Supplemental data

Extended Methods

1. Primary Culture of Neonatal Rat Ventricular Myocytes

Neonatal ventricular myocytes were isolated from 1-day-old Wistar rats (Charles River Laboratories North Wilmington) by digestion with trypsin-EDTA and type 2 collagenase as previously described [1]. This study was reviewed and approved by the Institutional Animal Care and Use Committee at New Jersey Medical School.

2. Kinetics of Phosphorylation

Reconstituted Tn complexes were incubated with active Mst1 in the presence of ^{32}P-ATP for 0 to 120 minutes at 30°C. Proteins were then resolved on 12% SDS-PAGE and the incorporation of radioactive phosphate was detected by autoradiography.

3. Expression and Purification of cTnI Mutants

In order to achieve high expression of cTnI in bacteria, two bases in the second and the fourth codons of cTnI cDNA (Ala2: GCG—GCC, and Gly4: GGG—GGT) were changed before cloning[2]. *In vitro* site-directed mutagenesis was performed to obtain the different mutated cTnI cDNA according to the instruction manual of the QuikChange multi-site-directed mutagenesis Kit (Stratagene). The modified and mutated cTnI cDNA was cloned into the *Nde* I and *Bam*H I sites of pET-21b vector. BL21(DE3) competent cells (Stratagene) was transformed with the pET-21b constructs and induced with 1 mM IPTG at 37 ºC for 3 hr. Recombinant cTnI was purified as previously described [3]. Briefly, the inclusion bodies were extracted by B-PER II bacterial protein extraction reagent (Pierce) and dissolved with the following buffer: 6 M urea, 25 mM MOPS, pH 7.0, 1 mM EDTA, 1 mM DTT. The supernatant fraction was applied to a DE52 anion exchange column. Flow-through was collected and applied to an SP-Sepharose column and eluted with a 0.5 M NaCl gradient. Proteins were further purified using an immobilized cTnC affinity column.

The cTnI protein was loaded to the cTnC column in 50 mM Tris, 0.5 M NaCl, 3 M urea, 2 mM $CaCl_2$, and 1 mM DTT, pH 7.5, and eluted with 6 M urea and 5 mM EDTA in 50 mM Tris, 0.5 M NaCl, and 2 mM DTT, pH 7.5, and dialyzed in 20 mM MOPS, 0.3 M KCl, 5 mM $MgCl_2$, and 2 mM DTT, pH 7.0.

4. mAb Epitope Analysis

The binding affinity between an antibody and its antigenic epitope depends on the three dimensional structural fit. Enzyme-linked immunosorbant assay (ELISA) epitope analysis [4] was employed to examine conformational differences between the non-phosphorylated and Mst1 phosphorylated cTnI. An mAb TnI-1 against an epitope in the C-terminal domain of cTnI [5], an mAb 4H6 against the central region of TnI, and a polyclonal rabbit ant-TnI serum RATnI [6] were used to monitor conformational changes that alter the antibody binding affinity. Non-phosphorylated and Mst1 phosphorylated cTnI were dissolved in Buffer A (0.1 M KCl, 3 mM $MgCl_2$, 20 mM PIPES, pH 7.0) at 1 μg/mL and 100 μL/well was used to coat microtiter plates by incubation at 4 °C overnight. After removing unbound cTnI and blocking the plates by washing with Buffer A containing 0.05% Tween-20 (Buffer T), the immobilized cTnI was incubated with 100 μL/well serial dilutions of the anti-TnI antibodies in Buffer T containing 0.1% BSA at room temperature for 2 h. Following three washes with Buffer T to remove the unbound first antibody, the plates were incubated with 100 μL/well horseradish peroxidase-conjugated anti-mouse (Santa Cruz) or anti-rabbit immunoglobulin second antibody (Sigma) in Buffer T containing 0.1% BSA at room temperature for 1 h. Unbound second antibody was removed by washes as above. The binding of antibodies to the non-phosphorylated or phosphorylated cTnI was detected by H_2O_2/2, 2’-azinobis-(3-ethylbenzthiazolinesulfonic acid) substrate reaction. The enzymatic reaction in each assay well was monitored at a series of time points by an automated microplate reader (BioRad Benchmark). A_{405nm} values in the linear course of the color development were

used to plot the antibody titration curves for quantifying the binding affinity of the antibodies to the cTnI epitopes. All experiments were done in triplet.

5. Protein Binding Assays

An ELISA-based solid phase protein binding assay [4] was used to investigate the interactions of the non-phosphorylated and Mst1 phosphorylated cTnI with TnT and TnC. Similar to that described above for the epitope analysis, cTnI or BSA control was dissolved in Buffer A at 5 μg/mL and 100 μL/well used to coat microtiter plates by incubation at 4°C overnight. Unbound TnT was removed and the plate was blocked by three washes with Buffer T before incubated with 100 μL/well of serially dilutions of purified TnT or TnC in Buffer T containing 0.1% BSA. Following incubation at room temperature for 2 h, the plates were washes three times with Buffer T. The bound TnT or TnC was quantified via an anti-TnT mAb CT3 [5] or an anti-TnC mAb 2C3 [7] followed by standard ELISA procedure as described above. The A_{405nm} values in the linear course of the color development were used to construct the protein binding curves for the quantification of binding affinity between the non-phosphorylated and phosphorylated cTnI to TnT and TnC. All experiments were done in triplicate.

References:

1. Sun J, Yan G, Ren A, You B, Liao JK. FHL2/SLIM3 decreases cardiomyocyte survival by inhibitory interaction with sphingosine kinase-1. *Circ Res* 2006;99(5):468-76.
2. al-Hillawi E, Minchin SD, Trayer IP. Overexpression of human cardiac troponin-I and troponin-C in Escherichia coli and their purification and characterisation. Two point mutations allow high-level expression of troponin-I. *Eur J Biochem* 1994;225(3):1195-201.
3. Guo X, Wattanapermpool J, Palmiter KA, Murphy AM, Solaro RJ. Mutagenesis of cardiac troponin I. Role of the unique NH2-terminal peptide in myofilament activation. *J Biol Chem* 1994;269(21):15210-6.

4. Wang J, Jin JP. Conformational modulation of troponin T by configuration of the NH2-terminal variable region and functional effects. *Biochemistry* 1998;37(41):14519-28.

5. Jin JP, Chen A, Ogut O, Huang QQ. Conformational modulation of slow skeletal muscle troponin T by an NH(2)-terminal metal-binding extension. *Am J Physiol Cell Physiol* 2000;279(4):C1067-77.

6. Jin JP. Alternative RNA splicing-generated cardiac troponin T isoform switching: a non-heart-restricted genetic programming synchronized in developing cardiac and skeletal muscles. *Biochem Biophys Res Commun* 1996;225(3):883-9.

7. Jin JP, Chong SM, Hossain MM. Microtiter plate monoclonal antibody epitope analysis of Ca2+- and Mg2+-induced conformational changes in troponin C. *Arch Biochem Biophys* 2007;466(1):1-7.

Figure S1. Identification of Mst1 Phosphorylation Sites in cTnI. Phosphorylated cTnI peptides were analyzed by MALDI/TOF MS and further identified by MS/MS spectra. The arrow-pointed residues were phosphorylated. *A*. MS/MS analysis of phosphorylated tryptic ions with mass/charge ratios (*m*/*z*) of 1067.4532 identified Mst1 catalyzed cTnI phosphorylation at Thr^{31}. *B.* MS/MS analysis of phosphorylated tryptic ions with mass/charge ratios (*m*/*z*) of 1589.97 identified Mst1 catalyzed cTnI phosphorylation at Thr^{51}. *C.* MS/MS analysis of phosphorylated tryptic ions with mass/charge ratios (*m*/*z*) of 1325.62 identified Mst1 catalyzed cTnI phosphorylation at Thr^{129}. *D.* MS/MS analysis of phosphorylated tryptic ions with mass/charge ratios (*m*/*z*) of 997.53 identified Mst1 catalyzed cTnI phosphorylation at Thr^{143}.

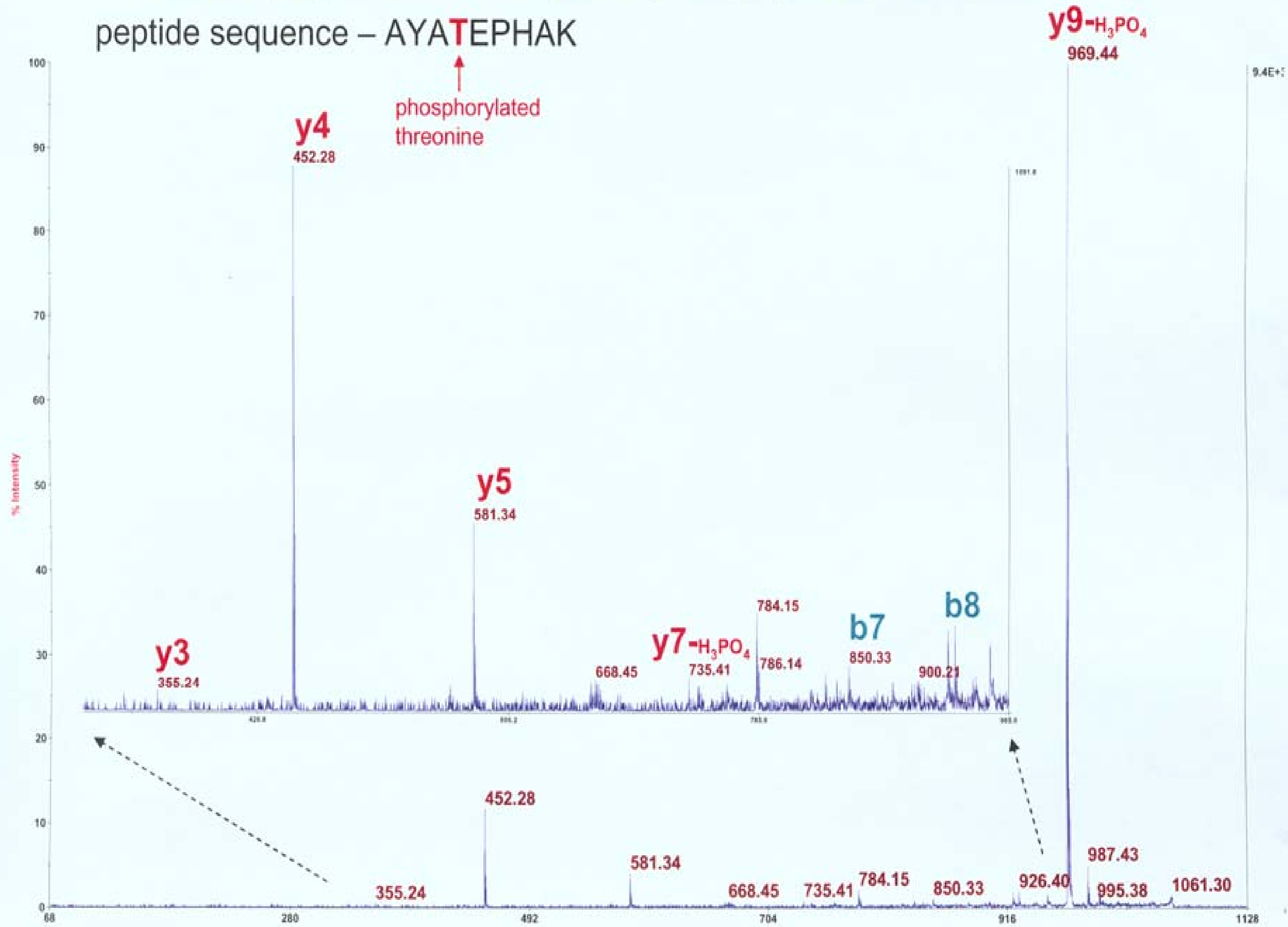

Fig. S1A

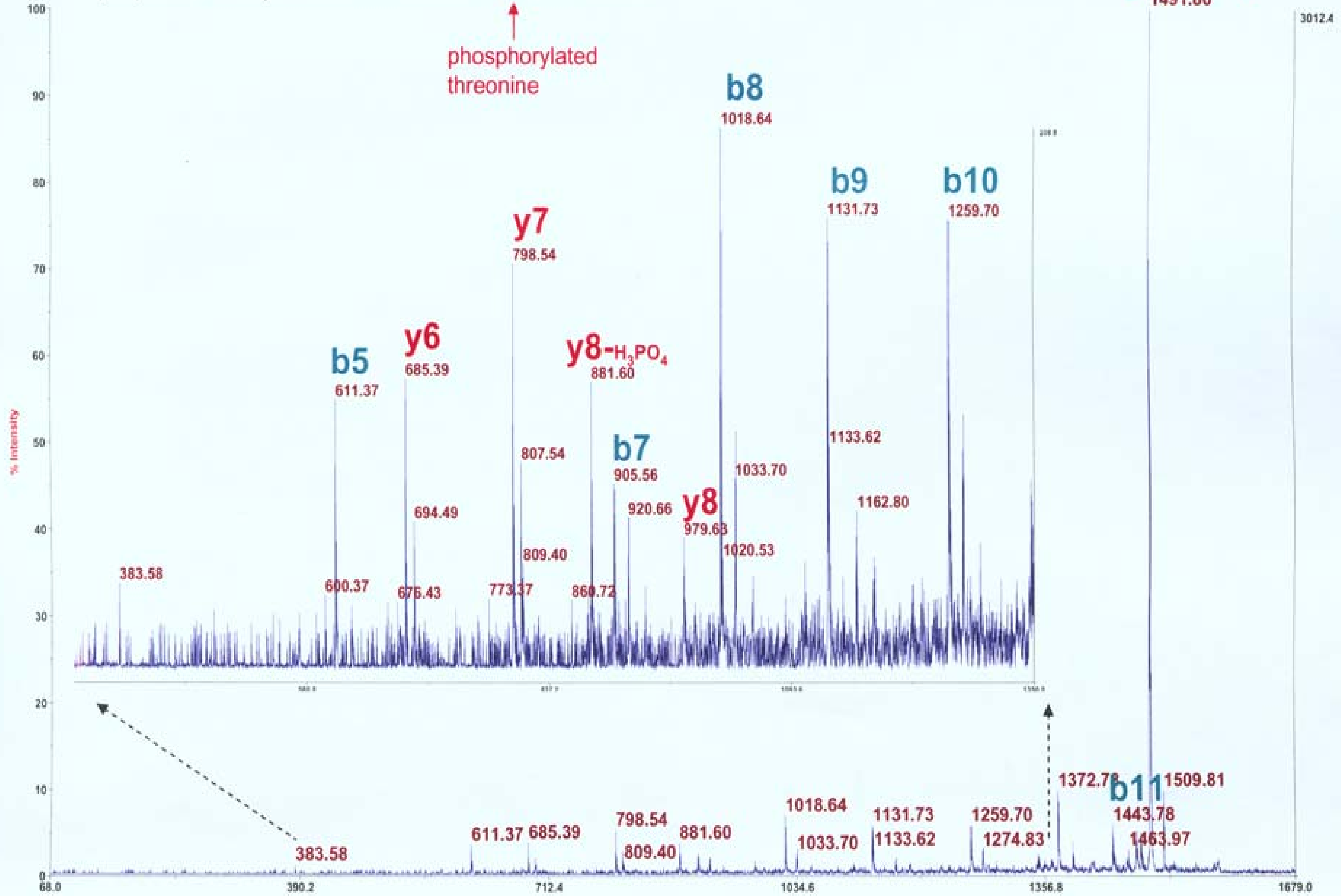

Fig. S1B

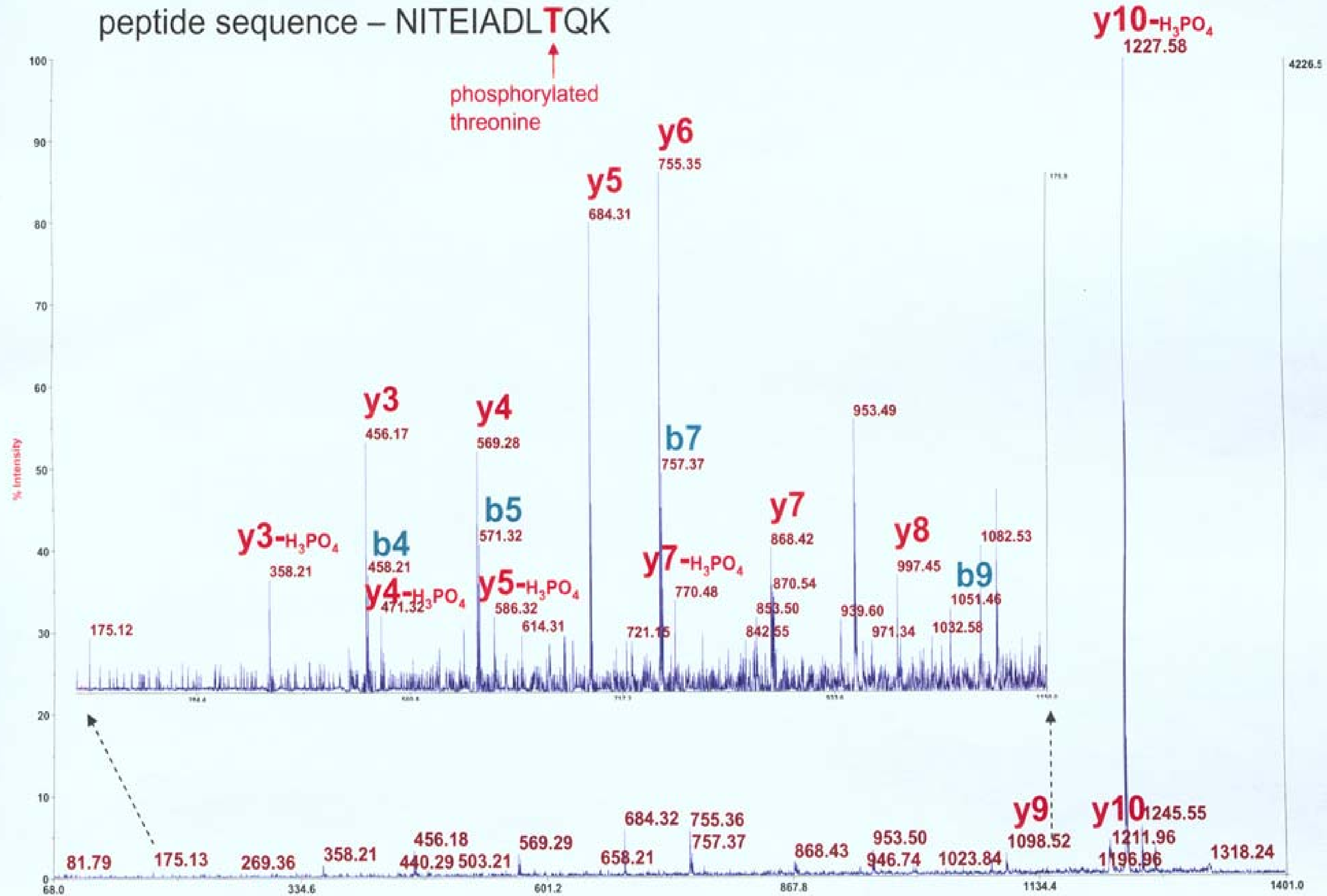

Fig. S1C

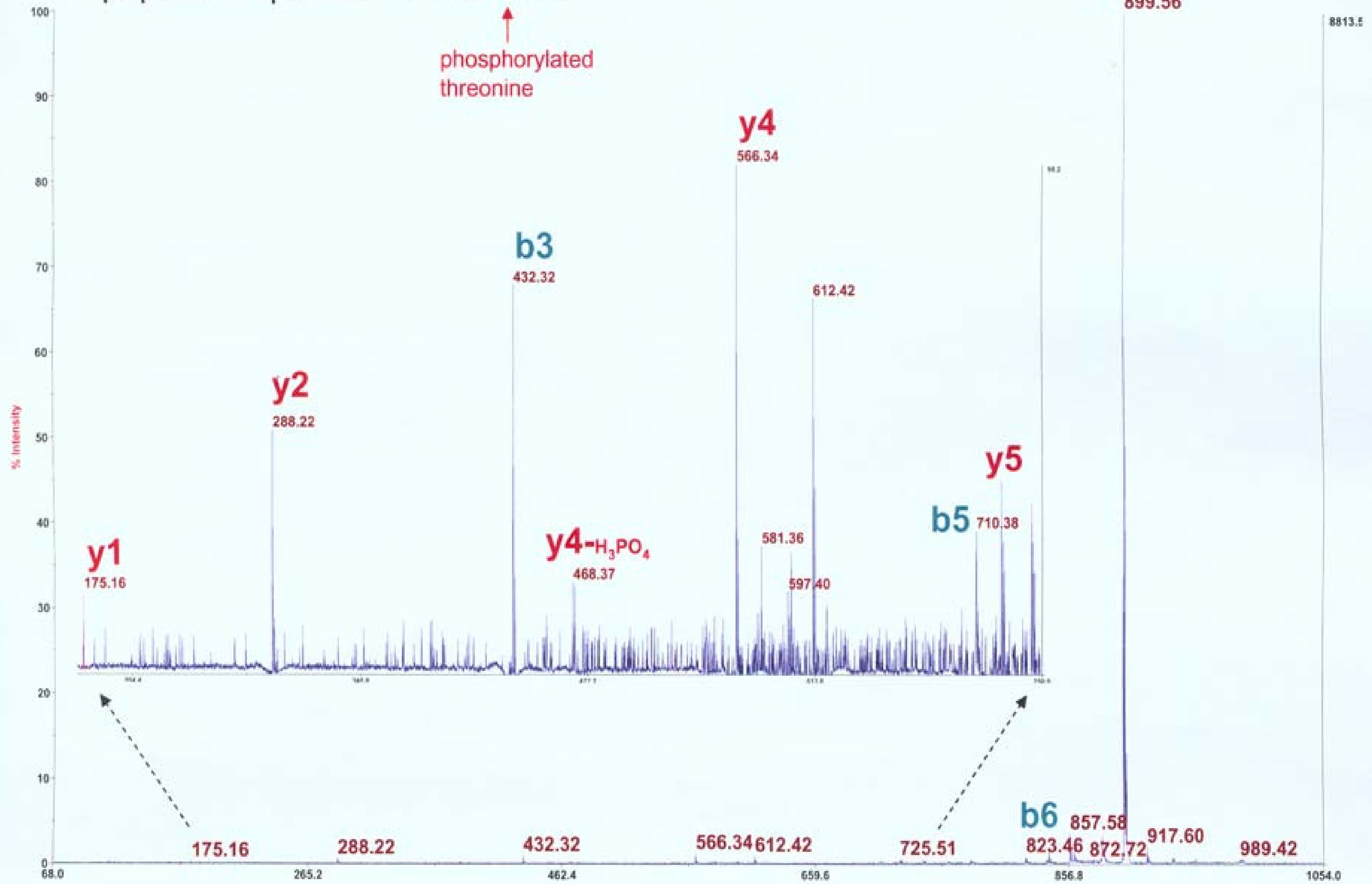

Fig. S1D