ Baseline</th>
<th>MuRF1 wildtype&lt;sup&gt;Tg+&lt;/sup&gt; Vehicle 1 Week</th>
<th>MuRF1 Tg+ Vehicle 1 Week</th>
<th>MuRF1 wildtype&lt;sup&gt;Tg+&lt;/sup&gt; Vehicle 2 Weeks</th>
<th>MuRF1 Tg+ Vehicle 2 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>AWTD (mm)</td>
<td>0.98± 0.03</td>
<td>0.98± 0.06</td>
<td>0.96± 0.04</td>
<td>0.93± 0.04</td>
<td>0.99± 0.04</td>
<td>0.97± 0.08</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.09± 0.23</td>
<td>2.80± 0.05</td>
<td>2.99± 0.21</td>
<td>2.87± 0.02</td>
<td>2.97± 0.11</td>
<td>3.02± 0.18</td>
</tr>
<tr>
<td>PWTD (mm)</td>
<td>1.01± 0.05</td>
<td>1.00± 0.06</td>
<td>1.06± 0.03</td>
<td>1.04± 0.05</td>
<td>1.05± 0.05</td>
<td>0.98± 0.07</td>
</tr>
<tr>
<td>AWTS (mm)</td>
<td>1.72± 0.10</td>
<td>1.51± 0.09</td>
<td>1.70± 0.02</td>
<td>1.59± 0.06</td>
<td>1.69± 0.08</td>
<td>1.55± 0.13</td>
</tr>
<tr>
<td>LVEDS (mm)</td>
<td>1.51± 0.22</td>
<td>1.45± 0.13</td>
<td>1.40± 0.22</td>
<td>1.35± 0.09</td>
<td>1.34± 0.15</td>
<td>1.53± 0.13</td>
</tr>
<tr>
<td>PWTS (mm)</td>
<td>1.50± 0.09</td>
<td>1.48± 0.10</td>
<td>1.54± 0.10</td>
<td>1.53± 0.12</td>
<td>1.60± 0.05</td>
<td>1.49± 0.12</td>
</tr>
<tr>
<td>LV Vol;d (µl)</td>
<td>38.7± 7.0</td>
<td>31.4± 2.0</td>
<td>35.7± 6.1</td>
<td>31.3± 4.0</td>
<td>34.4± 3.1</td>
<td>36.5± 5.2</td>
</tr>
<tr>
<td>LV Vol;s (µl)</td>
<td>4.4± 0.9</td>
<td>5.9± 1.1</td>
<td>5.8± 2.2</td>
<td>4.8± 0.8</td>
<td>4.9± 1.4</td>
<td>6.8± 1.4</td>
</tr>
<tr>
<td>EF%</td>
<td>83.6± 3.7</td>
<td>81.4± 3.6</td>
<td>85.4± 3.8</td>
<td>84.8± 2.6</td>
<td>86.5± 2.8</td>
<td>81.4± 3.5</td>
</tr>
<tr>
<td>FS%</td>
<td>51.8± 3.7</td>
<td>49.2± 3.6</td>
<td>54.2± 4.3</td>
<td>52.8± 3.0</td>
<td>55.2± 3.5</td>
<td>49.2± 3.7</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>107.6± 11.6</td>
<td>95.2± 7.5</td>
<td>105.8± 13.2</td>
<td>94.4± 5.8</td>
<td>104.8± 2.8</td>
<td>102.2± 13.6</td>
</tr>
<tr>
<td>LV Mass/BW (mg/g)</td>
<td>4.5± 0.8</td>
<td>4.1± 0.2</td>
<td>4.5± 0.6</td>
<td>4.2± 0.1</td>
<td>4.4± 0.3</td>
<td>4.1± 0.3</td>
</tr>
<tr>
<td>BW (g)</td>
<td>24.8± 1.0</td>
<td>23.0± 0.5</td>
<td>23.3± 1.1</td>
<td>22.3± 0.8</td>
<td>24.1± 1.1</td>
<td>24.4± 1.7</td>
</tr>
<tr>
<td>LV Mass/TL (mg/mm)</td>
<td>5.4± 1.3</td>
<td>5.6± 0.5</td>
<td>6.3± 0.8</td>
<td>5.3± 0.3</td>
<td>6.3± 0.2</td>
<td>6.0± 0.7</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>707± 24</td>
<td>703± 9</td>
<td>709± 16</td>
<td>711± 20</td>
<td>698± 13</td>
<td>738± 11</td>
</tr>
</tbody>
</table>
Data represent means ± SEM. HR, heart rate; ExLVD, external left ventricular diameter; bpm, heart beats per minute; AWTD, anterior wall thickness in diastole; AWTS, anterior wall thickness in systole; PWTD, posterior wall thickness in diastole; PWTS, posterior wall thickness in systole; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; FS, fractional shortening, calculated as (LVEDD-LVESD)/LVEDD x 100; EF%, ejection fraction calculated as (end Simpson’s diastolic volume – end Simpson’s systolic volume)/end Simpson’s diastolic volume * 100
TABLE 9 – Summary of ubiquitin-proteasome system expression in Golden retriever muscular dystrophy (GRMD) skeletal muscle and heart compared with age-matched controls.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>MuRF1 expression</th>
<th>MAFbx/atrogin-1 expression</th>
<th>CHIP expression</th>
<th>MDM2 expression</th>
<th>Ubiquitin expression</th>
<th>UNCA4/5 expression</th>
<th>UBC9 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long digital extensor</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>P = 0.14 (Inc)</td>
<td>—</td>
<td>P = 0.07 (Inc)</td>
</tr>
<tr>
<td>Lateral head gastrocnemius</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>P = 0.11 (Dec)</td>
<td>P = 0.19 (Inc)</td>
</tr>
<tr>
<td>Vastus lateralis</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Inc</td>
<td>—</td>
<td>—</td>
<td>P = 0.07 (Inc)</td>
</tr>
<tr>
<td>Biceps femoris</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>P = 0.10 (Inc)</td>
<td>—</td>
<td>—</td>
<td>P = 0.10 (Inc)</td>
</tr>
<tr>
<td>Left ventricle</td>
<td>—</td>
<td>—</td>
<td>Dec</td>
<td>Dec</td>
<td>—</td>
<td>Dec</td>
<td>—</td>
</tr>
<tr>
<td>Cranial sartorius</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>P = 0.14 (Inc)</td>
<td>—</td>
<td>—</td>
<td>Inc</td>
</tr>
</tbody>
</table>
‘‘Inc’’: mRNA expression or protease activity was significantly increased for the indicated GRMD muscle compared with wildtype; ‘‘—’’: protease activity was unchanged for the indicated GRMD muscle compared with wildtype; ‘‘Dec’’: expression was decreased in GRMD samples compared with controls. P-values given for relationships trending (<0.2); the direction of the trend is given in parentheses12

12 This table previously appeared in Muscle and Nerve. The original citation for the publication which includes this table is as follows: Muscle Nerve 44, no. 4 (October 2011): 553-62.
TABLE 10 – Summary of proteasome and calpain expression and activities in Golden retriever muscular dystrophy (GRMD) skeletal muscle and heart compared with age-matched controls

<table>
<thead>
<tr>
<th>Muscle</th>
<th>PSM46 exp.</th>
<th>PSM44 exp.</th>
<th>PSME1 exp.</th>
<th>Proteasome activity</th>
<th>Calpain 1 exp.</th>
<th>Calpain 2 exp.</th>
<th>Calpain1/2 activity</th>
<th>Atrophy/hypertrophy</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long digital extensor</td>
<td>Inc</td>
<td>—</td>
<td>P = 0.14 (Dec)</td>
<td>P ≤ 0.057 (Inc)</td>
<td>P ≤ 0.057 (Inc)</td>
<td>P = 0.07 (Inc)</td>
<td>P = 0.057 (Inc)</td>
<td>Severe atrophy</td>
<td>Extension of digits</td>
</tr>
<tr>
<td>Lateral head gastrocnemius</td>
<td>—</td>
<td>Inc</td>
<td>—</td>
<td>P ≤ 0.057 (Inc)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Atrophy</td>
<td>Extension of hock</td>
</tr>
<tr>
<td>Vastus lateralis</td>
<td>Inc</td>
<td>—</td>
<td>P = 0.10 (Inc)</td>
<td>P ≤ 0.057 (Inc)</td>
<td>P = 0.07 (Inc)</td>
<td>Inc</td>
<td>ND</td>
<td>ND</td>
<td>Extension of stifle</td>
</tr>
<tr>
<td>Biceps femoris</td>
<td>Dec</td>
<td>Dec</td>
<td>Dec</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Atrophy</td>
<td>Extension of stifle</td>
</tr>
<tr>
<td>Left ventricle</td>
<td>Inc</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cranial sartorus</td>
<td>P = 0.14 (Inc)</td>
<td>Inc</td>
<td>—</td>
<td>—</td>
<td>P = 0.14 (Inc)</td>
<td>Inc</td>
<td>P = 0.057 (Inc)</td>
<td>Hypertrophy</td>
<td>Flexion of hip</td>
</tr>
</tbody>
</table>
“Exp”: expression; Atrophy/hypertrophy data adapted from Kornegay et al.\textsuperscript{161}. “Inc”: mRNA expression or protease activity was significantly increased for the indicated GRMD muscle compared with the wildtype; “—“: protease activity was unchanged for the indicated GRMD muscle compared with wildtype; “Dec”: expression was decreased in GRMD samples compared with wildtype. “ND”: denotes that the atrophy/hypertrophy data was not previously determined. P-values given for relationships trending (<0.2); the direction of the trend is given in parentheses\textsuperscript{13}

\textsuperscript{13} This table previously appeared in \textit{Muscle and Nerve}. The original citation for the publication which includes this table is as follows: \textit{Muscle Nerve} 44, no. 4 (October 2011): 553-62.
TABLE 11 – The regulation of proteasome activity and role of proteasome inhibition in Duchenne muscular dystrophy.

<table>
<thead>
<tr>
<th>Study</th>
<th>Model</th>
<th>Muscle Studied</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kumamoto, et al., 2000</td>
<td>2 patients with DMD 2 controls</td>
<td>Biceps (Human)</td>
<td>Increased proteasome-positive fibers (4.9%) vs. controls (0.1%) determined by IHC of 20S proteasome</td>
</tr>
<tr>
<td>Bonuccelli, et al., 2003</td>
<td>mdx mice (8 months old) MG-132 injected into gastrocnemius</td>
<td>Gastrocnemius</td>
<td>Local gastrocnemius DGC improved, IHC and Western blot</td>
</tr>
<tr>
<td></td>
<td>Osmotic pump delivery (1 μg, 5 μg, 10 μg/kg/24 hours) x 8 days</td>
<td>Diaphragm Gastrocnemius</td>
<td>Evans blue dye demonstrated MG-132 improved diaphragm muscle integrity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Histologic improvement</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MG-132 does not alter calpain activity</td>
</tr>
<tr>
<td>Bonuccelli, et al., 2007</td>
<td>mdx mice (8 months old) Velcade 5 and 10 μmol/L doses</td>
<td>Gastrocnemius</td>
<td>Velcade Stabilized DGC proteins via Western and IHC</td>
</tr>
<tr>
<td></td>
<td>MLN273 (Another boronic acid proteasomal inhibitor), injected into gastrocnemius 20 μM, 100 μM, 160 μM; looked 24 hours later</td>
<td></td>
<td>MLN273 stabilized DGC proteins as per Western analysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Both Velcade and MLN273 stabilized NF-κB (inhibited activity)</td>
</tr>
<tr>
<td>Gazzero, et al., 2010</td>
<td>mdx mice (7 months old) 0.8 mg/kg Velcade IV q 72 hours x 2 weeks</td>
<td>Gastrocnemius Diaphragm</td>
<td>Proteasome inhibition improves: β-DG and α-SG membrane localization (Gastroc)</td>
</tr>
<tr>
<td></td>
<td>Human DMD Patients Muscle Explants for 16 hours Velcade 1 and 10 μmol/L doses</td>
<td></td>
<td>Proteasome Chymotrypsin-like activity enhanced 8 fold in mdx; Velcade inhibits 50% (Gastroc)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Proteasome inhibition:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1. Increases proteins in the DGC (Gastroc)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2. Decreases muscle degeneration and necrotic fibers (Gastroc)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3. Improves histology (Gastroc &amp; Diaphragm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4. Enhances muscle cell regeneration (Gastroc)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5. Reduces inflammation (decreased tissue macrophages, circulating IL-6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6. Decreases CK</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7. Decreases phospho-p65 (NF-κB activation)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+/- Histologic improvement of DGC complex x 8 DMD patients at 2 doses of Velcade. Increased DGC proteins by Western blot with controls</td>
</tr>
</tbody>
</table>
Studies using proteasome inhibitors are shaded in blue. Search criteria included: “muscular dystrophy proteasome inhibition” and “muscular dystrophy proteasome” in PubMed. “DGC”: dystrophin-glycoprotein complex\(^\text{14}\)

\[^{14}\text{This table previously appeared in } Muscle \text { and Nerve}. \text { The original citation for the publication which includes this table is as follows: } Muscle \text { Nerve } 44, \text { no. } 4 \text { (October } 2011) : 553-62.\]
TABLE 12 – The regulation of calpain activity and role of calpain inhibition in Duchenne muscular dystrophy

<table>
<thead>
<tr>
<th>Study</th>
<th>Model</th>
<th>Muscle Studied</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spencer and Tidball, 1992</td>
<td>Mdx mice 5 months to 1 year old</td>
<td>Gastrocnemius</td>
<td>Significant decrease in Calpain activity (Gastrocnemius, Quadriceps)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quadriceps &amp; Tibialis anterior</td>
<td>Calpain increased by Western blot (Gastrocnemius, Quadriceps, Tibialis anterior)</td>
</tr>
<tr>
<td>Spencer, et al., 1995</td>
<td>Mdx mice: 2 weeks (pre-necrotic), 4 weeks (peak necrotic), or 14 weeks</td>
<td>Gastrocnemius</td>
<td>Calpain expression by Western increases in mdx mice at 4 and 14 weeks</td>
</tr>
<tr>
<td></td>
<td>(regenerated) old at sacrifice</td>
<td>Quadriceps &amp; Hamstrings</td>
<td>Calpastatin levels by Western not affected</td>
</tr>
<tr>
<td>Badalamente, et al., 2000</td>
<td>14 day old mdx mice BID x 30 days 12 mg/kg &amp; 10 mg/kg</td>
<td>Gastrocnemius</td>
<td>Both doses improved Gastroc, AT, and Diaphragm Cross sectional area, Mdx mice had increased calpain activity in all 4 muscles; inhibited by both doses of Leupentin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soleus &amp; Tibialis anterior</td>
<td>Calpastatin reduces muscle necrosis at 4 weeks</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diaphragm</td>
<td>Membrane damage and CK levels unchanged with increased calpastatin expression</td>
</tr>
<tr>
<td>Spencer, et al., 2002</td>
<td>Mdx mice with transgenic calpastatin (endogenous inhibitor of m- and u-calpain) expression 4 weeks old</td>
<td>Quadriceps &amp; Triceps</td>
<td>Calpain activity significantly decreased in mdx muscle; altered distribution of activities in muscle fibers, Mdx muscle fibers exaggerate calpain activity in response to hypo-osmotic shock</td>
</tr>
<tr>
<td>Burdi, et al., 2006</td>
<td>Mdx (4-5 weeks of age) BN82270 calpain inhibitor/Exercise antioxidant</td>
<td>Diaphragm</td>
<td>Mdx have enhanced calpain activity; BN82270 inhibitor significantly inhibits mdx enhanced activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BN82270 decreased circulating CK in mdx mice</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gastrocnemius</td>
<td>No significant improvement histo logically with BN82270</td>
</tr>
<tr>
<td>Gailly, et al., 2007</td>
<td>60 and 120 day old mdx mice</td>
<td>Flexor digitorum brevis</td>
<td>Calpain activity significantly decreased in mdx muscle; altered distribution of activities in muscle fibers, Mdx muscle fibers exaggerate calpain activity in response to hypo-osmotic shock</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Calpastatin transgenic mice (3 weeks old)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diaphragm muscle</td>
<td>Mdx muscle fibers exaggerate calpain activity in response to hypo-osmotic shock, Improved cross sectional area distribution</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Western blot: Increased BDG.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EDL and soleus muscle function not</td>
<td>Improved cross section area distribution</td>
</tr>
<tr>
<td></td>
<td></td>
<td>improved after 4 weeks Rx C101</td>
<td>Mdx muscle fibers exaggerate calpain activity in response to hypo-osmotic shock, Improved cross sectional area distribution</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diaphragm tension not improved with</td>
<td>Compensatory mechanisms in place that enhance counteract calpain inhibition in mdx mice</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 months of C101 or Leupentin</td>
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</table>
Studies using calpain inhibitors are shaded in blue. Search criteria included: “muscular dystrophy calpain inhibitor”, “muscular dystrophy calpain”, and “mdx calpain” in PubMed\textsuperscript{15}

\textsuperscript{15} This table previously appeared in \textit{Muscle and Nerve}. The original citation for the publication which includes this table is as follows: \textit{Muscle Nerve} 44, no. 4 (October 2011): 553-62.
TABLE 13 – Sample information for Golden retriever muscular dystrophy (GRMD) and control dogs.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Gender</th>
<th>Phenotype</th>
<th>Long digital extensor</th>
<th>Lateral head gastrocnemius</th>
<th>Vastus lateralis</th>
<th>Bicep femoris</th>
<th>Left ventricle</th>
<th>Cranial sartoris</th>
<th>Biopsy/Necropsy</th>
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<tbody>
<tr>
<td>1</td>
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<td>GRMD</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>N</td>
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<td>F</td>
<td>GRMD</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
<td>X</td>
<td>N</td>
</tr>
<tr>
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<td>GRMD</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>N</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>7</td>
<td>M</td>
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<td></td>
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<tr>
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<td>X</td>
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<td>X</td>
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<td>X</td>
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<td>N</td>
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</table>
Skeletal muscle and heart samples were collected from both GRMD and control canines at 6 months of age. X=used in this study. N=samples collected at necropsy; B=samples collected by biopsy. Yellow indicates samples used for one muscle type only.\textsuperscript{16}

\footnotesize{\textsuperscript{16} This table previously appeared in \textit{Muscle and Nerve}. The original citation for the publication which includes this table is as follows: \textit{Muscle Nerve} 44, no. 4 (October 2011): 553-62.
FIGURE 1 – Molecular mechanisms driving physiological cardiac hypertrophy
FIGURE 2 – The ubiquitin proteasome system\textsuperscript{17}

\textsuperscript{17} Part of this figure has previously appeared in American Journal of Physiology Heart and Circulatory Physiology. The original citation for the publication which includes this figure is as follows: Am J Physiol Heart Circ Physiol 2012 Feb 1;302(3):H515-26.
FIGURE 3 – Knockdown of MuRF1 enhances and increased expression of MuRF1 represses insulin-like growth factor-1 (IGF-1)-induced cardiomyocyte hypertrophy. As of this writing, this figure appears in an Article in Press (published January 14, 2014) in American Journal of Physiology Endocrinology and Metabolism at the following website: http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013 DOI:10.1152/ajpendo.00326.2013
A. MuRF1 was knocked down using AdshMuRF1, with Adshscrambled as control, at MOI 60 for 24 hours in serum-free DMEM followed by treatment with 10nM IGF-1 for 18 hours. Cells were fixed and observed with a fluorescent microscope using a 40X objective lens. Shown are representative images of vehicle or IGF1-treated HL-1 cardiomyocytes transduced with either Adshscrambled or AdshMuRF1. B. Cardiomyocyte area (mm²) measurements of vehicle or IGF1-treated HL-1 cardiomyocytes transduced with either Adshscrambled or AdshMuRF1, averaged over at least 200 cardiomyocytes. Black bars represent vehicle and gray bars represent IGF-1-treated cells. C. MuRF1 was increased in expression using AdMuRF1, with AdGFP as a control, at MOI 25 for 24 hours in serum-free DMEM followed by treatment with IGF-1 for 18 hours. Shown are representative fluorescent images of vehicle or IGF1-treated HL-1 cardiomyocytes transduced with either AdGFP or AdMuRF1. D. Cardiomyocyte area (mm²) measurements of vehicle or IGF1-treated HL-1 cardiomyocytes transduced with either AdGFP or AdMuRF1. Black bars represent vehicle and gray bars represent IGF-1-treated cells. Cardiomyocyte area measurements are represented as mean area ± SEM. E. MuRF1 was increased in expression in NRVM using AdMuRF1, with AdGFP as a control, at MOI 25 for 24 hours in serum-free M199 followed by treatment with IGF-1 for 18 hours. Shown are representative fluorescent images of vehicle or IGF1-treated cardiomyocytes transduced with either AdGFP or AdMuRF1. F. Cardiomyocyte area (mm²) measurements of vehicle or IGF1-treated NRVM transduced with either AdGFP or AdMuRF1. Black bars represent vehicle and gray bars represent IGF-1-treated cells. Cardiomyocyte area measurements are represented as mean area ± SEM. A two-way ANOVA test was used to determine statistical significance. * indicates significance on level of adenovirus group, ** indicates significance on level of treatment group. % indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as # P < 0.05, ## P < 0.001 as determined using a pairwise post-test.
FIGURE 4 – Insulin-like growth factor-1 (IGF-1)-dependent expression of genes associated with Akt activity is inhibited by MuRF1.

As of this writing, this figure appears in an Article in Press (published January 14, 2014) in American Journal of Physiology Endocrinology and Metabolism at the following website:
http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013

DOI:10.1152/ajpendo.00326.2013
A. AdshMuRF1 and control Adshscrambled were used at MOI 60 for 24 hours in serum-free DMEM to knockdown MuRF1 in HL-1 cardiomyocytes and transduced cells were treated with 10nM IGF-1 for 18 hours. RNA was isolated and cDNA generated for use in measuring expression of MuRF1, MAPK13, RYR1, Igfbp5, and 18S (reference gene). Shown are RTPCR data for each of these genes in vehicle or IGF-1-treated HL-1 cardiomyocytes transduced with either Adshscrambled or AdshMuRF1. B. AdMuRF1 and control AdGFP were used at MOI 25 for 24 hours in serum-free DMEM to increase MuRF1 and transduced cells were treated with 10nM IGF-1 for 18 hours. Shown are RT-PCR data for MuRF1, MAPK13, RYR1, and Igfbp5 in cardiomyocytes transduced with either AdGFP or AdMuRF1 and treated with either vehicle or IGF-1. Raw CT values from three independent experiments were normalized to their respective 18S values, averaged over experimental group, and subsequently normalized to either to vehicle control (MAPK13, RYR1, and Igfbp5) or adenovirus control (MuRF1). Final data is represented as mean fold change ± SEM. A two-way ANOVA test was used to determine statistical significance. * indicates significance on level of adenovirus group. **indicates significance on level of treatment group. % indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P< 0.05, ##P< 0.001 as determined using a pairwise post-test. $ indicates significance (p<0.05) between groups within the treatment group, as measured by a Student’s t-test.
FIGURE 5 – MuRF1 inhibits protein expression of Akt and glycogen synthase kinase 3β (GSK3β) in insulin-like growth factor-1 (IGF-1)-stimulated HL-1 cardiomyocytes

As of this writing, this figure appears in an Article in Press (published January 14, 2014) in *American Journal of Physiology Endocrinology and Metabolism* at the following website:
http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013
DOI: 10.1152/ajpendo.00326.2013
A. HL-1 cells were transduced with AdMuRF1 or control AdGFP at MOI 25 for 24 hours in serum-free DMEM to increase MuRF1 expression, followed by treatment with 10nM IGF-1 for 30 minutes. Immunoblots using whole cell lysates from three independent experiments are shown for p-Akt S473, p-Akt T308, Akt, GSK3β, and p-GSK3β S9. Primary antibody against myc was used to assess adenovirus-dependent expression of myc-MuRF1 and immunoblot for MuRF1 was done to determine endogenous protein levels. β-actin was used as a loading control. Densitometry analysis of Akt, p-Akt S473, p-Akt T308, GSK3β, and p-GSK3β S9 are shown for vehicle or IGF-1-treated cardiomyocytes transduced with either AdGFP or AdMuRF1. Total protein levels were normalized to β-actin and phospho-protein levels were normalized first to β-actin and then to total protein levels. B. HL-1 cardiomyocytes were transduced with either AdshMuRF1 or control Adshscrambled at MOI 30 for 48 hours in serum-free DMEM, followed by treatment with 10nM IGF-1 for 30 minutes. Akt and GSK3β expression and phosphorylation in whole cell lysates from three independent experiments was assessed by immunoblot using primary antibodies raised against total Akt or GSK3β and p-Akt S473, p-Akt T308, and p-GSK3β S9, as indicated. Primary antibody against MuRF1 was used to confirm knockdown. β-actin was used as a loading control. Densitometry analysis of Akt, p-Akt S473, p-Akt T308, GSK3β, and p-GSK3β S9 are shown for vehicle or IGF-1-treated cardiomyocytes transduced with either Adshscrambled or AdshMuRF1. Densitometry analysis is represented as means ± SEM. A two-way ANOVA test was used to determine statistical significance. * indicates significance on level of adenovirus group. ** indicates significance on level of treatment group. % indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P < 0.05, ##P < 0.001 as determined using a pairwise post-test.
FIGURE 6 – MuRF1 inhibits protein expression of Akt and glycogen synthase kinase 3β (GSK3β) in insulin-like growth factor-1 (IGF-1)-stimulated NRVM²¹

<table>
<thead>
<tr>
<th>NRVM</th>
<th>AdGFP</th>
<th>AdMuRF1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>IGF-1</td>
</tr>
<tr>
<td>p-Akt T308</td>
<td></td>
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<tr>
<td>p-Akt S473</td>
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<td>p-GSK3beta S9</td>
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<td>beta-actin</td>
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</table>

²¹ As of this writing, this figure appears in an Article in Press (published January 14, 2014) in *American Journal of Physiology Endocrinology and Metabolism* at the following website: http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013 DOI:10.1152/ajpendo.00326.2013
NRVM were transduced with AdMuRF1 or control AdGFP at MOI 25 for 24 hours in serum-free M199 to increase MuRF1 expression, followed by treatment with 10nM IGF-1 for 30 minutes. Immunoblots using whole cell lysates from three independent experiments are shown for p-Akt S473, p-Akt T308, Akt, GSK3β, and p-GSK3β S9. Primary antibody against myc was used to assess adenovirus-dependent expression of myc-MuRF1. β-actin was used as a loading control. Densitometry analysis of Akt, p-Akt S473, p-Akt T308, and p-GSK3β S9 are shown for vehicle or IGF-1-treated cardiomyocytes transduced with either AdGFP or AdMuRF1. Total protein levels were normalized to β-actin and phospho-protein levels were normalized first to β-actin and then to total protein levels. Densitometry analysis is represented as means ± SEM. A two-way ANOVA test was used to determine statistical significance. * indicates significance on level of adenovirus group ** indicates significance on level of treatment group. % indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P< 0.05, ##P< 0.001 as determined using a pair-wise post-test.
FIGURE 7 – MuRF1 knockdown in HL-1 cardiomyocytes induces total mammalian target of rapamycin (mTOR) protein levels to increase upon insulin-like growth factor-1 (IGF-1) stimulation\textsuperscript{22}

\textbf{HL-1}

<table>
<thead>
<tr>
<th></th>
<th>Adh scrambled</th>
<th>AdhMuRF1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>IGF-1</td>
<td>Vehicle</td>
</tr>
<tr>
<td>p-mTOR S2481</td>
<td></td>
<td></td>
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<tr>
<td>mTOR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>beta-actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textbf{mTOR}

$\% \ F=5.922$

$\mathrm{DF}=1$

$\#$

\textbf{p-mTOR S2481}

$\% \ F=21.990$

$\mathrm{DF}=1$

$\#$

\textsuperscript{22} As of this writing, this figure appears in an Article in Press (published January 14, 2014) in \textit{American Journal of Physiology Endocrinology and Metabolism} at the following website: http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013 DOI:10.1152/ajpendo.00326.2013
HL-1 cardiomyocytes were transduced with either AdshMuRF1 or control Adshscrambled at MOI 30 for 48 hours in serum-free DMEM, followed by treatment with 10nM IGF-1 for 30 minutes. Total and phosphorylated (S2481) mTOR was measuring by immunoblot of whole cell lysates from three independent experiments. β-actin was used as a loading control. Densitometry analysis of mTOR and p-mTOR S2481 are shown for vehicle or IGF-1-treated cardiomyocytes transduced with either Adshscrambled or AdshMuRF1. Total protein levels were normalized to β-actin and phosphor-protein levels were normalized first to β-actin and then to total protein levels. Densitometry analysis is represented as means ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group. **indicates significance on level of treatment group. %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P< 0.05, ##P< 0.001 as determined using a pairwise post-test.
FIGURE 8 – MuRF1 inhibits HL-1 cardiomyocyte c-Jun protein levels and phosphorylation in the presence of insulin-like growth factor-1 (IGF-1) 23

As of this writing, this figure appears in an Article in Press (published January 14, 2014) in American Journal of Physiology Endocrinology and Metabolism at the following website: http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013 DOI:10.1152/ajpendo.00326.2013
A. MuRF1 expression was increased in HL-1 cells using AdMuRF1 and control AdGFP at MOI 25 for 24 hours in serum-free DMEM followed by treatment with 10nM IGF-1 for 30 minutes. c-Jun expression and phosphorylation in whole cell lysates from three independent experiments was assessed by immunoblot using primary antibodies raised against total c-Jun, p-c-Jun S63, p-c-Jun S73, and p-c-Jun T91, as indicated. Primary antibody against myc was used to confirm adenovirus-dependent expression of myc-MuRF1. β-actin was used as a loading control. Densitometry analysis of c-Jun, p-c-Jun S63, p-c-Jun S73, and p-c-Jun T91 are shown for vehicle or IGF-1-treated cardiomyocytes transduced with either AdGFP or AdMuRF1. Total protein levels were normalized to β-actin and phospho-protein levels were normalized first to β-actin and then to total protein levels. B. AdshMuRF1 and control Adshscrambled were used at MOI 30 for 48 hours in serum-free DMEM to knockdown MuRF1 in HL-1 cardiomyocytes followed by IGF-1 treatment for 30 minutes. Immunoblot using whole cell lysates from three independent experiments are shown for c-Jun, p-c-Jun S63, p-c-Jun S73, and p-c-Jun T91. MuRF1 primary antibody was used to confirm knockdown. β-actin was used as a loading control. Densitometry analysis of c-Jun, p-c-Jun S63, p-c-Jun S73, and p-c-Jun T91 are shown for vehicle or IGF-1-treated cardiomyocytes transduced with either Adshscrambled or AdshMuRF1. Total protein levels were normalized to β-actin and phospho-protein levels were normalized first to β-actin and then to total protein levels. Densitometry analysis is represented as means ± SEM. A two-way ANOVA test was used to determine statistical significance. * indicates significance on level of adenovirus group ** indicates significance on level of treatment group. % indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P < 0.05, ##P < 0.001 as determined using a pair-wise post-test.
FIGURE 9 – MuRF1 inhibits NRVM c-Jun protein levels and phosphorylation in the presence of insulin-like growth factor-1 (IGF-1)\textsuperscript{24}

<table>
<thead>
<tr>
<th>NRVM</th>
<th>AdGFP</th>
<th>AdMuRF1</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-c-Jun S63</td>
<td>Vehicle</td>
<td>IGF-1</td>
</tr>
<tr>
<td>p-c-Jun S73</td>
<td>Vehicle</td>
<td>IGF-1</td>
</tr>
<tr>
<td>p-c-Jun T91</td>
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</tr>
<tr>
<td>c-Jun</td>
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<td>Myc-MuRF1</td>
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<td>beta-actin</td>
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\textsuperscript{24} As of this writing, this figure appears in an Article in Press (published January 14, 2014) in *American Journal of Physiology Endocrinology and Metabolism* at the following website: [http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013](http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013) DOI:10.1152/ajpendo.00326.2013
NRVM were transduced with AdMuRF1 or control AdGFP at MOI 25 for 24 hours in serum-free M199 to increase MuRF1 expression, followed by treatment with 10nM IGF-1 for 30 minutes. Immunoblots using whole cell lysates from three independent experiments are shown for c-Jun, p-c-Jun S63, p-c-Jun S73, and p-c-Jun T91. Primary antibody against myc was used to assess adenovirus-dependent expression of myc-MuRF1. β-actin was used as a loading control. Densitometry analysis of c-Jun, p-c-Jun S63, p-c-Jun S73, and p-c-Jun T91 are shown for vehicle or IGF-1-treated cardiomyocytes transduced with either AdGFP or AdMuRF1. Densitometry analysis is represented as means ± SEM. A two-way ANOVA test was used to determine statistical significance. * indicates significance on level of adenovirus group ** indicates significance on level of treatment group. % indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P < 0.05, ##P < 0.001 as determined using a pair-wise post-test.
FIGURE 10 – Exaggerated insulin-like growth factor-1 (IGF-1)-dependent cardiomyocyte growth with MuRF1 knockdown required c-Jun N-terminal kinase (JNK) activity.\(^{25}\)

As of this writing, this figure appears in an Article in Press (published January 14, 2014) in American Journal of Physiology Endocrinology and Metabolism at the following website:
http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013  DOI:10.1152/ajpendo.00326.2013
MuRF1 was knocked down using AdshMuRF1, with Adshscrambled as control, at MOI 60 for 24 hours in serum-free DMEM followed by pre-treatment with 10µM SP600125 (JNK inhibitor) for 30 minutes. Cardiomyocytes were subsequently treated with 10nM IGF-1 for 18 hours. Cells were fixed and observed using with a fluorescent microscope using a 40X objective lens. Shown are representative images and quantification of cardiomyocyte area (mm2) averaged over at least 200 cells per group. Cardiomyocyte area measurements are represented as mean area ± SEM. A three-way ANOVA test was used to determine statistical significance. * indicates significance on level of adenovirus group ** indicates significance on level of pre-treatment group. % denotes significant interactions between either the adenovirus, pre-treatment, or treatment groups, as indicated. %%% indicates a significant interaction between all three groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as $P<0.05$, $P<0.001$ as determined using a pairwise post-test.
FIGURE 11 – Exaggerated insulin-like growth factor-1 (IGF-1)-dependent Akt-associated gene expression with MuRF1 knockdown required c-Jun N-terminal kinase (JNK) activity.

As of this writing, this figure appears in an Article in Press (published January 14, 2014) in American Journal of Physiology Endocrinology and Metabolism at the following website: http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013 DOI:10.1152/ajpendo.00326.2013
AdshMuRF1 and control Adshscrambled were used at MOI 60 for 24 hours in serum-free DMEM to knockdown MuRF1 in HL-1 cardiomyocytes. Transduced cells were pre-treated with 10µM SP600125 (JNK inhibitor) for 30 minutes, followed by treatment with 10nM IGF-1 treatment for 18 hours. RNA was isolated and cDNA generated for use in measuring expression of MuRF1, MAPK13, RYR1, and 18S (reference gene). Raw CT values from three independent experiments were normalized to their respective 18S values, averaged over experimental group, and subsequently normalized to either vehicle control (MAPK13, RYR1, and Igfbp5) or adenovirus control (MuRF1). Final data is represented as mean fold change ± SEM. Shown are RT-PCR data for each of these genes in cardiomyocytes transduced with either Adshscrambled or AdshMuRF1 and treated with vehicle or both JNK inhibitor and IGF-1. A three-way ANOVA test was used to determine statistical significance. * indicates significance on level of adenovirus group ** indicates significance on level of pre-treatment group. % denotes significant interactions between either the adenovirus, pre-treatment, or treatment groups, as indicated. %%% indicates a significant interaction between all three groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P< 0.05, ##P< 0.001 as determined using a pairwise post-test.
FIGURE 12 – MuRF1 regulates physiological cardiac hypertrophy induced by exercise training in MuRF1-/- mice\textsuperscript{27}

\textsuperscript{27} As of this writing, this figure appears in an Article in Press (published January 14, 2014) in American Journal of Physiology Endocrinology and Metabolism at the following website: http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013 DOI:10.1152/ajpendo.00326.2013
MuRF1/- and sibling-matched wildtype control mice (MuRF1 +/+) were randomly assigned to either sedentary or running groups, where running groups were provided with an in-cage exercise wheel for 5 weeks and sedentary groups were not. Cardiac growth and function were measured by echocardiography. A. Representative M-mode images, containing at least 10 waveforms, from echocardiography of sedentary and running MuRF1+/+ and MuRF1-/-; B. LV mass measurements, as measured by echocardiography, normalized to tibia length (TL) for MuRF1+/+ and MuRF1-/- mice assigned to either sedentary or running groups were also analyzed. C. Perfused and fixed paraffin-embedded heart sections from sedentary and running MuRF1/- and wild type control mice were stained with Lectin-TRITC, imaged using fluorescent microscopy to visualize cardiomyocytes, and cross-sectional area analyzed from >180 cardiomyocytes from at least three animals per group. D. Histological analysis of hemotoxylin and eosin (HE) was also performed. E. RNA was isolated from whole heart tissue from sedentary and running MuRF1+/+ and MuRF1-/-, cDNA generated, and BNP, ANF, skeletal muscle actin, TATA box-binding protein (Tbp), and hypoxanthine phosphoribosyltransferase (Hprt) expression determined, where both Tbp and Hprt were used as reference genes. Raw CT values were normalized to the average Ct value over all groups within each genotype pair to calculate ΔCT values. ΔCT values for each animal were then divided by their respective reference gene correction factor (geometric mean of Tbp and Hprt ΔCT values) to obtain final expression data for target genes (BNP, ANF, and skeletal muscle actin). Three animals (n=3) were used per group. A two-way ANOVA test was used to determine statistical significance. A two-way ANOVA test was used to determine statistical significance. * indicates significance on level of genotype group. ** indicates significance on level of exercise (sedentary or running) group. % indicates a significant interaction between genotype and exercise groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P<0.05, ##P<0.001 as determined using a pair-wise post-test.
FIGURE 13 – MuRF1 regulates physiological cardiac hypertrophy induced by exercise training in MuRF1Tg+ mice. As of this writing, this figure appears in an Article in Press (published January 14, 2014) in American Journal of Physiology Endocrinology and Metabolism at the following website: http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013 DOI:10.1152/ajpendo.00326.2013
MuRF1 Tg+ and sibling-matched wildtype control mice (WildtypeMuRF1Tg+) were randomly assigned to either sedentary or running groups, where running groups were provided with an in-cage exercise wheel for 5 weeks and sedentary groups were not. Cardiac growth and function were measured by echocardiography. A. Representative M-mode images, containing at least 10 waveforms, from echocardiography of sedentary and running MuRF1 Tg+ and WildtypeMuRF1Tg+ B. LV mass measurements, as measured by echocardiography, normalized to TL for MuRF1 Tg+ and WildtypeMuRF1Tg+ mice assigned to either sedentary or running groups were also analyzed. C. Perfused and fix paraffin-embedded heart sections from sedentary and running MuRF1 Tg+ and WildtypeMuRF1Tg+ control mice were stained with Lectin-TRITC, imaged using fluorescent microscopy to visualize cardiomyocytes, and cross-sectional area analyzed from >180 cardiomyocytes from at least three animals per group. D. Histological analysis of HE was also performed. E. RNA was isolated from whole heart tissue from sedentary and running WildtypeMuRF1Tg+ and MuRF1Tg+ mice cDNA generated and BNP, ANF, skeletal muscle actin, Tbp, and Hprt expression determined, where both Tbp and Hprt were used as reference genes (described above). A two-way ANOVA test was used to determine statistical significance. * indicates significance on level of genotype group. ** indicates significance on level of exercise (sedentary or running) group. % indicates a significant interaction between genotype and exercise groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups are represented as #P< 0.05, ##P< 0.001 as determined using a pair-wise post-test.
FIGURE 14 – High power histological analysis of MuRF1 -/-, MuRF1 Tg+, and sibling wildtype control hearts.29

29 As of this writing, this figure appears in an Article in Press (published January 14, 2014) in American Journal of Physiology Endocrinology and Metabolism at the following website: http://ajpendo.physiology.org/content/early/2014/01/09/ajpendo.00326.2013 DOI:10.1152/ajpendo.00326.2013
**Top:** HE staining of representative cardiac sections from **A.** MuRF1/- and **B.** MuRF1 Tg+ mice after 5 weeks sham or running challenge. **Bottom:** Masson's trichrome (MT) staining of representative cardiac sections from **C.** MuRF1/- and **D.** MuRF1 Tg+ mice after 5 weeks sham or running challenge.
FIGURE 15 – MuRF1 inhibits T3-induced cardiomyocyte hypertrophy \textit{in vitro}

<table>
<thead>
<tr>
<th>HL-1 Cardiomyocytes</th>
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</tr>
</thead>
<tbody>
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<tr>
<td>Vehicle</td>
<td>1μM T3</td>
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<tr>
<td>Adsh scrambled</td>
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<td>AdMuRF1</td>
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<td>AdMuRF1</td>
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</table>
MuRF1 was knocked down in HL-1 cardiomyocytes using AdshMuRF1, with Adshscrambled as control, at MOI 60 for 24 hours in serum-free DMEM followed by treatment with 1µM T3 for 18 hours. Shown are representative fluorescent images and area analysis for vehicle-and T3-treated cardiomyocytes transduced with either Adshscrambled or AdshMuRF1. B. MuRF1 was increased in expression in HL-1 cardiomyocytes using AdMuRF1, with AdGFP as a control, at MOI 25 for 24 hours in serum-free DMEM followed by treatment with 1µM T3 for 18 hours. Shown are representative fluorescent images and area analysis of vehicle- or T3-treated HL-1 cardiomyocytes transduced with either AdGFP or AdMuRF1. C. MuRF1 was increased in expression in NRVM using AdMuRF1, with AdGFP as a control, at MOI 25 for 24 hours in serum-free M199 followed by treatment with 1µM T3 for 18 hours. Shown are representative fluorescent images and area analysis of vehicle- or T3-treated NRVM transduced with either AdGFP or AdMuRF1. Data are represented as mean ± SEM. Black bars represent vehicle and gray bars represent T3-treated cells or animals. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus or genotype group, **indicates significance on level of treatment group. %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as \#P< 0.05, \##P< 0.001, as determined using a pairwise post-test.
FIGURE 16 – MuRF1 inhibits T3-induced cardiomyocyte hypertrophy in vivo
MuRF1−/−, MuRF1 Tg+, and sibling-matched wildtype control mice (MuRF1+/+, WildtypeMuRF1Tg+) were randomly assigned to either vehicle or T3 groups, where T3 groups were injected with 1mg/kg T3 and vehicle groups were injected with PBS intraperitoneally each day for 14 days. **A.** Heart weight (HW) measurements normalized to body weight (BW) for MuRF1+/+ and MuRF1−/− vehicle and T3 groups. **B.** Heart weight (HW) measurements normalized to body weight (BW) for WildtypeMuRF1Tg+ and MuRF1 Tg+ vehicle and T3 groups. **C.** Representative images of histological analysis of perfused and fixed heart sections stained with hemotoxylin and eosin (HE) from T3-treated MuRF1+/+, MuRF1−/−, WildtypeMuRF1Tg+, and MuRF1 Tg+. **D.** Representative 100x images of histological analysis of perfused and fixed heart sections stained with Masson’s Trichrome (MT) from vehicle- and T3-treated MuRF1+/+ and MuRF1−/− used for cardiomyocyte area measurements. Analysis of cardiomyocyte area of vehicle- and T3-treated MuRF1+/+ and MuRF1−/−. **E.** Representative 100x images of histological analysis of perfused and fixed heart sections stained with MT from vehicle- and T3-treated WildtypeMuRF1Tg+ and MuRF1 Tg+ used for cardiomyocyte area measurements. Analysis of cardiomyocyte area of vehicle- and T3-treated WildtypeMuRF1Tg+ and MuRF1 Tg+. Data are represented as mean ± SEM. Black bars represent vehicle and gray bars represent T3-treated cells or animals. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus or genotype group, **indicates significance on level of treatment group. %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as #P < 0.05, ##P < 0.001, as determined using a pairwise post-test.
FIGURE 17 – Gene expression associated with pathological hypertrophy is not altered in MuRF1-/- and MuRF1Tg+ mice treated with T3
A. RNA was isolated from whole heart tissue from vehicle- and T3-treated MuRF1+/+ and MuRF1−/−, cDNA generated, and BNP, skeletal muscle actin, ANF, and TATA box-binding protein (Tbp) expression determined, where Tbp was used as a reference gene. B. RNA was isolated from whole heart tissue from vehicle- and T3-treated Wildtype穆RF1Tg+ and MuRF1 Tg+, cDNA generated, and BNP, skeletal muscle actin, ANF, and TATA box-binding protein (Tbp) expression determined, where Tbp was used as a reference gene. *In vitro* cardiomyocyte area measurements were averaged over at least 200 cardiomyocytes. *In vivo* cardiomyocyte area measurements were averaged over at least 400 cardiomyocytes from three different animals per group. RT-PCR analysis was carried out using the ΔΔCT method, where raw CT values were first normalized to Tbp (ΔCT values) and then to vehicle-treated wildtype groups (ΔΔCT values). RT-PCR data were averaged over 3 animals per group. Data are represented as mean ± SEM. Black bars represent vehicle and gray bars represent T3-treated cells or animals.
FIGURE 18 – MuRF1 inhibits TH cardiac uptake
A. Serum T3 and T4 analysis of MuRF1-/ and sibling-matched wildtype control mice (MuRF1 +/+ ) injected with either 1mg/kg T3 or vehicle (PBS) intraperitoneally each day for 14 days. Black bars represent MuRF1+/+ and gray bars represent MuRF1-. B. Serum T3 and T4 analysis of MuRF1 Tg+ and sibling-matched wildtype control mice (Wildtype^{MuRF1Tg+}) injected with either 1mg/kg T3 or vehicle (PBS) intraperitoneally each day for 14 days. Black bars represent Wildtype^{MuRF1Tg+} and gray bars represent MuRF1 Tg+. Data are represented as mean ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group, **indicates significance on level of treatment group %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as #P< 0.05, ##P< 0.001 as determined using a pairwise post-test.
FIGURE 19 – MuRF1 inhibits cardiac gene expression of the TH transporter MCT10
A. RNA was isolated from whole heart tissue from vehicle- and T3-treated MuRF1+/+ and MuRF1−/−, cDNA generated, and monocarboxylate transporter 8 (MCT8), MCT10, and Tbp expression determined, where Tbp was used as a reference gene. Black bars represent MuRF1+/+ and gray bars represent MuRF1−/−. B. RNA was isolated from whole heart tissue from vehicle- and T3-treated WildtypeMuRF1Tg+ and MuRF1 Tg+, cDNA generated, and monocarboxylate transporter 8 (MCT8), MCT10, and Tbp expression determined, where Tbp was used as a reference gene. Black bars represent WildtypeMuRF1Tg+ and gray bars represent MuRF1 Tg+. RT-PCR analysis was carried out using the ΔΔCT method, where raw CT values were first normalized to Tbp (ΔCT values) and then to vehicle-treated wildtype groups (ΔΔCT values). RT-PCR data were averaged over 3 animals per group. Data are represented as mean ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group, **indicates significance on level of treatment group %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as #P< 0.05, ##P< 0.001 as determined using a pairwise post-test.
FIGURE 20 – MuRF1 inhibits TRα transcriptional activity, but does not target TRα for proteasomal degradation

A

* Plasmid Treatment

<table>
<thead>
<tr>
<th>Plasmid Treatment</th>
<th>Luciferase/βGal</th>
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<tr>
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<tr>
<td>TRα T3</td>
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</tr>
</tbody>
</table>

% Plasmid and Adenovirus F=8.046, DF=1

B

<table>
<thead>
<tr>
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<th>AdshMuRF1</th>
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<td>β-actin</td>
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C

<table>
<thead>
<tr>
<th>TRα</th>
<th>Control Adenovirus</th>
<th>AdMuRF1</th>
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</thead>
<tbody>
<tr>
<td>myc-MuRF1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
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</tr>
</tbody>
</table>
A. To assess the role of MuRF1 on TRα transcriptional activity, Cos-7 cells were co-transfected with luciferase plasmid driven by the thyroid response element (TRE) of the GH gene, as described previously\textsuperscript{122}, β-galactosidase plasmid (for transfection control), and FLAG-TRα plasmid, with empty FLAG as a control, transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 4 hours, and treated with 1µM T3 for 24 hours. Parallel plates were used to confirm expression of FLAG-TRα and myc-MuRF1 by immunoblot using FLAG and myc antibodies, respectively. Black bars represent AdGFP- and gray bars represent AdMuRF1-transduced cells. B. MuRF1 was knocked down in HL-1 cardiomyocytes using AdshMuRF1, with Adshscrambled as control, at MOI 60 for 24 hours. Immunoblot using whole cell lysates was used to access protein expression of endogenous TRα. MuRF1 antibody was used to confirm knockdown. β-actin was used as a loading control. C. MuRF1 was increased in expression in HL-1 cardiomyocytes using AdMuRF1, with AdGFP as a control, at MOI 25 for 24 hours. Immunoblot using whole cell lysates was used to access protein expression of endogenous TRα. Myc antibody was used to confirm expression of myc-MuRF1. β-actin was used as a loading control. Luciferase data is representative of three independent experiments. Data are represented as mean ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of plasmid group. %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as #P< 0.05 as determined using a pairwise post-test.
**FIGURE 21 – Localization of TRα in the nucleus of cardiomyocytes is increased by MuRF1**

<table>
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</tr>
<tr>
<td>Ad-myc-MuRF1</td>
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<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1μM T3</td>
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**A**

anti-FLAG (TRα)

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<tr>
<th></th>
<th>0.342±0.02</th>
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**B**

anti-MYC (MuRF1)

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<tr>
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<td>n=19</td>
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</table>

**C**

2° only [DAPI]

2° only [568 (TRα)]

2° only [647 (MuRF1)]
HL-1 cardiomyocytes were transfected with FLAG-TRα plasmid, or with empty FLAG as a control (see Figure 22), transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 4 hours, and treated with 1µM T3 for 2 hours. **A.** Nuclear localization of TRα was assessed using confocal microscopy, immunofluorescent staining of TRα (anti-FLAG, red) and nuclei (DAPI, blue), and quantitatively evaluated by determining the degree of TRα (red) and DAPI (blue) overlap via correlation coefficient measurements. **B.** The co-localization of MuRF1 and TRα in the nucleus was assessed using confocal microscopy, immunofluorescent staining of MuRF1 (anti-myc, green) and TRα (anti-FLAG, red), and quantitatively evaluated by determining the degree of MuRF1 (green) and TRα (red) overlap, isolated to the nucleus, via correlation coefficient measurements. **C.** Control confocal analysis of immunofluorescent staining with secondary antibodies (568 for TRα and 647 for MuRF1) alone. Quantitative immunofluorescence measurements were made using Volocity imaging software. For correlation coefficients, numeric range is from 0 to 1, where 0 indicates no correlation and 1 indicated perfect correlation. Data are represented as mean ± SEM. For TRα/DAPI co-immunofluorescence, a two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group, **indicates significance on level of treatment group. Significance between groups is represented as ##P< 0.001 as determined using a pairwise post-test. For TRα/MuRF1 coimmunofluorescence, a Student’s t-test was used to measure statistical significance where $ indicates P<0.001 between groups.
FIGURE 22 – Empty FLAG vector controls for FLAG-TRα immunofluorescence

<table>
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</tr>
<tr>
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<td>Empty Adenovirus</td>
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</tr>
<tr>
<td>Ad-myc-MuRF1</td>
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<td>+</td>
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<tr>
<td>1μM T3</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**IF:** FLAG (TRα)  
**DAPI/FLAG**
HL-1 cardiomyocytes were transfected with empty FLAG as a control, transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 4 hours, and treated with 1µM T3 for 2 hours. Control confocal analysis of immunofluorescent staining with anti-FLAG (red) and nuclei (DAPI, blue).
FIGURE 23 – Endogenous TRα accumulates in nucleus enriched fractions in a MuRF1- and T3-dependent manner

A

HL-1 cells

Nuclear Enrichment

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B

HL-1 cells

Cytoplasmic Enrichment

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<tbody>
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<td>Myc (MuRF1)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>p-Rb</td>
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</tbody>
</table>
MuRF1 was increased in expression in HL-1 cardiomyocytes using AdMuRF1, with AdGFP as a control, at MOI 25 for 24 hours in serum-free DMEM followed by treatment with 1µM T3 for 2 hours. Immunoblot using nuclear- and cytoplasmic-enriched fractions was used to access nuclear localization of endogenous TRα. p-Rb was used as a nuclear marker and GAPDH was used as a cytoplasmic marker. Densitometry analysis of TRα is shown for AdGFP- and AdMuRF1-transduced cardiomyocytes treated with vehicle (PBS) or T3. Data are represented as mean ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group, **indicates significance on level of treatment group %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as #P< 0.05 as determined using a pairwise post-test.
FIGURE 24 – MuRF1 interacts with TRα in the presence of T3

Cos-7 cells

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<th>1μM T3</th>
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**IP:** Myc   **IB:**

<table>
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**Input:**

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</thead>
<tbody>
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<td>Myc (MuRF1)</td>
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</table>

IgG heavy chain
Cos-7 cells were transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 24 hours, and treated with 1µM T3 for 2 hours. To determine if MuRF1 physically interacts with TRα, whole cell lysates were submitted to specific immunoprecipitation of myc-MuRF1 followed by immunoblot of endogenous TRα.
FIGURE 25 – MuRF1 mono-ubiquitates TRα, inhibiting TRα’s poly-ubiquitination

A

Cos-7 cells

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<td>AdMuRF1</td>
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B

Cos-7 cells

<table>
<thead>
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<th>Input 2</th>
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</tr>
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</tr>
<tr>
<td>AdMuRF1</td>
<td>-</td>
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</tbody>
</table>
Cos-7 cells were co-transfected with HA-Ub and FLAG-TRα plasmids, with empty HA and FLAG as controls, and transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 4 hours. To assess the effect of MuRF1 expression on TRα mono-ubiquitination (B), whole cell lysates were submitted to specific immunoprecipitation of FLAG-TRα followed by immunoblot of HA using anti-HA-horseradish peroxidase (HRP)-linked primary antibody (to prevent IgG heavy chain signal). To assess the effect of MuRF1 expression on TRα poly-ubiquitination (C), whole cell lysates were submitted to specific immunoprecipitation of FLAG-TRα followed by immunoblot of HA using primary anti-HA and HRP-linked secondary antibody.
FIGURE 26 – MuRF1 promotes the interaction of TRα and CAP350 in the nucleus

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Empty Plasmid</th>
<th>FLAG-TRα</th>
<th>Empty Adenovirus</th>
<th>Ad-myc-MuRF1</th>
<th>1μM T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
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</tbody>
</table>

A

- anti-FLAG (TRα)
- Anti-CAP350 (endogenous)
- FLAG/CAP350

TRα/CAP350 co-localization in nucleus:

<table>
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<tr>
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<th>n=23</th>
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<td>0.658±0.02</td>
<td>0.700±0.02</td>
<td>0.728±0.01</td>
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</table>

B

- 2° only [DAPI]
- 2° only [568 (TRα)]
- 2° only [488 (CAP350)]
HL-1 cardiomyocytes were transfected with FLAG-TRα plasmid, or with empty FLAG as a control (see Figure 22), transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 4 hours, and treated with 1µM T3 for 2 hours. **A.** The co-localization of TRα and endogenous centrosome-associated protein 350 (CAP350) in the nucleus was assessed using confocal microscopy, immunofluorescent staining of TRα (anti-FLAG, red) and CAP350 (green), and quantitatively evaluated by determining the degree of TRα (red) and CAP350 (green) overlap, isolated to the nucleus, via correlation coefficient measurements. **B.** Control confocal analysis of immunofluorescent staining with secondary antibodies (568 for TRα and 488 for CAP350) alone. Quantitative immunofluorescence measurements were made using Volocity imaging software. For correlation coefficients, numeric range is from 0 to 1, where 0 indicates no correlation and 1 indicated perfect correlation. Data are represented as mean ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group, **indicates significance on level of treatment group. Significance between groups is represented as ##P< 0.001 as determined using a pairwise post-test.
FIGURE 27 – Interaction between desmin and TRα at the cardiomyocyte nuclear membrane is MuRF1- and T3-dependent

<table>
<thead>
<tr>
<th>Condition</th>
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<th>+</th>
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<td>FLAG-TRα</td>
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<td>Empty Adenovirus</td>
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</tr>
<tr>
<td>Ad-/myc-MuRF1</td>
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<td>-</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>1μM T3</td>
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<td>-</td>
<td>+</td>
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</tr>
</tbody>
</table>

A

**anti-FLAG (TRα)**

**Desmin (endogenous)**

**FLAG/Desmin**

**TRα/Desmin co-localization at nuclear membrane**

<table>
<thead>
<tr>
<th>Condition</th>
<th>0.31±0.02</th>
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<th>0.39±0.02</th>
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<td>n=20</td>
<td>n=17</td>
<td>n=15</td>
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</tr>
</tbody>
</table>

B

**2° only [DAPI]**

**2° only [568 (TRα)]**

**2° only [488 (Desmin)]**
HL-1 cardiomyocytes were transfected with FLAG-TRα plasmid, or with empty FLAG as a control (see Figure 2), transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 4 hours, and treated with 1µM T3 for 2 hours. A. The co-localization of TRα and endogenous desmin at the nuclear membrane was assessed using confocal microscopy, immunofluorescent staining of TRα (anti-FLAG, red) and desmin (green), and quantitatively evaluated by determining the degree of TRα (red) and desmin (green) overlap, isolated to the nuclear membrane, via correlation coefficient measurements. B. Control confocal analysis of immunofluorescent staining with secondary antibodies (568 for TRα and 488 for desmin) alone. Quantitative immunofluorescence measurements were made using Volocity imaging software. For correlation coefficients, numeric range is from 0 to 1, where 0 indicates no correlation and 1 indicated perfect correlation. Data are represented as mean ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group, **indicates significance on level of treatment group % indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as #P< 0.05 as determined using a pairwise post-test.
FIGURE 28 – T3 promotes the interaction between laminβ1 and TRα, which is inhibited by MuRF1

<table>
<thead>
<tr>
<th>Condition</th>
<th>Empty Plasmid</th>
<th>FLAG-TRα</th>
<th>Empty Adenovirus</th>
<th>Ad-myc-MuRF1</th>
<th>1μM T3</th>
</tr>
</thead>
<tbody>
<tr>
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<td>+</td>
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</table>

**anti-FLAG (TRα)**

**Laminβ1 (endogenous)**

**FLAG/Laminβ1**

<table>
<thead>
<tr>
<th>TRα/Laminβ1 co-localization at nuclear membrane</th>
<th>0.265±0.01</th>
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</table>

**2º only [DAPI]**

**2º only [568 (TRα)]**

**2º only [488 (Laminβ1)]**
HL-1 cardiomyocytes were transfected with FLAG-TRα plasmid, or with empty FLAG as a control (see Figure 22), transduced with AdMuRF1, with AdGFP as a control, at MOI 25 for 4 hours, and treated with 1µM T3 for 2 hours. **A.** The co-localization of TRα and endogenous laminβ1 at the nuclear membrane was assessed using confocal microscopy, immunofluorescent staining of TRα (anti-FLAG, red) and laminβ1 (green), and quantitatively evaluated by determining the degree of TRα (red) and laminβ1 (green) overlap, isolated to the nuclear membrane, via correlation coefficient measurements. **B.** Control confocal analysis of immunofluorescent staining with secondary antibodies (568 for TRα and 488 for laminβ1) alone. Quantitative immunofluorescence measurements were made using Volocity imaging software. For correlation coefficients, numeric range is from 0 to 1, where 0 indicates no correlation and 1 indicated perfect correlation. Data are represented as mean ± SEM. A two-way ANOVA test was used to determine statistical significance. *indicates significance on level of adenovirus group, **indicates significance on level of treatment group %indicates a significant interaction between adenovirus and treatment groups. The F statistic and degrees of freedom (DF) were reported when dependence between groups was found to be a significant source of variation. Significance between groups is represented as #P < 0.05, ##P < 0.001 as determined using a pairwise post-test.
FIGURE 29 – Amino acid sequences of mouse TRα domain lysine mutants

**TRα Domains**

<table>
<thead>
<tr>
<th>A/B</th>
<th>C</th>
<th>D</th>
<th>E/F</th>
</tr>
</thead>
</table>

**TRα WT**

meqkpskvec gdpeensar spdgkrkrkn gceplkssms gyipsylkd eqevegdk a gyhyrcele eegkgffirr iqknhltypsy ckydscevid kiteqeqle rtkkciavym amdlvldsk rvarkkliq nrrrrkeem inlsqgrp peewdlikv ateahrstna gshswkqrrk fplpgqsp isvmppgdkv dleaesfslk itipairtvv dflakkpimfs elpeedqitllkgeceemis iaravrydpe sdltltsgem acvkrelqng ggvsvsdaif elgksafln lddtevalnq avllimstdrs gillevdkieq sqeayllafe hynrhkhnifi pwvplkmlk vtdlrnigae hasrflhmkv ecepfllpl flevfedqev

**TRα A/B K→R**

meqRpsRvec gdpeensar spdgRrRrRn gceplRssms gyipsylRd eqevegdk a gyhyrcele eegkgffirr iqknhltypsy ckydscevid kiteqeqle rtkkciavym amdlvldsk rvarkkliq nrrrrkeem inlsqgrp peewdlikv ateahrstna gshswkqrrk fplpgqsp isvmppgdkv dleaesfslk itipairtvv dflakkpimfs elpeedqitllkgeceemis iaravrydpe sdltltsgem acvkrelqng ggvsvsdaif elgksafln lddtevalnq avllimstdrs gillevdkieq sqeayllafe hynrhkhnifi pwvplkmlk vtdlrnigae hasrflhmkv ecepfllpl flevfedqev

**TRα C K→R**

meqkpskvec gdpeensar spdgkrkrkn gceplkssms gyipsylkd eqevegdk a gyhyrcele eegkgffirr iqknhltypsy ckydscevid kiteqeqle rtkkciavym amdlvldsk rvarkkliq nrrrrkeem inlsqgrp peewdlikv ateahrstna gshswkqrrk fplpgqsp isvmppgdkv dleaesfslk itipairtvv dflakkpimfs elpeedqitllkgeceemis iaravrydpe sdltltsgem acvkrelqng ggvsvsdaif elgksafln lddtevalnq avllimstdrs gillevdkieq sqeayllafe hynrhkhnifi pwvplkmlk vtdlrnigae hasrflhmkv ecepfllpl flevfedqev

**TRα D → R**

meqkpskvec gdpeensar spdgkrkrkn gceplkssms gyipsylkd eqevegdk a gyhyrcele eegkgffirr iqknhltypsy ckydscevid kiteqeqle rtkkciavym amdlvldsk rvarkkliq nrrrrkeem inlsqgrp peewdlikv ateahrstna gshswkqrrk fplpgqsp isvmppgdkv dleaesfslk itipairtvv dflakkpimfs elpeedqitllkgeceemis iaravrydpe sdltltsgem acvkrelqng ggvsvsdaif elgksafln lddtevalnq avllimstdrs gillevdkieq sqeayllafe hynrhkhnifi pwvplkmlk vtdlrnigae hasrflhmkv ecepfllpl flevfedqev

**TRα WT**

meqkpskvec gdpeensar spdgkrkrkn gceplkssms gyipsylkd eqevegdk a gyhyrcele eegkgffirr iqknhltypsy ckydscevid kiteqeqle rtkkciavym amdlvldsk rvarkkliq nrrrrkeem inlsqgrp peewdlikv ateahrstna gshswRqrR fplpgqsp isvmppgdRv dleaesfRl itipairtvv dflakkpimfs elpeedqitllkgeceemis iaravrydpe sdltltsgem acvkrelqng ggvsvsdaif elgRsasfln lddtevalnq avllimstdrs gillevRieR sqeayllafe hynrhRthi pwvplRlmk vtdlrnigae hasrflhmRv ecepfllpl flevfedqev
Final amino acid sequences of TRα WT, TRα A/B KtoR, TRα C KtoR, TRα D KtoR, and TRα E/F KtoR, as expressed via the p3XFLAG®-CMV-14 vector. Plasmid cloning described in data supplement.
FIGURE 30 – Mutation of the lysine residues within the E/F region of TRα inhibits MuRF1-dependent TRα nuclear accumulation

<table>
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<tr>
<th></th>
<th>WT</th>
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<th>C KtoR</th>
<th>D KtoR</th>
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HL-1 cardiomyocytes were transfected with indicated FLAG-TRα domain lysine to arginine (KtoR) mutant plasmids, or with empty FLAG as a control (see Figure 22), transduced with AdMuRF1 at MOI 25 for 4 hours, and treated with 1µM T3 for 2 hours. A. Nuclear localization of TRα was assessed using confocal microscopy, immunofluorescent staining of TRα (anti-FLAG, red) and nuclei (DAPI, blue), and quantitatively evaluated by determining the degree of TRα (red) and DAPI (blue) overlap via correlation coefficient measurements. B. The co-localization of MuRF1 and TRα in the nucleus was assessed using confocal microscopy, immunofluorescent staining of MuRF1 (anti-myc, green) and TRα (anti-FLAG, red), and quantitatively evaluated by determining the degree of MuRF1 (green) and TRα (red) overlap, isolated to the nucleus, via correlation coefficient measurements. C. The co-localization of TRα and endogenous CAP350 in the nucleus was assessed using confocal microscopy, immunofluorescent staining of TRα (anti-FLAG, red) and CAP350 (green), and quantitatively evaluated by determining the degree of TRα (red) and CAP350 (green) overlap, isolated to the nucleus, via correlation coefficient measurements. Quantitative immunofluorescence measurements were made using Volocity imaging software. For correlation coefficients, numeric range is from 0 to 1, where 0 indicates no correlation and 1 indicated perfect correlation. Data are represented as mean ± SEM. A one-way ANOVA test was used to determine statistical significance. Significance between groups is represented as ##P< 0.001 as determined using a pairwise post-test.
FIGURE 31 – Cardiac hypertrophy is associated with biasing the balance of protein synthesis and degradation towards synthesis\textsuperscript{30}

\textsuperscript{30} This figure was modified from Willis and Patterson\textsuperscript{178}
FIGURE 32 – Regions of TRα known to be modified by ubiquitination/SUMOylation
FIGURE 33 – Ubiquitin ligase (E3) expression in Golden retriever muscular dystrophy (GRMD)\textsuperscript{31}

\textsuperscript{31} This figure previously appeared in \textit{Muscle and Nerve}. The original citation for the publication which includes this figure is as follows: \textit{Muscle Nerve} 44, no. 4 (October 2011): 553-62.
Quantitative mRNA expression analysis of the ubiquitin ligases (A) muscle ring finger 1 (MuRF1), (B) muscle F-box protein (MAFbx), (C) carboxyl terminus of Hsp70-interacting protein (CHIP), and (D) mouse double minute-2 (MDM2) in GRMD lateral digital extensor, the lateral head of the gastrocnemius, vastus lateralis, biceps femoris, left ventricle, and cranial sartorius. mRNA expression is shown as the fold change compared with age-matched control animals. Data represent 3–5 dogs per group (outlined in Table 13), as indicated above each vertical boxplot. The vertical boxplot presents the median (50% percentile), indicated by the middle line inside the box, the 75th percentile indicated by the top of the box, and the 25th percentile indicated by the bottom of the box. A rank-sum test was used to determine the differences in mRNA expression. *P < 0.05.
FIGURE 34 – Expression of ubiquitin, uncoordinated phenotype 4/5 (UNC4/5), and the E2 ubiquitin-conjugating enzyme ubiquitin-like protein small ubiquitin-like modifier (SUMO)-1-conjugating enzyme (UBC9) in Golden retriever muscular dystrophy (GRMD) skeletal muscle and heart. \(^{32}\)

\(^{32}\) This figure previously appeared in *Muscle and Nerve*. The original citation for the publication which includes this figure is as follows: *Muscle Nerve* 44, no. 4 (October 2011): 553-62.
Quantitative mRNA expression analysis of (A) ubiquitin, (B) the protein chaperone UNC4/5, and (C) E2 UBC9 in the GRMD lateral digital extensor, the lateral head of the gastrocnemius, vastus lateralis, biceps femoris, left ventricle, and cranial sartorius. mRNA expression is shown as the fold change compared with age-matched control animals. Data represent 3–5 dogs per group (outlined in Table 13), as indicated above each vertical boxplot. The vertical boxplot presents the median (50th percentile), indicated by the middle line inside the box, the 75th percentile indicated by the top of the box, and the 25th percentile indicated by the bottom of the box. A rank-sum test was used to determine the differences in mRNA expression. *P < 0.05.
FIGURE 35 – Proteasome subunit expression in Golden retriever muscular dystrophy (GRMD) skeletal muscle and heart. This figure previously appeared in Muscle and Nerve. The original citation for the publication which includes this figure is as follows: Muscle Nerve 44, no. 4 (October 2011): 553-62.
(A) PSMA6, (B) PSMB4, and (C) PSME1 in GRMD long digital extensor, lateral head of the gastrocnemius, vastus lateralis, biceps femoris, left ventricle, and cranial sartorius. mRNA expression is shown as the fold change compared with age-matched control animals. Data represent 3–5 dogs per group (outlined in Table 13), as indicated above each vertical boxplot. The vertical boxplot presents the median (50th percentile), indicated by the middle line inside the box, the 75th percentile indicated by the top of the box, and the 25th percentile indicated by the bottom of the box. A rank-sum test was used to determine the differences in mRNA expression. *$P<0.05$. 
FIGURE 36 – Muscle-specific proteasome activities in Golden retriever muscular dystrophy (GRMD)\textsuperscript{34}

This figure previously appeared in Muscle and Nerve. The original citation for the publication which includes this figure is as follows: Muscle Nerve 44, no. 4 (October 2011): 553-62.

\textsuperscript{34}
(A) Trypsin-like, (B) caspase-like, and (C) chymotrypsin-like activities in GRMD long digital extensor, lateral head of the gastrocnemius, vastus lateralis, biceps femoris, left ventricle, and cranial sartorius. Proteasome activities are presented as arbitrary fluorescent units (AFU). Data represent 3–5 dogs per group (outlined in Table 13), as indicated above each vertical boxplot. The vertical box plot presents the median (50th percentile), indicated by the middle line inside the box, the 75th percentile indicated by the top of the box, and the 25th percentile indicated by the bottom of the box. A rank-sum test was used to determine the differences in proteasome activity. *$P < 0.05$. 
FIGURE 37 – Muscle-specific calpain 1 and 2 activities in Golden retriever muscular dystrophy (GRMD)\textsuperscript{35}

\textsuperscript{35} This figure previously appeared in \textit{Muscle and Nerve}. The original citation for the publication which includes this figure is as follows: \textit{Muscle Nerve} 44, no. 4 (October 2011): 553-62.
Quantitative mRNA expression analysis of (A) calpain 1, and (B) calpain 2 in GRMD long digital extensor, lateral head of the gastrocnemius, vastus lateralis, biceps femoris, left ventricle, and cranial sartorius. Expression levels are presented as a percentage of age-matched controls. (C) Calpain 1 and 2 activity determined by a fluorimetric assay based on GRMD long digital extensor, lateral head of the gastrocnemius, vastus lateralis, biceps femoris, left ventricle, and cranial sartorius. mRNA expression is shown as the fold change compared with age-matched control animals. Calpain 1 and 2 activity presented as relative fluorometric units. Data represent 3–5 dogs per group (outlined in Table 13), as indicated above each vertical boxplot. The vertical boxplot presents the median (50th percentile), indicated by the middle line inside the box, the 75th percentile indicated by the top of the box, and the 25th percentile indicated by the bottom of the box. A rank-sum test was used to determine the differences in mRNA expression and calpain 1 and 2 activity. *P< 0.05.
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