Removal of Abnormal Myofilament O-GlcNAcylation Restores Ca$^{2+}$ Sensitivity in Diabetic Cardiac Muscle

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Abstract

Contractile dysfunction and increased deposition of $O$-$\text{GlcNAc}$ylation in cardiac proteins are a hallmark of the diabetic heart. However, whether and how this post-translational alteration contributes to lower cardiac function remains unclear. Using a refined $\beta$-elimination/Michael addition with Tandem Mass Tags (TMT) labeling proteomic technique, here we show that CpOGA, a bacterial analogue of $O$-$\text{GlcNAc}$ase (OGA) that cleaves $O$-$\text{GlcNAc}$ *in vivo*, removes site-specific $O$-$\text{GlcNAc}$ylation from myofilaments, restoring $\text{Ca}^{2+}$ sensitivity in STZ-diabetic cardiac muscles. We report that in control rat hearts, $O$-$\text{GlcNAc}$ and $O$-$\text{GlcNAc}$ Transferase (OGT) are mainly localized at the Z-line, whereas OGA is at the A-band. Conversely, in diabetic hearts $O$-$\text{GlcNAc}$ levels are increased, and OGT and OGA delocalized. Consistent changes were found in human diabetic hearts. STZ-diabetic hearts display increased physical interactions of OGA with $\alpha$-actin, tropomyosin, and myosin light chain 1, along with reduced OGT and increased OGA activities. Our study is the first to reveal that specific removal of $O$-$\text{GlcNAc}$ylation restores myofilament response to $\text{Ca}^{2+}$ in diabetic hearts, and that altered $O$-$\text{GlcNAc}$ylation is due to the subcellular redistribution of OGT and OGA activities rather than to changes in their overall extent. Thus, preventing sarcomeric OGT and OGA displacement represents a new possible strategy for treating diabetic cardiomyopathy.

**Keywords:** Myocardial contractility; Diabetic Cardiomyopathy; Glucose; Contractile Proteins; Myofilament proteins; Myofilament $O$-$\text{GlcNAc}$ylation; Myofilament $\text{Ca}^{2+}$ Sensitivity; $O$-$\text{GlcNAc}$ase; O-$\text{GlcNAc}$ transferase.
In diabetic cardiomyopathy, the contractile and electrophysiological properties of the cardiac muscle are altered (1). Prior studies have mainly focused on alterations in Ca\(^{2+}\) handling (2-4). However, these perturbations alone unlikely account for lower force production and altered relaxation typically found in the heart of diabetic patients (5; 6). Indeed, the intrinsic properties of cardiac myofilaments appear to be altered too (7; 8). More specifically, Ca\(^{2+}\) sensitivity (EC\(_{50}\)), a measure of myofilament force production at near physiological Ca\(^{2+}\) levels, is reduced in the heart of diabetic patients (9-11). Yet the mechanisms responsible for Ca\(^{2+}\) desensitization in diabetic hearts remain incompletely understood.

O-GlcNAcylation is a post-translational modification (PTM) linked to glucose metabolism and centrally involved in regulating cellular homeostasis (12). This PTM consists of the addition of single O-linked \(\beta-D-N\)-acetylglucosamine (O-GlcNAc) sugar to serine and threonine residues of nuclear and cytoplasmic proteins. The reaction is catalyzed by O-GlcNAc transferase (OGT), whereas O-GlcNAc removal is under O-GlcNAcase’s control (OGA) (12). Excessive O-GlcNAcylation results from glucose- or other nutrient-induced overload of the hexosamine biosynthesis pathway (HBP). Alterations in HBP are increasingly recognized as major contributing factor for insulin resistance (12) and “glucose toxicity” during diabetes. Similar to phosphorylation, O-GlcNAcylation is a widely distributed and highly dynamic PTM (12). However, unlike phosphorylation that is regulated by a myriad of kinases and phosphatases, the extent of O-GlcNAcylation relies on two enzymes only, specifically OGT and OGA. OGT substrate specificity is regulated by transient protein:protein interactions that take place primarily at its tetratricopeptide repeat (TPR) domain (12) (Fig. 7A). Often OGT and OGA interact with each other and/or are found forming a holoenzyme complex with protein phosphatases and kinases (12). Modifications of Serine (Ser) and Threonine (Thr) by O-GlcNAc occur in myofilaments, and addition of exogenous
N-acetylglucosamine (GlcNAc) alters myofilament response to \(\text{Ca}^{2+}\) (13; 14). In addition, manipulation of cardiac O-GlcNAc levels influences \(\text{Ca}^{2+}\) cycling kinetics (4) and mitochondrial rates of respiration in diabetes (2; 15), functional recovery after ischemia-reperfusion injury (16; 17) or chronic pressure-overload (18-21). Despite all this evidence, whether and how altered O-GlcNAcylation contributes to myofilament dysfunction in diabetic cardiomyopathy (7; 8; 22-24) is currently unclear.

Here we proved that specific removal of O-GlcNAc excess from diabetic myofilaments ameliorates contractile dysfunction by linking force-\(\text{Ca}^{2+}\) relationships improvement to site-specific O-GlcNAc changes. We also determined a potential mechanism leading to altered O-GlcNAcylation, by comparing the status and sarcomeric distribution patterns of OGT and OGA in the heart of rats with streptozotocin-induced type 1 diabetes with that found in controls.

**RESEARCH DESIGN AND METHODS**

**Diabetes type 1 rat model**

Type 1 Diabetes was induced in male Sprague Dawley (SD Charles River) by an intra-peritoneal injection of streptozotocin (STZ) (65 mg/kg), control animals were injected with vehicle only. Animals were sacrificed 6-8 weeks after induction of diabetes, at the moment of tissue harvest STZ diabetic animal had blood glucose of 693.5±61.5 mg/dl and control had 123.9±9.5 mg/dl. All animal protocols were performed in accordance with institutional guidelines and approval of the Institutional Animal Care and Use Committee (IACUC).

**Isolated skinned fiber studies**
For skinned cardiac muscles studies, muscles were isolated and mounted as previously described (25; 26). Varied Ca\textsuperscript{2+} concentrations \([\text{Ca}^{2+}]_{o}\) were achieved by mixing the relaxing solution and activating solution in various ratios. After reaching the highest \([\text{Ca}^{2+}]_{o}\) concentration, trabeculae were washed in relaxing solution, and incubated at room temperature 1hr in 1 µg/ml of CpOGA (27) diluted in relaxing solution. Afterwards, a new Ca\textsuperscript{2+} activation protocol was repeated. Steady-state force-[Ca\textsuperscript{2+}]\textsubscript{o} relationships were determined experimentally, and fit to a modified Hill equation (25; 26).

**Immunofluorescence Confocal Microscopy**

Cold-acetone fixed cryosections (7-8 µm) from rat or human (BioChain® Inc. Office of Human Research Protection registered IRB00008283) myocardium were blocked and incubated over night with a primary antibody against O-GlcNAc (CTD110.6). In addition, isolated skinned myocytes were obtained from flash-frozen myocardium by homogenization in 0.03% Triton X-100 at low-speed, as previously described (28), seeded on eight chamber slides coated with 40 µg/ml of Laminin (Invitrogen) and fixed in 4% Formaldehyde-methanol free ultra-pure (Polysciences Inc). Crysections or isolated skinned myocytes were blocked and incubated over night with O-GlcNAcase (345), O-GlcNAc transferase (AL-25) (29) and Anti-α-actinin (Sigma) at 1 µg/ml, secondary antibodies were Alexa 647 Goat anti-mouse IgM (µ chain) for O-GlcNAc, Alexa 647 Goat anti-rabbit IgG for OGT, Alexa 594 Goat anti-chicken IgY for OGA and Alexa 488 Goat anti-mouse IgG for α-actinin. Prolong anti-fade with DAPI (Invitrogen) was use for mounting. Images were acquired on a Zeiss LSM710 upright microscope using a 25x or a 63x water-immersion objective (Nikon) and analyzed with Zen 9 Leica Zeiss software tools.

**Double Immuno-Electron Microscopy**
For gold immuno-labeling, a goat anti-Rabbit or a goat anti-Mouse, were labeled with 12 nm diameter particles to detect anti-OGT (AL-34) or anti-\(O\)-GlcNAc (CTD 110.6), and a goat anti-chicken was labeled with 6 nm diameter particles to detect anti-OGA (345). Labeled ultrathin sections (60 to 90 nm-thick) sections were examined under the transmission electron microscope (Hitachi 7600 TEM, Japan), 8 to 11 random field pictures were used for quantification of OGT, OGA and O-GlcNAc immune-gold particles with NIH ImageJ software.

**Co-immunoprecipitation for \(O\)-GlcNAc transferase and \(O\)-GlcNAcase**

For immuno-precipitation studies, anti-OGT (AL-28) or anti-OGA (345) (1 µg total) antibodies were added to 0.5 mg/ml protein samples. Immuno-precipitates were then separated by SDS-PAGE, transferred to PVDF membranes (Millipore, Bedford, MA), antibody probing against \(\alpha\)-cardiac actin (Sigma), \(\alpha\)-Tm (Sigma) and anti-MLC1 (Clone 1LC14, Spectral Diagnostics). Between different antibodies blots were stripped for 1 h at 25°C in 200 mM glycine (Sigma; pH 2.5) (29).

**OGT and OGA activity assays**

*OGT assays.* Heart homogenates were separated in cytosolic or myofilament fractions, desalted and subjected to OGT activity assays as described (30). Activity counts in disintegration per minute (d.p.m.) were normalized to total protein content (dpm/µg), then background activity without CKII peptide was subtracted (See Fig. 7C).

*OGAse assays.* OGAse activity was determined as previously described (31). Briefly, activity was expressed as the amount of enzyme catalyzing the release of 1 mmol/µg/min of pNP from pNP-GlcNAc, and then background activity in the presence of the most specific and potent OGA inhibitor (1µM Thiamet-G) was subtracted (See Fig. 7C).
**Myofilament isolation and TMT labeling**

Myofilament proteins were isolated as previously described (32). Protein concentration was determined by the Bradford assay and equal amount of proteins (~200 µg) were reduced with 5 mM DTT, alkylated with 15 mM iodoacetamide, and digested by trypsin (trypsin/protein ratio = 1/50). Tryptic peptides were labeled for quantitation with TMT 10plex labels (127, 128, 129, 130) following the manufactures guidelines (Thermo Fisher Scientific). The labeled peptides were combined and fractioned offline using XBridge HPLC column (Waters). Resulting 96 fractions were combined into 24 fractions for LC-MS/MS runs, while only reserving 10% for the pre-enriched analysis, and combining the remaining 90% for O-GlcNAc peptide enrichment (post enrichment).

**O-GlcNAc peptide enrichment**

The remaining pooled peptides were used to enrich O-GlcNAcylated peptides, by adapting a method described previously (33; 34), with some modifications. In brief, peptides were treated with alkaline phosphatase (50 units; New England Biolab) and PNGase F (1,000 units; New England Biolab) for 6 h, followed by desalting with a C18 spin column (Nest group). The dried peptides were resuspended in a buffer containing 20 mM DTT, 20% (v/v) EtOH, and 1.5% Triethanolamine (TEA pH 12.5) and incubated at 50 °C for 4 h with gentle shaking. Reaction was quenched by the addition of trifluoroacetic acid (final pH ~7.0). Peptides were desalted and then incubated with thiol-sepharose beads (Sigma) in PBS containing 1 mM EDTA (PBS/EDTA, pH 7.4) for 4 h. After 5 washes in a PBS/EDTA buffer supplemented with ACN 40% (v/v), beads were incubated in PBS/EDTA containing 20 mM DTT for 30 min. Released peptides were collected and desalted with a C18 spin column. Dried peptides were then analyzed by LC-MS/MS for O-GlcNAc site mapping.
and quantification.

**LC-MS/MS analysis**

The un-enriched and O-GlcNAc enriched fractions were analyzed with an LTQ-Orbitrap Velos (Thermo Fisher Scientific) attached to a Nano Acquity (Waters) chromatography system. Peptides were loaded on a 75 um x 2.5 cm C18 (YMC*GEL ODS-A 12nm S-10 μm) trap at 600 nl/min 0.1% FA (solvent A) and fractionated at 300 nL/min on a 75 μm x 150 mm reverse-phase column using a 2-90% acetonitrile in 0.1% formic acid gradient over 90 minutes. Eluting peptides were sprayed into the mass spectrometer through 1 μm emitter tip (New Objective) at 2.2 kV. Survey scans (full MS) were acquired within 350-1800 m/z with up to 8 peptide masses (precursor ions) individually isolated at IW1.9Da, and fragmented (MS/MS) using HCD 35 activation collision energy. Precursor and the fragment ions were analyzed at resolution 30,000 and 15000, respectively. Dynamic exclusion of 30 s, repeat count 1, MIPS (monoisotopic ion precursor selection) “on”, m/z option “off”, lock mass “on” (silocsane 371Da) were used. Tandem MS/MS mass spectra were processed by PEAKS Studio (Bioinformatics Solutions Inc.) using rattus norvegicus FASTA as proteome database, with concatenated decoy database, specifying all peptide species, trypsin as enzyme, missed cleavage 2, precursor mass tolerance 10 p.p.m., fragment mass tolerance 0.03Da, and Oxidation (M), Deamidation (NQ), Carbamidomethyl (C), and TMT labels 127, 128, 129 and 130 as variable modifications.

**TMT and O-GlcNAc Quantification**

Quantitation function of PEAKS Studio was used to export the raw intensity values of TMT peptides with or without O-GlcNAc enrichment. Only peptides with a positive identification value
or a cut-off value of 20 for peptide score threshold (-10 log P), quantification mass tolerance (0.2 Da) and 0.1 % FDR were considered. All Serine or Threonine residues from O-GlcNAcylated peptides were identified by a +136 mass shift given by DTT during BEMAD, and their MS/MS spectra manually inspected. The experimental designed consisted of twelve biological samples divided into three experiments with four isobaric mass-tags, each experiment comprising a STZ diabetic and a control sample with and without CpOGA treatment. The TMT pre-enrichment samples were analyzed to factor in potential changes in protein expression, the relative total protein load and to refine the comparisons among post-enrichment samples by accounting for technical/experimental variation. The median signal value for each modified peptide of each sample was first determined and converted to log2 notation (with 0.0 values excluded as nulls) for further processing. Data were then quantile normalized to achieve the same median, the difference between pre-normalized and normalized data provided a number that was further used as a correction factor for the post-enrichment peptide analysis. For the O-GlcNAc enriched peptides signals were treated as above, except when converted to log2 notation the 0.0 values were log-converted to 0.001 to be able to express ratios.

Detection of differential O-GlcNAcylation

We used a statistical generalized linear model approach for differential gene expression detection. Briefly, a mixed-effects linear model was fit for each individual modified peptide to estimate O-GlcNAcylation differences between the groups of samples being compared (i.e. Control vs STZ Diabetic, Control vs CpOGA-Control, STZ vs CpOGA-STZ). When distinct peptides for the same O-GlcNAcylated site were available, correlation coefficients were computed and the associated consensus correlation was added to the model (35). An empirical Bayes approach was applied to
moderate standard errors of log2 O-GlcNAcylation fold-change as previously described (36). Finally, for each analyzed feature moderated t-statistics, log-odds ratios of differential expression (B-statistics), raw and adjusted p-values (FDR control by the Benjamini and Hochberg method) were obtained. All analyses were performed using software packages available from the R/Bioconductor for statistical computing “limma” (36).

Statistics

Student's t-test, one-way ANOVA and two-way ANOVA with repeated measures, followed by post-hoc pair wise comparison, when appropriate, was used for statistical analysis of the data. A value of \( p < 0.05 \) was considered to indicate significant differences between groups. Unless otherwise indicated data were expressed as mean ± standard error of the mean (S.E.M.).

RESULTS

Removal of Site-Specific O-GlcNAcylation Excess Restores Myofilament Ca\(^{2+}\) Sensitivity in Diabetic Cardiac Muscle

Using a mass spectrometry approach, we have previously identified specific O-GlcNAcylation sites on five major cardiac myofilament proteins on normal hearts (14). We also showed that incubation of skinned cardiac muscles with N-acetyl-D-glucosamine (GlcNAc) reduces myofilament Ca\(^{2+}\) sensitivity, thus reproducing a hallmark of diabetic cardiac muscle (7; 14). Yet, site-specific O-GlcNAcylation changes on diabetic hearts remain unknown. Nor is clear whether removing endogenous O-GlcNAc from diabetic skinned cardiac muscles is sufficient to restore myofilament
function. To address these questions, we first confirmed that O-GlcNAcylation is enhanced in protein extracts from STZ diabetic hearts (Fig. 1A). Next, we used CpOGA to remove O-GlcNAc from diabetic skinned cardiac muscles (Fig. 1B). Finally, we employed a refined global quantitative proteomic technique that combines Tandem Mass Tags (TMT) labeling with β-elimination/Michael addition to quantify the O-GlcNAcylation changes in diabetic hearts and to identify the key amino acid residues where O-GlcNAc is reduced after CpOGA in STZ diabetic but not in controls (Fig. 1C).

O-GlcNAcase is the mammalian enzyme that removes O-GlcNAc in vivo, however its glycosidase activity is greatly reduced when expressed as a recombinant protein (31). To circumvent this issue, we utilized a bacterial glycosidase, CpOGA (27) (Clostridium perfringens N-Acetyl-glycosidaseJ, kindly provided by Dr. Daan van Aalten, University of Dundee). CpOGA is highly homologous to human O-GlcNAcase and displays potent and specific activity towards mammalian protein homogenates (37). Hence, skinned cardiac muscles from diabetic (STZ-induced) and control rats were incubated with CpOGA, and their contractile properties determined before and after this treatment. Steady-state force-[Ca\(^{2+}\)] relationships measurements revealed that CpOGA removal of abnormal O-GlcNAcylation restores myofilament Ca\(^{2+}\) sensitivity in diabetic skinned fibers (Fig. 1B). Remarkably, the EC\(_{50}\) of CpOGA-treated diabetic skinned fibers became similar to that found in control muscles, regardless of CpOGA presence (Fig. 1B). Upon CpOGA administration, no difference in maximal Ca\(^{2+}\) activated force (F\(_{\text{max}}\)) and Hill coefficient (n) was evident between control and diabetic muscles (Fig. 1B). Next, control and diabetic (STZ) rat heart myofilaments were compared before and after being incubated with recombinant CpOGA and analyzed for O-GlcNAcylation by tandem mass tagging and LC-MS/MS. In total we found 63 O-
GlcNAcylated sites, 39 in myosin heavy chain, 9 in alpha-sarcomeric actin, 2 in myosin light chain 1, 5 in tropomyosin alpha 1, 7 in cardiac troponin I, and 1 in myosin binding protein-C (Table I).

Since CpOGA removes O-GlcNAc in both, control and diabetic (STZ) rat muscles but only restores EC$_{50}$ in diabetic ones, we focused our proteomic analysis on identifying the site-specific O-GlcNAc changes that are statistically significant on CpOGA treated STZ diabetic vs. CpOGA untreated STZ diabetic myofilaments but not significant on CpOGA treated control vs. CpOGA untreated control myofilaments (Fig. 1C). Surprisingly, most of the sites that change significantly are located on myosin heavy chain (MHC S740, S844, S1414, S1465, S1471, S1472, S1598, T1601, S1602, S1778, S1917), alpha-sarcomeric actin (Actin S54, T326) and tropomyosin (Tm S87). Noteworthy, the sites that are significantly more O-GlcNAcylated in diabetic hearts, i.e. myosin heavy chain (MHC S844, S1471, S1472, T1601, S1917) and alpha-sarcomeric actin (Actin T326) (Fig. 1C red rectangles), are also part of the group of sites that change significantly upon CpOGA treatment of STZ diabetic myofilaments. Thus, abnormal O-GlcNAcylation of cardiac muscle proteins is sufficient to reduce myofilament Ca$^{2+}$ sensitivity and its removal is necessary to prevent this Ca$^{2+}$ desensitization.

**Diabetic Cardiac Muscle Display Sarcomeric O-GlcNAc Signal Increased and Delocalized from Z-Lines**

The α-cardiac actin O-GlcNAc signal intensity (by immunoblot) is augmented in animal models of diabetes (14). Moreover, in normal human myocardium O-GlcNAc predominantly modifies a Z-line protein called ZASP (Z-band alternatively spliced PDZ motif protein) (38). ZASP O-GlcNAcylation increases further in heart failure and hypertrophic cardiomyopathy (38). However, whether or not O-GlcNAc specific sub-cellular localization changes in diabetes is not
clear. To fill this gap, cryosections from normal and STZ-induced diabetic rat hearts were cold-acetone fixed and stained for O-GlcNAc (CTD 110.6) to determine the relative abundance and localization of O-GlcNAc (Fig. 2A). Consistent with previous findings, O-GlcNAc signal intensity was markedly increased in STZ rat diabetic myocardium when compared to control hearts (Fig. 2B). Also, immuno-electron microscopy revealed that in control myocardium O-GlcNAc was predominantly localized in clusters near the Z-lines, whereas in STZ-treated rats O-GlcNAc clusters were spread towards the A-band (Fig. 2C). Next, we performed a morphometric analysis of 9 random fields of control or STZ rat diabetic myocardium, utilizing immune-gold particles (12 nm diameter) to assess O-GlcNAc distance from the nearest Z-line (Fig. 2D). This approach allowed us to conclude that, in the sarcomeres of diabetic rats, overall O-GlcNAc signal is increased and moves away from Z-lines. To test whether similar alterations pertain to human pathology, O-GlcNAc immune-fluorescence was investigated in one sample from a normal (donor) and one from a type-2 diabetes patient. Consistent with findings in the rat, O-GlcNAc signal intensity was markedly increased in the human diabetic specimen (Fig. 2E).

**OGT and OGA Sarcomeric Distribution is Inverted in Diabetic Myocardium**

Next, we reasoned that the presence of enhanced O-GlcNAcylation in specific sarcomere compartments of diabetic hearts could be either due to enhanced activity of OGT, reduced OGA activity, abnormal enzyme’s localization, or both. To address this issue, we used anti-OGT (AL-28) or anti-OGA (345) antibodies and an immunofluorescence and confocal microscopy approach to analyze the signal intensity and co-localization of both enzymes. OGT exhibited a predominantly sarcomeric localization, whereas OGA displayed both a sarcomeric and reticular pattern (Fig. 3A, B). Similar to O-GlcNAc, OGT tended to co-localize mostly with α-actinin, at the proximity of the
Z-line (Fig. 3C,D). Although detectable at the Z-line too, OGA was instead predominantly distributed throughout the A-band, along the entire sarcomeric unit. To further consolidate this evidence, we used the co-localization tools of Zen 9 Image Analysis Software (Leica Zeiss), enabling us to quantify changes in OGT and OGA distribution. Representative images of normal and STZ diabetic hearts were used to generate XY pixel dot plots of Laser Scanning Signals. A series of co-localization quantification parameters are displayed as bar graphs for OGT and α-actinin (Fig. 3E) and OGT and OGA (Fig. 3F). Analysis of at least 3 random fields from 4 replicates on 3 different hearts showed clear differences between normal and STZ diabetic myocardium. OGT co-localization with α-actinin was decreased in STZ myocardium (Fig. 3E1-3), thus confirming a re-distribution apart from Z-line (p=0.0053). Interestingly, OGT/OGA co-localization was also reduced in STZ diabetic myocardium (*p≤0.05, Fig. 3 F1-3). α-Actinin, OGT and OGA immuno-fluorescence was also evaluated in human heart samples (Fig. 4A). Acetone pre-fixed frozen slides were triple-antigen stained by immunofluorescence for OGT (AL-28), OGA (345) and α-actinin. As expected, human control and diabetic myocardium exhibited a differential sarcomeric pattern for OGT and OGA signals. A selected area from control (Fig. 4A top panel) or diabetic (Fig. 4A bottom panel) is shown as a white square in the merged left picture, followed by enlarged areas showing the regions in single or combined channels for linear surface profile plots of ~6-8 sarcomere units (dashed line). Combined signals of OGT (magenta) or OGA (red) and α-actinin (green) for control and diabetic myocardium indicated that in control myocardium OGT labeling peaks correspond mostly with α-actinin signal. Conversely, OGA labeling showed variable signals, peaking both at A-band and Z-line regions (Fig. 4A top panel). In diabetic myocardium, however, OGT signal peaks tended to shift away from α-actinin signal, while OGA labeling centered more on α-actinin signal (Fig. 4A bottom panel). Since cryosections fixed with acetone
might reflect tissue architecture alterations, we decided to corroborate OGT and OGA staining pattern on isolated skinned myocytes obtained from flash frozen myocardium, derived from rat samples as described (28). Similar to rat or human myocardium cryosections, isolated skinned myocytes fixed with 4% formaldehyde displayed the expected OGT and OGA staining pattern in control (Fig. 4B top panel) or STZ diabetic (Fig. 4B bottom panel) myocytes. When sarcomeres in control hearts are compared with those found in diabetic hearts, these changes in OGT staining pattern mirror faithfully those found in rat STZ diabetic myocardium (Fig. 3), with an inverted pattern of OGT and OGA sarcomeric distribution. This inverted pattern of OGT/OGA localization may influence O-GlcNAcylation cycling rates, thus likely perturbing O-GlcNAc stoichiometry in specific sarcomere compartments.

Next, we analyzed OGT and OGA by double immuno-electron microscopy in hearts from STZ diabetic and control rats. Immuno-EM data fully corroborated the evidence obtained with the immune fluorescence approach, confirming the re-distribution of both enzymes within the sarcomere. Indeed, similar to O-GlcNAc, OGT immuno-gold labeled particles were mainly located at the Z-disk in normal hearts (Fig. 5A, top panel), whereas they were more diffused along the A-band in diabetic hearts (Fig. 5B, top panel). Instead of being localized mainly at the A-band (normal hearts), OGA formed sizable clusters in the vicinity of the Z-disk (diabetic hearts, Fig. 5B, bottom panel). Finally, we determined the frequency of OGT and OGA immuno-gold labeled particles, examining 9-10 random fields of normal or diabetic myocardium and quantifying OGT (12 nm, purple circle) and OGA (6 nm, red circle) immuno-gold particles confined to the myofilament apparatus. Our approach revealed that both O-GlcNAc cycling enzymes were detected at higher frequency in diabetic myocardium (Fig. 5C, D). Taken together, these data suggest that, in analogy
to cardiac kinases and phosphatases (39), mislocalized OGT and OGA activities can impact the function of contractile or regulatory proteins or both.

**Differential interactions of OGT and OGA with myofilament proteins in the diabetic heart**

Assessing OGT by Western blot in myofilament preparations (14) involves the use of high (1%) concentrations of Triton X-100 (32). This procedure may preclude the possibility of detecting weak interactions between OGT, OGA and their potential transient binding partner proteins, thus underestimating the extent of OGT and OGA abundance in the myofilaments. To properly evaluate these interactions, we used a co-immunoprecipitation approach, testing whether OGT and OGA are physically associated with myofilament proteins, and determining possible changes in their myofilament abundance imparted by diabetes. Fresh whole heart homogenates, from either control or diabetic (STZ treated) rats, were immuno-precipitated with anti-OGT (AL-28) or anti-OGA (345) antibodies (29), resolved by SDS-PAGE, and analyzed by Western blots against several myofilament proteins. Both OGT and OGA were associated with α-cardiac actin, α-tropomyosin (α-Tm), and myosin light chain 1 (MLC1) in normal hearts (Fig. 5E, F). A representative co-IP of OGT and OGA with interacting myofilament proteins is provided in Fig. 5E, F. In the diabetic hearts, the OGA immuno-precipitate interactions with α-cardiac actin, α-Tm and MLC1 were increased several fold as compared to the control ones (Fig. 5H) (n=5 vs. n=4, p< 0.05), whereas interactions with OGT were normal. On the other hand, although the OGT immuno-precipitate interactions with myofilaments tended to have increased interactions in diabetic samples they did not reach statistical significance. The heterogeneity of the signals for OGT and OGA Co-IPs on the control heart homogenates might be related to the feeding status *(ad libitum)* of the animals or to a
potential cyclic nature of these protein:protein interactions. Hence, in diabetic hearts O-GlcNAcase interactions with myofilament proteins are more abundant and they are likely contributing to reduce contractility.

**OGT and OGA Activity are altered in diabetic hearts**

In diabetic hearts, overall O-GlcNAc levels have been reported to change with or without reciprocal changes in OGT expression and UDP-GlcNAc levels (40). Protein specific O-GlcNAcylation, however, can change in either direction (40). With this paradox in mind, we hypothesized that the observed changes in OGT/OGA sarcomeric distribution and myofilament protein interactions might influence their enzymatic activity. To test this, fresh whole hearts from either control or STZ diabetic rats were homogenized in extraction buffer containing 1% Triton X-100. Total tissue homogenates were separated in cytosolic and myofilament fractions by centrifugation. For OGT assays, both cytosolic and myofilament fractions were either desalted into OGT desalting buffer (for activity assays) or stored at -80 °C for SDS-PAGE and Western Blots. After OGT expression was normalized to cardiac troponin I (cTn I) content, neither sub-cellular fraction showed significant differences between control and STZ diabetic hearts (myofilament and cytosolic fractions are shown in Fig.6A and B, respectively). OGT activity was assayed by the incorporation of UDP-[H³]N-acetyl-D-glucosamine into a synthetic peptide (CKII peptide); disintegration per minute (dpm) counts were normalized to µg of protein, and then OGT activity without peptide subtracted (31) (Fig. 7C). This approach revealed a significant reduction in myofilament associated OGT activity in STZ diabetic hearts (Fig. 6D, obtained in myofilament fractions). Conversely, OGT activity in the cytosolic fractions of STZ diabetic hearts was not significantly different (Fig. 6E). For OGA assays, myofilament fractions retained traces of
detergent; therefore, they were not suitable for measurement of enzymatic activity. However, total tissue homogenates became properly usable after protein precipitation with 30-50% ammonium sulfate and desalting into OGA assay buffer. After the latter procedure, OGA expression normalized to cTnI content was not significantly different between groups (0.54±0.02 for STZ vs. 1.14±0.29 for controls, \( n=3 \) each) (Fig.6C). OGA activity was assessed by the release of p-nitrophenol (pNP) from p-nitrophenol-N-acetylglucosamine (pNP-GlcNAc), a synthetic substrate. In addition, our approach accounted for OGA activity background more strictly because we subtracted OGA activity detected in presence of Thiamet-G (TMG), the most potent and specific OGA inhibitor available (41) (Fig. 7C). OGA specific activity was significantly increased in homogenates from STZ diabetic hearts as compared to controls (Fig.6F). Thus, changes in OGT/OGA protein:protein interactions and subcellular localization may influence OGT/OGA enzymatic activity.

**Discussion**

This study establishes that excessive \( O\text{-GlcNAcylation} \), in a specific subset of myofilament sites of myosin heavy chain, alpha-sarcomeric actin and tropomyosin, is sufficient to produce myofilament dysfunction in diabetic cardiomyopathy, and it does so by affecting Ca\(^{2+}\) sensitivity. These perturbations in myofilament \( O\text{-GlcNAcylation} \) result from OGT and OGA displacement within the sarcomere rather than from variations in enzymatic activity *per se*, resulting in an overall perturbed \( O\text{-GlcNAc} \) cycling. Important, from a translational point of view, is the finding that similar alterations occur in experimental and human diabetic hearts.

Depressed myofilament Ca\(^{2+}\) sensitivity is a hallmark of myofilament dysfunction in diabetic cardiomyopathy and heart failure (7; 11). Alterations in myofilament phosphorylation are
now recognized as important negative modulators of cardiac function, along with Ca\(^{2+}\) handling perturbations (39; 42-44). That alterations in cardiac protein O-GlcNAcylation are present in type-1 and type-2 diabetes models (2; 4; 24; 40; 45), and cardiac disease (21; 38) is well established; however, no studies have addressed yet the functional impact of cardiac myofilaments O-GlcNAcylation during the course of diabetic cardiac dysfunction (2-4). We have previously identified numerous sites within cardiac myofilament to be O-GlcNAcylated under normal conditions (14). We also showed that incubation of normal skinned cardiac muscles with N-acetyl-D-glucosamine (GlcNAc) reduced myofilament Ca\(^{2+}\) sensitivity (14). Moreover, adenoviral-based or inducible transgenic over-expression of human OGA, for which CpOGA is a very close homologue, is known to ameliorate contractile and energetic deficits associated with diabetic cardiomyopathy (4; 45). Here, we add important novel evidence that in diabetic myofilaments selective proteins, such as myosin heavy chain (MHC), alpha-sarcomeric cardiac actin (Actin) and cardiac tropomyosin alpha 1 (Tm), and some of their specific sites, such as MHC S844, S1471, S1472, T1601 and S1917, Actin T326 and Tm S87, have indeed excessive O-GlcNAcylation. More importantly, we demonstrated that specific removal of excessive O-GlcNAcylation, from a subset of myofilaments sites via CpOGA (see Fig. 1C), rapidly restores myofilament Ca\(^{2+}\) sensitivity, thus correcting myofilament dysfunction in STZ-diabetic skinned muscles. The advantage of our approach, i.e. the use of recombinant CpOGA on skinned cardiac muscles, resides in the ability of assessing the functional consequences of O-GlcNAcylation removal from myofilaments independently from other major adverse effects eventually imposed by O-GlcNAcylation on Ca\(^{2+}\) handling and mitochondrial key proteins (2; 4; 15; 24; 45-48). Thus, here we cement the view that excessive O-GlcNAcylation is a novel, negative modulator of myofilament function in the diabetic myocardium. At the same time, our data suggest, for the first time, that removing excess of O-
GlcNAc from diabetic myofilaments specific sites mimics what dephosphorylation does on Ca\textsuperscript{2+} sensitivity in experimental (49) and human heart failure (50-53).

Cardiac myofilament phosphorylation and function is regulated by multiple kinases and phosphatases (54; 55). They strategically dock at the Z-line and modify their activity and/or localization in response to mechanical forces and neuro-humoral stimuli (54; 55). The displacement of these enzymatic activities may have important functional repercussions on the contractile apparatus (39). For instance, at baseline, protein phosphatase 2A (PP2A) forms a complex with protein phosphatase 2B (PP2B) and p38-MAPK, but it moves away from the Z-line upon β-adrenergic stimulation (55). Whether similar effects can be attained by changes in the sarcomeric localization of OGT and OGA is currently known. Here we report that, in normal hearts, OGT and OGA distribute mainly at the Z-line and A-band, respectively. However, in diabetic hearts, OGT drifts away from the Z-line, whereas OGA appears even more clustered at this site. Importantly, these changes are similarly evident in rat and human diabetic myocardium. It is well known already that OGT localization and catalytic activity changes in response to metabolic cues (12; 56). OGT and OGA often occur in transient protein:protein complexes containing kinases and phosphatases (12; 56). These include kinases and phosphatases such as AMP-activated protein kinase (AMPK)(56), Ca\textsuperscript{2+}/calmodulin-dependent protein kinase IV (CaMKIV), p38-MAPK, protein phosphatase 1 (PP1) and myosin phosphatase targeting 1(MYPT1) that are key regulators of cardiac metabolism and function (12). Although, these protein interactions have not been confirmed in the heart, a recent report elegantly shows that CaMKII is O-GlcNAcylated during acute hyperglycemia and diabetes, leading to chronic activation, which contributes to diabetes cardiac mechanical dysfunction and arrhythmias (24). Present data show that, in the heart, OGT and OGA form complexes with α-cardiac actin, α-tropomyosin and myosin light chain 1, and that these
protein:protein complexes are enhanced for OGA in diabetes. These alterations are potentially relevant to the pathogenesis of diabetic systolic and diastolic dysfunction, given that transient protein:protein complexes regulate OGT substrate specificity (12). Since O-GlcNAcylation and phosphorylation signaling cascades have extensive cross-talk (12; 56), both at the level of site occupancy and at the level of modifying enzymes, it would be important to further dissect this phenomenon in the heart.

Previously, in diabetic hearts OGT expression (40; 57; 58) and activity (59) have been found to be normal, whereas OGA activity is reduced, with or without reciprocal changes in expression (57-59). Here, we analyzed myofilament and cytosolic fractions separately. We found that in diabetic hearts OGT expression is not different between diabetic and control hearts. Due to OGA inhibition by traces of Triton X-100, OGA expression and activity were analyzed only in total homogenates, in which OGA expression was not different between groups. In addition, our study accounts for OGT and OGA activity background in a more rigorous way. Indeed, OGT activity without CKII peptide and OGA activity with Thiamet-G (a specific OGAsel inhibitor) were subtracted. Our myofilament vs. cytosolic compared data showed that in diabetic hearts OGT enzymatic activity is reduced in the myofilament but not in the cytosolic fraction. In contrast, OGA activity in total homogenates is significantly increased in diabetic hearts (Fig.6F). This apparent paradoxical decrease of OGT activity in diabetic hearts could be explained by the way we accounted for the background activity, sub-cellar fractionation, or by changes in OGT substrate specificity in response to metabolic cues (30). Increased OGA activity may reflect compensation for excessive O-GlcNAcylation. Supporting this possibility is previous evidence showing that increased erythrocyte protein O-GlcNAcylation and OGA activity is present in samples from pre-diabetic and diabetic type 2 patients (60).
Cardiac contractility is regulated on a beat-to-beat basis by the Ca\textsuperscript{2+}-dependent modulation of myosin cross-bridge binding on actin by the tropomyosin-troponin complex. Cross-bridge cycling occur at the A-band (61), while most of the signaling arise from the Z-line (54; 55; 62). Thus, re-targeting of OGT and OGA may increase α-cardiac actin O-GlcNAcylation, at the A-band, potentially interfering with actin-tropomyosin modulation of cross-bridge cycling (Fig.7B). Altogether, these data suggest that in diabetic hearts OGT and OGA displacement rather than their catalytic activity is key in modulating excessive O-GlcNAcylation.

There are limitations in the present study. First, more work is needed to define the functional effect of myofilament O-GlcNAcylation at the site-specific level, along with their interplay with phosphorylation. Second, future studies should address whether excessive α-cardiac actin O-GlcNAcylation indeed perturbs cross-bridge cycling kinetics, actin-tropomyosin interactions or actin rate of polymerization. Finally, it is not known if O-GlcNAc modifications or phosphorylation of specific substrates or in OGT/OGA contribute to their altered pattern of localization. Answering this question would define more in detail the impact of this post-translational modification on function of diabetic hearts; however, it requires fully dedicated, separate studies.

**CONCLUSIONS**

Present work demonstrates that abnormal O-GlcNAcylation is sufficient to cause myofilament functional deficit in diabetic cardiomyopathy. Increased OGA interactions with sarcomeric proteins (α-cardiac actin, α-Tm, and MLC-1) are likely central to these alterations, showing that normal sarcomeric OGT and OGA sub-cellular localization is lost in myofilaments from diabetic hearts. This abnormal redistribution of O-GlcNAc cycling enzymes is similarly
present in experimental and human diabetic cardiomyopathy. On these grounds, here we propose that, in addition to direct targeting of abnormal site-specific phosphorylation of myofilament regulatory subunits, removing abnormal site-specific O-GlcNAcylation in myofilaments and/or preventing changes in O-GlcNAc cycling should be considered as another promising new therapeutic avenue for treating diabetic cardiomyopathy.

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FIGURE LEGENDS

Figure 1. Removing abnormal O-GlcNAcylation restores myofilament Ca\(^{2+}\) sensitivity in diabetic cardiac muscle. A, O-GlcNAc Western Blots demonstrate increased O-GlcNAcylation on STZ diabetic heart homogenates. B, Steady-state Force-[Ca\(^{2+}\)]\(_o\) relationship in skinned diabetic and control cardiac. STZ diabetic muscles Pre-CpOGA (n= 5) and STZ Post-CpOGA (n= 4), Control Pre-CpOGA (n= 6) and Control Post-CpOGA (n=5). (Lower panel) Comparison of EC\(_{50}\) for all groups. Maximal force (F\(_{\text{max}}\)) and Hill coefficient (n). C, Proteomic identification and quantification of site-specific O-GlcNAc changes that are statistically significant (generalized linear model approach) upon CpOGA treatment of STZ diabetic myofilaments but not on CpOGA treatment of control myofilaments. Highlighted in red rectangles are the sites that are significantly more O-GlcNAcylated in diabetic hearts. Statistical analysis employed were moderated t-statistics, log-odds ratios of differential expression (B-statistics), raw and adjusted p-values (FDR control by the Benjamini and Hochberg method).

Figure 2. Diabetic myocardium displays increased O-GlcNAcylation. A, Rat control and STZ diabetic myocardium cryosections were immunostained for O-GlcNAc and nuclei (DAPI). B, Quantification of O-GlcNAc immunofluorescence, channel intensity normalized to area (µm\(^2\)) shows significant increase in STZ diabetic. C, Immuno-electron microscopy for O-GlcNAc shows signal redistribution, from mainly Z-line towards A-Band. D, Relation of immuno-gold particles and distance from Z-Line. E, Human donor and diabetic myocardium cryosections display O-GlcNAc sarcomeric distribution and its increase in diabetic heart.

Figure 3. OGT and OGA mislocalization in STZ diabetic myocardium. A, Rat control and STZ diabetic myocardium cryosections were immunostained for α-actinin (green), OGT (magenta) and OGA (red). C, D, Regions of interest (white square and dashed line) were used to plot the signals of OGT or OGA and α-actinin, red arrows show Z-line. E, OGT and α-actinin co-localization parameters show significant differences 1) weighted coefficient, 2) overlap coefficient, 3) Pearson’s correlation R. F, OGT and OGA co-localization parameters show significant differences 1) overlap coefficient, 2) Pearson’s correlation R and 3) Correlation (R\(^2\)). Images were acquired with a Zeiss 710 LSM Meta confocal microscope. NIH Image J and Origin 8.0 software were used to plot OGT or OGA with α-actinin as relative intensity profiles.

Figure 4. OGT and OGA distribution profile in Human and Rat control and diabetic hearts. A, Human donor and diabetic myocardium cryosections were immunostained for α-actinin (green), OGT (magenta) and OGA (red). Regions of interest (white square and line) were chosen to plot OGT, OGA and α-actinin signals. Signals from donor or diabetic myocardium are display as double Y-axis graphs to illustrate the inversed distribution profile of OGT/ α-actinin and OGA/ α-actinin, respectively. B, Rat control and diabetic skinned myocytes were isolated from flash-frozen myocardium, fixed in 4% ultra pure formaldehyde and immunostained as above. Regions of interest and OGT, OGA and α-actinin signals were plotted as above and confirmed the abnormal distribution profile of OGT and OGA in diabetic hearts.
Figure 5. Differential OGT and OGA sub-cellular localization and myofilament interactions in control and diabetic myocardium. A, Representative transmission electron microscopy images of control and B, STZ diabetic rat myocardium. Ultrathin sections were immuno-EM with primary antibodies (anti-OGT AL-28 and anti-OGA), and secondary antibodies gold labeled (anti-rabbit 12 nm and anti-chicken 6 nm). Symbols; Z-line (green arrow head), OGT (purple circles), OGA (red circles), OGT and OGA in close vicinity (pink), OGT (purple arrow head) and OGA (red arrow head). C, D Quantification of OGA and OGT number of particles/field in 9 fields shows an increase for OGT (2± 0.6 vs 8.6± 2.6, *p ≤ 0.028) and OGA (9.4± 2.9 vs 37.6± 12.6, *p ≤ 0.05) immuno-EM in STZ diabetic myocardium. Images were analyzed in ImageJ (NIH). E, F, Representative co-immunoprecipitations of OGT and OGA followed by western blots for α-sarcomeric actin, tropomyosin and myosin light chain 1. A fraction of the inputs Controls (C1, C2) and STZ (S1, S2) and agarose beads with no antibody (M) or isotype specific normal antibody + agarose beads (1°). G, Analysis of integrated signal density of myofilament immunoreactivity normalized to total immunoprecipitated OGT displayed a trend towards increased interactions with Tm and MLC1, H, OGA co-IP shows that diabetic STZ rats displays several fold increased associations with α-actin, α-Tm and MLC1 (*p ≤ 0.05).

Figure 6. Sub-cellular expression and activity of OGT and OGA in diabetic hearts. A, Analysis of OGT expression in myofilament fractions, B, and in cytosolic fraction showed no significant differences. C, OGA expression tended to be lower in myocardium total homogenates from STZ, however did not reach statistical significance, D, OGT activity showed a significantly decrease in myofilament fractions from STZ. E, OGT activity assays in cytosolic fractions showed no significant difference. F, OGA assays for specific activity in total heart homogenates showed a significant increase in STZ.

Figure 7. Model of O-GlcNAc role in diabetic cardiomyopathy pathogenesis. A, General characteristics of OGT and OGA. B, OGT/OGA localization in normal and diabetic sarcomeres, functional effect of O-GlcNAc specific removal by OGA like CpOGA. C, Graphical representation of OGT activity towards CKII peptide or endogenous targets, and OGA total or Specific Activity when normalized to residual activity in the presence of ThiametG.
A

WB O-GlcNAc

| Co | STZ |
|----|-----|
|     |     |

MWM
170 kD
130 kD
100 kD
70 kD
40 kD
25 kD

WB Actin

O-GlcNAc/Actin

Con STZ

< 0.05

B

Con

STZ

$F_{\text{max}}$ (mN/mm$^2$)

$F_{\text{max}}$ (mN/mm$^2$)

Ca$^{2+}$ 10$^{-6}$ M

Pre-CpOGA

Post-CpOGA

Control

STZ

C

MHC

| S740 | S844 | S1414 | S1465 | S1471 | S1472 | S1598 | T1601 | S1602 | S1778 | S1917 | S54 | T326 | S87 |
|------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-----|-----|-----|
| 17.5 | 17.5 | 15.0  | 15.0  | 12.5  | 12.5  | 10.0  | 10.0  | 12.5  | 12.5  | 10.0  | 10.0 | 10.0 | 10.0 |

Actin

| Tm |
|----|
| S87 |

TMT Peptide Intensity

Con STZ

< 0.05
A. O-GlcNAc Transferase (OGT) and O-GlcNAcase (OGA) domains and their functions.

B. Euglycemic and Hyperglycemic conditions and their effects on O-GlcNAc transfer and myofilament Ca^{2+} sensitivity.

C. OGT Peptide Target and OGT Endogenous Targets with their respective activities and reactions.
Table I.- O-GlcNAcylated peptides and their corresponding amino acid sites in normal and diabetic cardiac myofilaments.

| Myosin heavy chain 6, P02563 | Actin, alpha cardiac, P68035 |
|-----------------------------|-----------------------------|
| **Peptide ID** | **AA** | **Peptide ID** | **AA** |
| R.*TNCFVPDDKEEYVK.A | T35 | R.AVFP*SIVGR.P | S34 |
| K.V*TAETENGK.T | T60 | K.D*SYVGDGAQSK.R | S54 |
| R.ENQ*SLITGESGAGK.T | S173/T177 | K.DSVYVGDGAQSK.R | S62 |
| Q.SILI*TGE*SGAGK.T | T177/S180 | R.GY*SFVTTEA.R.E | S201 |
| F.A*SIAAILG.DR.S | S197 | A.TAA*SSSSLEK.S | S234 |
| K.TVRNND*S*SRS.RF | S241/S242 | K.YELPQDGQVITIGNER.F | S241 |
| Y.A*S ADTGDSGKG. | S627 | K.EITALAP*S*T MK.I | S325/T326 |
| N.PAAIPQGFID*S R.K | S740 | K.QEYDEAGP*SIVHR.K | S370 |
| K.*SAETEK.E | S844 | **Myosin regulatory light chain 1, P16409** |
| R.IEDEQALG*SOLQK.K | S1102 | **Peptide ID** | **AA** |
| R.*S DLTR.E | S1139 | K.EAIEDA*SCK.I | S45 |
| R.ELEE*I*SER.L | S1149 | K.ALGQNP*TQAEVL.R | T93 |
| R.SVNDL*T*SQR.A | T1274/S1275 | **Tropomyosin alpha 1,** |
| K.LQTENGEL*S R.Q | S1288 | **Peptide ID** | **AA** |
| K.EALI*SOLQ.TR.G | S1301/T1304 | K.ATDADAV*SRLR.R | S87 |
| K.AN*SEVAQWR.T | S1368 | K.AAED*SER.G | S123 |
| K.C*S*SEKTK.H | S1414/S1415 | K.VIE*SR.A | S132 |
| R.*SNAAAALDK.K | S1437 | R.AE**SRG.K | S186 |
| K.YEE*SQ*S ELESSQK.E | S1465/S1467 | K.*SLEAQAEK.Y | S206 |
| E.SQSELE*S SQK.E | S1469 | **Cardiac Troponin I, P23693** |
| E.SQSELE*S SQK.E | S1471/S1472 | **Peptide ID** | **AA** |
| R.VVD*SLQ*T*S LDAETRS | S1598/T1601/S1602 | E.*SSDSAGEPPAPPRR.R | S5 |
| Q.*TSLDREATRS | T1601 | S.*SD*SAGEPPAPAPPR.R | S6/S8 |
| R.IA*SEAAK.RH | S1638 | K.ISA*SR.R | S43/S45 |
| R.AVEQ*T ER.S | T1697 | R.VL*STR.C | S78 |
| K.LAEQELIE*T*SER.V | T1711/T1712 | K.E*S LDLR.A | S167 |
| K.EQD*T*SAHLER.M | T1777/T1778 | **Cardiac Myosin Binding Protein C, P23693** |
| R.NAE*SVK.G | S1838 | **Peptide ID** | **AA** |
| R.ADIAE*SQVNKL | S1917 | R.DG*SDIAANDK.Y | S47 |