Supplemental Material

METHODS

Human heart samples

Left ventricular tissue samples from end-stage heart failure patients with idiopathic dilated cardiomyopathy (IDCM) or ischemic heart disease (ISHD) were obtained during heart transplantation surgery.[1] Tissue from donor hearts served as reference for non-failing myocardium. Samples were obtained after informed consent and with approval of the local Ethical Committee (St Vincent's Hospital Human Research Ethics Committee, Sydney, Australia: File number: H03/118; Title: Molecular Analysis of Human Heart Failure) and by The University of Sydney HREC number 12146. The investigation conforms with the principles outlined in the Declaration of Helsinki (1997).

Semi-purification of cMyBP-C from human heart tissue

cMyBP-C was isolated from donor as well as failing human cardiac tissue as previously described.[2]

Tandem mass spectrometry

1D SDS-PAGE gel lanes were cut into 2-mm slices using an automatic gel slicer and subjected to in-gel reduction with dithiothreitol, alkylation with iodoacetamide and digestion with trypsin (Promega, sequencing grade), essentially as described by Wilm *et al.*.[3] Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ-Orbitrap mass spectrometer (Thermo), operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm × 100 µm, packed in-house) at a flow rate of 8 µl/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm × 50 µm, packed in-house) using a linear gradient from 0 to 80% B (A = 0.1 % formic acid; B = 80% (v/v) acetonitrile, 0.1 % formic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode, either by CID or ETD. Peak lists were automatically created from raw data files using the Mascot Distiller software (version 2.3; MatrixScience). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against the IPI_human database (release IPI_human_20100821.fasta). The peptide tolerance was set to 10 ppm and the fragment ion tolerance was set to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 65. Individual peptide MS/MS spectra with Mascot scores below 25 were checked manually and either interpreted as valid identifications or discarded.

Antibody development

To quantify phosphorylation at Ser133 a custom phosphorylation site specific polyclonal antibody was raised in rabbits against the peptide (LGESAP-S(PO_3H_2)-PKGSS) corresponding to amino acids 127-138 of the human cMyBP-C sequence by Eurogentec (Eurogentec, the Netherlands). The antibodies were passed through on an affinity

chromatography column coated with the unphosphorylated peptide to remove antibodies that recognize unphosphorylated cMyBP-C.

Kinase prediction
Kinase prediction was performed using the publicly available NetPhosK program[4] (*http://www.cbs.dtu.dk/services/NetPhosK/*), using a 21 amino acid region (amino acids 120-140) as input.

Immunoblotting
Immunoblotting with phospho-specific antibodies was performed as described before.[5] Specific antibodies were used directed against known phosphorylation sites of cMyBP-C (Ser275, Ser284 and Ser304) and our custom made antibody against phosphorylated Ser133.

Phos-tag analysis β-catenin phosphorylation
Phos-tag™ acrylamide (FMS Laboratory; Hiroshima University, Japan) was used to visualize unphosphorylated and phosphorylated β-catenin species using alkoxide-bridged dinuclear metal (Mn^{2+}) complex as phosphate-binding tag (Phos-tag) molecule.[1, 6] Mn^{2+}-Phos-tag molecules preferentially capture phosphomonoester dianions bound to Ser, Thr and Tyr residues. Non-phosphorylated and phosphorylated β-catenin species were separated in 1D-PAGE with polyacrylamide-bound Mn^{2+}-Phos-tag and transferred to Western blots. Phosphorylated β-catenin species in the gel are visualized as slower migration bands compared to the corresponding unphosphorylated β-catenin form.

In vitro kinase assays
Recombinant C0C2 fragment was incubated for 2 h at 37°C in relax solution (pH 7.0; in mmol/L: free Mg^{2+} 1, KCl 145, EGTA 2, ATP 4, imidazole 10) with 750U PKA (Calbiochem) or 10 μl GSK3β (Sigma Aldrich). This reaction was stopped using 2D clean-up kit (GE Healthcare).

Preparation of a rough cytosolic extract from human heart tissue
Rough cytosolic extracts of donor heart tissue was prepared by dicing 100 mg of frozen human donor heart tissue in small pieces in relax solution (pH 7.0; in mmol/L: free Mg^{2+} 1, KCl 145, EGTA 2, ATP 4, imidazole 10) with protease inhibitor cocktail (Roche). To remove remaining blood, the diced tissue is centrifuged (10 min, 13k rpm) and supernatant is discarded. The pellet is resuspended in relax solution + protease inhibitor, homogenized and centrifuged (13k rpm, 10 min). The supernatant (containing all soluble components of the tissue) was considered the rough cytosolic extract.

Incubation of recombinant 40kDa cMyBP-C fragment with rough cytosolic extract
To test whether kinases from rough cytosolic extracts can phosphorylate cMyBP-C at Ser133, recombinant 40kDa cMyBP-C fragment (corresponding to amino acids 1-271 of the human cMyBP-C sequence. It was generated using the pET-system as previously described[7]). To determine the extent to which GSK3β from the rough cytosolic extract contributes to Ser133 phosphorylation, incubations were carried out with and without GSK3β antagonist CT99021 (Selleck Chemicals). CT99021 was used at a final concentration of 2 μM.[8] Incubations were carried out for 2 h at 37°C.

Force measurements
To assess if GSK3β alters kinetics of sarcomere force development, the rate of force redevelopment was measured before and after GSK3β treatment in Triton-permeabilized

cardiomyocytes as described previously.[9] Force measurements were performed at maximal [Ca^{2+}] and a sarcomere length of 2.2 µm before and after incubation of cells for 60 min at 20ºC in relaxing buffer supplemented with kinase buffer (pH 7.0; in mmol/L: Na phosphate 50, NaCl 300, imidazole 150, PMSF 0.1, DTT 0.2 and glycerol 25%(v/v)) with or without 20 µl GSK3β (total volume 100 µl). The maximal rate of force redevelopment (max k_{tr}) was determined from an exponential curve fit of force redevelopment after a slack test in activating solution with maximal [Ca^{2+}].[9]

References

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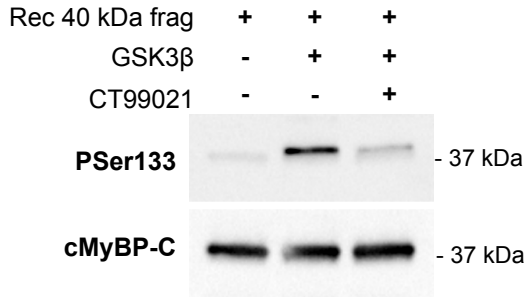
Online Figure I



Online Figure I. CT99021 blocks GSK3β mediated phosphorylation of Ser133. Incubation of recombinant human 40 kDa fragment with GSK3β leads to an increase in Ser133 phoshorylation. Addition of 2 μM CT99021 leads to an almost complete blockade of Ser133 phosphorylation, illustration CT99021's potency.