Supporting Information

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SI Materials and Methods

Reagents. DMEM and FBS were obtained from Life Technologies. Antibodies against β-actin and His G were purchased from Santa Cruz Biotechnology, and the antibody against Xpress was obtained from Invitrogen. The 10× kinase buffer and antibodies against RSK2, phosphor-Ser/Thr, and PCNA were purchased from Cell Signaling Technology. Active RSK2 was purchased from Upstate Biotechnology.

ELISA. PBMCs were isolated from peripheral blood samples. Cells (2×10^6/mL) were stimulated with 10 ng/mL PMA and 1 μM ionomycin (Sigma-Aldrich). The supernatant fractions were harvested at 5 h after stimulation and analyzed using a human IFNγ ELISA kit (Endogen) following the manufacturer's instructions.

Cell Culture. Human lymphoblast lines GM09621 and GM03317, containing an RKS2 WT gene (*RSK2*$^+$) and an RSK2 mutant gene (*RSK2*$^-$), respectively, were obtained from the National Institute of General Medical Sciences Human Genetic Cell Repository, Coriell Institute for Medical Research. Jurkat cells were obtained from American Type Culture Collection (ATCC) and cultured with antibiotics at 37 °C or 34 °C in a 5% CO_2 humidified incubator in accordance with ATCC protocols. All of the cells were cytogenetically tested and authenticated before being frozen and were thawed and maintained for approximately 2 mo (no more than 10 passages).

Flow Cytometry Analysis. Single immune cell populations in spleens or lymph nodes were cultured with PMA (5 ng/mL; Sigma-Aldrich) and ionomycin (500 ng/mL; Sigma-Aldrich) for 6 h and then harvested for surface and intracellular staining. Flow cytometry data were collected with a FACSCalibur flow cytometer (BD Biosciences). Data analyses were performed with FlowJo (Tree Star). The antibodies used for cell staining included anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD49b (clone DX5), and anti-IFNγ (clone XMG1.2).

Bone Marrow Cell Transplantation. Transplantation of bone marrow cells was performed using female RSK2 KO mice as recipients and male RSK2 KO mice as donors. For isolation of bone marrow cells, male RSK2 KO mice (age 10–12 wk) were euthanized, and their limbs were removed. Bone marrow cells were flushed from the medullary cavities of the tibias and femurs. Female RSK2 KO mice (age 10–12 wk) were sublethally irradiated (900 rad) using an X-ray generator, and bone marrow cells (1×10^6 cells in 0.25 mL) were transplanted i.v. within 3 h after irradiation.

Experimental Liver and Lung Metastasis Model. Littermate WT and RSK2 KO mice (age 8 wk, bred in-house) were used as recipients for the experimental liver and lung metastasis study. CT26 mouse colorectal cancer cells (1×10^6) were injected into each mouse spleen or tail vein. Mice were killed after 10–14 d, and liver and spleen or lungs were removed.

In Vivo Xenogen Imaging. Cells expressing firefly luciferase were implanted into mice by spleen or tail vein injection. On different days after tumor implantation, mice were injected with 150 μL of D-luciferin (15 mg/mL) by i.p. injection. Then in vivo imaging was performed and quantified.

Bacterial Expression of His-Tagged Fusion Proteins. For expression of the full-length His-fusion T-bet protein, the appropriate plasmids (pET46-His-T-bet and mutants pET46-His-T-bet$^{S498A/S502A}$) were expressed in BL21 *Escherichia coli*. The T-bet proteins were purified using a protein refolding kit (Novagen).

Computational Model. First, a N-terminal RSK2 crystal structure was derived from the Protein Data Bank (PDB ID code 3G51). This structure was fully prepared for docking using the Protein Preparation Wizard in Schrodinger Suite 2015. Then the homology modeling of T-bet fragment (residues 490–510) was built based on multiple-threading alignments by Prime from Schrodinger Suite 2015. Finally, the 3D First Fourier transform (FFT)-based protein docking algorithm of HEX 8.0 was used for protein–protein docking experiments to assess the possible binding mode between the RSK2 N-terminal and the T-bet fragment. We selected 100 sorted docked configuration possibilities for further analysis.

Quantitative Real-Time PCR. RNA was extracted from cultured or purified primary cells using the Qiagen RNeasy Mini Kit. cDNA synthesis was performed with the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative PCR was performed with SYBR Green PCR Master Mix using an ABI 7500 Real-Time PCR System (Applied Biosystems). The following primers were used: *RSK2* 5′ primer, CGAGGTCATACTCAGAGTGCTG; *RSK2* 3′ primer, ACTGTGGCATTCCAAGTTTGGCT; *Ifnγ* 5′ primer, ATCTGGAGGAACTGGCAAAA; *Ifnγ* 3′ primer, TTCAAGACTTCAAAGAGTCTGAGGTA; *T-bet* 5′ primer, CAACCAGCACCAGACAGAGA; *T-bet* 3′ primer, ACAAACATCCTGTAATGGCTTG; *IL-2* 5′ primer, TTGAGTGCCAATTCGATGATG, *IL-2* 3′ primer, AGATGATGCTTTGACAGAAGGCTAT; *gapdh* 5′ primer, ATGGTGAAGGTCGGTGTGA; *gapdh* 3′ primer, AATCTCTTTGCCACTGC.

Reporter Gene Assay. The RSK2 cells (8×10^4 per well) were seeded into 48-well plates and incubated for 24 h. Cells were then transfected with the *Ifnγ* reporter plasmid and T-bet with or without RSK2 and incubated for another 36 h. Firefly luciferase activity was measured using substrates provided in the reporter assay system (Promega). Transfection efficiency was normalized with a *Renilla* plasmid as an internal control.

Western Blot Analysis. Cellular proteins were extracted using cell lysis buffer (150 mM NaCl, 0.25% sodium deoxycholate, 50 mM Tris·HCl pH 8.0, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA, and protease inhibitor mixture). Protein concentration was measured using a Bio-Rad protein assay kit, and proteins were subjected to 10% SDS/PAGE. Proteins were then transferred onto PVDF membranes (Amersham Biosciences) and hybridized with the appropriate specific primary antibody and an HRP-conjugated secondary antibody. Antibody binding was conducted at 4 °C overnight, and proteins were visualized using an enhanced chemiluminescence reagent and the ImageQuant LAS4000 system (GE Healthcare).

Immunohistochemistry Staining. Mouse tissues were embedded in paraffin and subjected to immunohistochemistry analysis. Tissues were deparaffinized and hydrated, then permeabilized with 0.5% Triton X-100/1× PBS for 10 min. Tissues were hybridized with PANC (1:16,000) as the primary antibody and biotinylated goat anti-rabbit IgG as the secondary antibody. An ABC Kit (Vector Laboratories) was used to detect protein targets according to the manufacturer's instructions. After developing with 3,3′-diaminobenzidine, the sections were counterstained with hematoxylin and observed by light microscope (200× magnification) using Image-Pro Plus v. 6.1 (Media Cybernetics).

FACS Analysis. To assay the cell cycle distribution, mouse lymph node cells were harvested and fixed in 70% ethanol at −20 °C overnight, then stained with 20 μg/mL of propidium iodide and 200 μg/mL of RNase. The cells were treated with a final concentration of 30 μM BrdU for 30 min, and then fixed in 70% ethanol at 20 °C overnight. The fixed cells were denatured in 2 M HCl and then stained with anti-BrdU mouse IgG (Sigma-Aldrich), followed by incubation with anti-mouse IgG $F(ab')_2$ fragment–FITC (Sigma-Aldrich) and then DNA staining with 20 μg/mL of propidium iodide and 200 μg/mL of RNase. The stained cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences). Intact cells were gated in the FSC/SSC plot to exclude small debris. The cell cycle distribution was determined using ModFit LT version 3.2.1 (Verity Software House), and BrdU incorporation was analyzed using Cell Quest Pro version 6.0 (BD Biosciences).

Statistical Analysis. All quantitative data are expressed as mean ± SD or SE. Significant differences were determined by Student's *t* test or one-way ANOVA, with $P < 0.05$ as the criterion for statistical significance.

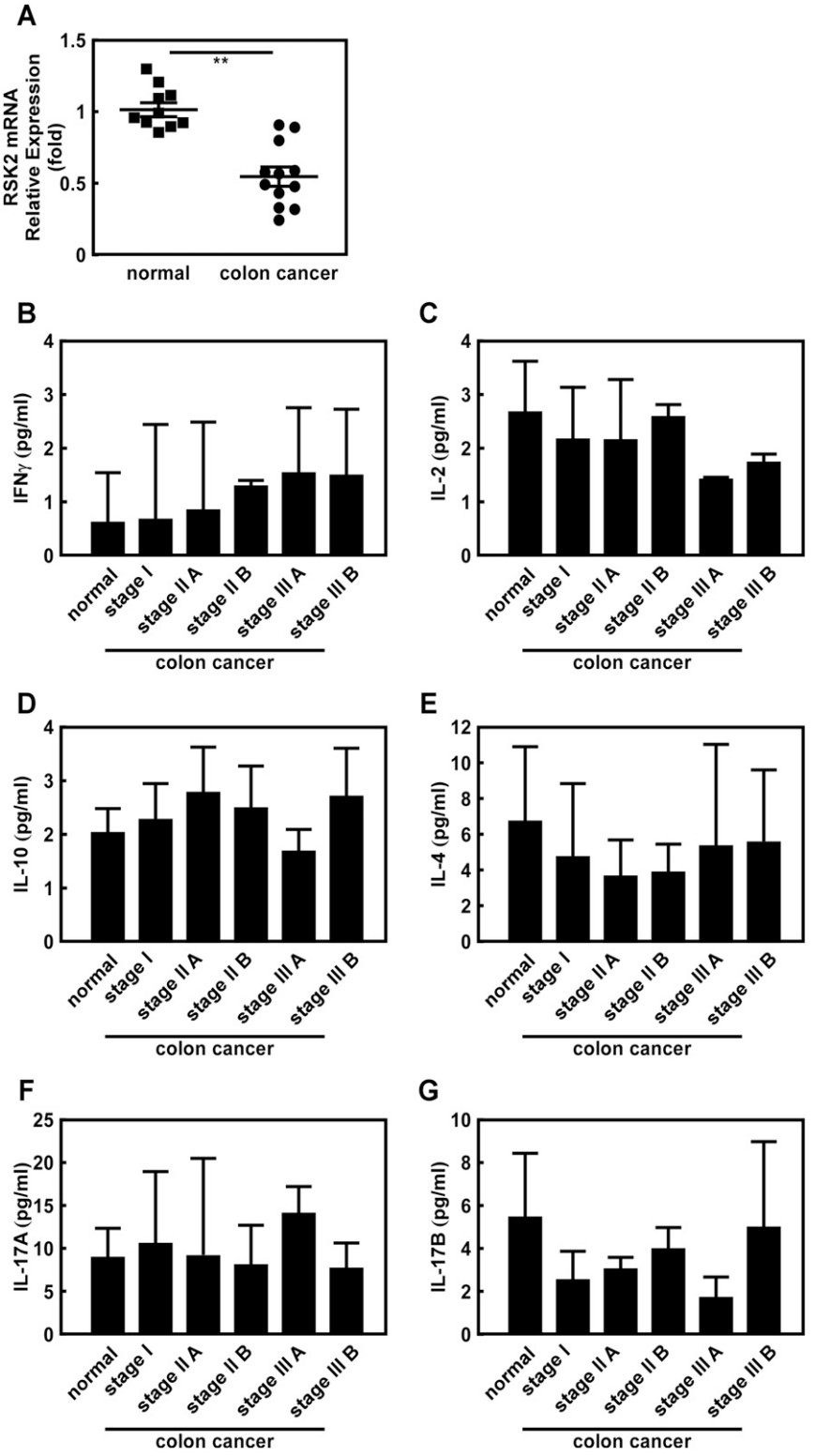

Fig. S1. (*A*) RSK2 mRNA expression analysis comparing normal healthy controls and colon cancer patients. (*B–G*) Serum cytokines in normal healthy controls and colon cancer patients detected using a human cytokine ELISA kit.

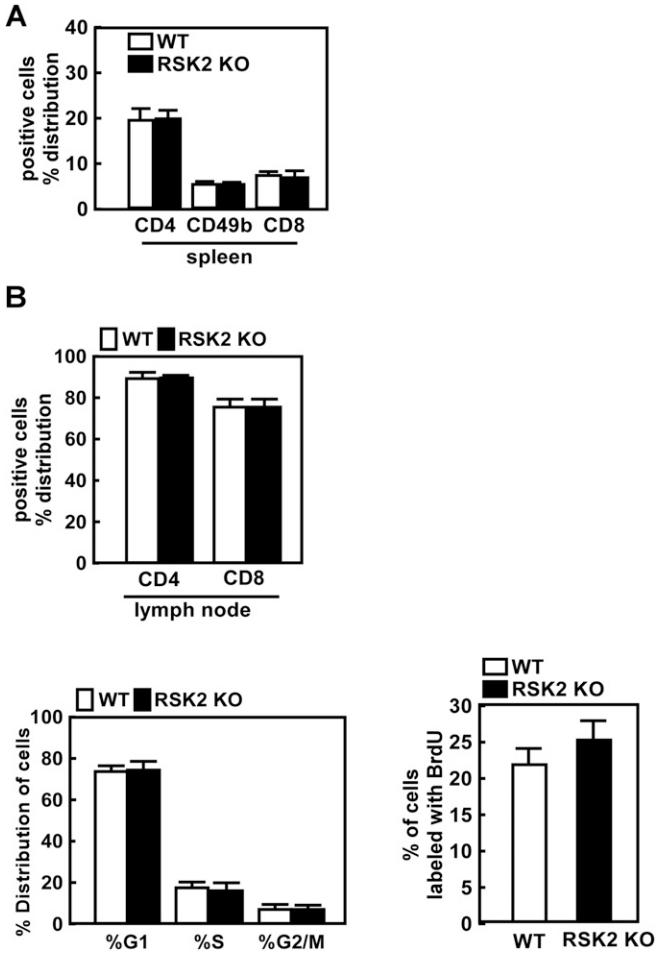

Fig. S2. (*A*) Flow cytometry analysis of the percentage of $CD4^+$, $CD49b^+$, $CD8^+$ cells in primary cells isolated from the spleen in WT and RSK2 KO mice. (*B*) Flow cytometry analysis of the percentages of $CD4^+$, $CD8^+$ cells in primary cells isolated from the lymph nodes in WT and RSK2 KO mice (*Upper*), cell cycle distribution of cells from WT and RSK2KO mice (*Lower Left*), and quantitative analyses of BrdU incorporation (*Lower Right*). Data are shown as mean ± SD percentage of labeling of three separate experiments.

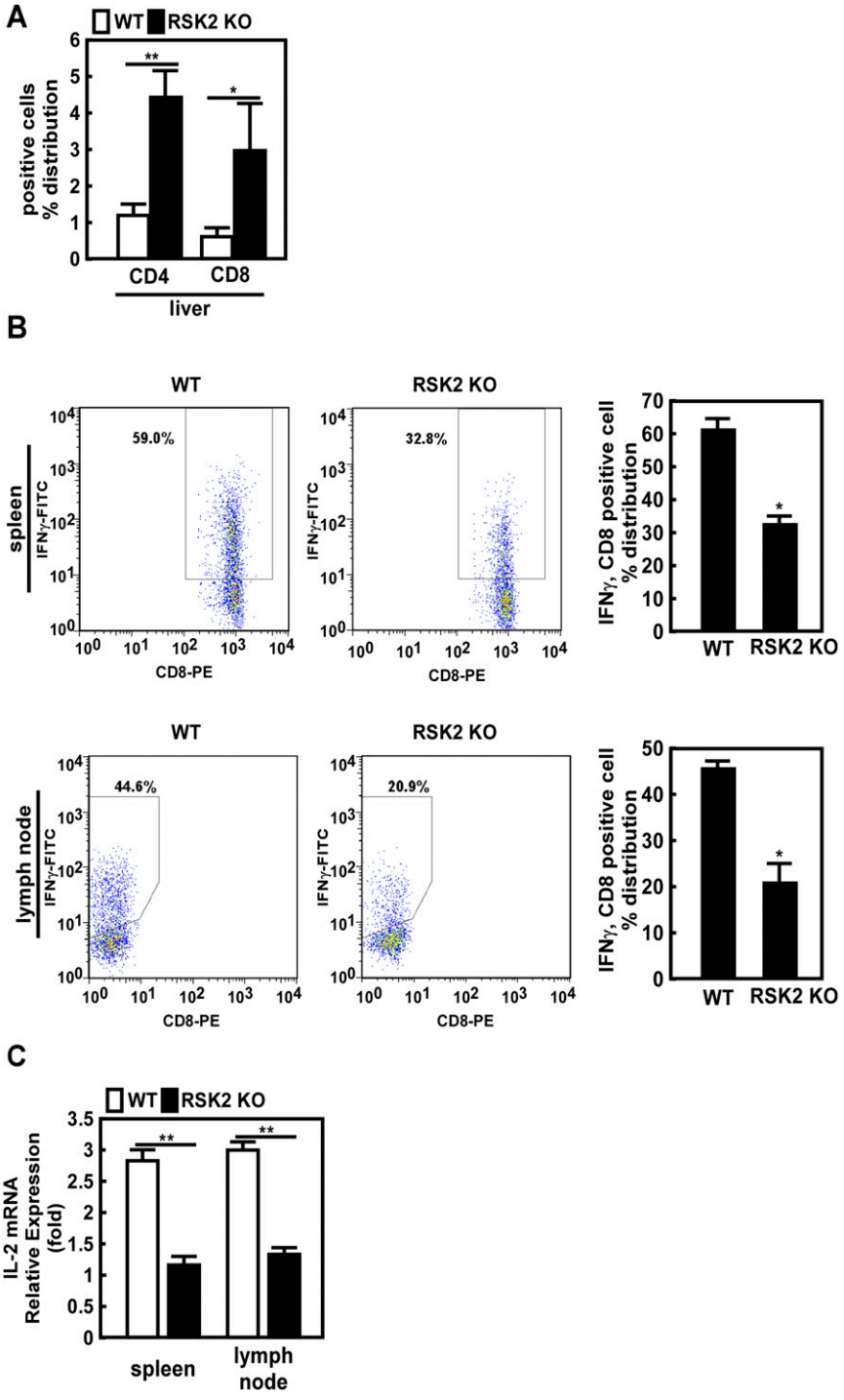

Fig. S3. (*A*) Flow cytometry analysis of the percentage of $CD4^+$, $CD49b^+$, $CD8^+$ cells in primary cells isolated from liver in WT and RSK2 KO mice under CT26 cell challenge. (*B*) Flow cytometry analysis of the percentage of IFNγ/$CD8^+$ cells in primary cells isolated from spleen or lymph nodes in WT and RSK2 KO mice. *$P < 0.05$, *t* test. (*C*) IL-2 mRNA expression analysis from spleen and lymph nodes in WT and RSK2 KO mice.

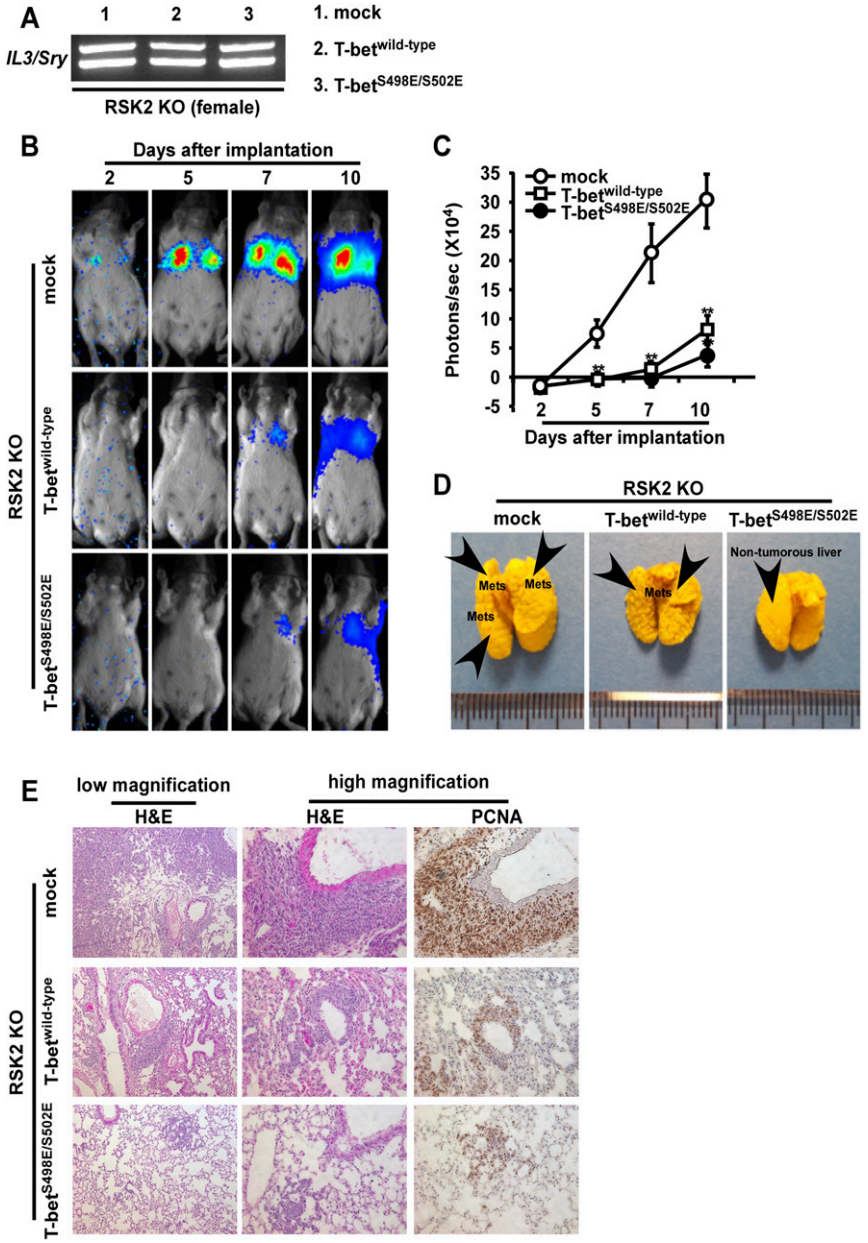

Fig. S4. Phosphorylation of T-bet$^{S498/S502}$ prevents colon cancer lung metastasis in RSK2 KO mice. RSK2 KO mice overexpressing mock, T-bet$^{wild\text{-}type}$, or T-bet$^{S498E/S502E}$ were established by bone marrow transplant assay. In these mice, CT26 cells (1×10^6) tagged with firefly luciferase were injected into the tail vein, and bioluminescence of CT26 cells was visualized using in vivo Xenogen imaging at different days after tumor implantation. (*A*) PCR analysis of *IL3/Sry* expression in RSK2 KO female mice overexpressing mock, T-bet$^{wild\text{-}type}$, or T-bet$^{S498/S502E}$. (*B*) Representative images for each group ($n = 5$). (*C*) Data analyzed using Bruker MI SE software. $^{**}P < 0.05$, ANOVA. (*D*) Representative photographs of lung metastases (Mets) of mice. (*E*) Immunohistochemical analysis of tumor tissues. Tumor tissues from each group were harvested, and slides were stained with H&E or a PCNA antibody for immunohistochemistry. Expression of PCNA was visualized by microscopy. Data were obtained from five separate areas on each slide and an average of three samples per group.

Table S1. Identification of phosphorylation sites on RSK2-phosphorylated T-bet

Confidence*	Score†	Precursor *m/z*‡	z§	Sequence	Theoretical MW¶	ΔMass#	Site
99	20	1,108.9429	2	IS[Pho]PY‖PS[Pho]SGDSSSPAGAPSPFDK	2,215.8711	0.1326	S498/S502

*The confidence for the peptide identification.
†The score for the peptide.
‡Precursor *m/z*.
§The charge for the fragmented ion.
¶Theoretical precursor molecular weight (MW) for peptide sequence.
#The difference between theoretical MW and experimental MW of the matching peptide sequence.
‖Dehydroalanine from tyrosine.