Supplemental Methods

Surgical Models of Pressure-Overload Hypertrophy and Failure
Wild type, inbred male C57BL6 mice (6-8 weeks old) were subjected to pressure overload by thoracic aortic constriction (TAC).[1] In this procedure a constriction was placed in the transverse aorta between the innominate and left common carotid arteries. Preanesthesia was achieved using ketamine (100mg/kg IP) plus xylazine (5 mg/kg IP). A warming pad was used to maintain body temperature with in a range of 34°C to 37°C. Respiratory rate, body temperature, and heart rate were monitored continuously during the procedure. The aorta was accessed via a left lateral thoracotomy and suture material (5−0 silk) used to ligate the transverse aorta along with an overlying 27G needle. After ligation, the 27G needle was removed leaving a discrete region of stenosis in the aorta. The chest was closed and the animals observed during recovery from anesthesia. At 3 weeks, when the hypertrophic response reaches steady state, integrity of aortic banding was confirmed by inspection of the surgical constriction and by visualization of marked differences in caliber of the right and left carotid arteries. TAC constriction to a 27G stenosis induced moderate hypertrophy (≈40% increase in heart mass) without clinical signs of heart failure or malignant ventricular arrhythmia. Severe, decompensated hypertrophy was induced by constricting the thoracic aorta to a 28G diameter (sTAC).[2]

Mini-osmotic pump implants
Animals were anesthetized with Avertin (0.02 ml/gram) and the hair shaved at the site of insertion. A 1 cm incision was made between the scapulae and extended with gentle blunt dissection into a pocket for the pump. The mini-osmotic pump (Alzet, Palo Alto, CA) was inserted under the skin and the incision closed using a wound clip.

Quantitative RT-PCR
Total RNA was isolated using Tripure reagent (Roche). Random hexamers were used to reverse-transcribe reactions using superscript II (Invitrogen). Real-time PCR was performed using SYBR green (Applied biosystems). Data for each transcript were normalized to both GAPDH and 18S rRNA as internal controls using the 2-ΔΔCt method. Primers used are listed in supplementary table 1.

Western Blot Analysis
Tissue was homogenized in a buffer containing 10 mmol/L Tris-HCl, pH7.5, 4% glycerol, 0.1% Triton-X, 0.5 mmol/L sodium bisulfite, 4mM sodium orthovandate, 100 mmol/L sodium fluoride, and protease inhibitors using a Dounce homogenizer then solublized by adding an equal volume of 2X SDS loading buffer. For resolving PLB pentamers, samples were run directly on SDS-PAGE without boiling. Total I-1 antibody was a generous gift from Dr, Angus Nairn (Yale University). Phospho-I-1[Ser67], and MCIP1/RCAN1 antibodies have been described previously.[3,4] Antibodies used included: PLB and NFATc1 (Affinity Bioreagents) tubulin (Sigma), pPLB-Ser[16] (Upstate), pPLB-Thr[17] (Barilla, Leeds, UK), ERK1/2 and pGSK3 (Ser9) (Cell Signaling Technology).

Quantification of Western Blot Analysis
Images for all westerns were obtained using the BIO-RAD Molecular Imager Gel Doc XR system and quantified using QuantityOne 1-D Analysis software (BIO-RAD). Values were then averaged for each time point and presented as ± sd. Statistical significance was determined (StatView) with Student's unpaired, 2-tailed *t* test.

RNA *In Situ* Hybridization: ^{35}S-labeled sense and antisense probes specific for exon 4 of the mouse *Rcan1* gene were generated using the Maxiscript kit (Ambion). Radioisotopic *in-situ* hybridization was performed as previously described.[5]

Immunohistochemistry: Monoclonal antibody against NFATc1 (clone 7a6; Affinity BioReagents) (diluted 1:300) was used for immunolabeling of paraformaldehyde-fixed paraffin-sections following Vector Laboratories' mouse-on-mouse detection system.[6]

Chromatin Immunoprecipitation From Heart Tissue was performed as described previously.[7]
Frozen tissue was pulverized in liquid N_2 then transferred to a 15 ml polypropylene tube and resuspended in five volumes of 1X PBS. Crosslinking was initiated by the addition of formaldehyde to a final concentration of 1%. Samples were incubated on a shaker at room temperature for 15 minutes. The crosslinking reaction was stopped by the addition of 0.125 mol/L glycine. Samples were then centrifuged (5000 rpm X 2 min at room temperature). Pellets were washed with 5ml of PBS and centrifuged again. Pellets were re-suspended in 250 ml of PBS containing protease inhibitor cocktail and PMSF (5 mmol/L) and homogenized using a hand-held homogenizer. Contents were transferred to an eppendorf tube and microfuged at 5000 rpm for 2 min at 4°C. 500 ml of cell lysis buffer (5 mmol/L PIPES pH 8.0, 85 mmol/L KCl, 0.5% NP40) supplement with 20 ml of protease inhibitor cocktail and 5 mmol/L PMSF was added to the pellet. Following 15 minutes of incubation on ice samples were centrifuged again at the above settings. Pelleted nuclei were lysed in 500 ml of nuclear lysis buffer (50 mmol/L TRIS-Cl pH 8.0, 10 mmol/L EDTA, 1% SDS) containing the usual protease inhibitor cocktail and PMSF. Samples were sonicated to shear chromatin to an average length of 500 bp (40% output, 15 sec continuous pulse for 5 times). Sonicated samples were then centrifuged at 15000 rpm for 10 min at 4°C. The supernatants were collected and 50 ml of the chromatin preparations were used for each immunoprecipitation (IP). The IP was performed in a final volume of 500 ml containing 10 mg salmon sperm DNA in immunoprecipitation buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L TRIS-Cl pH 8.0, 167 mmol/L NaCl). Anti-NFATc1 antibody (2 mg, clone 7a6, Affinity Bioreagents) was added and incubated gently rocking at 4°C for an hour. Normal mouse serum was used as negative control IgG. Protein A/G PLUS agarose (20 ml) was added and incubated over night at 4°C. Pellets were washed three times with IP buffer prior to isolation for real-time PCR. The primers used to assess NFATc1 binding to the *Rcan1.4* promoter amplified a 65 bp fragment between –282 and –217 in a region with the highest conservation between human, mouse and frog. Primers used for the *Rcan1.4* promoter were: forward 5'-TGGGAACTATGCCGCAAGAG-3' and reverse 5'- GGTGGAAAAGGCGCTAAGGT-3'. Q-PCR was performed using SYBR Green kit (Qiagen) on an i-Cycler (Bio-Rad) and analyzed by the 2-ΔΔC(T) method.[8] Data were normalized to the input DNA and plotted as fold values relative to the signal corresponding to a negative control primer specific to a non coding genomic DNA between *Gapdh* and *Cnap1* genes. Chromatin immunoprecipitations were performed three times in triplicate. Error bars represent the standard error of the mean. Similar results were obtained with either the 7A6 anti-NFATc1 antibody from Affinity BioReagents or the K-18 antibody from Santa Cruz.

Supplemental Table I: Primer pairs used for quantitative RT-PCR.

Gene | **Primer pair**

Rcan1.4
5'-CCC GTG AAA AAG CAG AAT GC –3'
5'-TCC TTG TCA TAT GTT CTG AAG AGG G-3'

Rcan1.1
5'-GAC CCG CGC GTG TTC- 3'
5'-TGT CAT ATG TTC TGA AGA GGG ATT C-3'

Per2
5'-ATG CTC GCC ATC CAC AAG A-3'
5'-GCG GAA TCG AAT GGG AGA AT-3'

b-MHC
5'-GCATTCTCCTGCTGTTTCCTT-3'
5'-TGGATTCTCAAACGTGTCTAGTGA

ANF
5'-GTA CAG TGC GGT GTC AAC CA-3'
5'-TCT CCT CCA GGT GGT CTA GCA-3'

I-1
5'-AAG ACC GGA TCC CCA ACT C -3'
5'-TCT CCT CCA GGT GGT CTA GCA-3'

GAPDH
5'-CAA GGT CAT CCA TGA CAA CTT TG-3'
5'-TCT CCT CCA GGT GGT CTA GCA-3'

18S rRNA
5'-ACC GCA GCT AGG AAT AAT GGA-3'
5'-TCT CCT CCA GGT GGT CTA GCA-3'
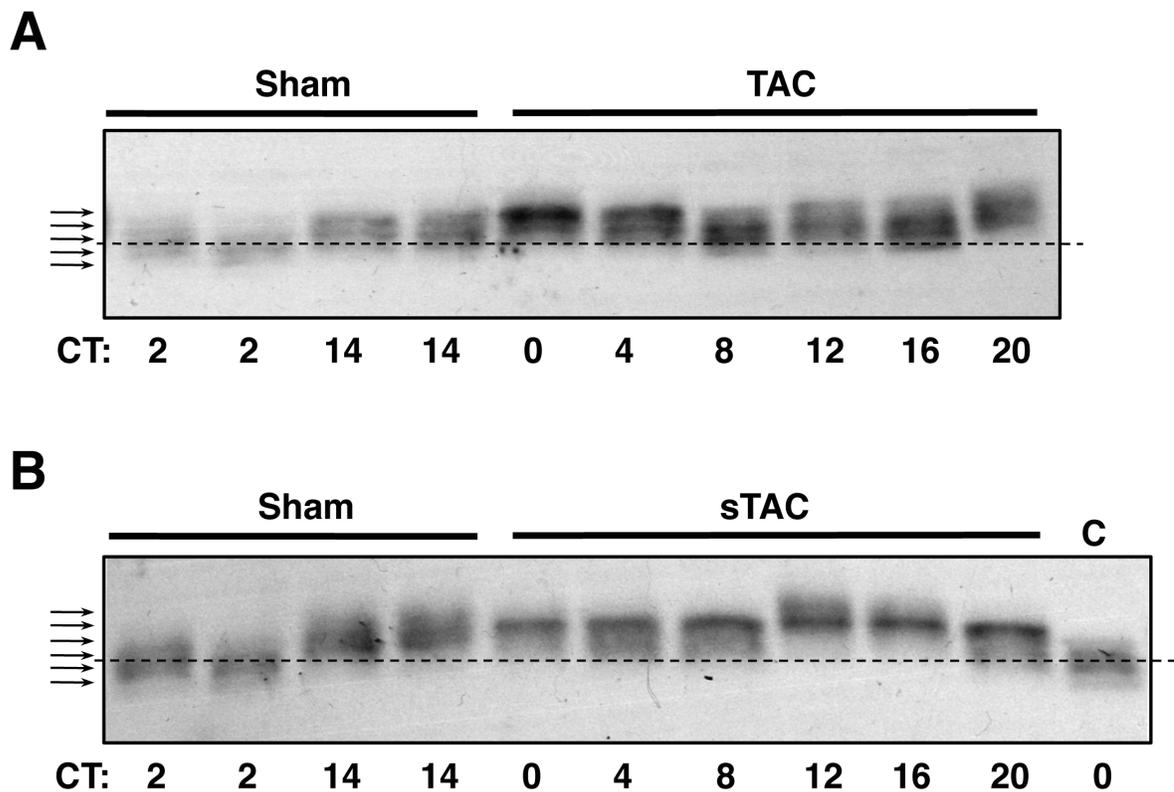
Online Figure I: Enlarged images of the western blots from Figure 7C and 7D showing changes in the migration of PLB pentamers from sham operated control, TAB (A), and sTAB (B) heart protein lysates isolated at the times indicated. Each monomer contains two potential phosphorylation sites, one at serine 16 and another at threonine 17, resulting in a total of ten potential sites for phosphorylation in the pentamer complex. The arrows to the left indicate the location of differentially phosphorylated complexes migrating to distinct locations in the SDS-PAGE gel. Five separate complexes can be seen in the TAC samples, whereas six could be resolved in the sTAC samples. The horizontal dashed line is drawn at mid-level migration of the Sham CT2 pentamer. Regardless of time of day, the mean distribution of all TAC and sTAC samples are above this line suggesting an increase in total phosphorylation. Because of the rapid-rate of dephosphorylation of PLB, all hearts were snap frozen precisely 30 seconds after cervical-dislocation.

Supplemental References

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