Hypertrophic cardiomyopathy mutations increase myofilament Ca$^{2+}$ buffering, alter intracellular Ca$^{2+}$ handling and stimulate Ca$^{2+}$ dependent signaling

Paul Robinson*, Xing Liu*, Alexander Sparrow, Suketu Patel, Yin-Hua Zhang#, Barbara Casadei, Hugh Watkins, Charles Redwood

Cardiovascular Medicine Division, Radcliffe Department of Medicine, University of Oxford, UK;

Running Title: HCM mutations increase Ca$^{2+}$ buffering and signalling

* These authors contributed equally.

# current affiliations: Department of Physiology & Biomedical Sciences, Ischemic/hypoxic Disease Institute, Seoul National University, College of Medicine, Seoul, Korea; Yanbian University Hospital, Yanji, Jilin Province, China; Institute of Cardiovascular Sciences, University of Manchester; Manchester, UK.

1 To whom correspondence should be addressed: Dr Paul Robinson, Cardiovascular Medicine Division, Level 6 West Wing, John Radcliffe Hospital, Headley Way, Headington, Oxford, UK; paulr@well.ox.ac.uk; Tel.: +44 1865 234646; Fax: +44 1865 234681.

Key words: cardiomyopathy; troponin; tropomyosin; calcium; Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII); extracellular-signal-regulated kinase (ERK); NFAT transcription factor, SERCA.

ABSTRACT

Mutations in thin filament regulatory proteins that cause hypertrophic cardiomyopathy (HCM) increase myofilament Ca$^{2+}$-sensitivity. Mouse models exhibit increased Ca$^{2+}$ buffering and arrhythmias, and we hypothesized that these changes are primary effects of the mutations (independent of compensatory changes) and that increased Ca$^{2+}$-buffering and altered Ca$^{2+}$-handling contribute to HCM pathogenesis via activation of Ca$^{2+}$-dependent signalling. Here, we determined the primary effects of HCM mutations on intracellular Ca$^{2+}$-handling and Ca$^{2+}$-dependent signalling in a model system possessing Ca$^{2+}$-handling mechanisms and contractile protein isoforms close to human in the absence of potentially confounding remodeling. Using adenovirus, we expressed HCM-causing variants of human troponin-T, troponin-I and α-tropomyosin (R92Q, R145G and D175N respectively) in isolated guinea pig left ventricular cardiomyocytes. After 48 hours, each variant had localized to the I-band and comprised ~50% of the total protein. HCM mutations significantly lowered the K_d of Ca$^{2+}$ binding resulting in higher Ca$^{2+}$ buffering of mutant cardiomyocytes. We observed increased diastolic [Ca$^{2+}$] and slowed Ca$^{2+}$ reuptake, coupled with a significant decrease in basal sarcomere length and slowed relaxation. HCM mutant cells had higher sodium/calcium exchanger activity, sarcoplasmic reticulum Ca$^{2+}$-load, and sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2) activity driven by Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) phosphorylation of phospholamban. The ryanodine receptor (RyR) leak-load relationship was also increased, driven by CaMKII-mediated RyR phosphorylation. Altered Ca$^{2+}$ homeostasis also increased signaling via both calcineurin/NFAT and extracellular-signal-regulated kinase pathways. Altered myofilament Ca$^{2+}$ buffering is the primary initiator of signalling cascades, indicating that directly targeting myofilament Ca$^{2+}$-sensitivity
Hypertrophic cardiomyopathy (HCM) is an autosomal dominant disorder, usually caused by a single heterozygous genetic variant shared by all affected family members. It is the most common inherited cardiac disorder with a prevalence of 1 in 500(1) and the leading cause of sudden death in young adults, and athletes in particular(2). Mutations underlying the disease are principally found in genes that encode components of the contractile apparatus(3). The most commonly affected genes encode the thick filament proteins myosin binding protein C (MyBPC) and β-myosin heavy chain (MyHC), while other HCM genes encode the thin filament regulatory proteins cardiac troponin T (cTnT), cardiac troponin I (cTnI) and α-tropomyosin (α-TM). We and others have established that HCM mutations in thin filament regulatory proteins increase myofilament Ca^{2+}-sensitivity of actomyosin ATPase activity, in vitro motility and force of skinned muscle fibres(4-7). Furthermore, we have shown that this is due to an increase in actual Ca^{2+} affinity of the low affinity regulatory Ca^{2+} binding site of cTnC(8,9). Other HCM mutations (e.g. in MYH7(10) and MYBPC3(11)) are also predicted to increase troponin C Ca^{2+} binding due to the effects of additional cross-bridge formation on cooperative thin filament activation. Thus, heightened myofilament Ca^{2+} sensitivity is likely to be a consistent feature of HCM mutations. Troponin C is the principal dynamic buffer of cytoplasmic Ca^{2+}, and has been estimated to bind approximately half of the Ca^{2+} ions released by the Sarcoplasmic Reticulum (SR) during systole(12). We predict that the increased myofilament Ca^{2+} affinity will directly alter intracellular Ca^{2+} homeostasis in patients with HCM via increasing myofilament Ca^{2+} buffering. Increased buffering would cause deleterious changes to intracellular Ca^{2+} cycling, which may trigger Ca^{2+}-dependent hypertrophic signalling and increase the probability of arrhythmic events. Recent work on transgenic mice containing troponin mutations has provided evidence of these outcomes although whether this is due to primary effects of the mutant protein or compensatory changes is unclear. Some studies have shown profound increases in basal [Ca^{2+}], in the presence of increased Ca^{2+} buffering (13,14), whilst others have found the opposite effect depending on the age of the mice (15,16). In this study we have systematically tested the changes to both Ca^{2+} cycling and Ca^{2+}-dependent signalling in a stable but short-term cardiomyocyte model of HCM. This approach allows evaluation of the direct cellular consequences of a HCM mutation, free of the secondary effects of pathological remodelling caused by compensatory (or maladaptive) gene expression. For example reduction of SERCA levels(17) and myofilament protein isoform switching(18), both well known hallmarks of heart failure and cardiomyopathy, would be expected to confound electrophysiological and contractile changes caused by the primary mutation in animal models and patients. We have used guinea pig left ventricular cardiomyocytes to model human cardiomyocyte Ca^{2+} cycling more accurately than previous studies published in transgenic mice., Mouse cardiomyocytes fundamentally differ from both human and guinea pig in structure and function. For example: they contain predominantly fast α-MyHC versus slow β-MyHC in human and guinea pig(19). The generation of Ca^{2+} transients relies almost entirely on Ca^{2+}-induced Ca^{2+} release from the SR with very little contribution from NCX current, whereas in humans and guinea pig the NCX contribution is ~30 % (20). Cardiac action potentials in mouse lack any appreciable plateau and differ markedly in waveform(21) indicating a restructured electrophysiological regulation.

We have engineered adenoviruses to express WT and R92Q cTnT, WT, and R145G cTnI, and WT and D175N α-TM. By measuring the impact of mutations affecting three different sarcomeric proteins, we aimed to identify hallmark changes in Ca^{2+} handling in HCM. Furthermore, we present for the first time, a novel analytical paradigm to fully incorporate the consequences of Ca^{2+} buffering on cardiomyocyte Ca^{2+} transients and Ca^{2+} handling protein activities. We consider the dynamics of total intracellular Ca^{2+} ([Ca^{2+}]_{i, tot}) derived from NCX integral measurements rather than free Ca^{2+} ([Ca^{2+}]_{i}) from fura2 fluorescence when assessing SR load, fractional SR release and SERCA activity. When applied, our data show that the primary effects of HCM mutant gene expression include altered Ca^{2+} transients and increased myofilament Ca^{2+} buffering, increased [Ca^{2+}] in both the SR and cytoplasm, and activation of Ca^{2+}-dependent signalling mediated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), calcineurin/NFAT and MAPK/ERK.

### Results

**Adenoviral expression of human troponin and tropomyosin in guinea pig cardiomyocytes**

Isolated guinea pig left ventricular cardiomyocytes were infected with ~1000 MOI of recombinant adenovirus. The level of infection was between 32.1±7.2 (WT TnI) and 87.6±9.1% (WT TM), calculated from the co-expression of hrGFP (Fig. 1A and C). The relative expression of thin filament
regulatory protein subunits was determined by western blot and subsequently adjusted by the infection levels for each cell preparation tested. Conjugation of an N-terminal (cTnT and cTnI) or C-terminal (α-TM) FLAG-tag increased the molecular weight of the recombinant proteins sufficiently to allow differentiation from the endogenous subunit (Fig. 1F). The relative expression level of recombinant protein in purified cardiomyocytes coexpressing hrGFP was found to be 54.3±9.5% (cTnT R92Q), 49.3±8.5% (cTnI R145G) and 57.6±7.5% (TM D175N) (Fig. 1B and D), thus likely to closely reflect the levels of mutant protein found in patients with autosomal dominant cardiomyopathy. The recombinant FLAG-tagged thin filament proteins were localised to the I-band of the myofilaments of infected cardiomyocytes (Fig. S3). We cultured for 1, 24 and 48 hours along with the extent of t-tubular de-differentiation (Fig. S3). We observed a small but significant reduction in the number of t-tubules and a prolongation in the relaxation and Ca\(^{2+}\) reuptake after 48 hours (Fig. S4 and Table S1).

The presence of the Ca\(^{2+}\) sensitising mutations increases myofilament Ca\(^{2+}\) buffering and drives altered Ca\(^{2+}\) homeostasis.

[Ca\(^{2+}\)]\(_{\text{total}}\) and [Ca\(^{2+}\)]\(_{i}\) were measured upon the application of 10 mM caffeine using simultaneous measurement of fura2 fluorescence ratio and whole cell voltage clamp current in a method adapted from Trafford et al(22) (Fig. S6). We postulated that increasing the Ca\(^{2+}\) affinity of myofilaments by introducing a Ca\(^{2+}\) sensitising HCM mutation will in turn increase total Ca\(^{2+}\) buffering in the intact cardiomyocytes. We fitted the calibrated total vs free [Ca\(^{2+}\)] data to a Michaelis–Menten type equation (Fig. 2A). The K\(_d\) of Ca\(^{2+}\) buffering was significantly decreased in the presence of each HCM causing mutation. (Fig. 2B and Table S2). However, the total Ca\(^{2+}\) occupancy of the myofilaments (B\(_{\text{max}}\)) was not significantly altered between groups (Table S2). The relative buffering at low [Ca\(^{2+}\)] is described by B\(_{\text{max}}\)/K\(_d\) and this ratio was increased for all three mutants compared to wild type (Fig. 2C). The calculated relationship between [Ca\(^{2+}\)]\(_{\text{total}}\) and [Ca\(^{2+}\)]\(_{i}\) over the full range of [Ca\(^{2+}\)] is shown in Fig. 2D. We have used these fits to convert observed Ca\(^{2+}\)\(_{\text{free}}\) to [Ca\(^{2+}\)]\(_{\text{total}}\) for each cell and calculate changes in SR load and SERCA activity. This incorporates the effect of altered Ca\(^{2+}\) buffering when considering these key Ca\(^{2+}\) handling parameters.

**Cardiomyocytes expressing Ca\(^{2+}\)-buffering HCM causing mutations exhibit altered Ca\(^{2+}\) transients and contractility.**

In analyses of cytosolic [Ca\(^{2+}\)]\(_{c}\) transients at 0.5 Hz pacing (Fig. 3A), cells expressing HCM-causing mutations had significantly higher diastolic [Ca\(^{2+}\)]\(_{c}\), \((Δ[Ca^{2+}]) = 0.175±0.007 to 0.307±0.011 \mu M\) (Fig. 3B and Table S3A) and significantly prolonged rates of Ca\(^{2+}\) reuptake (Δt = 0.027±0.003 to 0.041±0.002 sec\(^{-1}\)) (Fig. 3C and Table S3A) compared with cardiomyocytes expressing the corresponding WT protein. The time to 50% Ca\(^{2+}\) release from the SR and the Ca\(^{2+}\) transient amplitude were not significantly different between groups. In measurements of sarcomere shortening at 1 Hz pacing, all three HCM mutations were found to decrease basal sarcomere length compared with WT (ΔSL = -0.030±0.007 to -0.094±0.007 \mu m) in line with the higher levels of diastolic [Ca\(^{2+}\)]. Cardiomyocytes expressing HCM mutations also had significantly prolonged relaxation (AT\(_{\text{50}}\) = 0.011±0.002 to 0.037±0.003 sec) (Fig. 3D-F and Table S3B). Cardiomyocytes expressing the R145G mutation in cTnI displayed reduced fractional shortening compared to WT; these cells also had the shortest diastolic sarcomere length (1.776±0.007 vs 1.865±0.009 \mu m in WT) and this may impose some limit on the extent of shortening compared with the other two mutations. Due to fura2 accumulation(23) and resultant Ca\(^{2+}\) chelation in our loaded cardiomyocytes. The phenotype conferred by HCM mutations in fura2-loaded cardiomyocytes at 0.5 Hz was qualitatively the same, despite reduced fractional shortening due to the presence of the chemical dye (Fig. S7 and Table S3C).

**Increased myofilament Ca\(^{2+}\) buffering results in higher peak NCX current, SR load and RyR receptor leak/load relationship along with activation of CaMKII signalling.**

The NCX current was measured during the direct application of 10 mM caffeine by whole cell voltage clamp recordings (Fig. S6). The peak I\(_{\text{NCX}}\) current was significantly increased in cardiomyocytes expressing the HCM mutants. (Fig. 4-A and B and Table S4). Simultaneous measurement of L-Type Ca\(^{2+}\) current showed no difference between wild type and HCM mutant cardiomyocytes (Fig. S8 and Table S4).

Using a standard method (24) that utilises the fura2-based measurement of caffeine [Ca\(^{2+}\)], transients, SR load, fractional SR Ca\(^{2+}\) release and SERCA activity were calculated and found to be decreased, increased and unchanged respectively (Fig. 5A insets and Table S5A). Furthermore, we found that these alterations were independent of pause duration prior to caffeine spritz (Table S6).
However, we also calculated caffeine and Ca$^{2+}$-transients expressed as $[\text{Ca}^{2+}]_{\text{total}}$ derived from the NCX current integral and thus incorporating any alterations to Ca$^{2+}$ buffering, and used this approach to recalculate SR load, fractional SR Ca$^{2+}$ release and SERCA activity (Fig. 5A and Table S5B). HCM mutant cells consistently displayed a higher SR load ($\Delta[\text{Ca}^{2+}]_{\text{total}} = 34.1\pm3.2$ to $51.3\pm8.2$ µM) (Fig. 5B). The preceding $[\text{Ca}^{2+}]_{i}$ transient amplitude (Table S5A) was also converted to $[\text{Ca}^{2+}]_{\text{total}}$ (Table S5B) to calculate the fractional SR Ca$^{2+}$ release and SERCA2 activity. HCM cells had unchanged SR fractional release compared with wild type (Fig. 5C) and had increased SERCA2 activity ($\Delta$rate = 1.53±0.30 to 3.87±0.42 sec$^{-1}$) (Fig. 5D).

The increases in SR load and SERCA2 activity appear to be driven by increased CaMKII-dependent phosphorylation of phospholamban (PLN). Relative Phospho-Threonine 17 PLN levels were increased in HCM cells (between 87±27% and 2814 (20±11% to 87±27% greater than wild type) (Fig. 6C), the relative phosphorylation at the RyR CaMKII site Serine-2030, 2808 and 2814 (Fig. 6D), and found that consistent with changes in PLN phosphorylation, there was higher phosphorylation at the RyR CaMKII site Serine-2814 (20±11% to 87±27% greater than wild type) (Fig. 6E); phosphorylation at the PKA/PKG sites (Ser-2030 and 2808) did not differ between wild type and HCM mutant cardiomyocytes (Fig. S9).

Altered Ca$^{2+}$ homeostasis in HCM mutant cardiomyocytes activates NFAT and ERK signaling

Ca$^{2+}$-dependent signalling was assessed by the phosphorylation status and immunolocalisation of the nuclear factor of activated T-cells (NFAT) and extracellular signal-regulated kinase (ERK), two key mediators of HCM pathogenesis(26). Fig. 7 A and B show that both NFAT and ERK phosphorylation (NFAT at Serine 165 and ERK at Threonine 202/Tyrosine 204) are altered in paced cardiomyocytes containing HCM mutations. NFAT-c3 phosphorylation is unchanged in unpaced cells but is substantially dephosphorylated in paced (0.5 Hz for 8 hours) HCM mutant cardiomyocytes (phosphorylation reduced by 68±15% to 85±12% compared with wild type) (Fig. 7C). ERK phosphorylation is increased in wild type cells upon pacing but significantly more in the presence of HCM mutations (30±8% to 60±22% greater than wild type) (Fig. 7D). Concordant nuclear translocation of NFAT-c3 and ERK is shown in Fig. 7E and F. In the absence of pacing, the distribution of NFAT-c3 was equal between the nucleus and cytosol in both mutant and WT cells (Table S8). However, the presence of a Ca$^{2+}$-sensitising HCM mutation in cells that had been paced for 8 hours prior to fixation caused a 95.6±11.6% to 133.6±20.2% increase in the nuclear localisation of NFAT-c3 (Fig. 7G). Pacing of wild type cardiomyocytes resulted in a 13.1±11.6% to 31.4±4.2% increase in nuclear ERK, however HCM cardiomyocytes had significantly higher nuclear translocation (52.1±4.5% to 76.8±7.4%) (Fig. 6H and Table S8).

Discussion

This study set out to investigate the effects of Ca$^{2+}$-sensitising HCM mutations in thin filament regulatory proteins on myofilament Ca$^{2+}$ buffering and the resultant consequences to intracellular Ca$^{2+}$ handling and hypertrophic signalling. We examined mutants in three different regulatory proteins in adult guinea pig cardiomyocytes transfected with recombinant adenovirus, which resulted in ~50% incorporation of the mutant protein. Mutations in troponin and tropomyosin only comprise ~10% of all mutations found in HCM patients(27); however, they affect contractile regulation in a similar way to the more common mutations in βMyHC and MyBPC(3). The selected mutations cTnI R145G, cTnT R92Q, cTnI R145G, and α-TM D175N are also among the most prevalent in each gene and have similar HCM phenotypes(28,29). Characterisation of the infected cardiomyocytes showed that the presence of a Ca$^{2+}$-sensitising thin filament mutant doubled the Ca$^{2+}$ buffering of the myofilament and had a profound effect on Ca$^{2+}$ handling. These changes were consistent among the disease genes with the cTnI
R145G mutation having the largest effect in many of the assays. These functional alterations are likely to be maintained or even accentuated by the increased CaMKII-dependent phosphorylation of Ca\textsuperscript{2+} handling proteins (PLN and RyR). We also show the direct link between altered intracellular Ca\textsuperscript{2+} handling and activation of key regulators of cardiac hypertrophy. Both NFAT and ERK are translocated to the nucleus as a result of Ca\textsuperscript{2+} dysregulation in cardiomyocytes containing HCM mutations. These data emphasise the intimate link between myofilament Ca\textsuperscript{2+} buffering, Ca\textsuperscript{2+} handling and the initiation of Ca\textsuperscript{2+}-mediated hypertrophic signalling.

Our study builds on and extends earlier work, much of it using cardiomyocytes isolated from mouse models of HCM. Our experimental design provides the strongest evidence to date that altered Ca\textsuperscript{2+} buffering can be directly attributed to the primary effect of a mutation on myofilament Ca\textsuperscript{2+} affinity, due to the short-term transfection model used. Previous detailed electrophysiological characterisation of HCM cardiomyocytes used transgenic mice, in which the disease causing mutation is expressed from birth or earlier, and thus the observed functional changes reflect a mixture of primary effects due to the mutation and secondary compensatory alterations due to molecular and (in longer term studies) physiological remodelling. Furthermore, we opted to use adult guinea pig cardiomyocytes in which the myosin isofrom and Ca\textsuperscript{2+} handling more closely resemble that found in human. Murine cardiomyocytes contain principally \(\alpha\)-MyHC that has faster enzyme kinetics than the \(\beta\) isoform which predominates in both guinea pig and human cardiomyocytes(19), and has been shown to be an important determinant when assessing myocyte or cardiac function(30). Also, in the mouse Ca\textsuperscript{2+} reuptake during diastole is almost entirely dependent on SERCA2, whereas in humans and guinea pig the sarcolemmal NCX makes a substantial contribution(20). This, and the shorter action potential lacking an appreciable plateau(21), may make the guinea pig a more accurate model for assessing Ca\textsuperscript{2+} handling and the initiation of Ca\textsuperscript{2+}-mediated hypertrophic signalling.

Some recent work analysing Ca\textsuperscript{2+} handling in transgenic mice containing Ca\textsuperscript{2+} sensitising mutations has shown profound increases in basal [Ca\textsuperscript{2+}i], in the presence of increased Ca\textsuperscript{2+} buffering (13,14), whilst others have found the opposite effect depending on the age of the mice (15,16). One of the former experiments also shows a pause dependent increase in SR load when HCM variants are present (13); however using guinea pig cardiomyocytes, which have slower Ca\textsuperscript{2+} cycling, alterations to SR Ca\textsuperscript{2+} are seen independently of pausing prior to caffeine application. In the latter cases there are profound alterations in the levels of Ca\textsuperscript{2+} handling proteins such as SERCA and PLN, and age-dependent alterations to CAMKII phosphorylation levels. These findings are in agreement with our shorter-term study where Ca\textsuperscript{2+} buffering appears to directly affect SERCA2 activity via CaMKII activation and subsequent phosphorylation of PLN threonine 17. Adenoviral-mediated transfection of HCM mutations has previously been carried out using rat primary cardiomyocytes. The restrictive cardiomyopathy R193H TnI mutant increased myofilament Ca\textsuperscript{2+} sensitivity and decreased sarcomere length, as well as increasing both relaxation time and Ca\textsuperscript{2+} transient decay(31). Of note, cardiomyocytes isolated from a transgenic mouse model of the same mutant showed prolonged relaxation with no change in Ca\textsuperscript{2+} transient decay, suggesting secondary changes at the level of Ca\textsuperscript{2+} handling(32).

The observed increase in diastolic Ca\textsuperscript{2+} is likely to be caused by the slower release of Ca\textsuperscript{2+} from the myofilament during Ca\textsuperscript{2+} reuptake by SERCA, and maintained by increased RyR leak. Isolated cardiomyocytes from myectomy samples taken from HCM patients with different genetic mutations, showed increases in diastolic Ca\textsuperscript{2+}(33) analogous to those in our study. This may indicate that the primary defects in Ca\textsuperscript{2+} handling may again begin to predominate as disease pathogenesis progresses to end stage and the chronically remodelled myocardium begins to fail. More recent work from human samples shows that CaMKII signalling and functional effects on SERCA/PLN are preserved in end stage HCM; interestingly the study also shows compensatory changes of absolute SERCA levels(34). In contrast with our findings, RyR phosphorylation was unchanged in cardiomyocytes from ten patients. This suggests that some aspects of Ca\textsuperscript{2+} buffering in HCM are preserved throughout the natural history of the disease in non-murine HCM whilst others are compensated for to preserve long-term myocyte function during disease progression. It has also been suggested that CaMKII plays a nodal role in intracellular signalling(35); our study confirms that CaMKII mediates phosphorylation of both PLN and RyR and its activity is increased in HCM cardiomyocytes. Longer-term studies suggest secondary changes, potentially under the control of mechanochemotransduction pathways such as nitric oxide, which may shut off some of these nodes (35,36), as the myocardium attempts to achieve a homeostatic equilibrium of the Ca\textsuperscript{2+} pool to preserve contractile function.

Work on both human tissue and transgenic animals shows conflicting results when assessing SR Ca\textsuperscript{2+}, NCX and SERCA2 activity(13-15). This is the
first study to directly account for the myofilament buffering when calculating these parameters. Increased myofilament Ca$^{2+}$ occupancy will mask recordings that rely on cytoplasmic fura2 fluorescence, leading to under representations of the [Ca$^{2+}$] used to estimate NCX and SERCA2 activity. If the necessary adjustments for buffering are made (as in this study) a paradigm of Ca$^{2+}$-dependent myocardial dysregulation and signalling could be disease modifying in HCM and improve outcomes. Direct targeting of myofilament Ca$^{2+}$ sensitivity provides the most attractive potential therapeutic approach. Small molecules such as the green tea polyphenol epigallocatechin-3-gallate (EGCg) have been shown to bind to cTnC(43) and desensitise the myofilament(44). Although it should be noted, that EGCg currently lacks specificity and potency to be useful in itself(45). Such approaches using derivative compounds may provide a tractable method for drug treatment to prevent or even regress HCM disease pathology by targeting its primary cause.

**Experimental procedures**

Adenoviral design and production

Adenoviruses were engineered to contain either WT or HCM mutant FLAG-tagged thin filament proteins (cTnT R92Q, cTnI R145G, and α-TM D175N) using the AdEasy XL viral production system (Agilent Technologies). Viral particles were purified by CsCl gradient centrifugation, desalted by dialysis and the plaque forming units (PFU) per ml was determined using the manufacturer’s standard protocols.

Isolation of guinea pig left ventricular cardiomyocytes

This investigation was approved by the Animal Welfare and Ethical Review Board at the University of Oxford and conforms to the UK Animals (Scientific Procedures) Act, 1986, incorporating Directive 2010/63/EU of the European Parliament. Left ventricular cardiomyocytes were isolated from guinea pig heart, by standard collagenase perfusion and mechanical agitation(46). Cardiomyocytes were incubated in ACCITT$^\text{T}$ culture medium(47) at 37 °C and 5% CO$_2$ in the presence of ~1000 MOI of adenovirus for 48 hours. All subsequent functional experiments were carefully controlled for culture time, whilst viral MOI ratios were assessed throughout the duration of the study to ensure that the validity of the model was maintained. All experiments detailed herein, compare HCM mutant infected cardiomyocytes with similarly infected cardiomyocytes expressing human flag-tagged recombinant wild type protein. Uninfected control cardiomyocytes broadly resembled the WT infected controls; any exception to this is detailed in Tables S2-7.

**Determination of optimum fura2-AM-ester loading concentration**

Optimum fura2-AM-ester loading in isolated cardiomyocytes was determined by signal to noise analysis using Ionwizard software (IonOptix). 100,000-150,000 viable guinea pig left ventricular cardiomyocytes were plated in microplate wells in the presence of ~1000 MOI of adenovirus for 48 hours. All subsequent functional experiments were performed in the presence of ~1000 MOI of adenovirus for 48 hours. All subsequent functional experiments were carefully controlled for culture time, whilst viral MOI ratios were assessed throughout the duration of the study to ensure that the validity of the model was maintained. All experiments detailed herein, compare HCM mutant infected cardiomyocytes with similarly infected cardiomyocytes expressing human flag-tagged recombinant wild type protein. Uninfected control cardiomyocytes broadly resembled the WT infected controls; any exception to this is detailed in Tables S2-7.
cardiomyocytes were incubated with 5, 1, 0.5 or 0.1 μM fura2-AM ester (Life Technologies) in buffer containing 150 mM NaCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 7 mM glucose, 1 mM MgCl₂, 1 mM KCl, 0.3 mM NaH₂PO₄ and 250 μM CaCl₂. pH 7.4 with NaOH. F365/380 was determined using IonOptix μstep under electrical pacing at 40 V and 0.5 Hz to establish basal noise and peak stimulated fluorescence conditions. It was determined that 1 μM was the minimum concentration / time required to give a dynamic Ca²⁺ signal (with lower concentrations giving signal to noise ratios of 1 or lower) and was therefore used in all subsequent experiments (Fig. S5).

**Measurement of Ca²⁺ Buffering, NCX current and SR Content and SERCA activity**

Cardiomyocytes were loaded with fura2, attached to a whole cell voltage clamp pipette and spritzed with 10 mM caffeine. Intracellular Ca²⁺ buffering was calculated from the simultaneous measurement of total and free Ca²⁺ ([Ca²⁺]ₜₐ₉₉ and [Ca²⁺]ᵣₑ₉ₑ) respectively) using a previously reported technique(22). Specifically, caffeine caused the release of Ca²⁺ from SR and induced a brief Ca²⁺ transient measured using fura2 (“caffeine transient”) as well as Ca²⁺ efflux via NCX (NCX current), allowing [Ca²⁺]ᵣₑ₉ₑ and [Ca²⁺]ₜₐ₉₉ to be calculated. To exclude the other flux mechanism’s contribution to the extrusion of Ca, the measured NCX currents were integrated and corrected as previously described(48). After this correction, we now assume that extrusion of Ca²⁺ released from SR by caffeine is only via NCX. Based on this foundation, we then determined (at two time points) the change of integral of NCX current. This gave the Δ[Ca²⁺]ₜₐ₉₉ whilst the change of intracellular Ca²⁺ transient measured by fura2 gave Δ[Ca²⁺]ᵣₑ₉ₑ during caffeine application. Thus, the relationship between Δ[Ca²⁺]ᵣₑ₉ₑ and Δ[Ca²⁺]ₜₐ₉₉ represents the Ca²⁺ buffering regardless of differential bound myofilament Ca²⁺ in control and mutant cells at baseline. Plots of [Ca²⁺]ᵣₑ₉ₑ versus [Ca²⁺]ₜₐ₉₉ were fitted to a Michaelis-Menten equation to give estimates of buffering Kₐ₄ and Bₙ₆₉. NCX amplitudes were taken from currents obtained during the buffering measurement protocol. Relative changes in SR load were calculated using [Ca²⁺]ₜₐ₉₉ transients upon caffeine spritz to account for the Ca²⁺ buffering of each cell. SERCA2 activity was determined from the decay constants from the caffeine transient and the preceding Ca²⁺ transient using standard methods(49).

**Measurement of sarcomere shortening and cytoplasmic Ca²⁺ transients**

Fura2 loaded guinea pig left ventricular were then allowed to settle to the bottom of a perfusion chamber with a 0 thickness cover slip base, which was mounted on an inverted fluorescence microscope. Cells were perfused with buffer containing 150 mM NaCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 7 mM glucose, 1 mM MgCl₂, 1 mM KCl, 0.3 mM NaH₂PO₄ and 1.8 mM CaCl₂. pH 7.4 and electrically paced at 40 volts. Pacing frequency was set at 1 Hz for cells not loaded with Ca²⁺ indicator or 0.5 Hz for fura2.2 loaded cells in order to accurately measure resting diastolic [Ca²⁺]. The effects of HCM mutations on contractility were found to be qualitatively unaltered compared to their effect in cells not loaded with fura2 at 1 Hz (Fig S7 and Table S3). Sarcomere shortening was captured by fourier transform of the cardiomyocyte striations under phase contrast microscopy using a switching rate of 100 Hz. Ca²⁺ transients were captured simultaneously, using the ratio of fura2 fluorescence emission at 365/380 nm at a switching rate of 1000 Hz. All contracting cardiomyocytes were measured for contractility and fura2 Ca²⁺, any cells displaying asynchronous contractility, excessive blebbing/dysmorphology were ignored for acquisition.

**Measurement of RyR leak**

RyR leak was calculated using the RyR channel blocker tetracaine as previously reported(25). Briefly, contracting cardiomyocytes were identified under field stimulation at 0.5 Hz in the presence of 1.8 mM CaCl₂. RyR leak was measured for 50 seconds in the absence of field stimulation in a Na⁺ and Ca²⁺ free solution. Field stimulation and perfusion of 1.8 mM CaCl₂ was restarted to allow ensure basal functional conditions were as at the start of the experiment., Perfusion was again switched to Na⁺ and Ca²⁺ free solution containing 1 μM of tetracaine and field stimulation was stopped to make a baseline reading that was subtracted from the preceding RyR leak measurement. Leak/Load relationships were measured by 10 mM caffeine spritz following each perfusion switch.

**Western blotting and Immunolocalisation**

Western blots were performed to detect cTnI, cTnI α-TM and FLAG tag, PLN, phospho-serine 16 PLN, phospho-threonine 17 PLN, RyR, phospho-serine 2030 RyR, phospho-serine 2808 RyR, phospho-serine 2814 RyR, cTnI, phospho-serine 22/24, NCX, SERCA2A, NFAT, phospho-serine-165 NFAT, ERK or phospho-threonine 202/tyrosine 204-ERK in wild type and mutant infected cardiomyocytes. In immunolocalisation experiments, recombinant FLAG-tagged protein was detected versus α-actinin. NFAT and ERK .
nuclear localisation was also measured versus nucleoplasmic marker DAPI.

Statistics
The data are expressed as the average of n experiments±SEM throughout. Statistically significance was determined using unpaired Student Newman-Keuls analysis for non-normally distributed data (InStat, GraphPad Software), with significance values defined as $P<0.05$. 

HCM mutations increase Ca$^{2+}$ buffering and signalling
Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

References

1. Maron, B. J., Gardin, J. M., Flack, J. M., Gidding, S. S., and Bild, D. E. (1996) HCM in the general population. *Circulation* **94**, 588-589
2. Maron, B. J. (2003) Sudden death in young athletes. *N Engl J Med* **349**, 1064-1075
3. Watkins, H., Ashrafian, H., and Redwood, C. (2011) Inherited cardiomyopathies. *N Engl J Med* **364**, 1643-1656
4. Bing, W., Redwood, C. S., Purcell, I. F., Esposito, G., Watkins, H., and Marston, S. B. (1997) Effects of two hypertrophic cardiomyopathy mutations in alpha-tropomyosin, Asp175Asn and Glu180Gly, on Ca2+ regulation of thin filament motility. *Biochem Biophys Res Commun* **236**, 760-764
5. Robinson, P., Mirza, M., Knott, A., Abdulrazzak, H., Willott, R., Marston, S., Watkins, H., and Redwood, C. (2002) Alterations in thin filament regulation induced by a human cardiac troponin T mutant that causes dilated cardiomyopathy are distinct from those induced by troponin T mutants that cause hypertrophic cardiomyopathy. *J Biol Chem* **277**, 40710-40716
6. Burton, D., Abdulrazzak, H., Knott, A., Elliott, K., Redwood, C., Watkins, H., Marston, S., and Ashley, C. (2002) Two mutations in troponin I that cause hypertrophic cardiomyopathy have contrasting effects on cardiac muscle contractility. *Biochem J* **362**, 443-451
7. Elliott, K., Watkins, H., and Redwood, C. S. (2000) Altered regulatory properties of human cardiac troponin I mutants that cause hypertrophic cardiomyopathy. *J Biol Chem* **275**, 22069-22074
8. Lakdawala, N. K., Dellefave, L., Redwood, C. S., Sparks, E., Cirino, A. L., Depalma, S., Colan, S. D., Funke, B., Zimmerman, R. S., Robinson, P., Watkins, H., Seidman, C. E., Seidman, J. G., McNally, E. M., and Ho, C. Y. (2010) Familial dilated cardiomyopathy caused by an alpha-tropomyosin mutation: the distinctive natural history of sarcomeric dilated cardiomyopathy. *J Am Coll Cardiol* **55**, 320-329
9. Robinson, P., Griffiths, P. J., Watkins, H., and Redwood, C. S. (2007) Dilated and hypertrophic cardiomyopathy mutations in troponin and alpha-tropomyosin have opposing effects on the calcium affinity of cardiac thin filaments. *Circ Res* **101**, 1266-1273
10. Lowey, S. (2002) Functional consequences of mutations in the myosin heavy chain at sites implicated in familial hypertrophic cardiomyopathy. *Trends Cardiovasc Med* **12**, 348-354
11. Witt, C. C., Gerull, B., Davies, M. J., Centner, T., Linke, W. A., and Thierfelder, L. (2001) Hypercontractile properties of cardiac muscle fibers in a knock-in mouse model of cardiac myosin-binding protein-C. *J Biol Chem* **276**, 5353-5359
12. Smith, G. A., Dixon, H. B., Kirschenlohr, H. L., Grace, A. A., Metcalfe, J. C., and Vandenberg, J. I. (2000) Ca2+ buffering in the heart: Ca2+ binding to and activation of cardiac myofilaments. *Biochem J* **346 Pt 2**, 393-402
13. Schober, T., Huke, S., Venkataraman, R., Gryshchenko, O., Kryshtal, D., Hwang, H. S., Baudenbacher, F. J., and Knollmann, B. C. (2012) Myofilament Ca sensitization increases cytosolic Ca binding affinity, alters intracellular Ca homeostasis, and causes pause-dependent Ca-triggered arrhythmia. *Circ Res* **111**, 170-179
14. Coppini, R., Mazzoni, L., Ferrantini, C., Gentile, F., Pioner, J. M., Laurino, A., Santini, L., Bargelli, V., Rotellini, M., Bartolucci, G., Crocini, C., Sacconi, L., Tesi, C., Belardinelli, L., Tardiff, J., Mugelli, A., Olivotto, I., Cerbai, E., and Poggesi, C. (2017) Ranolazine Prevents Phenotype Development in a Mouse Model of Hypertrophic Cardiomyopathy. *Circ Heart Fail* **10**
15. Haim, T. E., Dowell, C., Diamanti, T., Scheuer, J., and Tardiff, J. C. (2007) Independent FHC-related cardiac troponin T mutations exhibit specific alterations in myocyte contractility and calcium kinetics. *J Mol Cell Cardiol* **42**, 1098-1110
HCM mutations increase Ca\(^{2+}\) buffering and signalling

16. Martins, A. S., Parvatiyar, M. S., Feng, H. Z., Bos, J. M., Gonzalez-Martinez, D., Vukmirovic, M., Turna, R. S., Sanchez-Gonzalez, M. A., Badger, C. D., Zorio, D. A., Singh, R. K., Wang, Y., Jin, J. P., Ackerman, M. J., and Pinto, J. R. (2015) In Vivo Analysis of Troponin C Knock-In (A8V) Mice: Evidence that TNNC1 Is a Hypertrophic Cardiomyopathy Susceptibility Gene. *Circ Cardiovasc Genet* 8, 653-664

17. Periasamy, M., and Huke, S. (2001) SERCA pump level is a critical determinant of Ca(2+) homeostasis and cardiac contractility. *J Mol Cell Cardiol* 33, 1053-1063

18. Hamdani, N., Bishu, K. G., von Frieling-Salewsky, M., Redfield, M. M., and Linke, W. A. (2013) Deranged myofilament phosphorylation and function in experimental heart failure with preserved ejection fraction. *Cardiovasc Res* 97, 464-471

19. Deacon, J. C., Bloemink, M. J., Rezavandi, H., Geeves, M. A., and Leinwand, L. A. (2012) Identification of functional differences between recombinant human alpha and beta cardiac myosin motors. *Cell Mol Life Sci* 69, 2261-2277

20. Bers, D. M. (2002) Cardiac excitation-contraction coupling. *Nature* 415, 198-205

21. Luo, C. H., and Rudy, Y. (1994) A dynamic model of the cardiac ventricular action potential. I. Simulations of ionic currents and concentration changes. *Circ Res* 74, 1071-1096

22. Trafford, A. W., Diaz, M. E., and Eisner, D. A. (1999) A novel, rapid and reversible method to measure Ca buffering and time-course of total sarcoplasmic reticulum Ca content in cardiac ventricular myocytes. *Pflugers Arch* 437, 501-503

23. Williams, D. A., Fogarty, K. E., Tsien, R. Y., and Fay, F. S. (1985) Calcium gradients in single smooth muscle cells revealed by the digital imaging microscope using Fura-2. *Nature* 318, 558-561

24. O'Neill, S. C., and Eisner, D. A. (1990) A mechanism for the effects of caffeine on Ca\(^{2+}\) release during diastole and systole in isolated rat ventricular myocytes. *J Physiol* 430, 519-536

25. Shannon, T. R., Ginsburg, K. S., and Bers, D. M. (2002) Quantitative assessment of the SR Ca\(^{2+}\) leak-load relationship. *Circ Res* 91, 594-600

26. Molkentin, J. D. (2004) Calcineurin-NFAT signaling regulates the cardiac hypertrophic response in coordination with the MAPKs. *Cardiovasc Res* 63, 467-475

27. Walsh, R., Thomson, K. L., Ware, J. S., Funke, B. H., Woodley, J., McGuire, K. J., Mazzarotto, F., Blair, E., Seller, A., Taylor, J. C., Minikel, E. V., Exome Aggregation, C., MacArthur, D. G., Farrall, M., Cook, S. A., and Watkins, H. (2017) Reassessment of Mendelian gene pathogenicity using 7,855 cardiomyopathy cases and 60,706 reference samples. *Genet Med* 19, 192-203

28. Thierfelder, L., Watkins, H., MacRae, C., Lamas, R., McKenna, W., Vosberg, H. P., Seidman, J. G., and Seidman, C. E. (1994) Alpha-tropomyosin and cardiac troponin T mutations cause familial hypertrophic cardiomyopathy: a disease of the sarcomere. *Cell* 77, 701-712

29. Kimura, A., Harada, H., Park, J. E., Nishi, H., Satoh, M., Takahashi, M., Hiroi, S., Sasaoka, T., Ohbuchi, N., Nakamura, T., Koyanagi, T., Hwang, T. H., Choo, J. A., Chung, K. S., Hasegawa, A., Nagai, R., Okazaki, O., Nakamura, H., Matsuzaki, M., Sakamoto, T., Toshima, H., Koga, Y., Imaizumi, T., and Sasazuki, T. (1997) Mutations in the cardiac troponin I gene associated with hypertrophic cardiomyopathy. *Nat Genet* 16, 379-382

30. Rice, R., Guinto, P., Dowell-Martino, C., He, H., Hoyer, K., Krenz, M., Robbins, J., Ingwall, J. S., and Tardiff, J. C. (2010) Cardiac myosin heavy chain isoform exchange alters the phenotype of cTnT-related cardiomyopathies in mouse hearts. *J Mol Cell Cardiol* 48, 979-988

31. Davis, J., Wen, H., Edwards, T., and Metzger, J. M. (2007) Thin filament disinhibition by restrictive cardiomyopathy mutant R193H troponin I induces Ca\(^{2+}\)-independent mechanical tone and acute myocyte remodeling. *Circ Res* 100, 1494-1502

32. Davis, J., Yasuda, S., Palpant, N. J., Martindale, J., Stevenson, T., Converso, K., and
HCM mutations increase Ca\(^{2+}\) buffering and signalling

Metzger, J. M. (2012) Diastolic dysfunction and thin filament dysregulation resulting from excitation-contraction uncoupling in a mouse model of restrictive cardiomyopathy. *J Mol Cell Cardiol* **53**, 446-457

33. Coppini, R., Ferrantini, C., Yao, L., Fan, P., Del Lungo, M., Stillitano, F., Sartiani, L., Tosi, B., Suffredini, S., Tesi, C., Yacoub, M., Olivotto, I., Belardinelli, L., Poggesi, C., Cerbai, E., and Mugelli, A. (2013) Late sodium current inhibition reverses electromechanical dysfunction in human hypertrophic cardiomyopathy. *Circulation* **127**, 575-584

34. Helms, A. S., Alvarado, F. J., Yob, J., Tang, V. T., Pagani, F., Russell, M. W., Valdivia, H. H., and Day, S. M. (2016) Genotype-Dependent and -Independent Calcium Signaling Dysregulation in Human Hypertrophic Cardiomyopathy. *Circulation* **134**, 1738-1748

35. Tardiff, J. C. (2016) The Role of Calcium/Calsensulin-Dependent Protein Kinase II Activation in Hypertrophic Cardiomyopathy. *Circulation* **134**, 1749-1751

36. Jian, Z., Han, H., Zhang, T., Puglisi, J., Izu, L. T., Shaw, J. A., Onofiok, E., Erickson, J. R., Chen, Y. J., Horvath, B., Shimkunas, R., Xiao, W., Li, Y., Pan, T., Chan, J., Banyasz, T., Tardiff, J. C., Chiamvimonvat, N., Bers, D. M., Lam, K. S., and Chen-Izu, Y. (2014) Mechnanochemotransduction during cardiomyocyte contraction is mediated by localized nitric oxide signaling. *Sci Signal* **7**, ra27

37. Helassa, N., Podor, B., Fine, A., and Torok, K. (2016) Design and mechanistic insight into ultrafast calcium indicators for monitoring intracellular calcium dynamics. *Sci Rep* **6**, 38276

38. Zhao, Y., Araki, S., Wu, J., Teramoto, T., Chang, Y. F., Nakano, M., Abdelfattah, A. S., Fujiwara, M., Ishihara, T., Nagai, T., and Campbell, R. E. (2011) An expanded palette of genetically encoded Ca(2)(+) indicators. *Science* **333**, 1888-1891

39. Davis, J., Davis, L. C., Correll, R. N., Makarewich, C. A., Schwaneckamp, J. A., Moussavi-Harami, F., Wang, D., York, A. J., Wu, H., Houser, S. R., Seidman, C. E., Seidman, J. G., Regnier, M., Metzger, J. M., Wu, J. C., and Molkentin, J. D. (2016) A Tension-Based Model Distinguishes Hypertrophic versus Dilated Cardiomyopathy. *Cell* **165**, 1147-1159

40. MacDonnell, S. M., Weisser-Thomas, J., Kubo, H., Hanscombe, M., Liu, Q., Jaleel, N., Berretta, R., Chen, X., Brown, J. H., Sabri, A. K., Molkentin, J. D., and Houser, S. R. (2009) CaMKII negatively regulates calcineurin-NFAT signaling in cardiac myocytes. *Circ Res* **105**, 316-325

41. Ashrafian, H., Redwood, C., Blair, E., and Watkins, H. (2003) Hypertrophic cardiomyopathy:a paradigm for myocardial energy depletion. *Trends Genet* **19**, 263-268

42. O'Hanlon, R., Grasso, A., Roughton, M., Moon, J. C., Clark, S., Wage, R., Webb, J., Kulkarni, M., Dawson, D., Sulaikeekh, L., Chandrasekaran, B., Bucciarelli-Ducci, C., Pasquale, F., Cowie, M. R., McKenna, W. J., Sheppard, M. N., Elliott, P. M., Pennell, D. J., and Prasad, S. K. (2010) Prognostic significance of myocardial fibrosis in hypertrophic cardiomyopathy. *J Am Coll Cardiol* **56**, 867-874

43. Robertson, I. M., Li, M. X., and Sykes, B. D. (2009) Solution structure of human cardiac troponin C in complex with the green tea polyphenol, (-)-epigallocatechin-3-gallate. *J Biol Chem* **284**, 23012-23023

44. Tadano, N., Du, C. K., Yumoto, F., Morimoto, S., Ohta, M., Xie, M. F., Nagata, K., Zhan, D. Y., Lu, Q. W., Miwa, Y., Takahashi-Yanaga, F., Tanokura, M., Ohtsuki, I., and Sasaguri, T. (2010) Biological actions of green tea catechins on cardiac troponin C. *British journal of pharmacology* **161**, 1034-1043

45. Feng, W., Hwang, H. S., Kryshdtal, D. O., Yang, T., Padilla, I. T., Tiwary, A. K., Puschner, B., Pessah, I. N., and Knollmann, B. C. (2012) Coordinated regulation of murine cardiomyocyte contractility by nanomolar (-)-epigallocatechin-3-gallate, the major green tea catechin. *Mol Pharmacol* **82**, 993-1000

46. Stemmer, P., Akera, T., Brody, T. M., Rardon, D. P., and Watanabe, A. M. (1989) Isolation and enrichment of Ca(2+)-tolerant myocytes for biochemical experiments from guinea-pig heart. *Life sciences* **44**, 1231-1237
HCM mutations increase \( Ca^{2+} \) buffering and signalling

47. Ellingsen, O., Davidoff, A. J., Prasad, S. K., Berger, H. J., Springhorn, J. P., Marsh, J. D., Kelly, R. A., and Smith, T. W. (1993) Adult rat ventricular myocytes cultured in defined medium: phenotype and electromechanical function. *Am J Physiol* 265, H747-754

48. Sears, C. E., Bryant, S. M., Ashley, E. A., Lygate, C. A., Rakovic, S., Wallis, H. L., Neubauer, S., Terrar, D. A., and Casadei, B. (2003) Cardiac neuronal nitric oxide synthase isoform regulates myocardial contraction and calcium handling. *Circ Res* 92, e52-59

49. Bode, E. F., Briston, S. J., Overend, C. L., O’Neill, S. C., Trafford, A. W., and Eisner, D. A. (2011) Changes of SERCA activity have only modest effects on sarcoplasmic reticulum \( Ca^{2+} \) content in rat ventricular myocytes. *J Physiol* 589, 4723-4729

**FOOTNOTES**

The work is supported by the British Heart Foundation (Programme grant RG/12/16/29939) and the British Heart Foundation Centre of Research Excellence (Oxford).

The following non-standard abbreviations and acronyms are used: \( \alpha \)-TM, Alpha tropomyosin; \([Ca^{2+}]_i \), free intracellular calcium; \([Ca^{2+}]_{\text{total}} \), total intracellular calcium; CaMKII, \( Ca^{2+} / \text{calmodulin} \) dependent protein kinase; cTnI, cardiac troponin I; cTnT, cardiac troponin T; ERK, Extracellular signal-regulated kinase; HCM, Hypertrophic cardiomyopathy; hrGFP, Human recombinant green fluorescent protein; MAPK, Mitogen-activated protein kinases; MOI, Multiplicity of infection; MyBPC, Myosin binding protein C; MyHC, Myosin heavy chain; NCX, Sodium / calcium exchanger; NFAT, Nuclear factor of activated T-cells; PLN, Phospholamban; RyR, Ryanodine receptor; SERCA2, Sarco-endoplasmic reticulum calcium ATPase 2; SR, Sarcoplasmic reticulum.
Figure 1. Recombinant adenoviral infection, protein expression and localisation in guinea pig left ventricular cardiomyocytes. A, shows the representative GFP expression in cardiomyocytes infected with recombinant adenovirus at ~1000 MOI 48 hours after transfection. B shows the relative protein expression of FLAG tagged human recombinant WT/R92Q cTnT, WT/R145G cTnl and WT/D175N α-TM compared to endogenous guinea pig subunits probed using anti-cTnT, anti-cTnI and anti-α-TM primary antibodies respectively. The third lane of each gel in B shows that endogenous protein in uninfected control cardiomyocytes is higher than endogenous levels from infected cardiomyocytes, therefore suggesting that recombinant protein replaces the endogenous at the myofilament. The bar graph on C shows the relative infection ratio of each virus as a percentage of green cells, whilst D shows the relative expression levels of endogenous (black bars) vs recombinant (coloured bars) protein, calculated from densitometry measurements of doublet bands in B multiplied by the relative expression levels of each virus calculated in C. E shows western blots for cell lysates expressing each different recombinant protein using anti-FLAG tag primary antibody. F shows a magnified image of adenovirally expressed FLAG tagged protein localised to the I band in cardiomyocytes. Co-localisation used anti-FLAG tag primary antibody (conjugated to Alexa568, red), with counterstain provided using an α-actinin antibody (conjugated to Alexa 633, false coloured green) to stain the z disks. Co-localisation was confirmed in the adjacent intensity profile plots. I band staining usually presents as a doublet, however, due to the contracted nature of isolated cells the signal from the I-band appeared as a single band with the limited resolution provided by the confocal microscope. (A full breakdown of the localisation of each recombinant protein used can be found in Fig S2).
Figure 2. HCM mutations acutely decrease the apparent $K_d$ of cytosolic Ca$^{2+}$ buffering.

Cardiomyocytes expressing WT or mutant cTnT / cTnI / α-TM were simultaneously assessed to measure [Ca$^{2+}$], by fura2 fluorescence and NCX current (to calculate [Ca$^{2+}$]$_{total}$) by voltage clamping upon the rapid application of 10 mM caffeine. A, plots of $\Delta$[Ca$^{2+}$]$_i$ vs $\Delta$[Ca$^{2+}$]$_{total}$ for cardiomyocytes containing WT cTnT (n=20) vs R92Q cTnT (n=23), WT cTnI (n=8) vs R145G cTnI (n=11), WT α-TM (n=14) vs D175N α-TM (n=12) reveal increased buffering capacity in cells containing HCM mutants red lines show best fit to the following: $\text{[Ca}^{2+}]_{total} = \frac{B_{\text{max}} \times \text{[Ca}^{2+}]_i}{(K_d + \text{[Ca}^{2+}]_i)} + B_{\text{min}}$. B, bar graph comparing mean $K_d$ s for the fits calculated in A. C, bar graph to show the relative estimated changes in buffering capacity (calculated from relative $K_d$ and $B_{\text{max}}$ values for individual cells undergoing the buffing protocol), where mutant calls have been compared to WT infected cells set to 1. D, cytosolic buffering curves over a wide range of Ca$^{2+}$ concentration drawn using the $K_d$ and $B_{\text{max}}$ values calculated in A. Comparison of wild type with HCM mutant: $p<0.01=^{**}$ and $p<0.05=^{*}$. 

Total [Ca$^{2+}$] = $B_{\text{max}} \times \text{[Ca}^{2+}]_i/(K_d + \text{[Ca}^{2+}]_i) + B_{\text{min}}$
Figure 3. Unloaded sarcomere shortening and Ca\(^{2+}\) transient measurements in adenovirally infected guinea pig left ventricular cardiomyocytes.

A, intracellular Ca\(^{2+}\) transients of cardiomyocytes loaded with 1 mM fura2 at 0.5 Hz pacing, expressing HCM causing mutations cTnT R92Q, cTnl R145G and α-TM D175N compared with cardiomyocytes infected with the equivalent WT protein. B and C, the average diastolic [Ca\(^{2+}\)]\(_i\) and Time to 50% Ca\(^{2+}\) reuptake respectively. D, the corresponding unloaded sarcomere shortening measurements at 1 Hz pacing. E and F, the average basal sarcomere length and Time to 50% relaxation respectively. The pairwise sarcomere shortening of fura2 loaded cardiomyocytes acquired at 0.5 Hz is shown in Fig S7. Each curve was averaged from multiple cells (n) taken from at least 4 separate cell preparations; total n numbers are given in the legends of each plot. Comparison of wild type with HCM mutant: p<0.001=***, p<0.01=** and p<0.05=*. 
Figure 4. HCM mutant infected cardiomyocytes have increased NCX current.

A, representative traces from voltage clamp recordings during the application of 10 mM caffeine. B, the average peak amplitude for wild type cTnT (n=24), cTnT R92Q (n=23), wild type cTnl (n=8), cTnl R145G (n=8), wild type α-TM (n=15) and α-TM D175N (n=19). Comparison of wild type with HCM mutant: p<0.001=***, p<0.01=** and p<0.05=*.
Figure 5. HCM mutant infected cardiomyocytes have increased SR load, fractional SR Ca\textsuperscript{2+} release and SERCA2 activity via CaMKII dependent PLN phosphorylation.

A, representative caffeine induced [Ca\textsuperscript{2+}]\textsubscript{total} transients generated by the direct perfusion of 10 mM caffeine after a 5 second pause of pacing comparing WT and HCM mutant transfected cardiomyocytes, where each transient amplitude has been adjusted from [Ca\textsuperscript{2+}]\textsubscript{i} (inset) to [Ca\textsuperscript{2+}]\textsubscript{total} using the buffering curves in Fig. 1A. Bar graphs show the SR load of [Ca\textsuperscript{2+}]\textsubscript{total} (B), fractional release of Ca\textsuperscript{2+} (calculated from the [Ca\textsuperscript{2+}]\textsubscript{total} caffeine transient amplitudes in B and Table S5B subtracted from the preceding [Ca\textsuperscript{2+}]\textsubscript{total} transient amplitude shown in Table S5B (C). The SERCA2 activity, calculated by the subtraction of [Ca\textsuperscript{2+}]\textsubscript{total} \(\tau\)-decay constants (total) and [Ca\textsuperscript{2+}]\textsubscript{total} caffeine transient (NCX) \(\tau\)-decay rates (Table S5B). The extracted parameters from [Ca\textsuperscript{2+}]\textsubscript{i} baseline and caffeine transients are also tabulated in Table S5A. E, representative western blots of total and phospho-Threonine 17 PLN, bar graph shows the densitometric quantification of phosphorylation increase (n=5), all preparations were paced for 8 hours at 0.5 Hz. p<0.001=***, p<0.01=**, p<0.05=* and p>0.05=ns.
SR Ca\(^{2+}\) load and Ca\(^{2+}\) leak via the RyR were assessed by the sequential perfusion of (a) 1.8mM CaCl\(_2\), (b) Na\(^+\) and Ca\(^{2+}\) free solution containing 1 mM tetracaine for 50 seconds; (c) the direct application of 10 mM caffeine, (d) 1.8mM CaCl\(_2\) for 100 seconds; (e) Na\(^+\) and Ca\(^{2+}\) free solution for 50 seconds; (f) a direct application of 10 mM caffeine. A, representative experimental traces for wild type cTnT (grey) and cTnT R92Q (purple) cardiomyocytes. All mutants tested were analogous to cTnT R92Q whilst all control and wild type infected cells tested resembled WT cTnT. B, the observed RyR dependent leak rate. C, the buffering-adjusted caffeine transient amplitude taken after perfusion with either 0Ca0Na (-) or with 0Na0Ca solution containing 1 μM tetracaine (+). D, the leak / load ratio, calculated as the leak rate divided by the caffeine transient amplitude at the end of each experiment. Each bar graph is an average of 30 cells, 15 of which were acquired as shown in A or B whilst a further 15 were acquired by reversing the sequence of 0Na0Ca solution with or without 1 μM tetracaine to prevent errors from fura2 signal degradation and cell fatigue. A full breakdown of D[Ca\(^{2+}\)]\(_i\) for each perfusion switch can be found in Fig. S10 and the extracted parameters are also tabulated in Table S7. Representative western blots of total and phospho-Serine 2814 RyR are given in E, with adjacent bar graph showing the average change in phosphorylation from densitometry measurements, all preparations were paced for 8 hours at 0.5 Hz. Unpaced preparations showed no significant changes at the same site (data not shown) p<0.001=***, p<0.01=** and p<0.05=* for comparing WT to HCM mutant and p<0.001= ###, p<0.01= ## and p<0.05= # for comparing the presence or absence of 1mM tetracaine.

**Figure 6.** HCM mutant infected cardiomyocytes have increased RyR leak / Load relationships and CAMKII activation of RyR.
Figure 7. HCM mutant infected cardiomyocytes have altered NFAT and ERK phosphorylation resulting in increased nuclear localisation.

A, Total NFAT and phospho-serine-165 NFAT were measured by western blot of transfected cardiomyocytes either paced at 0.5 Hz for 8 hours (P) or unpaced (UP). B, densitometric quantification of blots from paced cells and indicates that expression of HCM mutations results in a significant decrease in phospho-NFAT compared with WT (n=6). Similarly, C, ERK phosphorylated at Threonine-202 / Tyrosine-204 and total ERK were measured by western blot of transfected cardiomyocytes either paced (P) or unpaced (UP). D, densitometric quantification of blots from paced cells show that expression of HCM mutations results in a significant increase in phospho-ERK compared with WT (n=6). Relative changes in phosphorylation of NFAT and ERK in unpaced cardiomyocytes and the percentage change in phosphorylation caused by pacing for each group are tabulated in Table S7. E and F show representative cropped immunofluorescence images of single cardiomyocytes using anti-NFAT and anti-ERK antibodies respectively. Individual panels compare HCM mutant to wild type infected cardiomyocytes either unpaced or paced at 0.5 Hz for 8 hours. G and H, % change in NFAT or ERK nuclear localisation between paced and unpaced cardiomyocytes for each mutant and wild type infected group (n=30-40 cells in each group, p<0.001=***, p<0.01=**, p<0.05=* and p>0.05=ns). Normalised relative localisation verses background cytosolic NFAT and ERK comparing wild type versus mutant measurements are tabulated in Table S8.
Hypertrophic cardiomyopathy mutations increase myofilament Ca\textsuperscript{2+} buffering, alter intracellular Ca\textsuperscript{2+} handling and stimulate Ca\textsuperscript{2+} dependent signalling

Paul Robinson, Xing Liu, Alexander Sparrow, Suketu Patel, Yin Hua Zhang, Barbara Casadei, Hugh Watkins and Charles S. Redwood

*J. Biol. Chem.* published online May 14, 2018

Access the most updated version of this article at doi: [10.1074/jbc.RA118.002081](https://doi.org/10.1074/jbc.RA118.002081)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](https://www.jbc.org) to choose from all of JBC's e-mail alerts