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A Remarkable Adaptive Paradigm Of Heart Performance And Protection Emerges In Response To The Constitutive Challenge of Marked Cardiac-Specific Overexpression Of Adenylyl Cyclase Type 8.

A REMARKABLE ADAPTIVE PARADIGM OF HEART PERFORMANCE AND PROTECTION EMERGES IN RESPONSE TO THE CONSTITUTIVE CHALLENGE of MARKED CARDIAC-SPECIFIC OVEREXPRESSION OF ADENYLYL CYCLASE TYPE 8

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Title: Proteome Analysis of TGAC8 and WT mouse Left Ventricles.

Description:

Four LV samples from WT and TGAC8 mouse hearts were analyzed. A subset of 8 muscle samples each corresponding to 4 controls and 4 TGAC8 LVs and one averaged reference sample were labeled with 10-plex tandem mass spectrometry tags (TMT) using standard TMT labeling protocol (Thermo Fisher).

TGAC8 labeling: TMT-126; TMT-127N; TMT-127C; TMT-130N

WT labeling: TMT-128N; TMT-128C; TMT-129N TMT-129C

LV Proteome analysis.

Four LV samples from WT and TG^{AC8} mouse hearts were snap frozen in liquid nitrogen and stored at -80°C. On average, 2 mg of muscle tissue from each sample was pulverized in liquid nitrogen and mixed with a lysis buffer containing (4% SDS, 1% Triton X-114, 50 mM Tris, 150mM NaCl, protease inhibitor cocktail (Sigma), pH 7.6. Samples were sonicated on ice using a tip sonicator for 1 min with 3 sec pulses and 15 sec rest periods at 40% power. Lysates were centrifuged at +4°C for 15 min at 14000 rpm, aliquoted and stored at -80°C until further processing. Protein concentration was determined using commercially available 2-D quant kit (GE Healthcare Life Sciences). Sample quality was confirmed using NuPAGE® protein gels stained with fluorescent SyproRuby protein stain (Thermo Fisher).

In order to remove detergents and lipids 500 µg of muscle tissue lysate was precipitated using a methanol/chloroform extraction protocol (sample:methanol:chloroform:water – 1:4:1:3).[5] Proteins were resuspended in 50 ul of concentrated urea buffer (8M Urea, 150 mM NaCl (Sigma)), reduced with 50 mM DTT for 1 hour at 36°C and alkylated with 100 mM iodoacetamide for 1 hour at 36°C in the dark. The concentrated urea/protein mixture was diluted 12 times with 50 mM ammonium bicarbonate buffer, and proteins were digested for 18 hours at 36°C, using trypsin/LysC mixture

(Promega) in 1:50 (w/w) enzyme to protein ratio. Protein digests were desalted on 10 x 4.0 mm C18 cartridge (Restek, cat# 917450210) using Agilent 1260 Bio-inert HPLC system with a fraction collector. Purified peptides were speed vacuum dried and stored at -80°C until further processing.

A subset of 8 muscle samples (100 µg) each corresponding to 4 controls and 4 TGAC8 LVs and one averaged reference sample were labeled with 10-plex tandem mass spectrometry tags (TMT) using standard TMT labeling protocol (Thermo Fisher). 200 femtomole of bacterial beta-galactosidase digest (SCIEX) was spiked into each sample prior to TMT labeling to control for labeling efficiency and overall instrument performance. Labeled peptides from 10 different TMT channels were combined into one experiment and fractionated.

High-pH RPLC fractionation and concatenation strategy

High-pH RPLC fractionation was performed in an Agilent 1260 bio-inert HPLC system using a 3.9 mm X 5 mm XBridge BEH Shield RP18 XP VanGuard cartridge and a 4.6 mm X 250 mm XBridge Peptide BEH C18 column (Waters). The solvent contained 10mM ammonium formate (pH 10) as mobile phase (A), and 10mM ammonium format and 90% ACN (pH 10) as mobile-phase B 9.

TGAC8 labeling: TMT-126; TMT-127N; TMT-127C; TMT-130N

WT labeling: TMT-128N; TMT-128C; TMT-129N TMT-129C

TMT labeled peptides prepared from the ventricular muscle tissues were separated using a linear organic gradient from 5% to 50% B over 100 min. Initially, 99 fractions were collected at 1 min intervals. Three individual high-pH fractions were concatenated into 33 master fractions at 33 min intervals between fractions (fraction 1, 34, 67 = master fraction 1, fraction 2, 35, 68 = master

fraction 2 and so on). Combined fractions were speed vacuum dried, desalted and stored at -80°C until final LC-MS/MS analysis.

Capillary nano-LC-MS/MS analyses

Purified peptide fractions were analyzed using UltiMate 3000 Nano LC Systems coupled to the Q Executive HF Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA). Each fraction was separated on a 35 cm capillary column (3µm C18 silica, Hamilton, HxSil cat# 79139) with 200 µm ID on a linear organic gradient at a 500 nl/min flow rate. Gradient applied from 5 to 35 % in 205 min. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. Tandem mass spectra were obtained using Q Exactive HF mass spectrometer with a heated capillary temperature +280°C and spray voltage set to 2.5 kV. Full MS1 spectra were acquired from 300 to 1500 m/z at 120000 resolution and 40 ms maximum accumulation time with automatic gain control [AGC] set to $3x10^6$. Dd-MS2 spectra were acquired using a dynamic m/z range with fixed first mass of 100 m/z. MS/MS spectra were resolved to 30000 within of a maximum accumulation time, 120 ms with AGC target set to $2x10^5$. Twelve most abundant ions were selected for fragmentation using 28% normalized high collision energy. A dynamic exclusion time of 45 sec. was used to discriminate against the previously analyzed ions.

Bioinformatics Analysis of the LV Proteome

Acquired raw data files from Q Exactive HF were converted to mascot generic format (MGF) using MSConvert, an open-source software developed by ProteoWizard (http://proteowizard.sourceforge.net). Conversion filters were specified as follows: at MS level 1 with activation:HCD, threshold:count 900 most-intense, zeroSamples:remove Extra 1-, peakPicking;true 1-. Produced MGF files were searched in Mascot against the SWISS-PROT mouse database (02/06/2017) with the following parameters: enzyme trypsin/P, 2 missed

cleavages, MS1 tolerance 20 ppm, MS2 tolerance 0.08 Da, quantification TMT10plex. Variable modifications were set to methionine oxidation, carbamidomethylation of cysteines, deamidation at glutamine and asparagine, carbamylation of lysin. Searched mascot data files were processed using commercially available Scaffold Q+ software package (Proteome Software, Inc). Files were merged in to one summary file using MudPIT algorithm, and researched against SWISS-PROT mouse database (02/06/2017), using XTandem search engine for deeper protein coverage with both protein prophet scoring algorithm and protein clustering analysis turned on. Raw reporter ion intensities from unique peptides were extracted into excel file and used in the final analysis.

Minor variations in protein amounts between TMT channels was adjusted by calculating a ratio between signal intensity in each TMT channel (In). Adjusted intensity for each channel (Incorr) was calculated by taking a sum of all intensities in each TMT channel divided by the average (μ) of all calculated sum intensities and multiplied by the initial intensity in each TMT cannel for each peptide: $(\sum I126+\sum I127+\sum I128+\sum I129+\sum I130+\sum I131)/6=\mu$; $\sum In/\mu * In=Incorr$. Fold-change for each unique peptide in each experiment was calculated by dividing Incorr by the median of all intensities in all TMT channels. The fold change between genotypes for each expressed protein was calculated by taking a median of fold change for all unique peptides of a given protein detected by mass spectrometry. Genotype differences were compared via Student's t-test. A P value < 0.05 was considered to be significant