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Human Molecular Genetic and Functional Studies Identify *TRIM63*, Encoding Muscle RING Finger Protein 1, as a Novel Gene for Human Hypertrophic Cardiomyopathy

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Abstract

Rationale—A delicate balance between protein synthesis and degradation maintains cardiac size and function. *TRIM63* encoding Muscle RING Finger 1 (MuRF1) maintains muscle protein homeostasis by tagging the sarcomere proteins with ubiquitin for subsequent degradation by the Ubiquitin-Proteasome System (UPS).

Objectives—To determine the pathogenic role of *TRIM63* in human hypertrophic cardiomyopathy (HCM).

Methods and Results—Sequencing of *TRIM63* gene in 302 HCM probands (250 Caucasians) and 339 controls (262 Caucasians) led to identification of two missense (p.A48V and p.I130M) and a deletion (p.Q247*) variants exclusively in the HCM probands. These three variants were absent in 751 additional controls screened by TaqMan assays. Likewise, rare variants were enriched in the Caucasian HCM population (11/250, 4.4% vs. 3/262, 1.1%, respectively, p=0.024). Expression of the mutant TRIM63 was associated with mislocalization of TRIM63 to sarcomere Z disks, impaired auto-ubiquitination, reduced ubiquitination and UPS-mediated degradation of myosin heavy chain 6, cardiac myosin binding protein C, calcineurin (PPP3CB), and p-MTOR in adult cardiac myocytes. Induced expression of the mutant TRIM63 in the mouse heart was associated with cardiac hypertrophy, activation of the MTOR-S6K and calcineurin pathways and expression of the hypertrophic markers, which were normalized upon turning off expression of the mutant protein.

Conclusions—*TRIM63* mutations, identified in patients with HCM, impart loss-of-function effects on E3 ligase activity and are likely causal mutations in HCM. The findings implicate impaired protein degradation in the pathogenesis of HCM.

Keywords

Genetics; mutation; hypertrophic cardiomyopathy; ubiquitin; hypertrophy

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DISCLOSURES None

INTRODUCTION

A delicate balance between protein synthesis and degradation maintains muscle trophic homeostasis, including cardiac size and function. A shift in protein homeostasis in favor of synthesis, resulting in increased cell protein content, is an established mechanism for cardiac hypertrophy. [1] The molecular pathways responsible for enhanced protein synthesis and ensuing cardiac hypertrophy have been extensively characterized. [2] In contrast, the potential contributions of the protein degradation pathways, including the ubiquitin-proteasome system (UPS), in maintaining cardiac protein homeostasis and the pathogenesis of cardiac hypertrophy are less well understood. [3-7]

Hypertrophic cardiomyopathy (HCM) is a relatively common genetic disorder and a prototypic form of cardiac hypertrophy. [8, 9] Over a dozen causal genes, coding for thick, thin and Z disk proteins of sarcomeres, have been identified in probands and families with HCM. [8, 9] Cardiac hypertrophy, notwithstanding the genetic-based diagnosis, is the clinical diagnostic hallmark of HCM and a major determinant of mortality and morbidity. [10-12] HCM is the most common cause of sudden cardiac death (SCD) in the young athletes and an important cause of morbidity in the older adults. [9, 13]

Cardiac hypertrophy in HCM is considered secondary to activation of a diverse array of intracellular signaling pathways that collectively promote protein synthesis. [14, 15] The role of protein degradation, the opposite end of the spectrum from protein synthesis, in the pathogenesis of HCM is less well recognized. [16, 17] Identification and functional characterization of *TRIM63* encoding MuRF1 and *FBXO32* encoding F-box protein 32 (a.k.a. Atrogin 1 or MAFbx) have raised considerable interest in the role of these molecular regulators of cardiac protein degradation in maintaining cardiac structure and function in cardiomyopathies. [4, 5, 18-20]

TRIM63 (protein ID:Q969Q1), also known as muscle-specific RING finger protein 1 (MuRF1), is an E3 ubiquitin ligase that is expressed selectively in cardiac and skeletal muscles. [18] TRIM63 is capable of polyubiquitination and UPS-mediated degradation of thick filament proteins MYH6 and MYBPC3. [21-23] Over-expression of MuRF1 (TRIM63) antagonizes cardiac myocyte hypertrophic response to agonists and increases susceptibility to heart failure in response to pressure overload. [424] In contrast, deficiency of MuRF1 ($MuRF1^{-/-}$) exaggerates cardiac hypertrophic response to aortic banding. [6] In view of the increasing recognition of protein degradation pathways in regulating muscle trophic state, we set to delineate the pathogenic role of *TRIM63* in cardiomyopathies. Accordingly, we screened HCM probands for mutations in *TRIM63*, identified and functionally characterized three mutations that invoke impaired protein degradation as a likely pathogenic mechanism for cardiac hypertrophy in human HCM.

METHODS

An expanded METHODS section is provided as Online data.

Ethical approval

The Institutional Review Board approved the studies in humans. The participants signed informed consents. The use of mice conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the Institutional Animal Care and Use Committee.

Study population

The discovery study population was comprised of 302 (250 Caucasians) probands with HCM and 339 (262 Caucasians) controls individuals, all evaluated by history, physical examination, ECG and echocardiography. Additionally 751 control individuals were screened by the TaqMan assays for the specific rare variants identified in the HCM probands.

TRIM63 variants

The coding and exon-intron boundary regions of *TRIM63* were sequenced by Sanger sequencing (Online Table I). The sequence output was analyzed using Variant Reporter software and manually by two individuals and compared with the *TRIM63* reference sequence (hg19, Chromosome 1, NC_000001.10, 26377798..26394121, complement). Variant genotyping was performed using the Allelic Discrimination Assays on a 7900HT SDS (Online Table I).

Multiple sequence alignment

Regions encompassing the mutant amino acids from multiple species were aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Exclusion of known common causal genes for HCM

TRIM63 mutation carriers were screened for mutations in *MYH7*, *MYBPC3*, *TNNT2*, *TNNI3*, *TPM1* and *ACTC1*, known relatively common genes for HCM, by Sanger sequencing. [9]

Genotyping

Family members were genotyped for five short tandem repeat (STR) DNA markers located near the *TRIM63* locus on 1p34-p33 by PCR and capillary electrophoresis on an ABI 3730xl system [25] (Online Figure I).

Cloning and site directed mutagenesis

Trim63 cDNA was synthesized from mouse total cardiac RNA by reverse transcription and was tagged with a Flag at the 3′ end. The mutations were introduced by site directed mutagenesis (Online Table I).

Recombinant lenti- and adenoviruses

Flag-tagged WT, p.A48V, p.I130M and p.Q247* *Trim63* cDNAs were cloned into the lentiviral expression vector. The lentiviral plasmid containing the *Trim63* cDNA and the packaging plasmids were transfected into 293T cells to generate the viruses.

Flag-tagged *Trim63* cDNAs were cloned into adenovirus expression vector by homologous recombination. The plasmid DNA was mixed with Lipofectamine™ 2000 and transfected to 293A cells. Cells were harvested 7-10 days post-transfection, when reached ~80% cytopathicity. The crude adenovirus lysates were prepared by freeze/thaw cycles. Viruses were amplified to generate higher-titer viral stocks.

HeLa-His/Biotin-Ubiquitin cells

A MSCV retroviral plasmid expressing a His6-Biotin-Ubiquitin fusion protein and retrovirus packaging plasmids were co-transfected into 293T cells. Retroviruses were then used to infect the HeLa cells. Cells were then selected under blasticidin selection to generate stable HeLa-His/Bio-Ub cells.

Adult ventricular myocytes

Adult cardiac myocytes were isolated from four to six months old mice (FVB background) upon retrograde perfusion and enzymatic digestion with collagenase Type II and were reintroduced to calcium at a final concentration of 200μM $CaCl_2$, in the presence of 2mM ATP. Myocytes were counted in a hemocytometer and placed on laminin-coated plates in a CO_2 incubator at 37°C.

Adenoviral Infection

Adult cardiac myocytes were transduced with the recombinant adenoviruses at a multiplicity of infection of 100 for 4 hours. [26-28]

Doxycycline-inducible wild type and mutant TRIM63 transgenic mice

Cardiac-restricted inducible tet-off transgenic mice expressing $TRIM63^{WT}$, $TRIM63^{A48V}$, $TRIM63^{I130M}$ and $TRIM63^{Q247*}$ were generated by the conventional methods [29, 30]. The regulator mice (*Myh6*-tTA) and the tet-O target vector were kind and generous gifts from Dr. Robbins. [29, 30]

Immunoblotting

Immunoblotting was performed using aliquots of 30ug of protein extracts as published. [31]

Co-IP

To detect auto-ubiquitination, aliquots of 2μg/ml of Biotin were added to culture media of Hela-His/Bio-Ubiquitin cells transduced with the recombinant lentiviruses. Cells were cultured in the presence of MG132, a proteasome inhibitor, and a protease inhibitor cocktail. Cell lysates were sonicated to shear DNA and cell membranes. Strepavidin-Agarose Slurry was added to precipitate biotinylated proteins followed by electrophoresis and immunoblotting.

Likewise, 500ug aliquots of adult cardiac myocytes proteins, extracted in the presence of MG132, were pre-cleared with IgG and Protein A/G agarose beads, incubated with an anti Ubiquitin antibody and treated with Protein A/G agarose beads. The immunoprecipitates were used for immunoblotting.

Immunofluorescence

Immunofluorescence was performed per published methods. [31] Briefly, co-localization of ubiquitin and TRIM63 in HeLa-His/Bio-Ub cells was detected upon treating the cells with biotin to label ubiquitin. The cells were incubated with a rabbit polyclonal anti Flag antibody followed by incubation with a mixture of a donkey anti rabbit antibody conjugated with Alexa Fluor® 350 and Streptavidin conjugated with Texas Red. Co-localization in myocytes was performed using a monoclonal anti Flag antibody conjugated to Cy3 and the nuclei were counter-stained with DAPI. To detect co-localization of TRIM63 and α-actinin, adenovirally transduced adult cardiac myocytes were fixed, permeabilized and incubated with mouse monoclonal anti α-actinin. The secondary antibody was donkey anti mouse conjugated with Alexa Fluor 488 dye.

Creatine kinase (CK) enzymatic assay

Aliquots of protein (10μg) were mixed with the reconstituted reagent and incubated at room temperature. The optical density of each sample was read using a microplate reader at absorbance wavelength of 340nm at 10 min, 25 min and 40min. CK activities were calculated based on colorimetric readouts.

Echocardiography

Transthoracic (M mode and 2D) and Doppler echocardiography was performed to assess cardiac function in the age- and sex-matched non-transgenic and transgenic animals using an HP 5500 Sonos echocardiography unit equipped with a 15-MHz linear transducer. [31-33]

Cardiac morphology and histology

The heart was explanted, fixed by perfusion and photographed. Ventricular weight was determined after excising the heart at the atrioventricular groove to isolate atria and the great blood vessels. Thin myocardial sections were stained with H&E, and Masson trichrome. Collagen volume fraction was determined by morphometric analysis using ImageTool 3.0 analysis software (http://ddsdx.uthscsa.edu/dig/itdesc.html). [33, 34]

Myocyte cross sectional area (CSA) was determined after staining of the cryosections with 2μg/ml wheat germ agglutinin conjugated with Texas-Red. The sections were mounted in Hard Set^{TM} mounting medium and examined under fluorescence microscopy. [33]

Enrichment of ubiquitinated cardiac proteins

Heart protein lysates (250 μg) were incubated with Tandem Ubiquitin Binding Entities (TUBEs) agarose beads. Beads were collected by centrifugation, washed with TBS-T and the ubiquitin-enriched proteins were eluted for immunoblotting.

Turning off and on expression of the TRIM63^{Q247*} transgene

To shut down the expression of the transgene, three-month old non-transgenic and double transgenic mice were fed with doxycycline at a 500ug/ml concentration in water for 30 days. Doxycycline was then withdrawn in half of the treated animals in order to re-introduce expression of the transgene. After 72 hours of re-expression of the transgene, mice were euthanized for molecular analysis.

qPCR

The mRNA levels of selected markers were quantified by qPCR using specific TaqMan Gene expression assays, as published [31]. The list of the probes is shown in Online Table I.

Statistics

Differences among the groups (mean ±SD) were compared by one-way analysis of variance for normally distributed continuous variables. Bonferroni correction for multiple-comparisons was applied. Variables that did not follow a normal distribution pattern were compared by Kruskal-Wallis test. Differences in the categorical variables were compared by Chi Square test. All statistical analyses were performed using STATA v. 10.1.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

RESULTS

Characteristics of the study population

The discovery study population included 302 probands including 250 Caucasians with HCM and 339 (262 Caucasians) control individuals. Characteristics of the study population were largely similar to those published. [35] In brief, males comprised 54% of the HCM study population. The mean age of the HCM population was 51.4 ± 16.5 years and the mean interventricular septal thickness was 1.9 ± 0.47 cm (Online Table II).

TRIM63 variants

Five rare variants (MAF<0.01) and one common variant (MAF=0.17) were detected in the study population (Online Table III). All rare variants were detected in the Caucasians. The prevalence of the rare variants was enriched in the Caucasian probands with HCM, as the rare variants were present in 11/250 (4.4%) probands with HCM and 3/262 (1.1%) of the control individuals (X^2=5.1, p=0.024). The rare TRIM63 (RefSeq:NP_115977) variants p.A48V (Chr1:26393843C>T, p.Ala48Val), p.I130M (Chr1:26387768C>G, p.Ile130Met) and p.Q247* (Chr1:26384973C>T, p.Glu247Ter) were exclusive to five probands with HCM and absent in 339 control individuals that were sequenced and 751 control individuals that were screened by TaqMan assays. The p.A48V and p.Q247* occurred in two unrelated families and were present in the affected but not in unaffected members of the families (Figure 1, Panel A). The sizes of the families, nevertheless, were inadequate for co-segregation or a formal genetic linkage. Genotyping of the family members for five STR markers and 21 single nucleotide polymorphisms (SNPs) at the *TRIM63* locus (1p34-1p33) showed unique haplotypes for individuals with the p.A48V mutation. The finding suggests an independent origin of the mutation, assuming no recombination event at the region between the *TRIM63* and the marker. However, individuals with the p.Q247* shared the same haplotype for the STR markers and SNPs. Hence, an independent origin of the p.Q247* in these two families could not be established (Online Figure I). The p.E269K SNP (rs61749355, mRNA position 941, c.805G>A, NM_032588.2, **G**AG>**A**AΓ) was identified in 2 control individuals and 6 probands with HCM. A rare p.R195C variant was identified in a single control individual, who had no history of medical problems and had a normal ECG and echocardiogram. A known common SNP p.E237K (rs2275950, mRNA position 845, G>A) was detected at equal frequency in HCM probands and controls (MAF: 0.23 and 0.18, respectively, X^2=5.0, p=0.082). The complete list of non-synonymous and other variants is shown in Online Table III.

The p.A48V and p.I130M affected highly conserved amino acids and the p.Q247* variant led to deletion of 106 aa from the 353 aa long full-length protein (Figure 1 B-E). Residues 48 and 130 are located in RING-type (aa 23-79) and B-box-type (117-159) domains of Zinc finger motif in TRIM63 protein, respectively. Residue 130 is also located at interacting domain with titin (TTN), which includes residues 74 to 218. The p.Q247* mutation is located in the coil-coiled domain (aa 207-269), which typically provides mechanical stability to the proteins. PolyPhen2 prediction positioned the missense variants at the probably damaging category (highest). [36] Considering the human molecular genetic data, the p.A48V, p.I130M and p.Q247* were referred to as “mutations”.

Exclusion of known causal genes for HCM

None of the five HCM probands who carried the *TRIM63* mutations had a putative causal variant in *MYH7*, *MYBPC3*, *TNNT2*, *TNNI3*, *TPM1,* and *ACTC1,* known relatively common genes for HCM. [9]

Phenotypic expression of TRIM63 mutations

The phenotype in mutation carrier was notable for moderate to severe left ventricular hypertrophy associated with left ventricular outflow tract obstruction in four out of six individuals requiring either surgical septal myectomy or transcatheter septal ablation (Online Table IV).

Mutations impair auto-ubiquitination of TRIM63

E3 ligases are known to undergo auto-ubiquitination, particularly in the absence of their primary substrates. This intrinsic feature of E3 ligases afforded the opportunity to test the

effects of the p.A48V, p.I130M and p.Q247* mutations on TRIM63 ligase activity. [37] Transduction of the HeLa-His/Biotin-Ubiquitin cells with the recombinant lentiviruses expressing either Flag-tagged $TRIM63^{WT}$, $TRIM63^{A48V}$, $TRIM63I^{130M}$ or $TRIM63^{Q247*}$ followed by co-immunoprecipitation (Co-IP) showed near complete loss of auto-ubiquitination in cells transduced with the $TRIM63^{Q247*}$ lentiviral construct (Figure 2A-C). Likewise, immunofluorescence staining of the transduced HeLa-His/Biotin-Ubiquitin cells for ubiquitin and TRIM63 (Flag) showed no discernible auto-ubiquitinated TRIM63 in the cells transduced with the $TRIM63^{Q247*}$ viruses (Figure 2D). Quantitative analysis also showed 60 to 70% reductions in the levels of auto-ubiquitinated $TRIM63^{A48V}$ and $TRIM63^{I130M}$ (Online Figure II).

Mutations impair ubiquitination of TRIM63 substrates in adult cardiac myocytes

To detect whether mutations affected binding to and ubiquitination of TRIM63 known substrates, cardiac myocytes were transduced with the recombinant adenoviruses and levels of ubiquitinated and total MYH6 and MYBPC3 were determined by Co-IP and immunoblotting. Co-IP of ubiquitinated MYH6 and MYBPC3 with an anti ubiquitin antibody, performed in the presence of MG132 to prevent degradation of ubiquitinated proteins, showed a near complete absence of ubiquitinated MYH6 and MYBPC3 in cardiac myocytes transduced with Ad5/CMV/$TRIM63^{Q247*}$ viruses (Figure 3, A and B). As compared to $TRIM63^{WT}$, level of co-precipitated MYH6 and MYBPC3 were modestly reduced in cardiac myocytes transduced with recombinant viruses expressing $TRIM63^{A48V}$ protein but was largely unchanged in myocytes transduced with the $TRIM63^{I130M}$ viruses (Figure 3, A and B). Because FBXO32 (Atrogin 1 or MAFbx), a muscle E3 ligase, is known to target calcineurin, we determined ubiquitination of PPP3CB (protein phosphatase 3 catalytic subunit β) in cardiac myocytes transduced with the WT or mutant TRIM63 constructs. [38] As shown in Figure 3, A and B, level of ubiquitinated PPP3CB was increased in myocytes transduced with the $TRIM63^{WT}$ construct as compared to non-transduced myocytes. In contrast, level of ubiquitinated PPP3CB was drastically reduced in cardiac myocytes transduced with $TRIM63^{Q247*}$ viruses and moderately in the $TRIM63^{I130M}$ group.

Mutations impair UPS-mediated degradation of MYH6 and MYBPC3 in adult cardiac myocytes

To determine effects of impaired ubiquitination of mutant TRIM63 on protein levels of known substrates, we performed immunoblotting to detect and quantify MYH6 and MYBPC3 protein levels in cardiac myocytes transduced with the recombinant viruses in the presence or absence of MG132. As shown in Figure 3C, levels of MYH6 and MYBPC3 were reduced significantly in cardiac myocytes transduced with the adenoviruses expressing $TRIM63^{WT}$ in the absence of MG132, as compared to non-transduced cells. The findings confirm the previous data identifying thick filament proteins as targets of TRIM63. [39] In contrast, levels of MYH6 and MYBPC3 protein were increased in cells treated with the adenoviruses expressing $TRIM63^{A48V}$, $TRIM63^{I130M}$ and $TRIM63^{Q247*}$ as compared to myocytes expressing $TRIM63^{WT}$ (Figure 3, C and E). Treatment with MG132 equalized levels of MYH6 and MYBPC3 in the experimental groups, supporting UPS mediated degradation of MYH6 and MYBPC3 in $TRIM63^{WT}$ group (Figure 3, D and E). Protein level of cardiac troponin I was unchanged in cells expressing the $TRIM63^{WT}$ protein and was similar in all experimental groups.

Mutations reduce localization of TRIM63 to sarcomere Z disk

To determine whether TRIM63 mutations affected its localization in the sarcomere, cardiac myocytes were transduced with the recombinant viruses and co-stained with anti Flag (TRIM63) and anti α-actinin antibodies. As shown in Figure 4, $TRIM63^{WT}$ was co-localized with α-actinin at the Z disk (r=0.814). In contrast, $TRIM63^{I130M}$ and

TRIM63^{Q247*} had a diffuse expression pattern with minimal co-localization with α-actinin (r=0.115 and r=0.199, respectively). TRIM63^{A48V} showed a moderate reduction in co-localization with α-actinin (r=0.518).

Mutations impair UPS-mediated degradation of MTOR-S6K hypertrophic signaling pathway in adult cardiac myocytes

To identify the mechanism(s) by which TRIM63 mutations cause HCM, we focused on the mammalian target of rapamycin (MTOR) pathway, not only because it is a major cardiac hypertrophic signaling pathway but also because F-box proteins, such as FBXW7 are known to target phosphorylated MTOR (p-MTOR) for ubiquitination and degradation. [40] Immunoblot analysis of protein extracts of adult cardiac myocytes transduced with the recombinant adenoviruses in the absence of the UPS inhibitor MG132 showed reduced level of p-MTOR in myocytes expressing the TRIM63^{WT} protein (Figure 5A). In contrast, p-MTOR protein level in cardiac myocytes transduced with the mutant TRIM63 constructs was significantly higher as compared to the TRIM63^{WT} group. Inhibition of the UPS by MG132 led to equalization of the p-MTOR level in in all experimental groups including in the TRIM63^{WT} group. Total MTOR protein level was unchanged in all experimental groups in both MG132 treated and untreated cells.

To further investigate reduced p-MTOR levels in the TRIM63^{WT} group, we detected and quantified levels of total and p-S6K1 and p-AKT1, downstream targets of MTORC1 and MTORC2, respectively, by immunoblotting. In accord with the reduced level of p-MTOR, level of p-S6K1 but not total S6K1 was also significantly reduced in cardiac myocytes expressing TRIM63^{WT} but not in any of the mutant TRIM63 groups (Figure 5B). In contrast, protein level of p-AKT1 and total AKT1 were unchanged in cardiac myocytes transduced with the WT or mutant *Trim63* constructs. Treatment with MG132 normalized reduced level of p-S6K1 in the TRIM63^{WT} group and equalized in all groups.

To determine whether p-MTOR was the direct target of TRIM63, we performed Co-IP using an anti Flag antibody in immunoprecipitation and an anti p-MTOR antibody for immunoblotting and repeated the Co-IP in the reverse order. Despite six independent Co-IP reactions and using different antibodies, no significant Co-IP of p-MTOR with TRIM63 in any of the experimental groups was detected. Likewise, no significant Co-IP of p-S6K1 with TRIM63 was detected in any of the experimental groups. The null results of Co-IP studies might simply reflect the experimental conditions and yet might also indicate an indirect targeting of p-MTOR by TRIM63 through targeting other components of the MTORC1 complex. Therefore, we determined levels of p-Raptor, total Raptor, and GβL by immunoblotting. Levels of p-Raptor and GβL were largely unchanged and there were not significant differences in their levels among the experimental groups (Online Figure III).

Mutations had no discernible effects on CK activity

TRIM63 is implicated in regulating CK enzymatic activity. [41] To determine whether mutations affected CK enzymatic activity, CK enzymatic activity was quantified serially in adult mouse cardiac myocytes transduced with the recombinant adenoviruses. TRIM63 mutations had no significant effects on CK protein levels or CK enzymatic activity (Online Figure IV).

Inducible expression of mutant TRIM63 leads to cardiac hypertrophy

Because each human genome contains about 100 loss-of-function variants [42], to further substantiate the causal role of the *TRIM63* mutations identified in human patients with HCM, we generated Doxycycline-inducible double transgenic mice, transcriptionally regulated by the *Myh6* promoter [29, 30]. Expression of the transgene was detected by

immunoblotting of cardiac protein extracts in three tTA:TRIM63^{A48V}, one tTA:TRIM63^{I130M}, two tTA:TRIM63^{Q247*} and one tTA:TRIM63^{WT} independent lines (Figure 6A). To determine relative levels of the Flag-tagged transgene TRIM63 and endogenous TRIM63 proteins, cardiac protein extracts were probed by immunoblotting using a pan-TRIM63 antibody. Level of the transgene protein was lower than that of the endogenous TRIM63 protein in tTA:TRIM63^{WT}, tTA"TRIM63^{A48A} and tTA:TRIM63^{Q247*} but was higher in the tTA:TRIM63^{I130M} double transgenic mice (Online Figure V). Morphological and histological analysis showed increased ventricular weight/body weight by approximately 15 to 20% (Online Figure VI) and myocyte CSA by about 1.6 to 2-fold in the three mutant TRIM63 groups, particularly in tTA:TRIM63^{Q247*} group, as compared to tTA:TRIM63^{WT} or non-transgenic mice (Figure 6B). There was also an approximately 3-fold increase in interstitial fibrosis in the tTA:TRIM63^{I130M} group as compared to non-transgenic mice (Online Figure VII). Interstitial fibrosis was not increased significantly in other mutant TRIM63 groups.

Echocardiographic evaluation of cardiac size and function showed increased left ventricular wall thickness, mass and mass index in the mutant TRIM63 groups, except for the TRIM63^{I130M}, as compared to non-transgenic or tTA:TRIM63^{WT} mice (Online Table V). Left ventricular fractional shortening was similar to that in the non-transgenic mice, except in the TRIM63^{I130M}, which was decreased modestly. Expression of the TRIM63^{WT} was associated with increased left ventricular end diastolic diameter and modestly decreased wall thickness, as compared to non-transgenic mice. However, left ventricular fractional shortening was preserved.

Mutations reduce ubiquitination of total protein, MYH6 and MYBPC3 and activate the MTOR-S6K pathway in the heart

To assess the effects of the mutations on cardiac protein ubiquitination, we enriched myocardial protein extracts for ubiquitinated proteins by passing the extracts through Tandem Ubiquitin Binding entities (TUBEs) agarose beads and probing the enriched proteins by immunoblotting using an anti Ubiquitin antibody. The findings were notable for a greater than two-fold increase in the level of ubiquitinated cardiac proteins in the tTA:TRIM63^{WT} mice (Figure 7A). In contrast, levels of ubiquitinated total protein were reduced in the mutant tTA:TRIM63 groups, as compared to TRIM63^{WT} mice and was near absent in the tTA:TRIM63^{Q247*} mice. These findings are in accord with the *in vitro* data in the HeLa-His/Biotin-Ubiquitin cells and Co-IP studies in virally transduced adult cardiac myocytes. Likewise, level of ubiquitinated TRIM63^{A48V} and TRIM63^{Q247*} were reduced significantly but less so in the TRIM63^{I130M} as compared to TRIM63^{WT} (Figure 7A, lower panel).

To analyze levels of the specific sarcomere proteins, we determined protein levels of TRIM63, MYH6 and MYBPC3 in the cardiac protein extracts by immunoblotting. As shown in Figure 7, panels B and C, MYH6 level were reduced in the tTA:TRIM63^{WT} mice, as compared to non-transgenic mice but were higher in the mutant tTA:TRIM63 groups as compared to the tTA:TRIM63^{WT} mice but equal to that in the non-transgenic mice, in accord with the *in vitro* data in cultured cardiac myocytes. MYBPC3 level was only increased in the tTA:TRIM63^{Q247*} mouse heart.

Immunobloting was performed to detect and quantify levels of the main components of TORC1 complex. There was an approximately 30% reduction in p-MTOR level in the tTA:TRIM63^{WT} group as compared to non-transgenic mice. Notably, p-S6K1 level in the heart was dramatically increased in the mutant tTA-TRIM63 group, as compared to non-transgenic or TRIM63^{WT} mice (Figure 7, D-F).

Because over-expression of TRIM63 has been shown to affect *Mybpc3* mRNA level, we also assessed mRNA levels of *Myh6*, *Mybpc3*, *Mtor*, *Ppp2cb* and *Rps6kb1* (S6k) in the heart of WT and mutant TRIM63 transgenic animals. [20] There were no significant differences in the mRNA levels of the selected molecules (Online Figure VIII).

Switching on expression of mutant TRIM63 proteins in the heart activates the MTOR-S6K and calcineurin-NFAT1 pathways

The switch on and off bigenic mouse approach afforded the opportunity to turn off expression of the mutant TRIM63 protein and then analyze induction of expression of the molecular regulators and markers of cardiac hypertrophy. In this set of experiments tTA:TRIM63^{Q247*} was used as this mutation was associated with the most dramatic phenotype in isolated adult cardiac myocytes and in transgenic mice. Accordingly, expression of the TRIM63^{Q247*} was turned off in the tTA:TRIM63^{Q247*} mice upon daily administration of Doxycycline for 30 days and then it was turned on in half of the animals – by withdrawing Doxycycline (Online Figure IX). Immunoblotting performed three days after turning on expression of the mutant transgene showed concordant increase in the expression levels of p-MTOR, pS6K1 and RCAN1.4 and normalization of these proteins upon turning off in the mutant TRIM63 bigenic mice (Figure 8, A and B). In conjunction with activation of the hypertrophic signaling pathways, mRNA levels of *Myh7*, *Nppa* and *Nppb*, quantified by qPCR, were increased three days after turning on and were normal upon turning off expression of the mutant TRIM63^{Q247*} protein (Figure 8, C). Echocardiographic assessment of cardiac size and function showed normal wall thickness and systolic function during the period when the transgene expression was shut down as well as three days after turning on expression of TRIM63^{Q247*} protein.

DISCUSSION

We provide human molecular genetic and *in vitro* and *in vivo* functional evidence to implicate *TRIM63*, encoding MuRF1, an E3 ubiquitin ligase, as a likely causal gene for human HCM. The p.A48V, p.I130M and p.Q247* variants were exclusive to the HCM study population (Caucasians) and were not detected in over 500 control Caucasian individuals. The small size of the families was not permissive to genotype-phenotype co-segregation analysis, which is a limitation of the study. The nonsense (p.Q247*) and the p.A48V variants recurred in two families. The nonsense variant led to premature truncation and loss of approximately 1/3rd of the protein. The p.A48V and p.I130M variants affected highly conserved amino acids and were predicted – *in silico* - to be probably damaging to protein structure and function. Functionally, the variants had loss-of-function effects on E3 ubiquitin ligase activity, as detected in specialized Ubiquitin-tagged HeLa cells, virally transduced adult cardiac myocytes and transgenic hearts. Furthermore, expression of the mutant TRIM63 in the heart through inducible transgenesis led to cardiac and myocyte hypertrophy in mice, activation of the MTOR-S6K and calcineurin-RCAN1.4 pathways and expression of the hypertrophic markers. The truncating TRIM63^{Q247*} had the most prominent *in vitro* and *in vivo* phenotypic effects. Collectively, the human molecular genetic data along with the functional studies in adult cardiac myocytes and transgenic mice support the causal role of *TRIM63* in the pathogenesis of human HCM. The findings implicate impaired degradation of cardiac proteins as a pathogenic mechanism for cardiac hypertrophy and activation of the MTOR-S6K and calcineurin-RCAN1.4 pathways as the responsible pathways involved in the pathogenesis of HCM caused by *TRIM63* mutations.

The main biological function of UPS in the heart is clearance of the misfolded and damaged proteins. Likewise, TRIM63 (MuRF1) and FBX032 (commonly known as atrogin-1 or MAFbx), the major components of the UPS in the heart, are implicated in the removal of mutant sarcomere proteins in HCM. [20] In addition, a mismatch between UPS capacity and

accumulation of substrates is commonly observed in various forms of cardiac hypertrophy and dysfunction, which might actively participate in the pathogenesis of cardiomyopathies. [3, 5, 16, 17, 43] The findings of the present study extend the biological functions of UPS in the heart and provide direct genetic and functional evidence to implicate defective protein degradation as the initial impetus for induction of cardiac hypertrophy in HCM. These discoveries advocate the notion that cardiac hypertrophy might also be a disease of impaired protein degradation.

TRIM63 mutations led to loss of E3 ligase activity, as reflected by reduced auto-ubiquitination and ubiquitination of the known substrates. The mutations, however, exhibited variable functional effects and cardiac phenotypes, which are not unanticipated but rather inherent to phenotypic expression of mutations in various genes, as best illustrated for the phenotypic plasticity of *LMNA* mutations. [44] The phenotype was more prominent for the truncating TRIM63^{Q247*} mutation, which apparently, at least in part, had escaped the Nonsense Mediated Decay (NMD) mechanism, an established mechanism for haploinsufficiency resulting from the nonsense mutations in HCM. [17, 45] Stable expression of the truncated TRIM63, observed in HeLa cells transduced with a lentiviral construct, in adult cardiac myocytes infected with an adenoviral construct and in the heart of transgenic mice, might reflect the use of cDNA in these experiments as opposed to the genomic fragment. Alternatively, the relatively distal location of the p.Q247* mutation from the translation initiation site might afford the opportunity for the stable expression of a 246 aa protein. The truncated TRIM63 protein exhibited a near total loss of the E3 ligase activity, as demonstrated by near total loss of auto-ubiquitination and ubiquitinylation of TRIM63 substrates MYH6 and MYBPC3. In addition, the level of ubiquitinated calcineurin (PPP3CB) was drastically lower in cardiac myocytes transduced with the TRIM63^{Q247*} viruses. Thus, the biological and functional data are the strongest for the TRIM63^{Q247*}.

Expression of TRIM63^{WT} was associated with reduced levels of MYH6 and MYBPC3, known substrates for TRIM63. [39] However, protein level of cardiac troponin I was unchanged, which has been observed in some but not other studies. [3923] Reduced MYH6 and MYBPC3 levels and increased level of ubiquitinated PPP3CB, were associated with mild thinning of the interventricular septum and left ventricular enlargement as compared to non-transgenic mice but no significant change in left ventricular mass, mass index or fractional shortening at the baseline. Over-expression of TRIM63^{WT} in the heart is known to render the heart susceptible to failure in response to stress. [4]

The findings also implicate activation of MTOR-S6K (MTORC1 pathway) and calcineurin in the pathogenesis of HCM caused by the *TRIM63* mutations and hence, links the protein degradation and synthesis pathways. These findings suggest MTORC1 and PPP3CB but not MTORC2 pathways, as the potential direct or indirect targets of TRIM63 in the heart. Despite reduced levels of p-MTOR and PPP3CB upon over-expression of TRIM63, we could not demonstrate co-precipitation of TRIM63 and p-MTOR or PPP3CB. Thus, the changes in the p-MTOR, p-S6K1, and RCAN1.4 levels might be secondary to activation of the hypertrophic program instigated by the *TRIM63* mutations. Nonetheless, activation of p-MTOR/p-S6K and calcineurin/RCAN1.4 pathways in isolated myocytes and the mouse heart support the causal role of *TRIM63* variants in the pathogenesis of HCM. The precise mechanism(s) by which a homeostatic shift in favor of reduced protein degradation instigates a cardiac hypertrophic response remain unknown.

Several alternative mechanisms might also account or influence the phenotypic consequences of *TRIM63* mutations. The truncating mutation, which eliminates a major part of the coiled coil domain, might interfere with the mechanical stability of the protein and affect its interactions with proteins other than MYH6 and MYBPC3. The p.A48V mutation

is located in RING type domain, which mediates ubiquitin-ligase activity by binding to ubiquitination enzymes as well as the substrates. [46] The p.I130M and p.Q247* also affected subcellular distribution of TRIM63 and its co-localization with the α-actinin. The p.I130M mutation is located in the B-box type domains of Zinc finger motif, which is also the interacting domain with titin (residues 74 to 218 on TRIM63 and repeats A168/A169 in titin), adjacent to the titin kinase domain. [47, 48] TRIM63 is involved in regulating muscle trophic homeostasis through interactions with a number of proteins including glycogen phosphorylase and FOXO (Forkhead box, subgroup O). [47] Whether *TRIM63* mutations influence titin kinase activity or ubiquitination of other substrates is unknown. The crystal structure of the B-Box domain of TRIM63 has been resolved. [49] The isoleucine at position 130 resides in a groove near the interacting domain with the muscle type CK. [50] Substitution of methionine, which has a side branch, for isoleucine might affect its interactions with CK. However, under the conditions of our experiments, we detected no significant effects of over expression of wild type or mutant TRIM63 on CK enzymatic activity in virally transduced cardiac myocytes. It is also possible that reduced E3 ligase activity of the mutant TRIM63 proteins impairs clearance of misfolded and damaged sarcomere proteins, which might partially incorporate into the sarcomere, and cause sarcomere dysfunction leading to cardiac hypertrophy and dysfunction. Likewise, reduced clearance of damaged and misfolded TRIM63 substrate proteins could lead to their accumulation in the endoplasmic reticulum, inducing an unfolded protein response, which might contribute to the pathogenesis of cardiac phenotype. [51] Therefore, various alternative or additive mechanisms – beyond those delineated in the present studies - might be responsible for the hypertrophic effects of *TRIM63* mutations in humans. Although we did not detect significant differences in the levels of WT and mutant TRIM63 proteins in transduced cardiac myocytes and in the transgenic hearts, differences, beyond the sensitivity of the immunoblotting, could exist in the expression levels of WT and mutant TRIM63 that might confound data interpretation.

In conclusion, we provide molecular genetic evidence in humans and functional data in isolated cardiac myocytes and in inducible transgenic mouse models to implicate *TRIM63* as a likely causal gene for human HCM. The discoveries provide evidence for impaired degradation of sarcomere proteins as a mechanism for the pathogenesis of HCM in humans. Consequently, sarcomeres are not only the contractile units of striated muscles but are also signaling hubs for regulation of muscle trophic homeostasis and hence, cardiac size and function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations

HCM	Hypertrophic cardiomyopathy
TRIM63	Tripartite motif containing 63

UPS	Ubiquitin proteasome system
MuRF1	Muscle-specific RING Finger 1
SCD	Sudden cardiac death
MYH6	Myosin heavy chain 6 (or alpha)
MYBPC3	Myosin binding protein C 3
PPP3CB	Protein phosphatase 3 catalytic subunit, Beta isoform
MTOR	Mammalian target of rapamycin
WT	Wild type

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Novelty and Significance

What Is Known?

- Hypertrophic cardiomyopathy (HCM), a single gene disorders with Mendelian patterns of inheritance, is a major cause of sudden cardiac death in the young and morbidity in the adult.
- Mutations in over a dozen genes, encoding for sarcomere proteins are known to cause HCM.
- The molecular mechanisms responsible for the phenotypic expression of HCM are diverse but largely involve impaired sarcomere structure and function.
- Impaired ubiquitin-proteasome system (UPS) is implicated in the pathogenesis of HCM phenotype.

What New Information Does This Study Contribute?

- We identified three rare variants in *TRIM63* as likely causal mutations in small families with HCM and showed that rare variants in TRIM63 were enriched in HCM cases.
- *TRIM63* rare variants were loss-of-function variants and impaired E3 ubiquitin ligase activity of TRIM63 against its known substrates MYH6 and MYBPC3.
- The findings also implicated MTOR and PPPC3B as potential new targets of TRIM63.

With identification of the common causal genes for HCM, the focus has been shifted to identification of the uncommon causal genes, typically in sporadic cases and small families. However, molecular genetic data in sporadic cases and small families are often inconclusive because of the abundance of the common and rare including functional variants in each genome. The genetic approaches need to be complemented with molecular mechanistic studies to strengthen the causal role of the novel or rare variants identified in sporadic cases or small families. In the present study, we provide human molecular genetic data in conjunction with the mechanistic studies in adult cardiac myocytes and inducible transgenic mice to implicate *TRIM63* as a likely causal gene for human HCM. The findings illustrate the diversity of the molecular genetic causes of HCM and emphasize the complexity of the mechanisms involved in the pathogenesis of HCM. Successful prevention and treatment of HCM is likely to necessitate delineation of the specific genes and the molecular pathways involved in its pathogenesis. Such will provide the opportunity for specific interventions targeted to specific genes and pathways in order to prevent the evolving phenotype and reverse the established HCM.

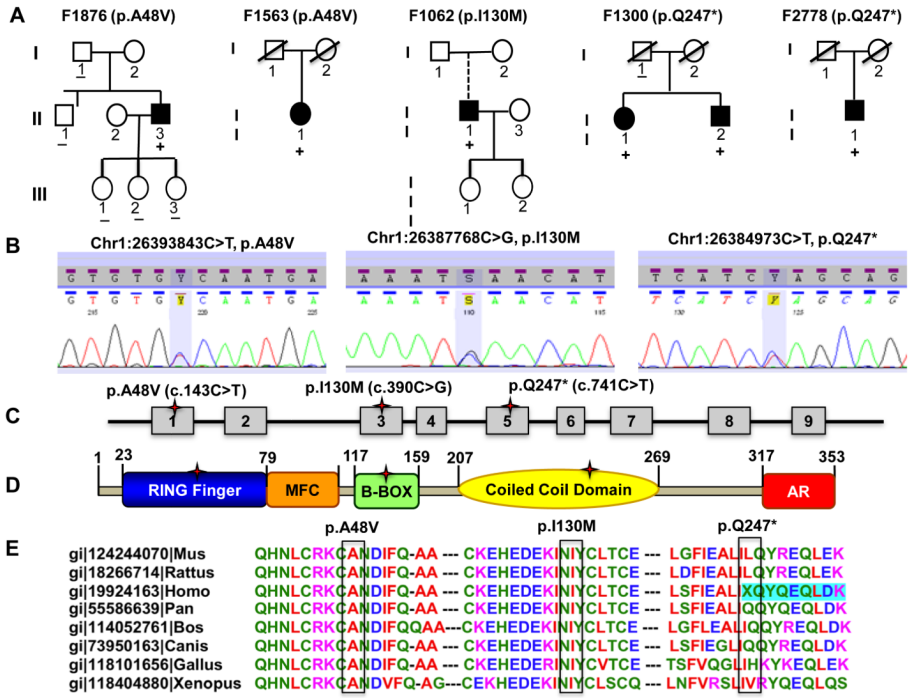

Figure 1. *TRIM63* mutations

A. Pedigrees of families with *TRIM63* mutations. The full circles (female) and squares (male) indicate the affected individuals. Those with the mutations are identified with the + sign. A broken connecting line indicates an adopted person. The slash through sign indicates a deceased person. **B.** Electrophoregram of three *TRIM63* mutations identified in HCM probands. **C** and **D**. Topographic location of the *TRIM63* mutations on exons and protein, respectively. **E.** Multiple sequence alignment showing evolutionary conservation of the affected amino acids.

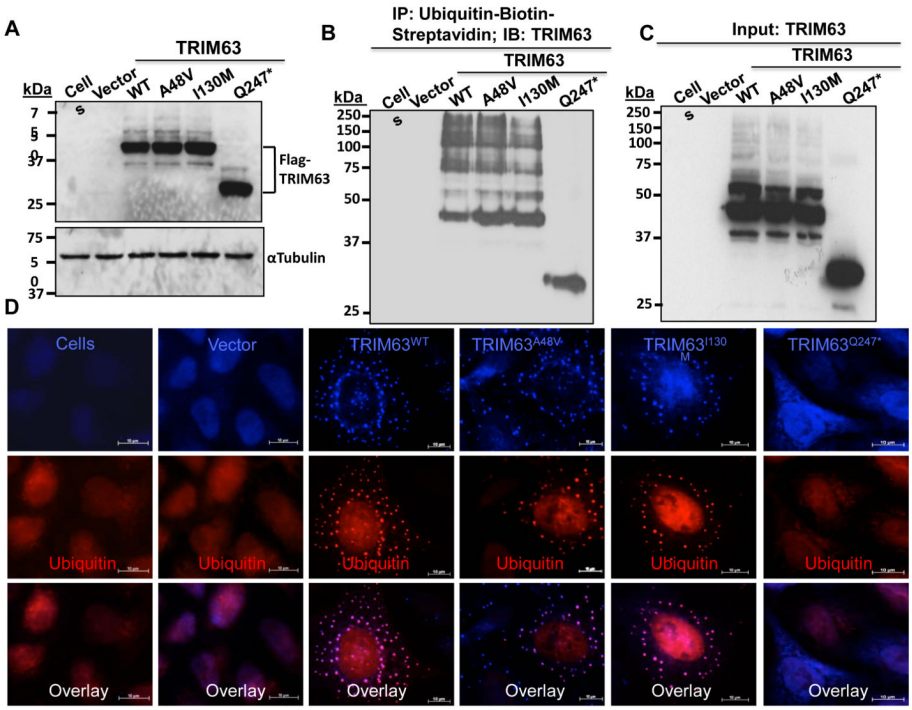

Figure 2. Effects of the mutations on auto-ubiquitination of TRIM63

A. Immunoblot showing expression of the wild type (WT) and mutant TRIM63 proteins in HeLa cells transduced with the recombinant lentiviruses. **B.** Auto-ubiquitination of the WT and mutant TRIM63 in MG132 treated HeLa-His/Bio-Ub cells detected by Co-immunoprecipitation (Co-IP). Biotinylated ubiquitin was precipitated with streptavidin-conjugate agarose beads and probed with an anti Flag antibody. **C.** Immunoblot of the input protein probed with an anti Flag (TRIM63) antibody. **D.** Immunofluorescence staining of auto-ubiquitinated TRIM63 in HeLa-His/Bio-Ub cells treated with MG132. TRIM63 is detected using an anti Flag antibody and biotinylated ubiquitin with a streptavidin-conjugated anti ubiquitin antibody. Percent auto-ubiquitinated TRIM63 aggregates, as determined by the percentage of co-localized ubiquitin and TRIM63 proteins, were 76.7 ± 13%, 31.5 ± 8%, 21.0 ± 5% and 0% in the $TRIM63^{WT}$, $TRIM63^{A48V}$, $TRIM63^{I130M}$ and $TRIM63^{Q247*}$, respectively (N= 20 cells per group, ANOVA $p<0.001$ and $p<0.05$ any of the mutant TRIM63 vs $TRIM63^{WT}$).

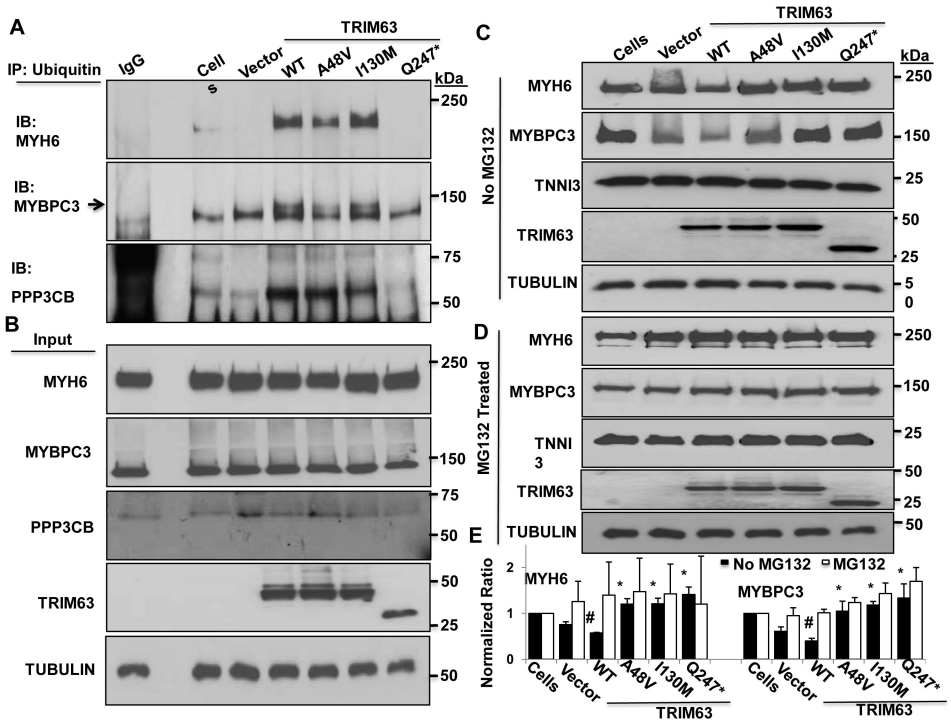

Figure 3. Effects of the TRIM63 mutations on ubiquitination of MYH6, MYBPC3 and PPP3CB in adult cardiac myocytes

A. IP was performed using an anti ubiquitin antibody and immunoblotting with anti MYH6, anti MYBPC3 and anti PPP3CB antibodies. **B**. Blots representing input proteins and α-TUBULIN (loading control). **C** and **D**. Immunoblots of MYH6 and MYBPC3 in the absence or presence of MG132, respectively. **E**. Quantitative data for MYH6 and MYBPC3 levels in the absence (black columns) or presence (open columns) of MG132 (N=3 per group, # $p<0.05$ compared to cells alone and *<0.05 compared to $TRIM63^{WT}$).

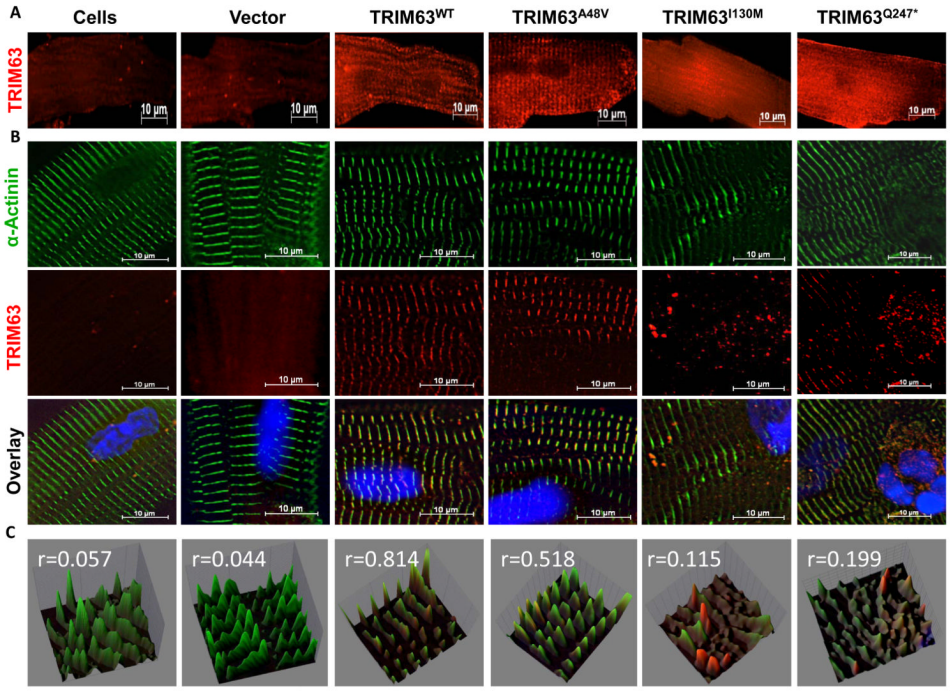

Figure 4. Reduced localization of mutant TRIM63 proteins to Z disks in cardiac myocytes

A. Low-magnification immunofluorescence images of transduced adult cardiac myocytes expressing either a WT or a mutant TRIM63 protein. **B**. Deconvolution images of myocytes at Z disk regions after co-staining with anti α-ACTININ (green) and anti Flag (red) antibodies and the corresponding overlay images. **C**. Quantitative spectral display of Z disks stained for α-ACTININ and TRIM63 in the overlay images. Correlation coefficient (r value) between the two colors in each image is shown (A value of 0 indicates no correlation and 1 a perfect co-localization).

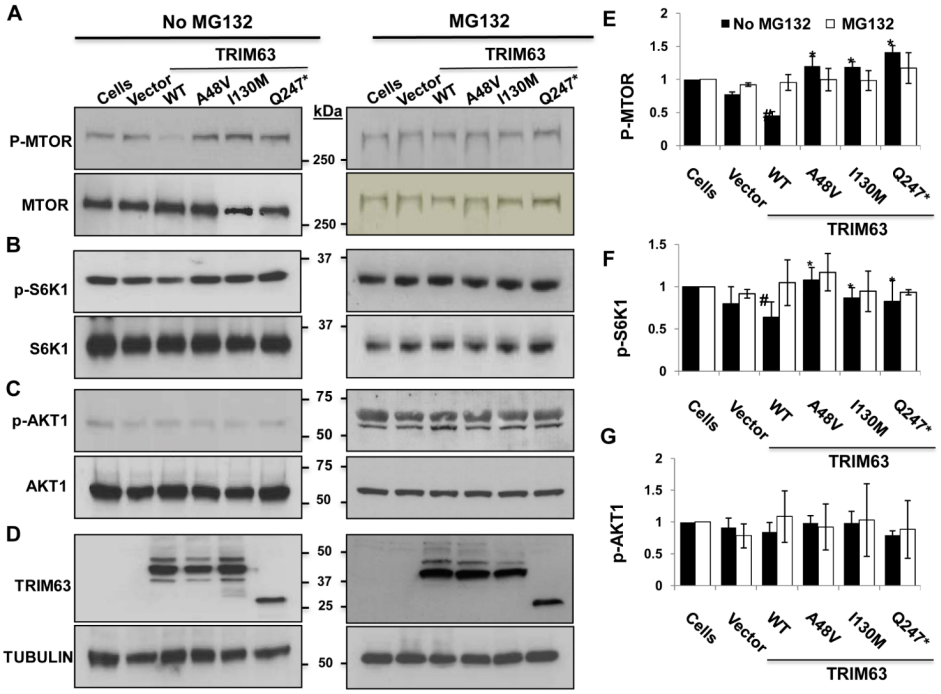

Figure 5. Levels of selected signal regulators of cardiac hypertrophy in adult cardiac myocytes

Immunoblots showing levels of p-MTOR and total MTOR (**A**), p-S6K1 and total S6K1 (**B**), and p-AKT1 and total AKT1 (**C**) in the presence and absence of MG132. **D**. Represents expression of WT and mutant TRIM63 in the experimental groups. **E**, **F** and **G**. show quantitative levels of p-MTOR, p-S6K1 and p-AKT1, respectively, in the absence (black columns) and presence (open columns) of MG132 (N=3 per group, # $p<0.05$ $TRIM63^{WT}$ compared to control cells; * $p<0.05$ Mutant TRIM63 groups compared to $TRIM63^{WT}$).

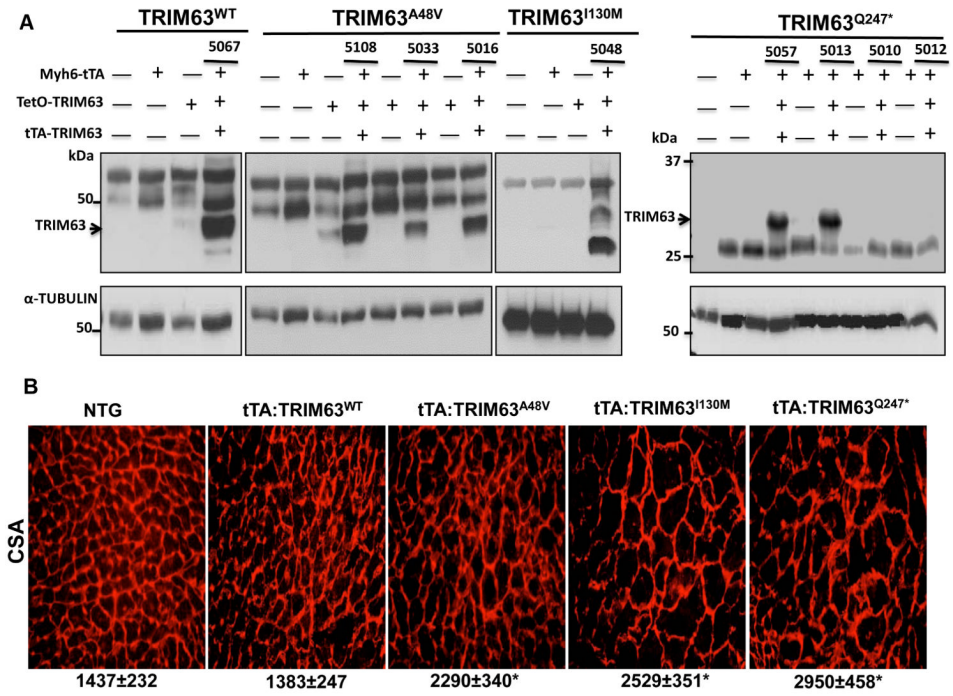

Figure 6. Cardiac and myocyte hypertrophy in Doxycycline-inducible WT and mutant TRIM63 transgenic mice

A. Immunoblots showing expression of the transgene protein detected using an anti Flag antibody in double transgenic lines (without Doxycycline). Bands other than those corresponding to TRIM63 are thought to represent nonspecific antibody reactivity. There were no discernible differences in the expression levels of the transgene among different lines of the same gene, with the exception of the line 5033 in the TRIM63^{A48V} group, which had a lower level of transgene protein. Therefore, TRIM63^{WT} line 5067, TRIM^{A48V} line 5016, TRIM63^{I130M} line 5048 and for TRIM63^{Q247*} line 5057 were used in the analysis. **B**. Gross cardiac morphology. **C**. Left ventricular weight/body weight ratio (mean ±SD, N=6 to 12 mice per group). **D.** Representative WGA stained thin myocardial sections reflective of myocyte cross-sectional area. **E.** Myocyte cross sectional area (mean ± SD), was measured in over 2,000 myocytes per mouse and three to four mice per group. # $p<0.05$ compared to non-transgenic mice, * $p<0.05$ compared to TRIM63^{WT}. Molecular size markers on the right side of the panels relate only to the TRIM63^{Q247*} group.

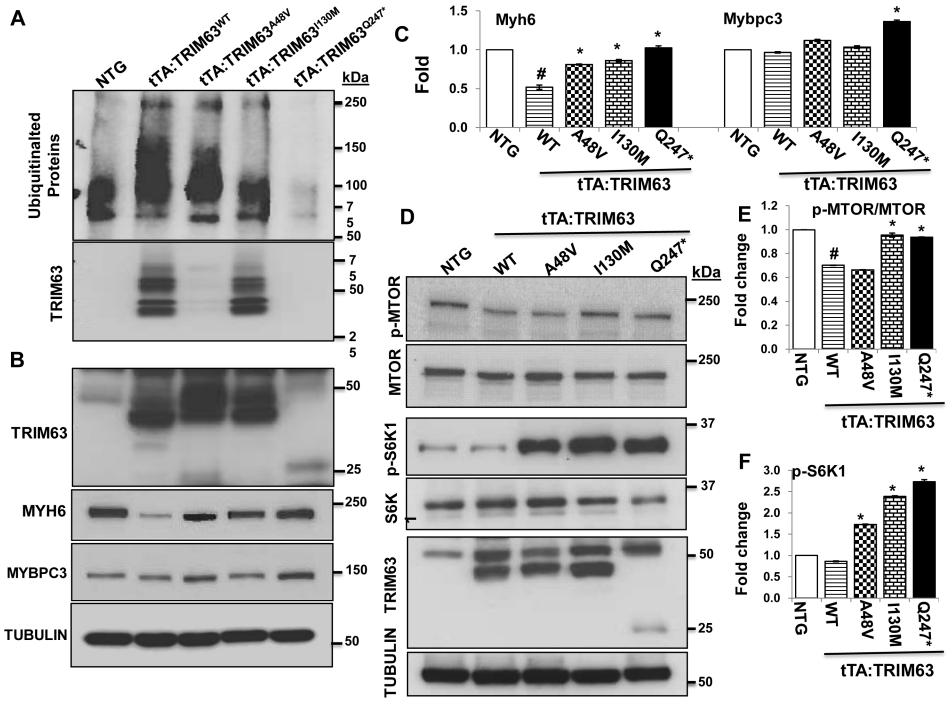

Figure 7. Reduced ubiquitination of cardiac proteins in mutant TRIM63 transgenic mice

A. Immunoblot showing levels of ubiquitinated cardiac proteins detected probed with an anti ubiquitin antibody. The lower panel represents ubiquitinated TRIM63 enriched for ubiquitination and probed with an anti Flag antibody. **B.** Immunoblots showing levels of TRIM63, MYH6, MYBPC3 and α-TUBULIN proteins, the latter as a control for loading conditions. **C**. Quantitative data on MYH6 and MYBPC3 levels. **D**. Levels of p-MTOR, total MTOR, p-S6K1 and total S6K1 along with panels showing expression of the transgene proteins and α-TUBULIN. **E** and **D**. Quantitative data on protein levels of p-MTOR and p-S6K1. N=3-4 mice per group, # $p<0.05$ compared to non-transgenic mice, * $p<0.05$ compared to $TRIM63^{WT}$.

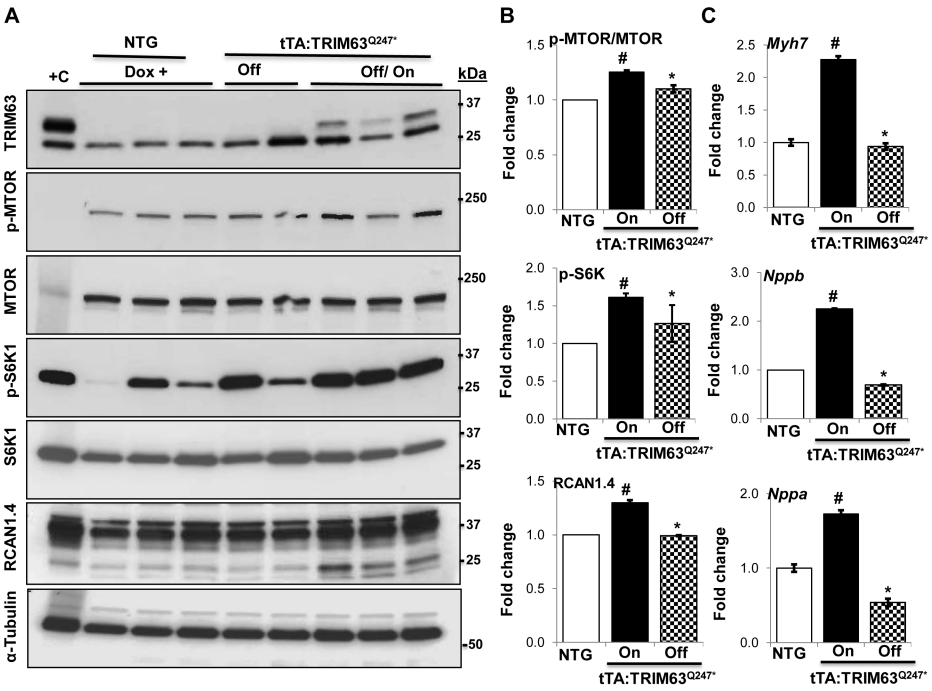

Figure 8. Induction of cardiac hypertrophic markers upon turning off and on expression of the mutant TRIM63 protein

A. Immunoblots representing levels of the transgene TRIM63, p-MTOR, total MTOR, p-S6K1, total S6K1, RCAN1.4 and α-TUBULIN upon withdrawal (turning on) and administration of Doxycycline (turning on) expression of the TRIM63^{Q247*}. **B.** Quantitative data for the p-MTOR, p-S6K1 and RCAN1.4. The comparisons were made between off and on states of the transgene and between transgenic on mode and non-transgenic groups. TRIM63^{WT} mice were not included in these experiments. **C**. qPCR data on relative mRNA levels of selected hypertrophic markers *Myh7*, *Nppa* and *Nppb*. (N= 3 per group, # $p<0.05$ compared to non-transgenic mice, * $p<0.05$ compared to TRIM63^{WT}).