Methods:

Animal model

Hartley guinea pigs (~300 g; HillTop Lab Animals) were housed in the animal facility at the Johns Hopkins University. This study conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Johns Hopkins Animal Care and Use Committee. Male animals were anesthetized with 4% isoflurane in a closed box for 4 min, and then intubated. Animals were ventilated with oxygen and 2% isoflurane. Ascending aortic constriction (AC) was produced by tying a suture around the ascending aorta using an 18-gauge needle as a spacer, which was then removed. Sham operation was performed following the same procedure without the ligation. Daily bolus of Isoproterenol (ISO) was administered via a programmable iPRECIO® pump (SMP-200, Data Science International, St. Paul MN) that was implanted in the peritoneal cavity. The pump was programmed for 1-hour daily delivery of Isoproterenol for a dose of 1 mg/kg/day. For the treatment of SB203580, an osmotic pump (Alzet Osmotic pump, model 2004, (Cupertino, CA) was implanted in the abdominal cavity. SB203580 was delivered continuously at 0.5 mg/kg/day DA. A vehicle filled osmotic pump was implanted for control in ACi group. The following treatment group were studied: 1. Sham-operated, serving as controls 2. ACi (Aortic constriction +Isoproterenol treatment till end point) 3. ACi-SB (Aortic constriction +Isoproterenol treatment+ SB203580 treatment) All personnel involved in data collection and analysis were blinded to the treatment and non-treatment groups. Both groups received similar incisions and thus, could not be distinguished based on these interventions. Each animal was assigned a unique computer-generated numeric ID.

31 *Echocardiography*

32

33 Transthoracic echocardiography was performed on conscious non-anesthetized guinea
34 pigs by using a Vevo 2100 high-resolution in vivo imaging system with 24 MHz transducer
35 (VisualSonics, Toronto, ON, Canada) and analyzed with the Advanced cardiovascular
36 package software (VisualSonics).

37

38 *Data and statistical analysis*

39

40 For heart weight, lung weight and FS% analysis between groups, one-way analysis of
41 variance (ANOVA) followed by Tukey's post hoc analysis was used.

42

43 *Proteomic Studies*

44 *Protein extraction and Immunoblotting*

45

46 Ventricles were harvested. Tissues were rinsed in cold PBS, rapidly heat-stabilized
47 (Stabilizor™, Denator, Inc.), snap-frozen in liquid nitrogen, and stored in a -80 freezer. To
48 extract protein, stabilized tissues were homogenized with RIPA buffer in the presence of
49 2% SDS, solubilized, and boiled in 1x LDS sample buffer for SDS-PAGE. The protein
50 mixture was separated on a 4-12% NuPAGE gel (1 mm, Invitrogen). Samples were run
51 at room temperature for 35 min at 200 V. Proteins were transferred to nitrocellulose
52 membranes with iBlot (Invitrogen, Inc.), using program 3 for 7 min. Membranes were
53 stained with Ponceau S solution (Sigma-Aldrich) to evaluate the transfer efficiency.
54 Membranes were blocked for 1 h using Odyssey® blocking buffer (Li-Cor Biosciences)
55 and incubated with primary antibody overnight at 4°C. Antibody binding was visualized
56 with an infrared imaging system using IRDye secondary antibodies and quantification of
57 band intensity was performed using the Odyssey Application Software 3.0.

58

Antibodies	**Brand**	**Cat #**	**Species**	**Type**	**Dilution**

p38 MAPK Antibody	Cell Signaling Technology	9212	Rabbit	primary	1:1,000
Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb	Cell Signaling Technology	4511	Rabbit	primary	1:1,000
IRDye® 800CW Goat anti-Rabbit IgG Secondary Antibody	Li-Cor	926-32211	Goat	secondary	1:10,000

Densitometric analysis of p38 and phosphorylated p38 (p-p38) signals in three distinct groups was conducted using Image J software, with data derived from digitized .tif image files. These signals were subsequently normalized to total protein levels detected via Ponceau staining. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, to evaluate the comparison of p-p38/p38 ratios across the different groups.

Sample Preparation, Proteolysis and TMT labeling

The experiment compared 3 experimental groups: 1) Sham-operated controls, 2) ACi failing hearts (at 4 weeks) and 3) ACi animals treated concomitantly with SB203580 (0.5 mg/kg/day).

Sample Preparation, Proteolysis and TMT labeling

Hearts were harvested and washed with ice cold phosphate-buffered saline. The left ventricle free wall was isolated and immediately subjected to heat denaturation to abolish all enzyme activity using the Denator Stabilizor System (Denator) according to the manufacturer's instructions. Denatured samples were then stored at -80° C until the next step. The left ventricle of each guinea pig heart was homogenized with a handheld Polytron tissue disrupter in filtered and deionized, Tris-buffered 9M urea (5mL), pH 7.5 Samples were allowed to solubilize for 30 min at room temperature. Soluble homogenates

82 were subjected to methanol/chloroform/water extraction and protein precipitation by the
83 method of Wessel & Flugge(1). Samples were dried under nitrogen gas to remove
84 residual chloroform before resolubilizing for 30 min in 9M urea. Aggregates were
85 disrupted by brief bursts of sonication (< 30s total). Peptides were diluted 6-fold into 60
86 mM HEPES, 0.6 mM DTT, pH 7.5 such that the final reaction buffer contained 50 mM
87 HEPES, 1.5 M urea, 0.5 mM DTT, pH 7.5. All samples were diluted further with reaction
88 buffer to a common final protein concentration of 1mg/mL. Samples (1mg) were subjected
89 to proteolytic digestion with proteomics grade Trypsin (1µg/100µg protein; Promega) at
90 room temperature, overnight. The following morning, samples were supplemented with
91 Trypsin (1µg/100µg protein; Promega). At that time, DTT was added to samples at a
92 concentration of 5 mM and the digest was allowed to proceed for 1 hour prior to peptide
93 alkylation by addition of iodoacetamide to a final concentration of 15 mM. Alkylation was
94 allowed to proceed 1 hour at room temperature in the dark. Peptides were subsequently
95 acidified by addition of trifluoroacetic acid to a final concentration 0.5% (v/v) and purified
96 by solid phase extraction using SepPak tC18 cartridges (Waters) on a vacuum manifold.
97 Purified peptides were eluted with 60% (v/v) acetonitrile in aqueous 0.1% (v/v) formic
98 acid. Peptides were evaporated to dryness on an Eppendorf Vacufuge. Peptide samples
99 were resolubilized in triethylammonium bicarbonate (TEAB) pH 8.5 and labeled with TMT
100 reagents according to the manufacturer's instructions.

101

102 *Chromatography & Mass Spectrometry*

103 Following TMT labeling of individual samples, peptides were pooled and subjected to
104 high-pH reversed-phase liquid chromatography (bRP-HPLC) (2), as detailed by Foster et
105 al. (3). Briefly, samples were fractionated at 250ul/min on a Waters BEH C18 column with
106 a gradient running from 10mM TEAB to 90% Acetonitrile, 10mM TEAB over 105 minutes.
107 The fractions were concatenated into 24 fractions of which 20% of each was taken for the
108 expression proteome determination. The remaining 80% was combined into 11 fractions
109 and TiO_2 enrichment was performed to assess the phosphoproteome.

110

111 Following concatenation, samples were analyzed using a nanoAquity nanoLC system
112 (Waters) interfaced with an Orbitrap Fusion Lumos Tribrid Mass Spectrometer

113 (ThermoFisher Scientific). Peptides were injected onto a 2-cm trap column at 5 μL/minute
114 for 6 minutes before being eluted onto a 75 μm×15 cm in-house packed column (Michrom
115 Magic C18AQ, 5 μm, 100Å) operating at 300 nL/min. Each sample was run on a 90-
116 minute gradient. Data were acquired using a 3 second cycle between MS1 scans in FT-
117 FT acquisition mode. The survey full-scan MS (400-1600 Th) was performed at a
118 resolution 120,000 with an automatic gain control target ion intensity of $4x10^5$, while MS^2
119 scans were performed at a resolution of 50,000 with a target value of $1.25x10^5$. Mass
120 tolerances for were +/- 10ppm for MS1. Maximum injection times were set to "Auto" for
121 MS1 and 86 ms for MS^2. A 0.7 Dalton isolation window was used and HCD fragmentation
122 was performed with a normalized collision energy of 36. Internal calibration was set to
123 Easy-IC. Spectra whose charge was unassigned or +1 were not tabulated. Dynamic
124 exclusion was set to a repeat count of 1 with a 15s exclusion time.
125
126 *Protein Identification*
127
128 The .Raw files for both the enriched and unenriched (12 + 24 respectively) were searched
129 against a guinea pig database of predicted protein sequences (NCBI RefSeq, taxonomy:
130 Cavia porcellus, date:01/20/2021, FASTA format, 37727 sequences), using Mascot
131 Version: 2.8.0 (Matrix Science) interfaced through Proteome Discoverer 2.4.0.305
132 (Thermo). Peaks were filtered at a signal to noise ratio of 3, deisotoped, and searched
133 with a parent ion mass tolerance of 5 ppm and an MS2 mass tolerance of 0.02 Da.
134 Precursors that had co-isolation of 30% or greater were discarded from quantification.
135 Trypsin was specified as the enzyme and 1 missed cleavage was allowed. N-terminal
136 labeling with TMTpro reagent and carbamidomethyl were specified as a fixed
137 modifications and dynamic modifications included deamidated NQ, oxidized M,
138 phosphorylation on STY, and TMTpro on lysine. All searches were conducted with the
139 reversed database search mode engaged. Percolator software was used for peptide FDR
140 (q-value) calculations. Mascot output files (.dat) tabulated were in Proteome Discoverer.
141 Only high confidence peptides only ($q<0.01$) were used for protein identification and
142 quantification.
143

144 *Spectral Inclusion Criteria for Quantitation*

145

146 Analysis was confined to uniquely- and unambiguously assigned spectra (1% peptide
147 FDR). Missingness of ion intensities for a single spectrum across reporter channels, was
148 low (< 2% of unique spectra with intensities missing from one or more channels),
149 indicative of efficient TMT labeling and fragmentation.

150

151 *Protein Quantification by TMT and Statistical Analysis*

152

153 Reporter ion intensities were integrated over 20 ppm using the most confident centroid
154 method and corrected for purity in Proteome Discoverer 2.5 (Thermo). Spectral TMT
155 signals were quantified using the median sweep algorithm originally described by
156 Herbrich et al.(4) essentially as implemented recently by Foster et al.(3) with a minor
157 modification. TMT reporter ion intensities were 1) logarithmically-transformed (base 2), 2)
158 median-centered within each channel prior to 3) median-centering each individual
159 spectrum across channels, 4) determining protein abundance by taking the median value
160 of the logarithmically-transformed median-centered intensities for all spectra belonging to
161 that protein in a given channel (median summarization) For phosphopeptide analysis, all
162 spectra (phosphorylated and unphosphorylated) median-centered, as above, and median
163 summarized at the peptide level. Unphosphorylated peptides were filtered from the final
164 table.

165

166 Following the median sweep, differential protein abundance between experimental
167 groups was assessed using a Bayesian statistical framework, specifically, linear modeling
168 of microarrays(5) (LIMMA) with multi-group comparison. Pairwise contrasts were also
169 performed. The resulting moderated p-values were used to assess the positive false
170 discovery rate (q-value) method of Storey (6, 7). The code used for the median sweep
171 procedure and statistical analysis can be found at https://github.com/Frostman300/p38-
172 upload. P-values derived from pairwise-comparisons to were utilized to determine specific
173 differences between the groups, as demonstrated in the boxplots presented in the figures.

174

Finding Homologous Human Peptides

An Excel macro was written to find human peptides homologous to the detected guinea pig peptides using the command line version of Protein-Protein BLAST (2.15.0+). In brief, for each guinea pig peptide detected, blastp was used to compare the full length sequence of the corresponding guinea pig protein with a fasta format database of all Homo Sapiens refseq proteins downloaded from the NCBI protein database. Blastp was then used to align the guinea pig peptide with the corresponding highest scoring human protein target. The best homologous peptide match was then output to the spreadsheet. Macro code is available at https://github.com/Frostman300/p38-upload.

Ingenuity Pathways Core Analysis Search Parameters

For IPA core analysis of the proteome, uploaded data fields included the gene name and log2(ACi/ACiSB) value. Data were compared to the Ingenuity Knowledge Base reference set, which included genes only. The search space was limited to consideration of database relationships derived from primary tissues; data arising from cancer cell lines was excluded from analysis.Both direct(transcriptional) and indirect(signaling) relationships were probed. Highlighted pathways were among those with Benjamin-Hochberg-corrected p-values of <0.05. Pathways are colored according to their z-score. The z-score integrates, not only gene over-representation, but the degree of concordance between supplied data (direction of change, magnitude, and p-value) and a database of curated relationships compiled from scientific literature and publicly-available datasets. IPA provides an inference about whether upstream gene-regulatory and signaling pathways may be activated or inhibited. Higher scores, whether positive or negative, reflect the strength of the inference(8).

Network Analysis

Functional protein association/interaction networks were constructed by loading the gene identifiers of up and downregulated proteins into stringApp 2.0.3 (9) embedded in

Cytoscape 3.10.2 (10). The default association/interaction threshold (STRING score > 0.4) was used to map relationships between proteins. Network modularity was assessed with the Markov clustering function in the clusterMaker2 app (v.2.3.4) (11) using the STRING score (>0.6) for edge weighting. The granularity parameter (inflation value) was set empirically. Modules were rearranged for clarity and named consistent with STRING's multipathway enrichment terms (e.g. Gene Ontology, Reactome, Kegg, among others). Singletons that were ontologically consistent with a module were grouped with it. The phosphorylation association network was further annotated using Omics Visualizer 1.3.1. (12) embedded in Cytoscape.

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