

SHORT COMMUNICATION

The inhibitory subunit of cardiac troponin (cTnI) is modified by arginine methylation in the human heart

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Keywords: arginine methylation; inhibitory/switch peptide; mass spectrometry; post-translational modifications; troponin inhibitory subunit

Acknowledgement of Grant Support: DOO acknowledges Rivers state university of Science & Technology Port Harcourt Nigeria, and TETFund Nigeria (Academic staff Training and development unit). Sabrina Samuel acknowledges an Allam PhD scholarship. We acknowledge grant support from the Hull and East Riding Cardiac Trust.

No conflict of interest is declared.

Abstract:

Background

The inhibitory subunit of cardiac troponin (cTnI) is a gold standard cardiac biomarker and also an essential protein in cardiomyocyte excitation-contraction coupling. The interactions of cTnI with other proteins are fine-tuned by post-translational modification of cTnI. Mutations in cTnI can lead to hypertrophic cardiomyopathy.

Methods and Results

Here we report, for the first time, that cTnI is modified by arginine methylation in human myocardium. Using Western blot, we observed reduced levels of cTnI arginine methylation in human hypertrophic cardiomyopathy compared to dilated cardiomyopathy biopsies. Similarly, using a rat model of cardiac hypertrophy we observed reduced levels of cTnI arginine methylation compared to sham controls. Using mass spectrometry, we identified cTnI methylation sites at R74/R79 and R146/R148 in human cardiac samples. R146 and R148 lie at the boundary between the critical cTnI inhibitory and switch peptides; PRMT1 methylated an extended inhibitory peptide at R146 and R148 *in vitro*. Mutations at R145 that have been associated with hypertrophic cardiomyopathy hampered R146/R148 methylation by PRMT1 *in vitro*. H9c2 cardiac-like cells transfected with plasmids encoding for a methylation-deficient R146A/R148A cTnI protein developed cell hypertrophy, with a 32% increase in cell size after 72 h, compared to control cells.

Discussion

Our results provide evidence for a novel and significant cTnI post-translational modification. Our work opens the door to translational investigations of cTnI arginine methylation as a biomarker of disease, which can include e.g. cardiomyopathies, myocardial infarction and heart failure, and offers a novel way to investigate the effect of cTnI mutations in the inhibitory/switch peptides.

Abbreviations: Arginine methylation (ArgMe), collision induced dissociation (CID), dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), matrix-assisted laser desorption ionisation - time of flight (MALDI-TOF), protein arginine methyltransferases (PRMTs), S-adenosyl-L-methionine (SAM).

Introduction

Myosin, actin, titin and the troponins are among the major proteins composing the sarcomere, the elemental contractile unit of cardiomyocytes. The troponin complex consists of three proteins: Ca²⁺ binding (gene: *TNNC1*, protein: cTnC), tropomyosin binding (*TNNT2*, cTnT) and inhibitory (*TNNI3*, cTnI) subunits. The paramount role of troponins in cardiomyocyte contraction is highlighted by the fact that mutations in *TNNT2* and *TNNI3* can lead to hypertrophic and dilated cardiomyopathy (HCM and DCM), diseases associated with sudden cardiac death and heart failure [1].

Structurally, the cTnI subunit comprises of a protein *N* terminus (residues 1-33), IT arm (40-136), inhibitory (137-146) and switch (147-163) peptides, and *C* terminus (164-210) [2]. Inhibitory and switch peptides are hot-spots for mutations associated with HCM [3], of which R145G, R145Q and R145W are prominent examples [4]. cTnI is known to undergo post-translational modifications and there are 16 known phosphorylation sites in cTnI. Although cTnI phosphorylation is sub-stoichiometric and only 1-5% of the protein may be phosphorylated at a given site, cTnI phosphorylation is relevant to cardiac disease because the relative abundance of phosphorylation sites changes in DCM [5, 6].

Arginine methylation (ArgMe) is a stable post-translational modification consisting on the transfer of methyl groups from *S*-adenosyl-L-methionine (SAM) to Arg residues. ArgMe increases the hydrophobicity and size of the Arg residue and can affect hydrogen bonding and protein interactions. ArgMe is catalysed by Protein Arginine Methyl Transferases (PRMTs) and PRMT1 is responsible for most ArgMe in cells [7].

Within the cardiac proteome, ArgMe appears to be enriched in proteins involved in cardiomyocyte contraction [8]. Here, we report ArgMe of cTnI; this is relevant to cardiology because of the use of cTnI as a cardiac biomarker and the growing evidence indicating that cTnI post-translational modifications play a role in cardiomyopathies.

1. Methods

1.1 Cardiac samples

Cardiac septum samples from patients clinically and pathologically diagnosed with HCM or DCM were from Papworth Hospital Tissue Bank (Supplemental Table 1). HCM and DCM septum samples (n = 4) were obtained from explanted hearts of patients undergoing cardiac transplantation. Protocols were approved by Papworth Hospital NHS Foundation Trust (T02239) and University of Hull (FEC_50_2017_H_FEC) ethical committees.

Male Sprague Dawley rat cardiac samples were obtained in accordance with procedures authorised by license PPL 70/7966. Pressure-overload hypertrophy was induced by abdominal aortic constriction as described previously [9]. Control animals underwent surgery without aortic constriction. Cardiac samples were collected and snap-frozen 9 weeks after surgery.

1.2 *Detection of cTnI ArgMe using Western blot*

Sixty µg cardiac protein (1% SDS lysates) were analysed by Western blot using antibodies targeting ArgMe and α -cTnI (Abcam). Band density was measured using Image J. Significance was estimated by two-tailed Student's t-test. Purified cTnI was purchased from Abcam.

2.2. *Identification of cTnI ArgMe by mass spectrometry*

Four HCM samples were pulverised under liquid N₂ and homogenised (8 M urea). Ten mg proteins were reduced (2 mM TCEP, 37 °C, 30 min), alkylated (10 mM iodoacetamide, 37 °C, 30 min) and digested (0.5 AU LysC, 30 °C, overnight). Methylated peptides were immunoprecipitated using antibodies recognising monoArgMe (CST) [10].

Peptides were separated by liquid chromatography and analysed using a LTQ-Orbitrap Velos Pro (Thermo). Top 20 intensity ions were selected for fragmentation and analysed in the ion trap. Proteins were identified using Proteome Discoverer (v.1.4.0.288) against the Swissprot human database (version 2017_03), allowing 2 missed cleavages and 10 ppm and 0.6 Da tolerance for parent ion and MS/MS spectra, respectively. Cys carbamidomethylation was set as fixed modification. Met oxidation and ArgMe were set as variable modifications. False discovery rate was 0.01.

2.3. *ArgMe assays in vitro*

Methylation assays contained 1 µg peptide, 10-fold excess SAM, and 1 ng PRMT1 (Abcam) in Tris buffer (pH 7.4). Reactions were incubated at 30 °C for 6 h, acidified (formic acid) and mixed

with 5 mg/mL α -Cyano-4-hydroxycinnamic acid. Peptides were analysed using Bruker Autoflex and Ultraflex III mass spectrometers in reflectron mode. MS/MS analyses were performed in LIFT mode at a minimum signal-to-noise ratio of 3. Bruker flexAnalysis 3.3 and Mascot 2.5.1 were used for spectral processing, peak list generation and interpretation using peptide and MS/MS tolerances of 25 ppm and 1 Da.

2.4. H9c2 hypertrophy assays

Site directed mutagenesis was performed using the Quikchange kit. H9c2 cells were transfected (Lipofectamine) with plasmids encoding for FLAG-tagged WT and R146A/R148A cTnI. After 72h, cell area was measured by confocal microscopy as described previously [8]. At least 2 independent transfections were done and the area of 35-90 cells was measured for each condition. Statistical significance was calculated using ANOVA tests.

2. Results

2.1 *cTnI is modified by ArgMe in the heart*

Available algorithms predict extensive ArgMe of cTnI, including at R74, R79, R146 and R148 (Supplemental Table 2), [12]. Here, we tested the hypothesis that cTnI is modified by ArgMe in the heart. Peptides modified by ArgMe were immunoprecipitated from HCM cardiac lysates using α -monoArgMe antibodies [10], and identified by LC-MS/MS. We confidently identified two peptides in cTnI as methylated in four biological replicates. The peptide RPTLRRVRISADAMMQALLGARAK (residues 141-164) was identified as monomethylated at R146 and R148 (Figure 1A, Supplemental Figure 1 and Tables 3 and 4), residues which lie at the interface between the cTnI inhibitory and switch peptides.

We also identified the peptide GRALSTRCQPLELAGLGFAELQDLCRQLHARVDK (residues 73-106) as methylated at R74 and/or R79 (Figure 1B, Supplemental Figure 1 and Tables 3 and 5). R74 and R79 are included within the first half of the IT arm of cTnI that interacts with the C-terminal lobe of cTnC [13].

To examine the relevance of cTnI ArgMe in the setting of cardiac disease, we compared cTnI ArgMe in HCM *vs.* DCM using Western blot. Our results suggest a substantial decrease in the levels of cTnI ArgMe in HCM relative to DCM (Figure 1C). Control human samples were

not available to us, but commercial cTnI purified from normal human hearts was also recognised by α -ArgMe antibodies (Figure 1D). In support of the idea that cTnI ArgMe is decreased in HCM, we observed strongly reduced cTnI ArgMe in a rat model of cardiac hypertrophy (Figure 1E).

3.2 An extended cTnI inhibitory peptide is methylated by PRMT1 at R146 and R148

To further demonstrate cTnI recognition by PRMTs, we performed ArgMe assays *in vitro* using synthetic peptides [14]. We incubated an extended inhibitory peptide spanning cTnI residues 137-151, GKFKRPTLRRVRISA (R146 and R148 underlined), with PRMT1. We analysed the peptide by MS/MS and detected new species at +14 and +28 Da, strongly suggesting production of mono- and dimethylated peptides (Supplemental Figure 2). The site of monomethylation of the peptide at 1799 Da was R146 (Figure 2A and Supplemental Table 6). We assigned a methyl group to R146 and R148 in the dimethylated peptide on the basis of a weak $b_{11}^+ - H_2O$ diagnostic ion (Supplemental Figure 3) and a Mascot expectation value of 0.014 (Supplemental Table 6).

Mutations at cTnI inhibitory/switch peptide positions R141, T143, L144, S150 and R145 have been associated with HCM and some of these mutations could affect levels of R146/148 methylation. Consistent with this hypothesis, we observed reduced ArgMe of peptides including mutations R145G, R145Q and R145W (Supplemental Figure 4). The fact that there have been no reports of mutations at R146 or R148 associated with cardiac disease would either suggest the essential nature of R146 and R148 or a degree of flexibility at those positions; the former is supported by the absolute conservation of R146 and R148 across species (Supplemental Figure 5).

3.3 H9c2 cells expressing R146A/R148A cTnI develop cell hypertrophy

We hypothesised that reduced cTnI ArgMe is associated with cardiomyocyte hypertrophy. To test this hypothesis, we transfected H9c2 cardiac-like cells, one of the most frequently used cell models of cardiomyocyte hypertrophy [15, 16], with plasmids encoding methylation-deficient and wild type cTnI proteins. We measured cell size of H9c2 cells expressing R146A/R148A cTnI and observed an increase in size by 32% after 72 h when compared to cells expressing wild type cTnI (Figure 2B and Supplemental Figure 6). Taken together, our results using human and rat

cardiac samples and cardiac-like rat cells indicate that cardiomyocyte hypertrophy is associated with a reduction in cTnI ArgMe.

3. Discussion

ArgMe is recognised as one of the most important protein post-translational modifications [17], which is highlighted by its involvement in disease and its high energy cost to the cell [18]. Here, we report for the first time that cTnI is modified by ArgMe in the human heart. Our work could help explain some of the minor, unidentified peaks previously observed by top-down mass spectrometry analysis of cTnI [6]. We show evidence supporting the notion that cardiac cell hypertrophy is associated with reduced cTnI ArgMe. The interpretation of our results is limited by the fact that no disease-free human cardiac specimens were available to us, by the low number of biological replicates in both the human and the rat models and by the lack of molecular tools (e.g. specific antibodies and inhibitors) to manipulate cTnI ArgMe *in vivo*. Although preliminary, the present work is the first step towards and enables the investigation of the role of cTnI ArgMe in sarcomere interactions, cardiomyocyte physiology and, from a translational standpoint, cardiac disease. It will also be interesting to see if cTnI released to circulation in the setting of cardiac damage (myocardial infarction, heart failure [19]) is modified by ArgMe, as this could provide a new twist in the field of cardiac biomarkers.

Acknowledgements

DOO acknowledges Rivers state university of Science & Technology Port Harcourt Nigeria, and TETFund Nigeria (Academic staff Training and development unit) for funding. Sabrina Samuel acknowledges an Allam PhD scholarship and Pedro Beltran-Alvarez acknowledges support from The Hull & East Riding Cardiac Trust Fund, without which this work would have not been possible. We are very grateful to Dr Anne-Marie Seymour (University of Hull, now retired) for animal provision and surgery. We thanks Prof Dr Thomas Zimmer (Universitaetsklinikum Jena) for comments on the manuscript. Mass spectrometry analyses were done in the mass spectrometry facility at the University of Hull (Rob McDonald), in the Metabolomics and Proteomics Facility at the University of York (Adam Dowle), and in the CRG/UPF and University of Barcelona

Proteomics Units (Cristina Chiva, Eduard Sabido, Antonia Odena, Eliandre de Oliveira). Peptides were synthesised by the Peptide Synthesis Facility, UPF, Barcelona (Javier Valle, David Andreu). We thank Kath Bulmer for outstanding technical assistance.

Disclosures

The authors report no relationships that could be construed as a conflict of interest.

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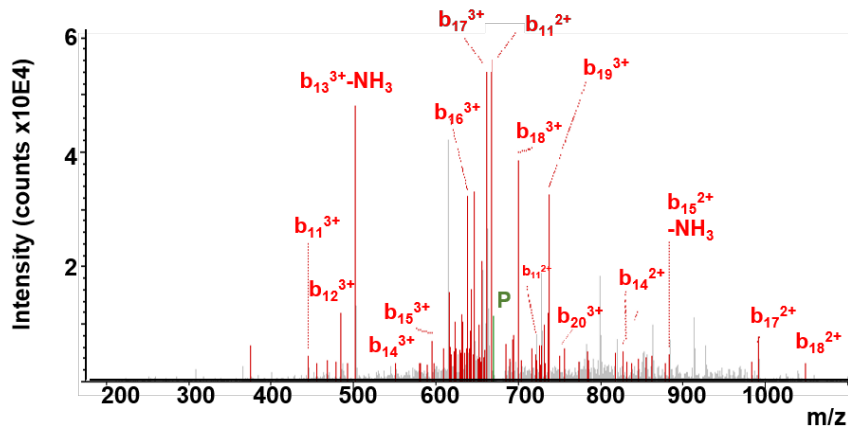
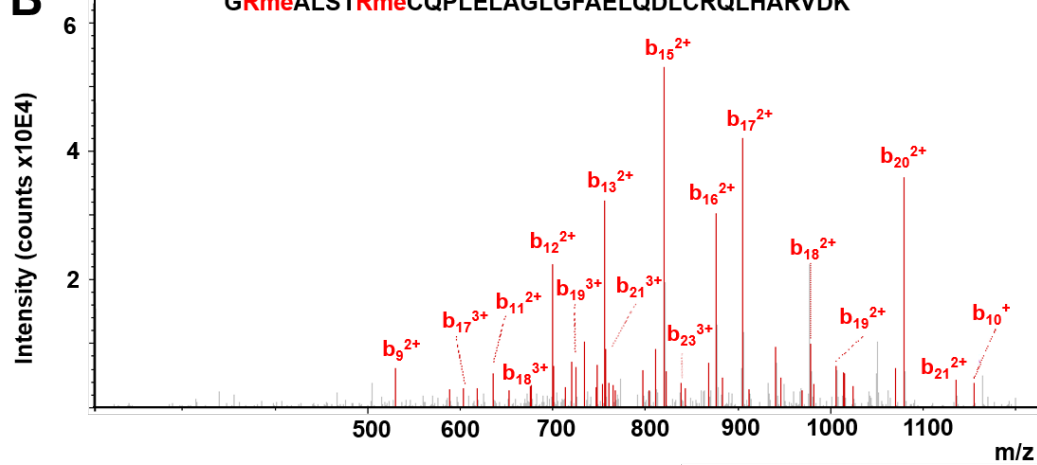
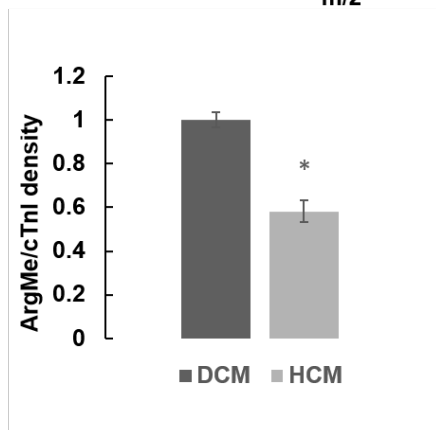
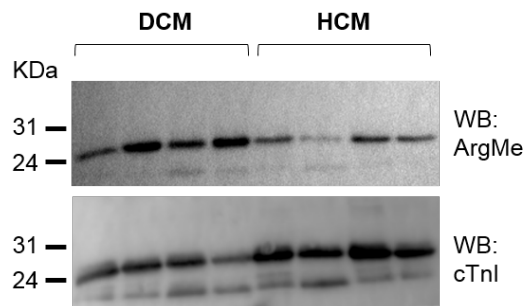
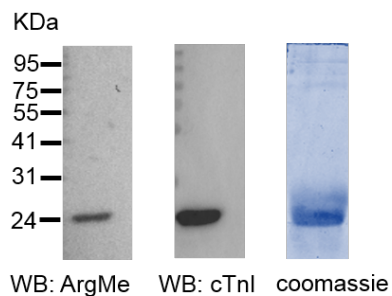
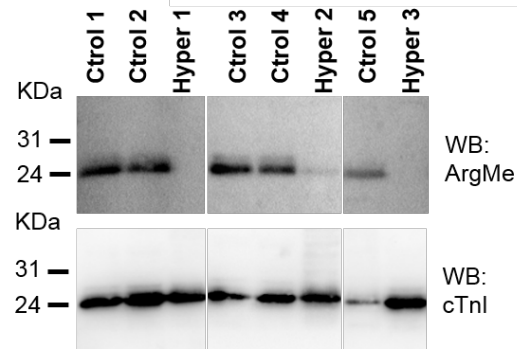
ARPTLR**Rme**VR**me**ISADAMMQALLGARAK**B**GR**me**ALST**Rme**CQPLELAGLGFAELQDLRCRLHARVDK**C****D****E**

Figure 1. **A.** Mass spectrum of the dimethylated cTnI peptide containing R146 and R148. **B.** Mass spectrum of the dimethylated cTnI peptide containing R74 and R79. Only *b* and precursor ions are coloured (in red and green, respectively). **C.** (Left) Representative western blot (of at least 3 repeats) showing cTnI recognition by an α -ArgMe antibody in DCM and HCM lysates. The second band on the cTnI blot probably corresponds to a degraded cTnI protein [6], note that this band is also recognised by the α -ArgMe antibody. (Right) Intensity of ArgMe bands compared to intensity of cTnI bands and normalised to DCM. Values are \pm SEM. Star relates to statistical significance ($p = 0.0002$). **D.** Western blots and coomassie staining of 1.5 μ g cTnI purified from normal human hearts (purchased from Abcam), showing recognition of cTnI by an α -ArgMe antibody (representative of 3 technical repeats). The coomassie-stained gel is shown to allow assessment of cTnI purity. **E.** Western blot showing much reduced cTnI ArgMe in cardiac lysates from rats that have undergone aortic banding (Hyper, $n = 3$) compared to sham animals (Ctrl, $n = 5$).

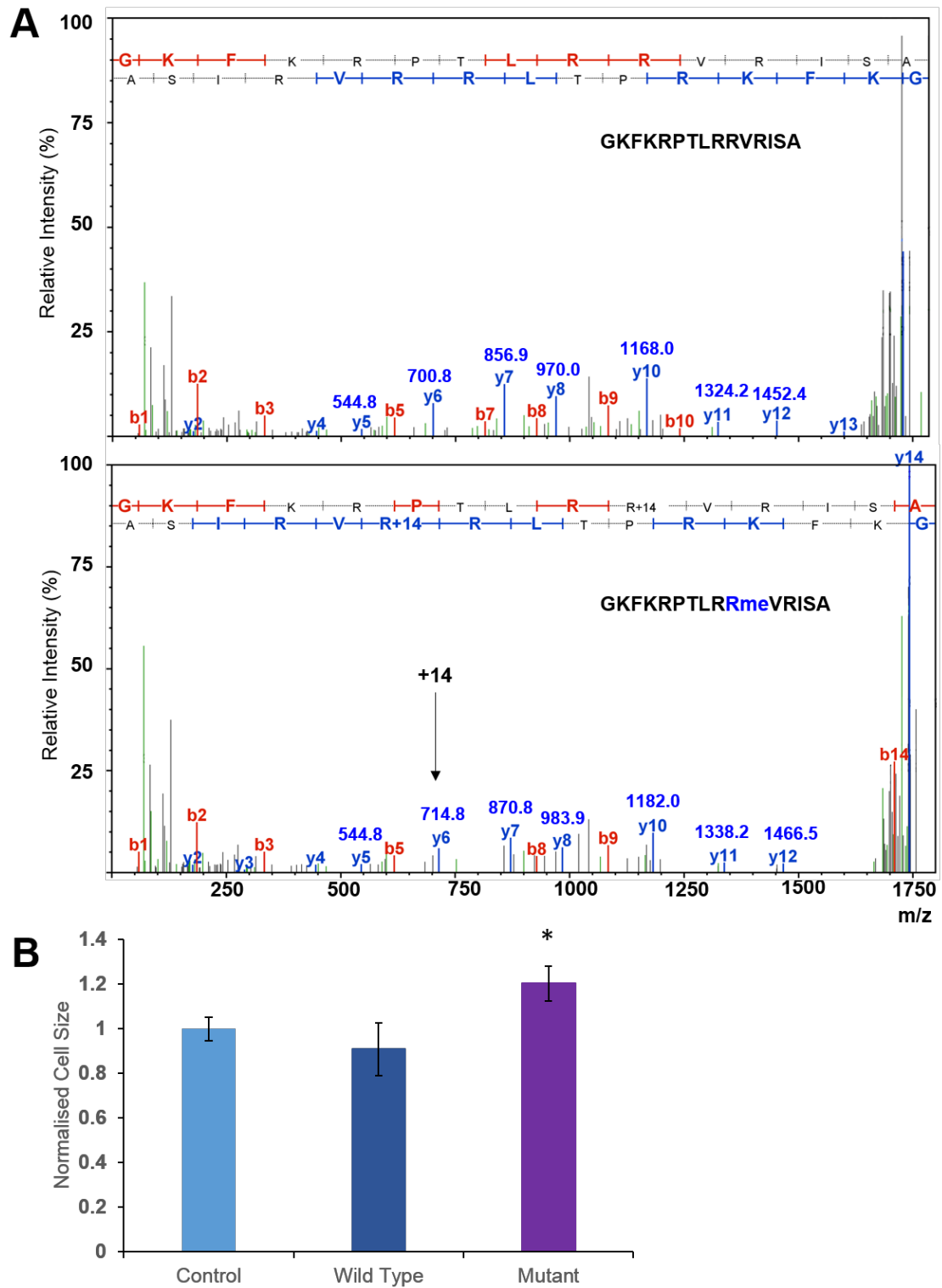


Figure 2. **A.** MS/MS spectrum of unmodified (top) and monomethylated (bottom) cTnI peptides. Note the shift in +14 Da from y6 to y12, indicating R146 monoArgMe (in blue). **B.** H9c2 cells expressing the double mutant R146A R148A cTnI were 32% larger than cells expressing wild type cTnI. Star relates to statistical significance ($p = 0.003$ with respect to cell expressing wild type cTnI).

Supplemental Tables

Supplemental Table 1. (see enclosed Excel file). Relevant clinical data of patients suffering from HCM (green) and DCM (orange) including age, gender, comorbidities, time of transplant from diagnosis, drug prescription and cardiac physiology.

Position	Sequence	Probability
10	MADGSSDAAREPRPAPAPI	0.960092
13	GSSDAAREPRPAPAPIRRR	0.964337
20	EPRPAPAPIRRRSSNYRAY	0.886081
21	PRPAPAPIRRRSSNYRAYA	0.795793
22	RPAPAPIRRRSSNYRAYAT	0.827292
27	PIRRRSSNYRAYATEPHAK	0.73918
68	QELERAEERRGEKGRALS	0.784937
69	ELERAEERRGEKGRALST	0.937995
<u>74</u>	AEERRGEKGR <u>R</u> ALSTRCQPL	0.943868
<u>79</u>	GEKGRALSTR <u>R</u> CQPLELAGL	0.83242
136	DLTQKIFDLRGKFKRPTLR	0.772542
141	IFDLRGKFKRPTLRRVRIS	0.707748
<u>146</u>	GKFKRPTLR <u>R</u> VRISADAMM	0.583457
<u>148</u>	FKRPTLRR <u>V</u> RISADAMMQA	0.639459
162	AMMQALLGARAKESLDLRA	0.849551
192	KENREVGDWKRNIDALSGM	0.775724
204	IDALSGMEGRKKKFESXXX	0.77948

Supplemental Table 2. Prediction of ArgMe sites in cTnI by ref. 12. Note the inclusion of R74, R79, R146 and R148 (in bold and underlined) as probably modified by methylation.

Supplemental Table 3 (see enclosed Excel file). LysC peptides identified as modified by ArgMe by Proteome Discoverer. All peptides are shown, including cTnI peptides at the top.

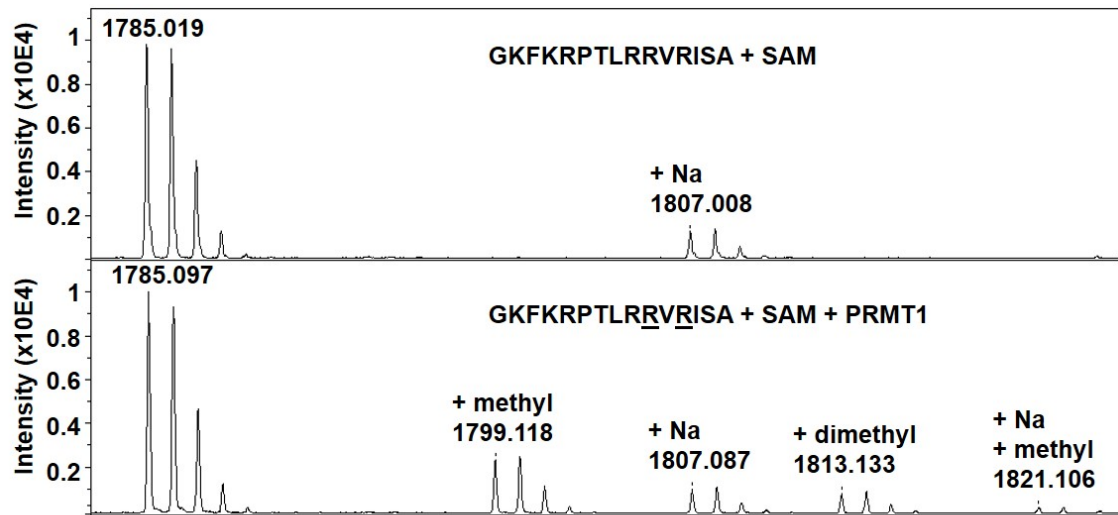
Supplemental Table 4 (see enclosed Excel file). Details of mass spectrometry identification of cTnI peptide containing R146 and R148. The list of identified *b* (red) and *y* (blue) series ions is shown. Not detected ions are in black.

Supplemental Table 5 (see enclosed Excel file). Details of mass spectrometry identification of cTnI peptide containing R74 and R79. The list of identified *b* (red) and *y* (blue) series ions is shown. Not detected ions are in black.

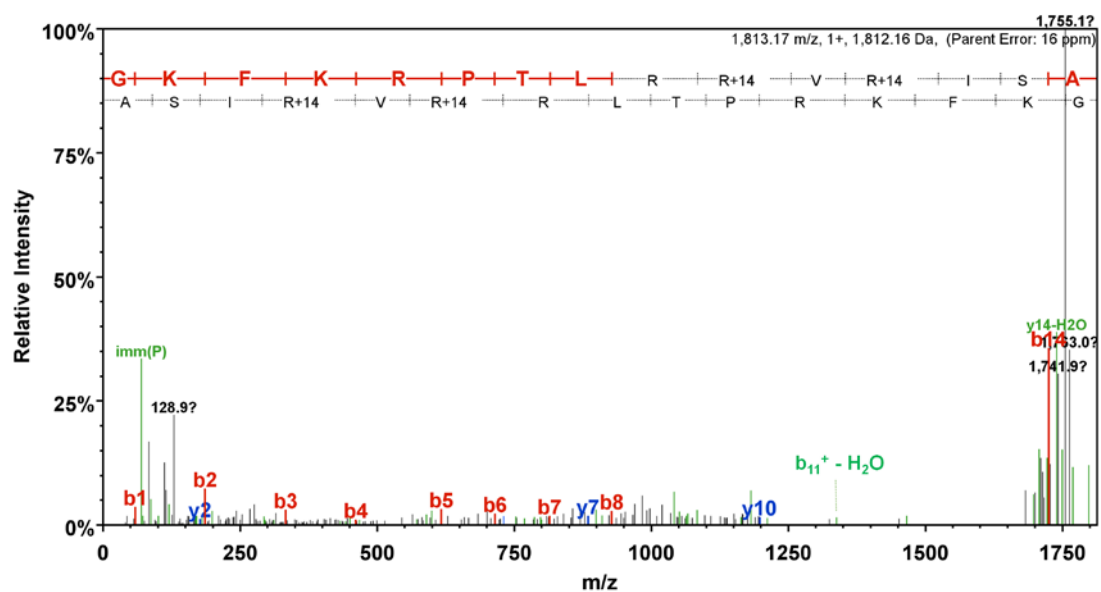
Supplemental Table 6 (see enclosed Excel file). Details of MS/MS identification of a synthetic cTnI inhibitory peptide containing R146 and R148. Different sheets are included for the unmodified, mono- and di-methylated peptides. The list of identified *b* and *y* series ions is shown in black. Diagnostic ions indicating mono- or di-methylation are in bold. Diagnostic ions indicating R146 (as opposed to R145 or R148) monomethylation in the species at 1799 Da are highlighted in yellow. Diagnostic ions indicating R146 and R148 monomethylation in the species at 1813 Da are highlighted in yellow. Not detected ions are not shown. Mascot scores and expectation values are included for each peptide species.

Supplemental Figures

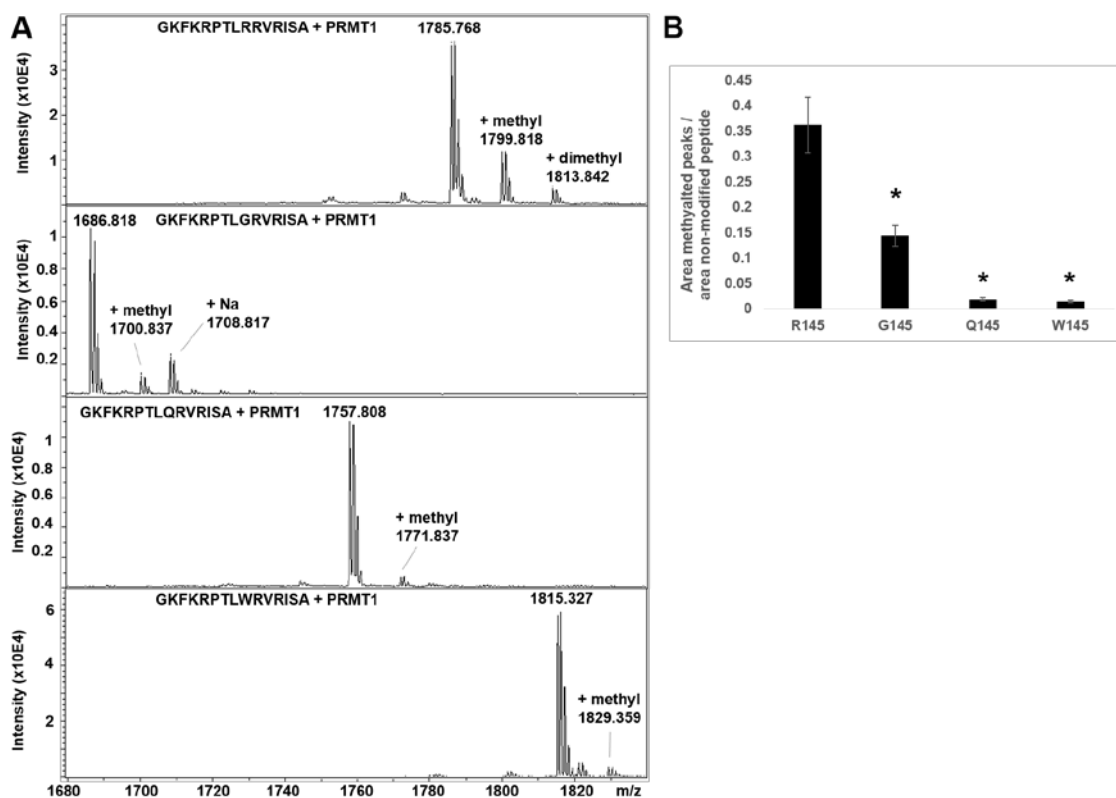
Supplemental Figure S1. (see enclosed ppt file) **A.** Mass spectrum of the dimethylated cTnI LysC peptide containing R146 and R148 in three more biological replicates. **B.** Mass spectrum of the dimethylated cTnI LysC peptide containing R74 and R79 in three more biological replicates.



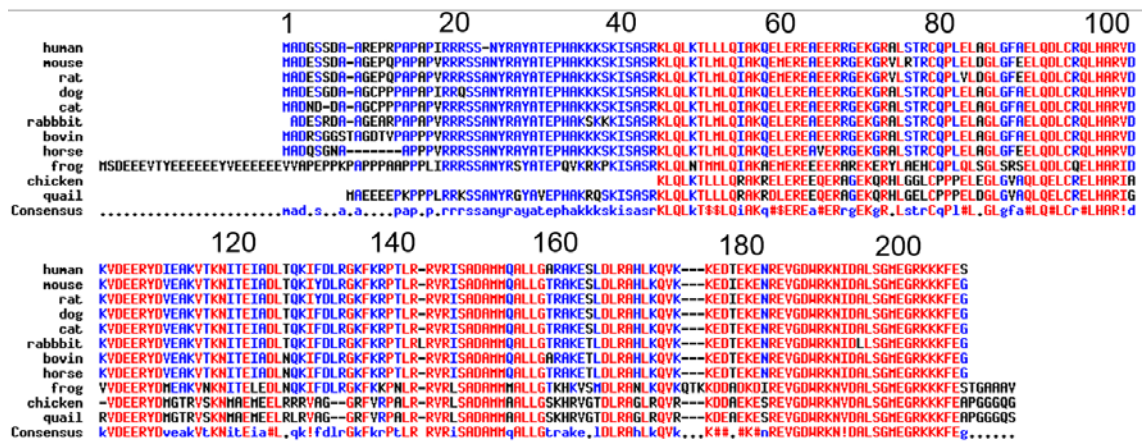
Supplemental Figure S2. MALDI-TOF mass spectrum of GKFKRPTLRRVRISA (R146 and R148 underlined) in the absence (top) and presence (bottom) of PRMT1. Expected peptide mass for $[M+H]^+$ is 1785.10 Da. Note the production of mono- and dimethylated species at +14 and +28 Da.



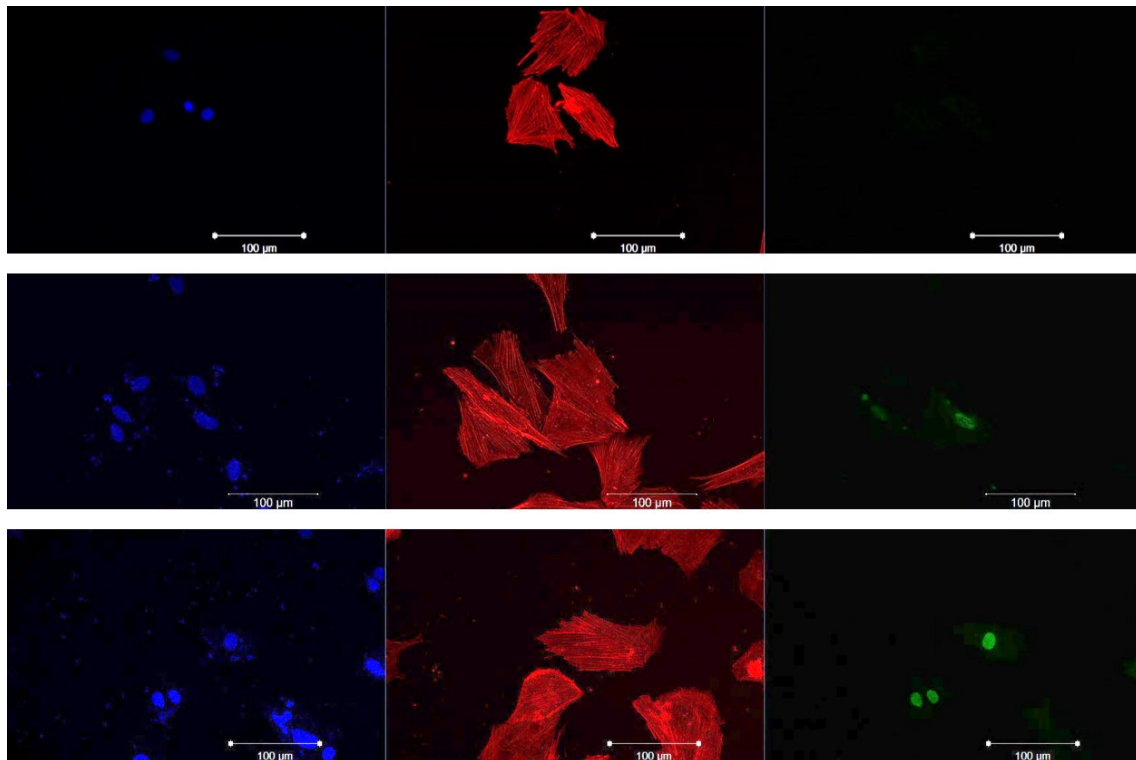
Supplemental Figure S3. MS/MS spectrum of peptide GKFKRPTLRVRISA dimethylated by PRMT1 *in vitro*. We assigned a methyl group to R146 and R148 on the basis of a weak $b_{11}^+ - H_2O$ diagnostic ion.



Supplemental Figure S4. A. MALDI-TOF mass spectra of GKFKRPTLGVRISA (expected $[M+H]^+$ 1686 Da), GKFKRPTLQVRISA (expected $[M+H]^+$ 1757 Da) and GKFKRPTLWVRISA (expected $[M+H]^+$ 1815 Da) peptides incubated with PRMT1, compared to methylation of GKFKRPTLRVRISA (top panel, R146 and R148 underlined). Note the production of mono- and di-methylated species at +14 and +28 Da. Representative spectra are shown for peptides bearing R145 and G145. Spectra showing highest levels of ArgMe are shown for peptides containing Q145 and W145. **B.** Estimation of fraction of methylated peptides compared to unmodified peptides. Area under peaks was used to semiquantify reaction yields [14]. Shown is the ratio of mono- and di-methylated peak areas with respect to unmodified peptide area, for peptides containing R, G, Q and W at position 145. Values are shown as mean \pm SEM. Star denotes statistical significance compared to R145 peptide ($p < 0.05$, Student's t-test, $n = 3$).



Supplemental Figure S5. Sequence alignment of all cTnI sequences available in Uniprot. Arrows indicate absolute conservation of R146 and R148 (human numbering).



Supplemental Figure S6. Example images of H9c2 cells mock-transfected (top), transfected with a plasmid encoding for wild type cTnI (middle) and transfected with a plasmid encoding for the double methylation-deficient mutant R146A/R148A cTnI (bottom). At least 2 independent transfections were done and the size of 35-90 cells was measured for each condition. Images are shown, from left to right, DAPI staining (blue), F-actin staining (phalloidin, red) and cTnI signals (green). Phalloidin signals were used to delineate cells and cell sizes determined using ImageJ as we have recently described [8]. Wild type and mutant cTnI signals were detected in both cell nuclei and cytoskeleton. It is now widely accepted that typically sarcomeric proteins including cTnI can also localise and have functions in cell nuclei, especially at neonatal stages (see reference [20] and cites therein) and it is worth noting at this point that H9c2 cells are of embryonic origin. The precise mechanism(s) leading to increased size of cells expressing methylation-deficient cTnI will need investigation by further work.