

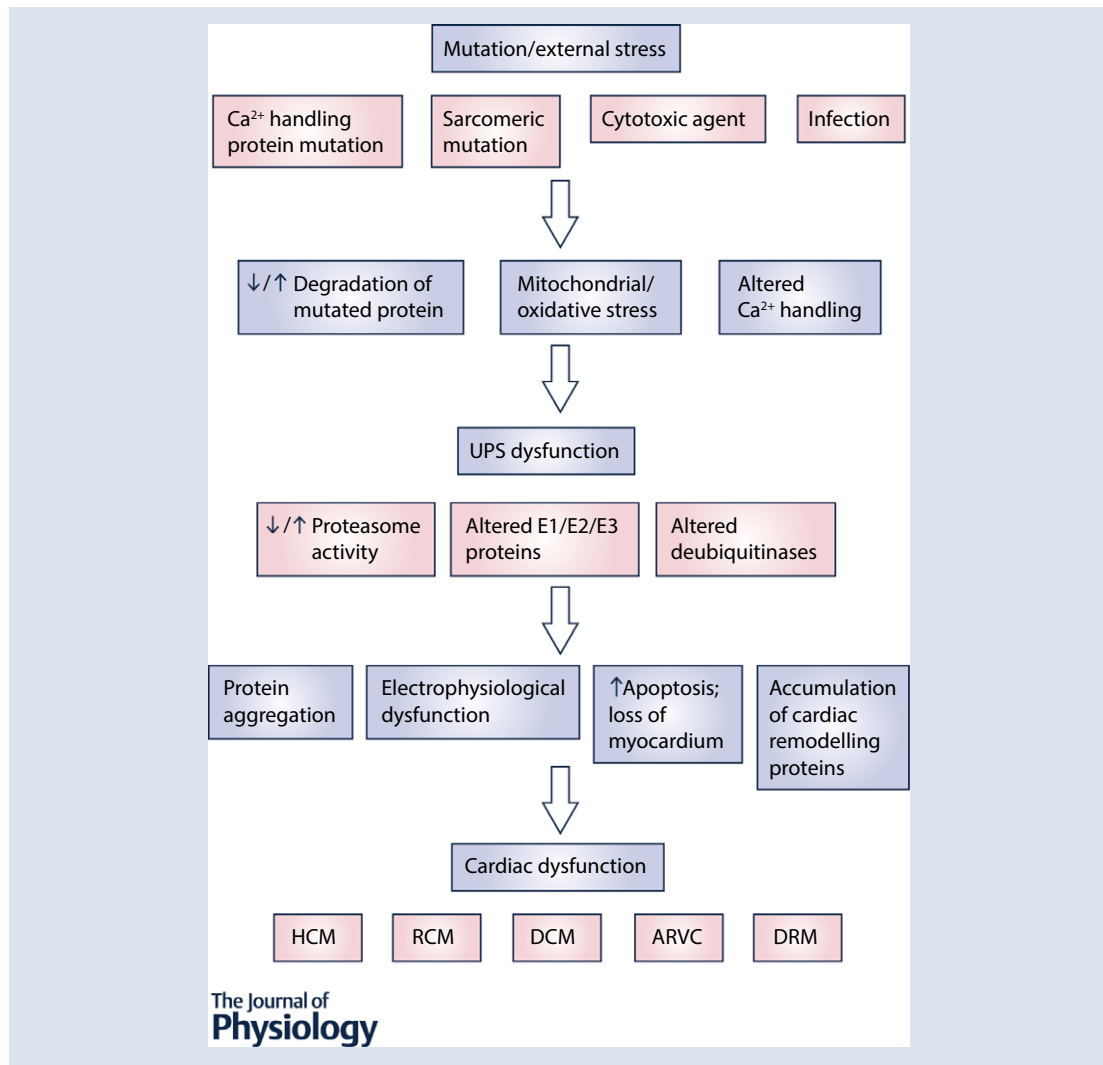
TOPICAL REVIEW

Proteasome dysfunction in cardiomyopathies

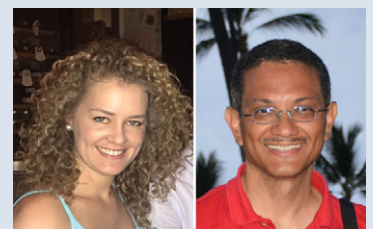
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Jennifer Gilda is a doctoral candidate at the University of California, Davis. One of her major research interests is the involvement of the proteasome in familial cardiomyopathies. Using proteomics, metabolomics, mouse models, and biochemical techniques, she has contributed to our understanding of how the ubiquitin-proteasome system and other pathways are affected in troponin-related cardiomyopathies. **Aldrin Gomes** is an Associate Professor at the University of California, Davis. His major interest is determining the molecular mechanisms of signal transduction, particularly in the role of proteostasis (protein homeostasis) in cardiovascular diseases. His background is in biochemistry, proteomics, and striated muscle physiology.



Abstract The ubiquitin–proteasome system (UPS) plays a critical role in removing unwanted intracellular proteins and is involved in protein quality control, signalling and cell death. Because the heart is subject to continuous metabolic and mechanical stress, the proteasome plays a particularly important role in the heart, and proteasome dysfunction has been suggested as a causative factor in cardiac dysfunction. Proteasome impairment has been detected in cardiomyopathies, heart failure, myocardial ischaemia, and hypertrophy. Proteasome inhibition is also sufficient to cause cardiac dysfunction in healthy pigs, and patients using a proteasome inhibitor for cancer therapy have a higher incidence of heart failure. In this Topical Review we discuss the experimental data which suggest UPS dysfunction is a common feature of cardiomyopathies, with an emphasis on hypertrophic cardiomyopathy caused by sarcomeric mutations. We also propose potential mechanisms by which cardiomyopathy-causing mutations may lead to proteasome impairment, such as altered calcium handling and increased oxidative stress due to mitochondrial dysfunction.

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Abstract figure legend Possible effects of sarcomeric mutations and/or external stress on ubiquitin–proteasome system (UPS) dysfunction and subsequent cardiac dysfunction observed in cardiomyopathies. ARVC, arrhythmogenic right ventricular cardiomyopathy; DCM, dilated cardiomyopathy; DRM, desmin-related myopathy; HCM, hypertrophic cardiomyopathy; RCM, restrictive cardiomyopathy.

Abbreviations ARVC, arrhythmogenic right ventricular cardiomyopathy; cMyBP-C, cardiac myosin binding protein C; DCM, dilated cardiomyopathy; DRM, desmin-related myopathy; FHC, familial hypertrophic cardiomyopathy; HCM, hypertrophic cardiomyopathy; NRCM, neonatal rat cardiac myocytes; PKG, protein kinase G; RCM, restrictive cardiomyopathy; SCD, sudden cardiac death; TnC, troponin C; TnI, troponin I; TnT, troponin T; UPS, ubiquitin–proteasome system.

Introduction

Cardiomyopathy refers to a group of diseases that affect the heart muscle. Types of cardiomyopathies include hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM), dilated cardiomyopathy (DCM), and arrhythmogenic right ventricular cardiomyopathy (ARVC). Familial HCM is a genetic disease that is inherited in an autosomal dominant manner and is characterized by thickening of the heart muscle, arrhythmias and sudden cardiac death (SCD) (Maron, 2002). Familial HCM is common; it is estimated to affect 1 in 500 people and is the leading cause of sudden death in young athletes (Maron, 2002, 2003). HCM is often caused by mutations in sarcomeric proteins such as myosin heavy chain (MHC), cardiac myosin binding protein C (cMyBP-C), and cardiac troponin T (TnT) (Gomes *et al.* 2004; Wu *et al.* 2012). In DCM the ventricles become dilated and weakened, and cannot pump blood effectively (Araco *et al.* 2017). Familial DCM is usually inherited in an autosomal dominant manner (Hershberger & Morales, 1993; McNally *et al.* 2013; Sisakian, 2014). RCM is rare and is characterized by stiffening of the heart muscle that leads to impaired filling (Wu *et al.* 2015). ARVC is another rare form of cardiomyopathy in which myocardium is replaced by fatty or fibrous tissue, primarily in the right ventricle (Gigli *et al.* 2016).

The mechanism by which sarcomeric protein mutations cause cardiomyopathies is unclear. Proposed pathological mechanisms of familial cardiomyopathies involve altered cardiac contractility, changes in Ca^{2+} handling, and altered energy homeostasis (Gomes & Potter, 2004; Frey *et al.* 2012). Current treatments include β -adrenergic receptor blockers, Ca^{2+} channel antagonists, myocardial reduction by surgical or septal ablation, and implanted defibrillators (Maron, 2002). There is growing evidence that ubiquitin–proteasome system (UPS) dysfunction may play a pathogenic role in cardiomyopathies (Predmore *et al.* 2010; Schlossarek & Carrier, 2011; Day, 2013). Proper UPS function is critical to the function of the cell, as it plays important regulatory roles through degradation of proteins involved in signal transduction as well as quality control through breakdown of misfolded or damaged proteins. UPS dysfunction is believed to play a pathogenic role in many cardiac diseases (Wang & Robbins, 2006; Patterson *et al.* 2007) and may be a unifying factor of cardiomyopathies.

The sarcomere is the basic contractile unit of striated muscle (Marques & de Oliveira, 2016). It is made up of thick filaments, composed mainly of myosin and cardiac myosin binding protein C (cMyBP-C), and thin filaments, composed mainly of actin, tropomyosin and troponin. Cardiac muscle contraction is initiated by an action potential that causes an influx of Ca^{2+} into the

cytoplasm (Bers, 2000). At resting Ca^{2+} levels, the thin filament proteins troponin I (TnI) and tropomyosin block myosin binding sites on actin. Upon an increase in cytosolic Ca^{2+} levels the sarcomere is activated: TnI and tropomyosin (Tm) are shifted out of myosin binding sites and the head of myosin binds to the thin filaments, forming cross-bridges. ATP is hydrolysed by myosin to activate the high energy conformation of the myosin head. The myosin head interacts with actin, allowing the head to rotate and resulting in sarcomere shortening. In addition to TnI and tropomyosin, the interaction between myosin and actin is mediated by other proteins such as troponin C (TnC), troponin T (TnT) and cMyBP-C (de Tombe, 2003).

The UPS is responsible for degrading most intracellular proteins, both native and misfolded, in a process that normally involves polyubiquitination followed by degradation by the proteasome (Li *et al.* 2011a). Polyubiquitination occurs via the ubiquitination pathway (Fig. 1). The E1 ubiquitin-activating enzyme utilizes ATP to activate ubiquitin and transfers it to an E2 ubiquitin-conjugating enzyme. An E3 ubiquitin ligase, which may be a multi-protein complex, interacts with both the E2 enzyme and the target protein, assisting or directly catalysing the transfer of ubiquitin from the E2 to a lysine residue on the protein substrate. The proteasome is composed of a multi-subunit 20S core that contains the catalytic activity in the $\beta 1$, $\beta 2$, and $\beta 5$ subunits. Each of these subunits has a different type of proteolytic

activity; $\beta 1$ has caspase-like activity, $\beta 2$ has trypsin-like activity, and $\beta 5$ has chymotrypsin-like activity. These subunits work together to cleave proteasome substrates into short peptides, which may be further processed by downstream proteases (Reits *et al.* 2004). The catalytic sites are located inside the barrel-like structure of the 20S core, and substrate entry into the proteasome is regulated by caps that bind the core particle. 19S regulatory particles associate with one or both ends of the 20S core to form the 26S proteasome, the most common form of the proteasome (Stadtmueller & Hill, 2011). The 19S cap recognizes ubiquitinated proteins and threads them into the core in an ATP-dependent manner (Stadtmueller & Hill, 2011). The 20S core can also be capped by alternative regulatory particles such as the 11S regulatory particle, which is ATP-independent and thought to be involved in the degradation of peptides or partially unfolded proteins. The 11S-capped proteasome plays an important role in the degradation of oxidized proteins (Jung *et al.* 2014). Through targeted degradation of proteins that are no longer required by the cell, the proteasome plays a role in virtually all cellular processes (Wang *et al.* 2011).

Ubiquitin–proteasome system dysfunction

UPS dysfunction in heart disease. The UPS is especially important in the heart, as cardiomyocytes are particularly prone to protein damage due to constant mechanical and

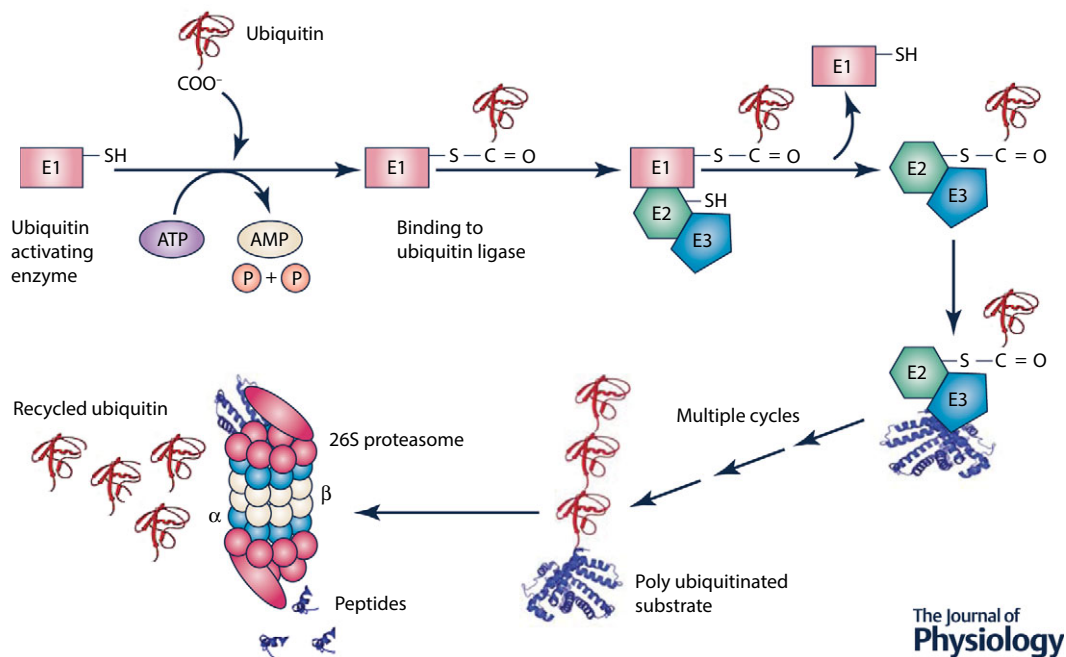


Figure 1. Schematic diagram of the UPS

Ubiquitin is conjugated to target proteins by an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase. The ubiquitinated substrate is delivered to the 26S proteasome, which degrades the protein.

metabolic stress (Wang & Robbins, 2006; Patterson *et al.* 2007). Additionally, the heart has very limited capacity for self-renewal, meaning that cell death, which may result from impaired proteasome function, can be highly detrimental to the health of the organ (Patterson *et al.* 2007; Wang, 2013). The importance of proper proteasome function is highlighted by the fact that proteasome inhibition is sufficient to cause cardiac dysfunction in healthy pigs (Herrmann *et al.* 2013). Accumulation of ubiquitinated proteins, increases and decreases in proteasome activity, and changes in expression of the proteasome and other UPS proteins such as E2 and E3 enzymes have been observed in several experimental and human heart diseases (Weekes *et al.* 2003; Mearini *et al.* 2008; Schlossarek & Carrier, 2011; Tian *et al.* 2012; Day, 2013). Many E3 ligases are known to target proteins associated with HCM and DCM (Table 1). The E3 enzyme, MuRF1 (TRIM63), has also been implicated in the pathogenesis of HCM, as transgenic mice expressing mutations in MuRF1 identified in HCM patients (A48V and I130 M, and a deletion variant Q247*) develop cardiac hypertrophy (Chen *et al.* 2012). Expression of these MuRF1 mutants in adult cardiomyocytes caused reduced ubiquitination and UPS-mediated degradation of the thick filament proteins myosin heavy chain 6 and cMyBP-C. While mutations in MuRF1 and MuRF2 have been identified that contribute to the severity of HCM (Su *et al.* 2014), a patient with protein accumulation in muscle fibres and HCM had a homozygous *TRIM63* (encodes MuRF1) null mutation in combination with the heterozygous *TRIM54* (encodes MuRF3) mutation (Olive *et al.* 2015). The results from Olive *et al.* 2015 also suggested that MuRF1 and MuRF3 are important for positioning thick filaments in the sarcomeres.

Proteasome inhibition is sufficient to cause cardiac dysfunction. Hearts of 3-month-old healthy pigs treated with twice-weekly injections of the proteasome inhibitor MLN-273 had 77% lower chymotrypsin-like proteasome activity and increased levels of ubiquitinated proteins relative to the control group. After 11 weeks of treatment, cardiac output was lower, left ventricular mass was higher, and the hearts showed perivascular and interstitial fibrosis in the inhibitor-treated group. Overall, the hearts showed alterations consistent with a hypertrophic–restrictive cardiomyopathy phenotype (Herrmann *et al.* 2013). Proteasome inhibition is also sufficient to promote maladaptive cardiac remodelling and cardiac dysfunction in stressed mouse hearts (Tang *et al.* 2010). In humans, the use of the proteasome inhibitor bortezomib (Velcade) for cancer treatment has been associated with an increased risk of adverse cardiac events, including congestive heart failure (Voortman & Giaccone, 2006; Enrico *et al.* 2007; Hacıhanefioglu *et al.* 2008). Even the second generation of proteasome inhibitors (e.g. carfilzomib) has also been

reported to show clinical cardiotoxicity (Grandin *et al.* 2015). Together these results indicate a causative role of UPS impairment in cardiac dysfunction.

UPS dysfunction in cMyBP-C-related HCM. Cardiac myosin binding protein C (cMyBP-C) is a thick filament-associated protein that interacts with myosin, actin and titin, and plays a role in regulating muscle contraction (Barefield & Sadayappan, 2010). Mutations in cMyBP-C are the leading cause of familial hypertrophic cardiomyopathy (FHC), accounting for an estimated 42% of FHC cases, and are typically associated with a favourable prognosis (Richard *et al.* 2003). Several recent studies have shown that proteasome function is impaired in HCM caused by several cMyBP-C mutations (Table 2). In HCM caused by certain cMyBP-C mutations, the mutated protein is degraded by the proteasome at a faster rate than the wild-type protein, which leads to UPS dysfunction (Sarikas *et al.* 2005; Bahrudin *et al.* 2008; Vignier *et al.* 2009; Schlossarek *et al.* 2012a). This phenomenon was discovered after several studies on cMyBP-C mutations predicted to produce truncated cMyBP-C showed none or low levels of the expected protein in tissues or cells (Rottbauer *et al.* 1997; Flavigny *et al.* 1999; Yang *et al.* 1999; Moolman *et al.* 2000). This finding contradicted the poison polypeptide hypothesis, which suggested that the mutated protein incorporates into the sarcomere and acts as a dominant negative (Rottbauer *et al.* 1997). It was later demonstrated that the absence of truncated cMyBP-C is due to its rapid degradation by the proteasome. Sarikas *et al.* (2005) showed that the low levels of two cMyBP-C truncation mutations, M6t (3% truncated) and M7t (80% truncated), which were present at 70% and 11%, respectively, of WT levels when expressed in neonatal rat cardiac myocytes (NRCMs), was due to rapid proteasomal degradation. M6t incorporated weakly into the sarcomere, while M7t was misincorporated at the Z disk and was present in ubiquitin aggregates. Treating with a proteasome inhibitor (lactacystin or MG132) raised levels of the two truncated proteins to WT levels, while the lysosomal inhibitor bafilomycin had little effect (Sarikas *et al.* 2005).

Vignier, *et al.* developed a cMyBP-C knock-in mouse with a point mutation associated with poor prognosis in humans (Vignier *et al.* 2009). The mutation, a G→A transition in a donor splice site sequence, resulted in 50% and 80% lower total cMyBP-C mRNA levels than in WT for heterozygotes and homozygotes, respectively. Interestingly, the single G→A change gave rise to three mRNAs: missense, nonsense, and deletion/insertion. Inhibition of nonsense-mediated mRNA decay increased the levels of nonsense mRNAs only, but not the other mRNAs. Protein levels of cMyBP-C were lower in heterozygous and homozygous mice, and proteasome inhibition increased the protein levels of cMyBP-C. These results suggested that nonsense-mediated mRNA decay as well as the proteasome

Table 1. Muscle specific E3 ligases and their putative targets in heart

Cardiac E3 ubiquitin ligases	Specific targets	Known pathways affected	References
Arkadia	Smad7	Myocardial fibrosis, TGF- β signalling	He <i>et al.</i> (2011)
Ankyrin repeat- and SOCS box-containing protein 2 β (Asb2 β)	Desmin	—	Thottakara <i>et al.</i> (2015)
Atrogin1/muscle atrophy F-box (MAFbx)	Akt, calcineurin, truncated M7t-cMyBP-C	Calcineurin, FoxO, JNK, p53 signalling, hypertrophy pathway	Li <i>et al.</i> (2004, 2007), Mearini <i>et al.</i> (2010)
Carboxy terminus of Hsp70-interacting protein (CHIP)	p53, oestrogen receptor- α	Apoptosis	Fan <i>et al.</i> (2005), Naito <i>et al.</i> (2010), Le <i>et al.</i> (2012)
Casitas b-lineage lymphoma (c-Cbl)	FAK, paxillin, troponin I	—	Rafiq <i>et al.</i> (2012)
Cellular inhibitor of apoptosis 1 (ciAP1)	Caspase 3/9	Apoptosis	Zhao <i>et al.</i> (2015b)
Cellular inhibitor of apoptosis 2 (ciAP2)	Caspase 3/7	Apoptosis	Huang <i>et al.</i> (2000)
F-box and leucine-rich repeat protein 22 (Fbxl22)	α -Actinin-2, filamin C	—	Spaich <i>et al.</i> (2012)
E3 ubiquitin-protein ligase Itchy homolog (ITCH)	Thioredoxin-interacting protein (TXNIP)	Reactive oxygen species-induced cardiotoxicity through the thioredoxin system	Otaki <i>et al.</i> (2016)
Muscle ring finger protein 1 (MuRF1)	Troponin I, β -MHC, MLC-2	JNK signalling, atrophy and hypertrophy pathways	Kedar <i>et al.</i> (2004), Witt <i>et al.</i> (2005), Pan <i>et al.</i> (2016)
Muscle ring finger protein 2 (MuRF2)	ND	—	Witt <i>et al.</i> (2005)
Muscle ring finger protein 3 (MuRF3)	β -MHC, four-and-a-half LIM domain (FHL2) and γ -filamin	—	Fielitz <i>et al.</i> (2007)
Murine double minute 2 (MDM2)	T cap, P53	Apoptosis, hypertrophy pathway	Tian <i>et al.</i> (2006), Toth <i>et al.</i> (2006)
Neural precursor cell expressed developmentally down-regulated protein 4-2 (Nedd 4-2)	Human ether- α -go-go-related gene (hERG) potassium channels, VEGF, Na ⁺ and K ⁺ channels	—	Abriel <i>et al.</i> (2000), Murdaca <i>et al.</i> (2004), Jespersen <i>et al.</i> (2007), Cui & Zhang (2013), Lamothe & Zhang, (2013)
Parkin	VDAC1	Mitochondrial homeostasis	Watanabe <i>et al.</i> (2014)
RING finger protein RNF207	ND	Action potential duration	Roder <i>et al.</i> (2014)
Tripartite motif 21 (TRIM21)	p62 (ubiquitination prevents its dimerization and sequestration)	Hypertrophy pathway	Pan <i>et al.</i> (2016)
Tripartite motif 32 (TRIM32)	ND	Hypertrophy pathway	Chen <i>et al.</i> (2016)
Seven in absentia homolog 2 (Siah2)	PHD1, PHD3	Hypoxia pathway	Nakayama <i>et al.</i> (2004)
SMAD ubiquitination regulatory factor 1 (Smurf1)	TGF β receptor	Myocardial fibrosis	Wang <i>et al.</i> (2012)
SMAD ubiquitination regulatory factor 2 (Smurf2)	ND	FoxO signalling	Pramod & Shivakumar (2014)
Mothers against decapentaplegic homolog 7 (Smad7)/Smurf2	TGF β receptor	Myocardial fibrosis	Cunnington <i>et al.</i> (2009)
TNF receptor-associated factor 6 (TRAF6)	TGF β receptor	Myocardial fibrosis	Kavsak <i>et al.</i> (2000)
E3 component N-recogin (UBR) 3	ND	—	Zhang <i>et al.</i> (2014)
UBR 6	Voltage-gated Na ⁺ channel Nav 1.5	—	Zhao <i>et al.</i> (2015a)
X-linked inhibitor of proteolysis (XIAP)	Voltage-gated Na ⁺ channel Nav 1.5	—	Zhao <i>et al.</i> (2015a)
ND, not determined.	Caspase 3	Apoptosis	Suzuki <i>et al.</i> (2001)

Table 2. Summary of cMyBP-C mutants on UPS function

Mutation	Model/tissue	Effect on cMyBP-C	Effect on UPS	Reference
Splice donor site mutation (G→A at position 1 of 5' splice donor sequence) resulting in deletion of 160 bp exon and premature termination of translation	Endomyocardial biopsies from left ventricular myocardium of family members with FHC	Western blots showed none of the expected truncation mutant and somewhat reduced levels of WT protein	Not investigated	Rottbauer et al. (1997)
Guanine nucleotide insertion in exon 25 (codon 791) resulting in the loss of 40 bases	Myectomy tissue from family members with FHC	Western blots showed none of the expected truncation mutant	Not investigated	Moolman et al. (2000)
cMyBP-C lacking the 240 nucleotides at the 3' end of the cDNA that constitute the myosin binding domain	Cardiac tissue from transgenic mice	Very low levels of the truncated protein were expressed	Not investigated	Yang et al. (1999)
Glu258Lys	Myectomy tissue from FHC patient	The mutant mRNA level was moderately decreased relative to WT. The cMyBP-C content relative to actin was significantly lower (present at 85% of donor control levels)	Not investigated	Marston et al. (2009)
Arg502Trp	Myectomy tissue from FHC patient	The cMyBP-C content relative to actin was significantly lower (present at 82% of donor control levels).	Not investigated	Marston et al. (2009)
Intron17 donor site A>T+4 resulting in truncation in C3 domain	Myectomy tissue from FHC patients	None of expected the 52 kDa protein was detected by Western blot with antibody to N terminus. The cMyBP-C content relative to actin was significantly lower (present at 68–83% of donor control levels)	Not investigated	Marston et al. (2009)
InsG2374 resulting in truncation in C5 domain	Myectomy tissue from FHC patient	The mutant mRNA level was moderately decreased relative to WT. None of the expected 90kDa protein was detected by Western blot with antibody to N terminus. The cMyBP-C content relative to actin was significantly lower (present at 81% of donor control levels)	Not investigated	Marston et al. (2009)
T>A 2604, delC 2605 resulting in truncation in C7 domain	Myectomy tissue from FHC patient	The mutant mRNA level was moderately decreased relative to WT. None of the expected 97 kDa protein was detected by Western blot with antibody to N terminus. The cMyBP-C content relative to actin was significantly lower (present at 65% of donor control levels)	Not investigated	Marston et al. (2009)

(Continued)

Table 2. Continued

Mutation	Model/tissue	Effect on cMyBP-C	Effect on UPS	Reference
delCT 2864/5 resulting in truncation in C7 domain	Myectomy tissue from FHC patient	None of the expected 114 kDa protein was detected by Western blot with antibody to N terminus. The cMyBP-C content relative to actin was significantly lower (present at 65% of donor control levels)	Not investigated	Marston <i>et al.</i> (2009)
Arg1271stop resulting in truncation in C10 domain	Myectomy tissue from FHC patient	The mutant mRNA level was not affected relative to WT. None of the expected 140 kDa protein was detected by Western blot with antibody to N terminus. The cMyBP-C content relative to actin was significantly lower (present at 77% of donor control levels)	Not investigated	Marston <i>et al.</i> (2009)
c.2373dupG resulting in truncation after C5 domain	Cardiac tissue from the left ventricular septum of FHC patients	The mutant mRNA made up 23% of the total cMyBP-C mRNA. None of the expected 93kDa protein was detected by Western blot with antibody to N terminus	Not investigated	van Dijk <i>et al.</i> (2009)
c.2864.2865delCT resulting in truncation at the end of the C8 domain	Cardiac tissue from the left ventricular septum of FHC patients	The mutant mRNA made up 20% of the total cMyBP-C mRNA. None of the expected 116 kDa protein was detected by Western blot with antibody to N terminus	Not investigated	van Dijk <i>et al.</i> (2009)
c.2373dupG, c.2864.2865delCT, splice site mutation c.927 to 2A>G, splice site mutation c.1458 to 1G>C	Cardiac tissue from the left ventricular septum of FHC patients	Samples from patients with FHC due to one of four different cMyBP-C mutations had 33% lower levels of cMyBP-C protein relative to non-failing donor hearts	Not investigated	van Dijk <i>et al.</i> (2012)
G→A transition on the last nucleotide of exon 6	Homozygous knock-in mice	Homozygous KI mice show 80% lower mRNA than in WT mice	The mRNA levels of five E3 ubiquitin ligases were lower in KI mice relative to WT mice: Neddd4, Ube3c, Mdm2, Trim32, and Asb2β, which showed the greatest reduction (37% lower than WT)	Thottakara <i>et al.</i> (2015)

(Continued)

Table 2. Continued

Mutation	Model/tissue	Effect on cMyBP-C	Effect on UPS	Reference
12bp duplication/4bp deletion in exon 33 which causes frameshift that results in 19 novel amino acids and premature stop codon (3% truncation) G→A transition at the last nucleotide of exon 6 that leads to skipping of exon 6 (80% truncated)	Expressed in neonatal rat cardiac myocytes (NRCMs) Expressed in NRCMs	The resulting mutant protein (M6t) was present at 70% of WT levels and incorporated weakly into the sarcomere The resulting mutant protein (M7t) was present at 11% of WT levels, misincorporated at the Z disk, and was present in ubiquitin aggregates	Treating with a proteasome inhibitor raised levels of the truncated protein to WT levels The mutant formed aggregates and inhibited the breakdown of other proteasome substrates, leading to proteasome dysfunction. Treating with a proteasome inhibitor raised levels of the truncated protein to WT levels	Sarikas <i>et al.</i> (2005) Sarikas <i>et al.</i> (2005)
G→A transition on the last nucleotide of exon 6	Heterozygous knock-in mice	50% and 80% lower mRNA levels than in WT for heterozygotes and homozygotes, respectively. The single G>A change gave rise to three mRNAs: missense, nonsense, and deletion/insertion. Levels of one of these mRNAs were increased by inhibition of nonsense-mediated mRNA decay	Levels of the proteins encoded by the other mRNAs were increased by proteasomal inhibition	Vignier <i>et al.</i> (2009)
G→A transition on the last nucleotide of exon 6	Ventricular tissue from heterozygous knock-in mice	Described above	The proteasome was impaired with age as shown by accumulation of the UPS reporter UbG76V-GFP and lower chymotrypsin-like activities. Ubiquitinated proteins were higher than in WT at 1 year of age. Adrenergic stress led to proteasome dysfunction	Schlossarek <i>et al.</i> (2012a,b)
Glu334Lys	Mutation identified in Japanese FHC patient. Expressed in COS-7, NRCMs, and HL-1 cells	Protein level was approximately half that of WT-cMyBP-C, despite similar mRNA levels. Treatment with proteasome inhibitors restored protein levels to WT levels	Expression of E334K-cMyBP-C led to decreased chymotrypsin-like proteasome activity by approximately 50%. Another study showed that the trypsin- and caspase-like activities were decreased by E334K expression as well	Bahrudin <i>et al.</i> (2008), Bahrudin <i>et al.</i> (2011)
Δ2864-2865GC (resulting in 16.2% truncation), ΔLys814, Gln998Glu, Thr1046Met	Mutation identified in Japanese FHC patient	Protein levels not affected	No effect on proteasome activity. Other UPS functions not investigated	Bahrudin <i>et al.</i> (2008)

was responsible for lower levels of mutant cMyBP-C for this mutation.

An HCM-causing missense mutation in cMyBP-C (E334K, identified in a Japanese HCM patient) has also been identified that is degraded at an accelerated rate (Bahrudin *et al.* 2008). When E334K-cMyBP-C was expressed in COS-7 and NRCMs, the protein level was approximately half that of WT-cMyBP-C, despite similar mRNA levels (Bahrudin *et al.* 2008). Treatment with proteasome inhibitors (lactacystin or MG132) restored protein levels of E334K-cMyBP-C to WT levels, while treatment with the lysosomal inhibitor chloroquine had no effect. In NRCMs, expression of E334K-cMyBP-C led to decreased chymotrypsin-like proteasome activity by approximately 50%. Another study showed that the trypsin- and caspase-like activities of the proteasome were decreased by E334K expression as well (Bahrudin *et al.* 2008). E334K expression also led to increased levels of the pro-apoptotic proteins p53, Bax and cytochrome *c* and lower levels of anti-apoptotic proteins. Staining with annexin V suggested more E334K-transfected cells were apoptotic than WT-transfected cells. It may be noted that not all cMyBP-C mutants are degraded at an accelerated rate by the proteasome; different mechanisms are in play depending on the specific mutation (Table 2).

Schlossarek *et al.* investigated effects on the UPS using the heterozygous knock-in (KI) for the mutated cMyBP-C developed by Vignier *et al.*, which reflects what occurs genetically in HCM patients, and a heterozygous MyBP-C knock-out (KO) (Vignier *et al.* 2009; Schlossarek *et al.* 2012a). The KO mice have left ventricular hypertrophy and reduced fractional shortening and serve as a model of cMyBP-C insufficiency (Carrier *et al.* 2004). Both models were compared with WT mice and exhibited cardiac hypertrophy; the KO mice at 2 weeks and later, and KI at birth and later. The proteasome was shown to be impaired with age in the KI mice only, as shown by the accumulation of the UPS reporter Ub^{G76V}-GFP and lower chymotrypsin-like activities. Ubiquitinated proteins were higher in both KO and KI than in WT at 1 year of age. Accumulation of autophagy-related proteins such as beclin-1 suggests defects in the autophagy-lysosome pathway in both KO and KI mice (Schlossarek *et al.* 2012a; Lin *et al.* 2015). Adrenergic stress due to treatment with a combination of isoprenaline and phenylephrine led to septal hypertrophy in both models, but proteasome dysfunction only in the KI mice (Schlossarek *et al.* 2012b).

UPS dysfunction in troponin T-related HCM. Mutations in TnT account for approximately 7% of HCM cases (Richard *et al.* 2003). We recently showed that UPS function is impaired in hearts of 3-month-old mice with severe TnT-related HCM (Gilda *et al.* 2016). Transgenic mice expressing human cardiac TnT with the I79N or R278C mutation were compared with mice expressing WT human

TnT. The I79N mutation is myofilament Ca²⁺ sensitizing and associated with severe, early onset HCM and sudden cardiac death. The R278C mutation does not affect myofilament Ca²⁺ sensitivity and is associated with mild, late onset heart disease. Proteomics carried out on these transgenic mouse models showed that expression of several proteasome subunits was affected, with decreases in subunit levels in R278C hearts relative to WT and higher levels in I79N than R278C hearts, suggesting differential regulation of the proteasome in I79N and R278C hearts. Western blotting was used to verify these changes, and showed a similar pattern of expression. When proteasome activity was measured, 20S and 26S chymotrypsin-like proteasome activity and immunoproteasome activity (β 1i and β 5i) were lower in hearts of I79N mice relative to WT mice. The immunoproteasome is an alternative form of the proteasome in which the catalytic β 1, β 2 and β 5 subunits are swapped out for the β 1i, β 2i and β 5i inducible subunits in response to pro-inflammatory cytokines or oxidative stress. The inducible subunits have altered cleavage specificity that favours the generation of antigenic peptides and an increased ability to degrade oxidized proteins. Decreased proteasome activity in I79N hearts was accompanied by higher levels of ubiquitinated and oxidized proteins. The only change in proteasome activity in R278C hearts was decreased β 1i immunoproteasome activity.

In I79N mice, proteomics and metabolomics also suggested accelerated energy production, as shown by increased levels of enzymes and metabolites in the glycolytic pathway, tricarboxylic acid cycle and the electron transport chain, and upregulated expression of antioxidant enzymes. Previous studies have shown that energy depletion occurs in HCM, and impaired energy metabolism has been suggested as an important factor in HCM. These findings suggest that I79N heart cells are subject to higher stress and may upregulate antioxidants to cope with these changes. It is possible that accelerated energy production may lead to mitochondrial dysfunction and increased ROS production. This could lead to higher levels of oxidized proteins for the protein to degrade, as well as direct damage to proteasome subunits if they are oxidatively modified.

The proteasome is also impaired in mice with the HCM-causing F110I TnT mutation. The F110I mutation increases myofilament Ca²⁺ sensitivity, though to a lesser extent than the I79N mutation (Hernandez *et al.* 2005). In hearts of 3-month-old F110I mice, 20S proteasome activity was decreased and the levels of ubiquitinated proteins were increased (Gomes, 2009). Although no other research related to proteasome dysfunction in TnT-related cardiomyopathies has been published, one study in *Drosophila* showed that TnT with a mutation that causes muscle defects was rapidly degraded (Fyrberg *et al.* 1990). In flies homozygous for three different mutations

in TnT, a splice donor mutation, an intronic deletion, and S311F, levels of TnT protein were reduced. Reduced TnT protein levels of the splice donor and intronic deletion mutants may be due to lower mRNA levels as a result of splicing defects. Lower levels of the S311 F mutant may be due to accelerated proteasomal degradation, but this study did not investigate the role of the proteasome.

UPS perturbations in dilated cardiomyopathy. In DCM the chambers of the heart become enlarged, and systolic function is typically reduced (McNally *et al.* 2013). In the past DCM has been associated with a poor prognosis, though advances in diagnosis treatment have improved outlook for DCM patients (Keeling *et al.* 1995; Franklin & Burch, 2000; Kubo *et al.* 2008; Hazebroek *et al.* 2012). Mutations in titin, a large sarcomeric protein that acts as a stretch sensor, are a major cause of DCM (Herman *et al.* 2012; McNally *et al.* 2013; Begay *et al.* 2015). While few studies have been conducted on the UPS in DCM, certain investigations indicate proteasome impairment as well as alterations in the ubiquitination pathway in DCM hearts. Interestingly, loss or mutation of different proteins in the ubiquitination pathway has been implicated in the pathogenesis of DCM (Xiong *et al.* 2007; Al-Yacoub *et al.* 2016). A homozygous mutation (Gly243Arg) in the cardiac E3 ligase, FBXO32 (Atrogin 1/MAFbx), was recently found to be associated with DCM (Watanabe *et al.* 2014; Al-Yacoub *et al.* 2016). There are also reports of protein aggregation in DCM, which may be linked to UPS function (Hamada *et al.* 2004; Gianni *et al.* 2010).

A study on bovine DCM revealed that levels of ubiquitin carboxyl-terminal hydrolase (UCH), a deubiquitinating protein, were greatly elevated in DCM hearts, and levels of ubiquitinated proteins were generally higher (Weekes *et al.* 1999). Hearts of human DCM patients had higher levels of E1 and E2 enzymes, and levels of ubiquitinated proteins were twofold higher than ischaemic hearts and fivefold higher than control donor hearts (Weekes *et al.* 2003). Levels of UCH were also higher in human DCM hearts, in agreement with the study on bovine DCM. Proteasome activity was not measured in this study, but these findings showed UPS alterations at the level of the ubiquitination pathway. In other patients with DCM, proteasome levels were increased, and oxidative stress appeared to be increased (Otsuka *et al.* 2010). Immunohistochemistry showed that expression of proteasome and ubiquitin was enhanced, and they were present in large granular structures relative to control hearts. Ubiquitin-positive granular structures were likely to be due to the accumulation and aggregation of ubiquitinated proteins. Products of lipid and carbohydrate oxidation were also higher in DCM hearts. Levels of superoxide dismutase-1 (SOD1) were higher as well, consistent with increased oxidative stress in DCM hearts. A separate study comparing cardiac tissue from DCM patients and donors

showed that levels of polyubiquitinated proteins were increased in DCM hearts, as well as the chymotrypsin-like activity of the proteasome (Birks *et al.* 2008).

Inflammation, autoimmunity, and compromised protein quality control have been linked to DCM. As the UPS is involved in the immune response and inflammation, Voigt *et al.* (2010) investigated anti-proteasomal immunity in hearts of DCM patients. They found that autoimmune responses to proteasome in the heart were increased in DCM patients, predominantly towards 20S α subunits, and the levels of antibodies to proteasome were particularly enhanced in advanced heart failure (Voigt *et al.* 2010). This suggests that in DCM patients altered proteasome structures are resulting in the generation of antibodies to these 'foreign' proteins.

Desmin-related myopathy. Desmin-related myopathy (DRM) is a disease associated with mutations in desmin or associated proteins (Paulin & Li, 2004; Goldfarb & Dalakas, 2009). Desmin is an intermediate filament expressed in cardiac, skeletal and smooth muscle that plays an important role in maintaining the contractile apparatus (Paulin & Li, 2004; Goldfarb & Dalakas, 2009). DRM patients typically have skeletal muscle weakness and cardiomyopathy, and respiratory weakness is also commonly observed (van Spaendonck-Zwarts *et al.* 2011). Causes of death in DRM patients include sudden cardiac death and heart failure (van Spaendonck-Zwarts *et al.* 2011). DRM-causing mutations in desmin cause improper folding, which contributes to the accumulation of unfolded proteins and pre-amyloid oligomer formation (McLendon & Robbins, 2011). In line with this, DRM can also be caused by mutations in α B-crystallin (CryAB), a chaperone that folds desmin (McLendon & Robbins, 2011). The proteasome plays an important role in removing misfolded proteins, and can itself be inhibited by protein aggregates. In mice expressing a mutated form of desmin that is linked to DRM (deletion of 7 amino acids, R173–E179), cardiac UPS function is impaired, as shown by increased levels of the UPS reporter GFPdgn (Liu *et al.* 2006a). UPS impairment was likely to be due to impaired delivery of substrates into the proteasome, as the proteolytic activities were found to be increased rather than decreased, and subunits of the 19S cap were depleted (Liu *et al.* 2006a). In cultured neonatal rat ventricular myocytes, overexpressing mutated desmin caused desmin-positive aggregates to form and disrupted proteasome function, as shown by the accumulation of the proteasome reporter GFPu (Liu *et al.* 2006b). Co-expressing the chaperones CryAB or Hsp70 significantly reduced desmin aggregation and reduced or completely abolished GFPu accumulation, demonstrating that desmin aggregation is necessary to cause proteasome dysfunction (Liu *et al.* 2006b). The proteasome is also

impaired in DRM mice with the R120G mutation in CryAB (Chen *et al.* 2005). There was an accumulation of ubiquitinated proteins and GFPdgn reporter in the heart, while proteolytic activities of the proteasome were increased and subunit expression was decreased (Chen *et al.* 2005). This is similar to findings in DRM mice with desmin mutations and suggests the defect occurs in substrate delivery. These studies demonstrate that desmin mutants impair the proteasome, which may contribute to further protein aggregation and cardiac dysfunction in DRM.

UPS perturbations in restrictive cardiomyopathy. The R145W mutation in TnI causes restrictive cardiomyopathy and has been associated with sudden cardiac death (van den Wijngaard *et al.* 2011). This mutation increases the Ca^{2+} sensitivity of force and ATPase activity (Gomes *et al.* 2005; Wen *et al.* 2009). Proteomic analysis of hearts from 3-month-old mice expressing R145W-TnI found that several pathways were affected, including increases in enzymes involved in ATP production and stress-related proteins. No change was observed in the levels of two proteasome subunits: PSMA6 and Rpt1. Importantly, proteolytic assays showed that proteasome activity was lower in R145W mice than WT mice, indicating proteasome impairment (Cui *et al.* 2013b).

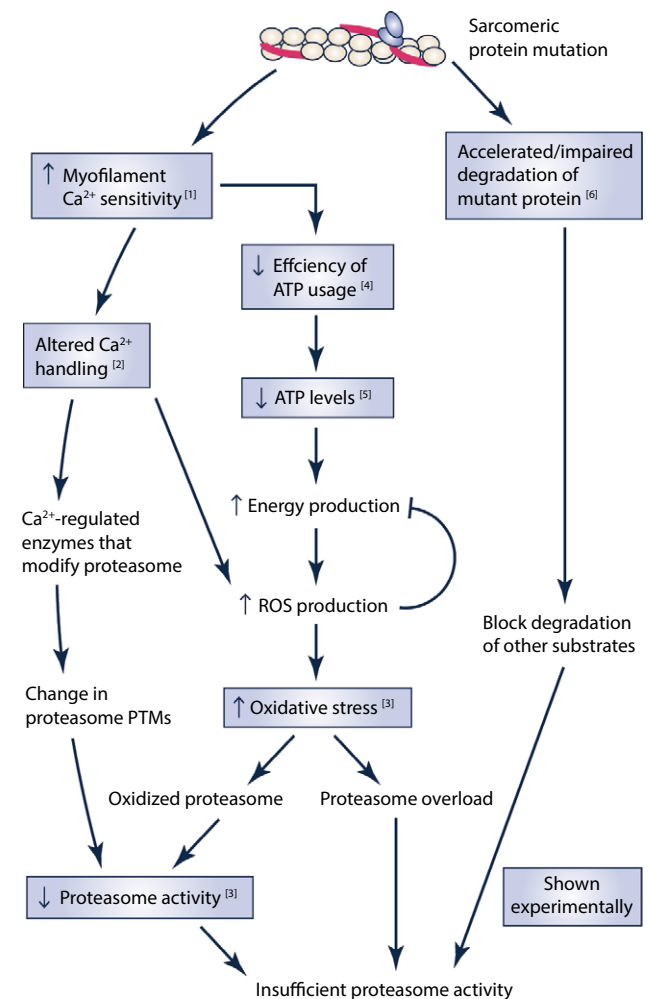
UPS dysfunction in right ventricular dysfunction. Effects on the UPS were investigated in mice with right ventricular hypertrophy/failure due to pulmonary artery constriction, and the chymotrypsin-like activity of the proteasome was found to be decreased, while the proteasome subunit Rpt5 as well as UCHL1 deubiquitinase and Smurf1 E3 ligase were increased (Rajagopalan *et al.* 2013). Levels of poly-ubiquitinated proteins were also increased in conjunction with impaired proteasome function (Rajagopalan *et al.* 2013). Levels of the pro-apoptotic protein Bax were higher, while the anti-apoptotic protein Bcl-2 was lower (Rajagopalan *et al.* 2013). An increase in ubiquitinated proteins as well as changes in several E3 ligases, including Mdm2 and E6AP, was observed in a feline right ventricular pressure overload model (Balasubramanian *et al.* 2006).

Potential mechanisms for UPS dysfunction

There are multiple mechanisms by which mutations in sarcomeric proteins could lead to UPS dysfunction, such as altered degradation of the mutated protein, altered calcium handling, and mitochondrial dysfunction. Possible mechanisms to explain how HCM mutations in sarcomeric proteins cause proteasome dysfunction are shown in Fig. 2 and discussed below.

Altered proteasomal degradation of mutant sarcomeric protein

Abnormal sarcomeric proteins may be degraded by the proteasome at an increased rate, thereby overloading the proteasome and leading to insufficient activity to break down other UPS substrates (Sarikas *et al.* 2005; Bahrudin *et al.* 2008). Mutants may also be resistant to degradation, and clog the proteasome, preventing entry of other proteasome substrates. Sarikas *et al.*'s investigation showed that the cMyBP-C M7t mutant formed aggregates and inhibited the breakdown



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Figure 2. Schematic diagram showing the potential mechanisms by which familial cardiomyopathy mutations in sarcomeric proteins may cause proteasome dysfunction

Boxes show changes that have been observed experimentally and the numbers in these boxes correspond to the following references: 1, Baudenbacher *et al.* (2008); 2, Schober *et al.* (2012); 3, Gilda *et al.* (2016); 4, Miller *et al.* (2001); 5, JE Gilda & AV Gomes, unpublished data; 6, Bahrudin *et al.* (2008). PTMs, post-translational modifications.

of other proteasome substrates, leading to proteasome dysfunction (Sarikas *et al.* 2005). These aggregates were ubiquitin-positive suggesting that they contained the mutant cMyBP-C that may be ubiquitin bound, but not degraded. The presence of ubiquitin-positive aggregates is a prominent feature of most neurodegenerative disorders, where misfolded proteins and aggregates are proposed to overload the proteasome and/or block the pore (Ciechanover & Brundin, 2003). Misfolded proteins may accumulate due to increased abundance as the result of genetic mutations, aging and/or cellular stress and are associated with proteotoxicity (McLendon & Robbins, 2015). Both cytosolic and nuclear proteasomes participate in the degradation of misfolded proteins (von Mikecz *et al.* 2008; McLendon & Robbins, 2015). Since excellent recent reviews concerning the presence of aggregates and proteotoxicity in cardiomyopathies exist this aspect will not be covered in significant detail in this review (Willis & Patterson, 2013; McLendon & Robbins, 2015). In addition to the UPS, several mechanisms have evolved to remove or repair misfolded proteins, such as autophagy, which clears misfolded proteins and aggregates, and chaperones, which repair or protect misfolded proteins from degradation. It is not known if mutations in sarcomeric proteins other than cMyBP-C, such as actin and troponin I, affect their rate of degradation by the proteasome.

Altered calcium handling

Another potential mechanism is that alterations in intracellular Ca^{2+} concentrations may affect proteasome activity. Most cardiomyopathy-causing sarcomeric protein mutations increase Ca^{2+} sensitivity of muscle contraction, and mutations that sensitize the myofilament to the effects of Ca^{2+} are associated with a high risk of SCD (Huke & Knollmann, 2010). TnC, the Ca^{2+} -binding subunits of the troponin complex, is a major cytosolic Ca^{2+} buffer, and increases in myofilament Ca^{2+} sensitivity have been shown to alter Ca^{2+} handling in the cell. Increased myofilament Ca^{2+} sensitivity leads to slow Ca^{2+} transient decays and increased diastolic Ca^{2+} concentrations (Knollmann *et al.* 2003; Sirenko *et al.* 2006; Schober *et al.* 2012; Wu *et al.* 2012). One potential mechanism by which altered Ca^{2+} concentrations could affect proteasome activity is by affecting the activity of Ca^{2+} -regulated proteins that associate with and modify the proteasome. Calmodulin, a Ca^{2+} -binding protein, has been shown to interact with 26S proteasome subunits non-ATPase regulatory subunit 2 and non-ATPase regulatory subunit 12 (Shen *et al.* 2005). Interaction of calmodulin with either or both proteasome subunits could potentially directly affect proteasome activity. Calmodulin also interacts with the proteasome associating protein RAD23 homolog B (Shen *et al.* 2005).

Post-translational modifications of the proteasome, and phosphorylation in particular, are very important in regulating the activity and stability of the proteasome. Many subunits of the proteasome are endogenously phosphorylated, and over 400 proteasome phosphorylation sites have been identified in total (Gomes *et al.* 2006, 2009; Drews *et al.* 2007; Cui *et al.* 2013a). Proteasome activity is regulated by enzymes such as protein phosphatase 2A (PP2A), which decreases proteasome phosphorylation and activity, and protein kinase A (PKA), which enhances proteasome phosphorylation and activity (Marambaud *et al.* 1996; Zong *et al.* 2006; Zhang *et al.* 2007). In addition to these enzymes, several other kinases have been identified that phosphorylate the proteasome and modulate its activity and/or stability, such as casein kinase 2, p38 MAPK, polo-like kinase, c-Abl tyrosine kinase, and Arg tyrosine kinase (Castano *et al.* 1996; Feng *et al.* 2001; Bose *et al.* 2004; Liu *et al.* 2006c; Lee *et al.* 2010). Certain Ca^{2+} -dependent enzymes have been shown to associate with and modify the proteasome, such as calcium/calmodulin-dependent protein kinase II (CaMKII) and calcineurin (Li *et al.* 2011b; Djakovic *et al.* 2012). In neurons, CaMKII α phosphorylates Rpt6, a subunit of the 19S proteasome regulatory particle, leading to increased proteasome activity (Djakovic *et al.* 2012). The regulatory subunit of calcineurin, a phosphatase regulated by Ca^{2+} /calmodulin, has been shown to interact with the 20S proteasome subunit PSMA7 (α 4) and stimulate proteasome activity (Li *et al.* 2011b). In cardiomyocytes, protein kinase G (PKG) has been shown to positively regulate proteasomal function (Ranek *et al.* 2013). The same group found that parasympathetic muscarinic 2 receptor activation stimulates cardiac proteasome activity in a PKG dependent manner (Ranek *et al.* 2014). Reduced parasympathetic tone as well as reduced PKG activity are linked to cardiac malfunction, and may serve as a possible cause of impaired proteasomal degradation in cardiomyopathies. It is possible that the altered Ca^{2+} levels in the cell, which occur as a result of myofilament Ca^{2+} -sensitizing mutations such as the I79N mutation in TnT, may affect the activity of these and other Ca^{2+} -regulated enzymes that modify the proteasome.

Mitochondrial dysfunction and oxidative stress

Another potential mechanism by which increased Ca^{2+} concentrations could affect proteasome activity is by inducing mitochondrial dysfunction, which could lead to increased reactive oxygen species (ROS) production (Celsi *et al.* 2009). Calcium plays an important role in regulating mitochondrial function. Studies have shown that when mitochondria are exposed to high concentrations of Ca^{2+} in the cell, Ca^{2+} is pumped into the mitochondria through the Ca^{2+} uniporter (Bianchi *et al.* 2004). Increases in

cytosolic Ca^{2+} levels can lead to mitochondrial matrix Ca^{2+} overload which results in increased production of ROS (Peng & Jou, 2010). Mitochondria produce ROS such as superoxide, hydrogen peroxide, and hydroxyl radicals, as well as reactive nitrogen species. Mitochondrial ROS is mainly produced from complexes I and III of the electron transport chain during oxidative phosphorylation. High levels of ROS are detrimental to the cell, as they can react with proteins and other molecules in the cell and damage them. Mitochondrial dysfunction has been reported in mice and patients with HCM (Vakrou & Abraham, 2014). In cardiac cells with altered Ca^{2+} handling, it is possible that mitochondrial Ca^{2+} levels and ROS production are affected. We found that in 3-month I79N mouse hearts, levels of oxidized proteins were increased (Gilda *et al.* 2016). Increased levels of ROS could lead to oxidative protein damage and an overload of proteasome substrates, and consequent proteasome functional insufficiency. ROS may also directly react with proteasome subunits and damage the proteasome, leading to decreased activity.

Subunits of the proteasome can be oxidatively damaged, leading to lower proteasome activity (Aiken *et al.* 2011). Oxidation of the Rpt3 subunit of the 19S regulatory particle leads to decreased proteasome activity (Ishii *et al.* 2005). Oxidation of the 20S proteasome has also been shown to impair the proteolytic activity of the proteasome (Bulteau *et al.* 2002). Breusing *et al.* (2009) showed that with age, protein oxidation increases and proteasome activity decreases. Impaired docking of the 19S to the 20S, which would reduce polyubiquitin-dependent degradation, was observed in human end-stage heart failure (Day *et al.* 2013). Our lab recently showed that treating H9c2 cardiac cells with the NSAID meclufenamate sodium resulted in elevated levels of mitochondrial ROS, increased oxidation of 19S proteasome subunits, altered 19S and 20S association, and decreased proteasome activity (Ghosh *et al.* 2016).

Impaired energy metabolism has also been suggested as a unifying factor of HCM, and myofibrillar Ca^{2+} -sensitizing mutations lead to decreased efficiency of ATP usage. Recent proteomics and metabolomics data suggest that energy production pathways are accelerated in hearts of mice with the I79N mutation in TnT (Gilda *et al.* 2016), which may lead to enhanced energy production via the electron transport chain and subsequently increased mitochondrial ROS production. It may also be noted that the UPS utilizes ATP; hence ATP depletion could potentially impair the UPS by a more direct route, though further investigation is needed. Unpublished results from our laboratory suggest that ATP levels in the hearts are significantly lower in I79N transgenic mice compared with WT mice. Hearts from mice with the R92W and R92L mutations in TnT utilize more ATP during muscle contraction and have lower levels of ATP and higher levels of ADP and P_i (He *et al.* 2007). H9c2 cardiac cells

subjected to severe ATP depletion showed stress-associated proteotoxicity and cell death (Kabakov *et al.* 2002). As such, lower ATP levels as observed in the I79N hearts are also likely to contribute to UPS dysfunction.

Can increasing proteasome activity improve cardiac function? Enhancing proteasome function by overexpression of the proteasome subunit PA28 α decreased cardiac hypertrophy and increased the lifespan of R120G-CryAB mice (Li *et al.* 2011a). Enhancement of proteasome function by PA28 α overexpression also reduces the size of infarct and protects against cardiac dysfunction in mice with ischaemia-reperfusion injury (Li *et al.* 2011a). Enhancing proteasome function in mice by overexpression of the proteasome regulatory subunit PA28 α partially attenuated right ventricular failure and improved survival (Rajagopalan *et al.* 2013). Activation of the proteasome by pharmacological means (e.g. via PDE5 inhibition/PKG activation) was found to protect against DRM disease progression in mice (Ranek *et al.* 2013). These results all suggest that enhancing proteasome function can have a cardioprotective effect.

Mechanistic links between proteasome dysfunction and cardiomyopathy

The UPS plays an important role in most cellular processes, and is particularly important in the heart; therefore UPS impairment may lead to cardiac dysfunction through multiple mechanisms.

Electrophysiological dysfunction. Bahrudin *et al.* hypothesized that since UPS impairment led to an accumulation of pro-apoptotic proteins, it may also lead to accumulation of other proteins that it normally degrades, such as ion channels and Ca^{2+} handling proteins, and this may contribute to the electrophysiological dysfunction and the arrhythmias observed in patients with the E334K mutation in cMyBP-C. In HL-1 cells and NRCMs, expression of E334K-cMyBP-C led to lower proteasome activity and significantly higher levels of Kv1.5, Nav1.5, Cav3.2, Hcn4, Cav1.2, Serca, RyR2 and Ncx1 compared with their levels in WT-cMyBP-C-expressing cells, despite similar mRNA levels (Bahrudin *et al.* 2011). Expression of E334K-cMyBP-C led to higher Ca^{2+} transients and longer action potential durations (Bahrudin *et al.* 2011). Thus, proteasome impairment could in part explain some of the electrophysiological dysfunction in cardiomyopathy patients.

Proteasome inhibition and apoptosis. Proteasome inhibition has been shown to induce apoptosis in several different cell types (Ding *et al.* 2007; Pandit & Gartel, 2011; Tsuchiya *et al.* 2011). Proteasome

inhibition is often associated with an increase in the levels of pro-apoptotic proteins. Levels of pro-apoptotic proteins have been shown to be increased in cardiomyopathies. The apoptosis regulator p53 was shown to be upregulated in DCM (Birks *et al.* 2008). The E334K mutation in cMyBP-C, which leads to reduced proteasome activity, increased the levels of pro-apoptotic proteins (p53, Bax and cytochrome *c*) and decreased levels of anti-apoptotic proteins (Bcl-2 and Bcl-XL) (Bahrudin *et al.* 2008). Sarikas *et al.* (2005) observed an increased rate of apoptosis in M7t-cMyBP-C-expressing NRCMs.

Besides other models described earlier, a cMyBP-C KI mouse model of HCM showed reduced proteasome activity and decreased mRNA levels of the muscle-specific E3 ligases, Asb2 β (Thottakara *et al.* 2015). In neonatal mouse cardiomyocytes Asb2 β was found to target desmin for degradation (Thottakara *et al.* 2015). Reduced proteasome activity has also been observed in explanted failing hearts and myectomy samples from HCM patients when compared with samples from non-failing hearts (Predmore *et al.* 2010). Increased protein expression of p53 in patient HCM samples suggest that apoptosis may play a key role in HCM pathogenesis (Predmore *et al.* 2010). In these human heart samples from HCM patients, reduced proteasome activity was not due to changes in proteasome protein content suggesting that post-translational modifications of proteasomes may be responsible for the impaired protein degradation in HCM (Predmore *et al.* 2010). Mutations in cMyBP-C accounted for most of the HCM patients used in the Predmore *et al.* studies, but one TnT mutant (D86A) patient and one Tm (I284V) mutant patient were also part of the HCM group investigated (Predmore *et al.* 2010). These studies provided the first suggestion that patients with HCM associated with TnT and Tm mutations may also affect proteasome function. As even small increases in apoptosis can cause cardiac dysfunction, it is possible that one mechanism by which proteasome impairment leads to cardiac dysfunction is through increased apoptosis. Altered Ca²⁺ handling may also be linked to the increased apoptosis; increased Ca²⁺ influx may lead to sarcoplasmic reticulum Ca²⁺ overload and cell death by the mitochondrial death pathway.

Proteasome inhibition and calcineurin. Another mechanism could be the activation of the calcineurin–nuclear factor of activated T-cells (NFAT) pathway by proteasome inhibition (Tang *et al.* 2010). Both calcineurin and NFAT are degraded by the UPS, and in hearts with impaired proteasome activity, such as in the D7-desmin mouse hearts, the levels of calcineurin and NFAT may be increased (Tang *et al.* 2010). Increased calcineurin activity is physiologically important since calcineurin has been shown to be a key

regulator of cardiomyocyte hypertrophy (Chen *et al.* 2016).

Conclusions

The current experimental data suggest that UPS dysfunction may be a hallmark of cardiomyopathies. It is likely that in some cases UPS dysfunction in cardiomyopathies may be caused directly by changes in the ability of the UPS system to degrade the mutant protein (as observed with some cMyBP-C mutants). It is possible that direct effects of changes in Ca²⁺ signalling resulting in changes in proteasome activity through interacting partners or post-translational modifications may also be important in cardiomyopathies. Limited experimental data also suggest that indirect effects of changes in Ca²⁺ signalling could lead to mitochondrial dysfunction that increases ROS and affects proteasome function via post-translational modifications. Increased levels of ROS and/or decreased ATP levels may also affect UPS components other than the proteasome. UPS dysfunction could lead to myriad cellular problems including proteotoxicity, mitochondrial dysfunction and apoptosis. Proteotoxicity, mitochondrial dysfunction and apoptosis are each individually associated with cell death and cardiovascular disease, but cardiomyopathies are likely to involve more than one and possibly all three of these processes.

Future studies

Studies have shown that familial cardiomyopathy-causing mutations in cMyBP-C, TnT and TnI impair proteasome activity (Bahrudin *et al.* 2008; Cui *et al.* 2013b; Gilda *et al.* 2016). Further studies are needed to determine whether the UPS is affected in cardiomyopathies caused by other mutations in these sarcomeric proteins as well as other sarcomeric proteins, such as α -tropomyosin and myosin heavy chain. As experimental evidence for the cause of UPS dysfunction as well as the mechanism(s) by which UPS dysfunction may cause cardiomyopathies is still in its infancy, significant studies to further investigate the cause and effect of UPS dysfunction in cardiomyopathies are needed. In particular, the molecular effects of altered Ca²⁺ levels in cardiac cells as well as the role of mitochondrial dysfunction need to be investigated in cardiomyopathies. Studies are needed to determine if the interaction between calmodulin and proteasome affects proteasome function, and if this plays a role in cardiomyopathies. It is unknown if cardiomyopathy-causing mutations in other sarcomeric proteins besides cMyBP-C affect their rate of degradation by the proteasome. A major area of future study should be devoted to investigating the effects of enhancing proteasome function on cardiovascular function in cardiomyopathies, especially under

conditions related to increased stress, such as under elevated adrenergic stress.

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Additional information

Competing interests

The authors have no competing interests.

Author contributions

Both authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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