SUPPLEMENTS

Experimental procedures

RT-PCR analysis

Total RNA was prepared from HEK293 and BeWo cells using the TRIzol reagent (Invitrogen), and the cDNA were synthesized by Superscriptase II (Invitrogen) with an oligo (dT) primer. To determine the expression of each CNN isoform, PCR was performed with specific primers (5'-3'): gcgaagacgaaaggaaac and gcggaattgtagtagttg for CNN1, gagcggggtggacattgg and ggtagtaaggggatatt for CNN2, ccaaagtgtaattggtctg and atgccttggtcgctatat for CNN3, catttgcaggggggagcc and gatgaccttgcccacagc for GAPDH. The reaction condition was as follows; 30 cycles of denaturation at 94℃ for 30 sec, annealing at 58ºC for 30 sec, and extension at 72ºC for 30 sec. Amplified cDNA fragment were confirmed by electrophoresis on an 1% agarose gel.

Expression and purification of His-CNN3 furion protein

rCNN3 was produced by the *E. coli* BL21 which was transformed with pET19b containing a human CNN3 coding region. After induction by IPTG and incubation for 3h, recombinant His-tagged CNN3 were purified by nickel affinity chromatography according to the manufacture's protocol (Bio-Rad).

In vitro CNN3 phosphorylation assay

rCNN3 (10 μg) was phosphorylated *in vitro* with either 100 ng ROCKII active (Millipore) or 50 ng casein kinase1 active (Millipore) in an assay solution (50 μl) containing 50 mM Tris-HCl pH 7.5, 2 mM EGTA, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 6 mM KCl, 10 mM $MnCl_2$ and 100 μM ATP. Reaction was terminated by addition of SDS-PAGE sample buffer. Phosphorylated rCNN3 was visualized by either ProQ Diamond phospho-specific staining or Western blotting using antibodies raised against phosphorylated S293 of CNN3.

In-gel digestion and peptide mass fingerprinting

The gel pieces were washed twice in 300 μl of CH_3CN for 30 min and dried. The gel pieces were then rehydrated in 100 μl of reduction buffer (10 mM DTT and 100 mM NH_4HCO_3) and left standing at 56ºC for 1 h. After removal of supernatant, the gel pieces were incubated in 100 μl of 50 mM iodoacetamide in 100 mM NH_4HCO_3 at room temperature for 45 min. The gel pieces were then washed in 100 μl of 100 mM NH_4HCO_3 and dehydrated in 300 μl of CH_3CN. After washing and dehydration, the dried gel pieces were rehydrated on ice in

digestion buffer containing 50 mM Tris-HCl pH 8.0, 10 ng/μl each of lysylendopeptidase (WAKO, Osaka, Japan) and modified trypsin (Promega) for 45 min. The supernatant was replaced with 10 μl of 50 mM Tris-HCl, pH 8.0, and the gel pieces were incubated at 37ºC overnight. The supernatant was collected and the digested peptides were extracted repeatedly with a 50 μl solution of 5% (v/v) formic acid and 50% (v/v) CH_3CN by vortexing. The combined supernatant was evaporated to dryness in a vacuum centrifuge. Resulting peptides were absorbed onto a ZipTip C18 (Millipore), and bound peptides were eluted with 50% CH_3CN and 0.1% trifluoroacetic acid (TFA). Equal amounts of the resulting peptide solution and a sample matrix (saturated α-cyano-4-hydroxycinnamic acid dissolved in 50% CH_3CN and 0.1% TFA) for matrix-assisted laser desorption/ionization (MALDI) were mixed on the sample target for mass spectrometry (MS). Peptide mass fingerprinting was carried out with Mascot search for the peptide masses obtained by a Voyager DE-Pro time-of-flight mass spectrometer (Applied Biosystems).

Phospho-peptide enrichment and MS

Purified FlagCNN3 was reduced with DTT followed by carbamidomethylation, and the alkylated samples were digested with a mixture of trypsin and lysylendopeptidase in a buffer containing 0.1 M Tris HCl (pH 8.0) at 37ºC overnight. Phosphopeptides from the digests were enriched using a MonoTip TiO (Larsen *et al.*, 2005; Liang *et al.*, 2006; Thingholm *et al.*, 2006; Sugiyama *et al.*, 2007) (GL Science, Tokyo, Japan) according to the manufacturer's protocol. Dephosphorylation reactions were performed in 50 mM NH_4HCO_3 containing 0.1% alkaline phosphatase at 37ºC for 15 min. Alternatively, to collect the phosphopeptides, HPLC of the digests was carried out on a C18 column (150 × 1.0 mm) with a linear gradient elution of CH_3CN (1–70%, v/v) in 0.1% (v/v) TFA. The phosphopeptides isolated by HPLC or MonoTip TiO were subjected to MS or further digested with endoproteinase AspN (Roche Diagnostics, Basel, Switzerland) in a buffer containing 50mM Tris-HCl at pH 7.4, and then dried for MS. The measurements for phosphopeptides were carried out in linear TOF mode. Tandem mass spectrometry (MS/MS) was performed on a MALDI multiple-stage tandem mass spectrometer AXIMA-QIT (Shimadzu, Kyoto, Japan).

Legends of supplemental figures

Figure S1 Verification of lentivirus-mediated CNN3 gene knockdown in BeWo cells, and cellular localization of CNN3 in HEK293 cells. (A) Identification of endogenous CNN isoforms in HEK293 and BeWo cells by RT-PCR. mRNA expression of endogenous CNN isoforms was examined by RT-PCR. Total RNA was isolated from HEK293 and BeWo cells

and reverse transcriptase reactions were performed. The cDNA were amplified by PCR as describes in the supplemental experimental procedure section. **(B)** Western blotting of CNN3 to verify the knockdown in GFP-expressing ("G" in the panel) or CFP-Nuc-expressing ("C" in the panel) BeWo cells. Transgenes were introduced into BeWo cells using a lentivirus with both U6-dependent shRNA and CMV-dependent marker gene expression. Two independent shRNA sequences for RNAi were used. **(C)** Cell lysate of BeWo cells after forskolin treatment at indicated times was analyzed by Western blotting using indicated antibodies. The amount of loaded samples was adjusted according to the GAPDH levels in ech sample. **(D)** Flag-CNN3 was transduced using a lentivirus and the HEK293 cells were treated with 50 μM forskolin for the indicated times. Flag-CNN3 was recovered by immunoprecipitation using anti-FLAG agarose and co-IP assay was carried out using the indicated antibodies. **(E)** EYFP-CNN3 distribution was not changed by forskolin treatment in HEK293 cells alone. Scale bar: 50 μm. **(F)** Wild type (WT) or ΔC Flag-CNN3 expressing HEK293 cells were treated with forskolin for the indicated times and CNN3-actin association was analyzed by co-IP assay using anti-Flag agarose. **(G)** *In vitro*-phosphorylation assay was performed using rCNN3 as substrate. rCNN3 phosphorylation was induced by addition of either active form of ROCKII or casein kinase1 for indicated times. Phosphorylated rCNN3 was detected by ProQ Diamond phospho-specific staining (upper panels) or Western blotting using indicated antibodies (lower panels). **(H)** The CAPMP fraction extracted from BeWo cells was separated by 2-DE and transferred onto PVDF membranes for immunoblotting analysis. The spots indicated by arrowheads were those at the same isoelectric point.

Figure S2 Analysis of phosphopeptides derived from Flag-CNN3. (A) MALDI linear TOF mass spectrum of a tryptic digest from FlagCNN3 expressed in HEK293 cells. FlagCNN3 was purified using anti Flag-agarose and concentrated by TCA precipitation. After alkylation, samples were digested with a mixture of trypsin and lysylendopeptidase. **(B)** MALDI linear TOF mass spectra of phosphopeptides enriched by TiO_2, before (a and c) and after (b and d) alkaline phosphatase (AP) treatment. Trypsinised samples from (A) were further purified using TiO_2. Verification of phosphopeptide was analyzed by dephosphorylation assay using alkaline phosphoatase. **(C)** MALDI linear TOF mass spectrum of an HPLC fraction containing phospho-peptides. **(D)** MALDI QIT TOF mass spectrum of an HPLC fraction containing phosphopeptides. **(E)** Product ion mass spectra of the protonated peptides at *m/z*2437, *m/z*3228 and *m/z*3930. **(F, G)** MALDI linear TOF mass spectra of an HPLC fraction containing phosphopeptides from additional endoproteinase AspN digestion, before (upper panel) and after (lower panel) AP treatment, and assignment of peptides. Note that the protonated peptide at *m/z*2750.9 contains two phosphorylation sites as shown in **(G)**. Average

or exact mass is used for the linear TOF or QIT mass spectrum, respectively. **(H)** Putative phosphorylation sites based on mass spectrometric results from F. **(I, J)** MALDI linear TOF mass spectra of tryptic digests of various CNN3 mutants expressed in HEK293 cells, with or without AP treatment.

Figure S3 Charactarization of phospho-specific CNN3 antibodies. (A-D) ELISA of phospho-S293 **(A, C)** and phospho-S296 **(B, D)** antibodies. Phosphorylated **(A, B)** and non-phosphorylated **(C, D)** peptides were covalently couples to N-oxysuccinimide plate and probed with various dilutions of normal rabbit serum (■), antibodies which were affinity-purified using phospho-peptide-conjugated sepharose and pre-absorbed with (▲) or absorbed (●) with non -phospho peptide-conjugated sepharose and an non-phospho peptide-adsorbed fraction (x). Note that phospho-S293 antibody has no reactivity with non-phospho peptide but that of phospho-S296 antibody remained weakly. **(E)** FlagCNN3s were transiently transfected to HEK293 cells by calcium phosphate. Equal amount of TCL samples were subjected to SDS-PAGE and analysed by western blotting using indicated antibodies. The antibody against phospho-S293 works well, but that against phospho-S296 has residual affinity to non-phosphorylated CNN3 peptide as shown in D(▲) and displays non-specific binding to the overexpressed CNN3. **(F)** BeWo TCL samples were prepared with or without forskolin treatment for indicated times and analyzed by western blotting using indicated antibodies. Since the specificity of the antibody raised against phospho-S296 was poor, this antibody was applied to the cytosolic fraction in Figure 6A.

Figure S4 Distribution of the YFP-tagged CNN3 mutants in BeWo cells. EYFP-CNN3-expressing cells were cultivated in the presence **(B)** or absence **(A)** of 50 μM forskolin for 72 h. After fixation, the cells were stained by Alexa Fluor 568-conjugated phalloidin (red) and DAPI (blue). Note that the syncytium expressing WT and S293,296D EYFP-CNN3 did not have F-actin bundles after forskolin treatment, whereas ΔC and S293,296A EYFP-CNN3 still overlapped with F-actin even after forskolin treatment. The dotted lines show the periphery of syncytium. Scale bar: 50 μm.

Supplemental Table I

List of proteins, from CAPMP fraction, accompanying a qualitative or quantitative change by fusion induction.

Expression (decrease after fusion induction)
• chaperonin containing TCP1, subunit 3
• chaperonin containing TCP1, subunit 7
Expression (increase after fusion induction)
• calreticulin precursor variant
Change isoelectric point
• laminin receptor 1
• protein phosphatase 1, catalytic subunit, alpha
• protein phosphatase 1, catalytic subunit, beta
Dephosphorylation
• adenylyl cyclase-associated protein
• GAPDH/uracil DNA glycosylase
• eukaryotic translation elongation factor 2
• septin 2
• calponin 3

Supplemental Table II

Tryptic peptides of CNN3 identified by peptide mass fingerprinting

Start-End	Sequence
7 - 17	K.GPSYGLSAEVK.N
54 - 64	K.DGIILCELINK.L
133 - 143	K.GFHTTIDIGVK.Y
159 - 172	K.AGQSVIGLQMGTNK.C Oxidation (M)
193 - 212	K.MQTDKPFDQTTISLQMGTNK.G Oxidation (M)
193 - 212	K.MQTDKPFD Oxidation (M)
257 - 265	K.GMSVYGLGR.Q Oxidation (M)

Supplemental reference

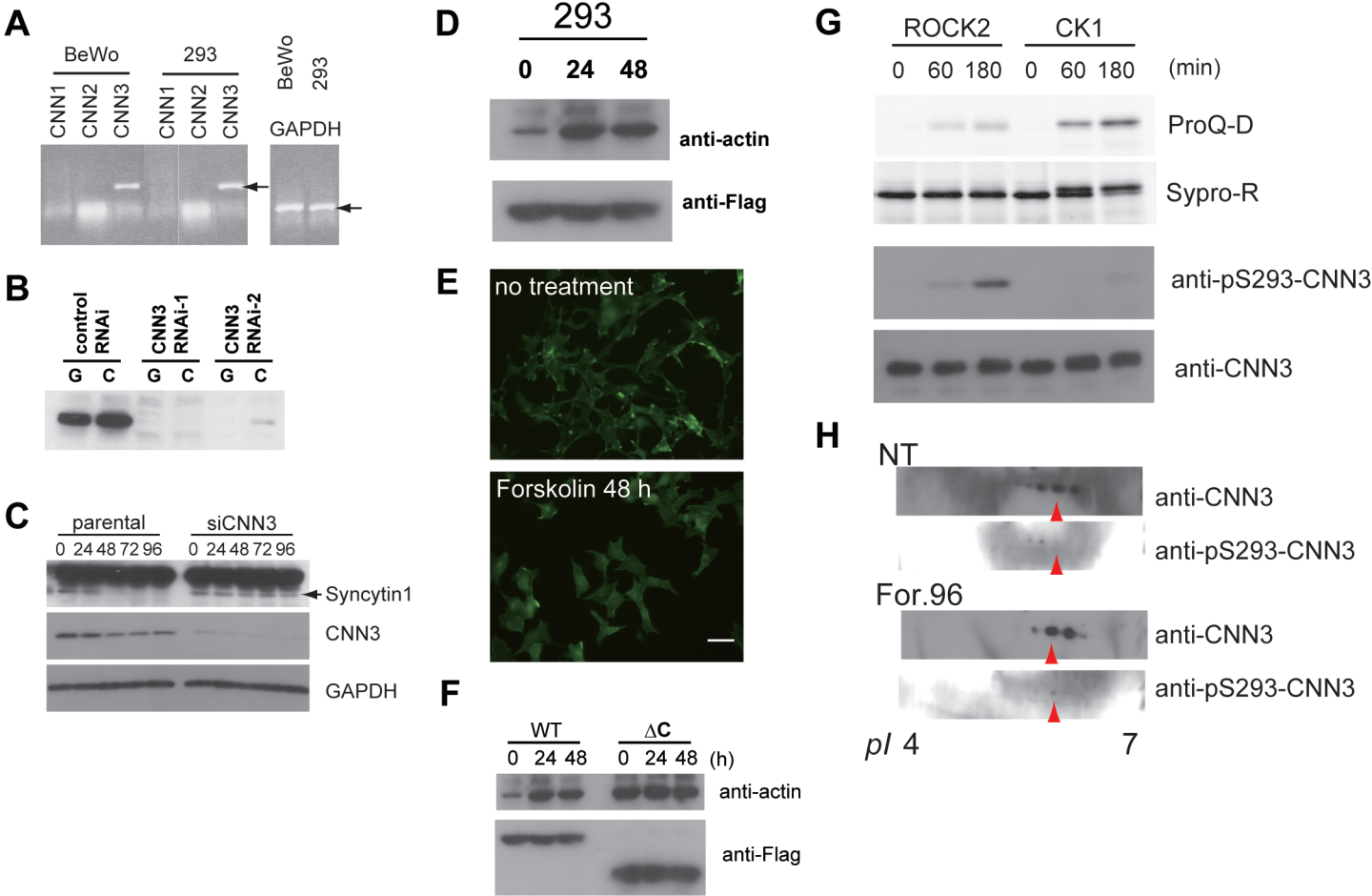
Larsen, M.R., Thingholm, T.E., Jensen, O.N., Roepstorff, P., and Jorgensen, T.J. (2005). Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. Mol Cell Proteomics. *4*, 873-886. Epub 2005 Apr 2027.

Liang, S.S., Makamba, H., Huang, S.Y., and Chen, S.H. (2006). Nano-titanium dioxide composites for the enrichment of phosphopeptides. J Chromatogr A. *1116*, 38-45. Epub 2006 Mar 2030.

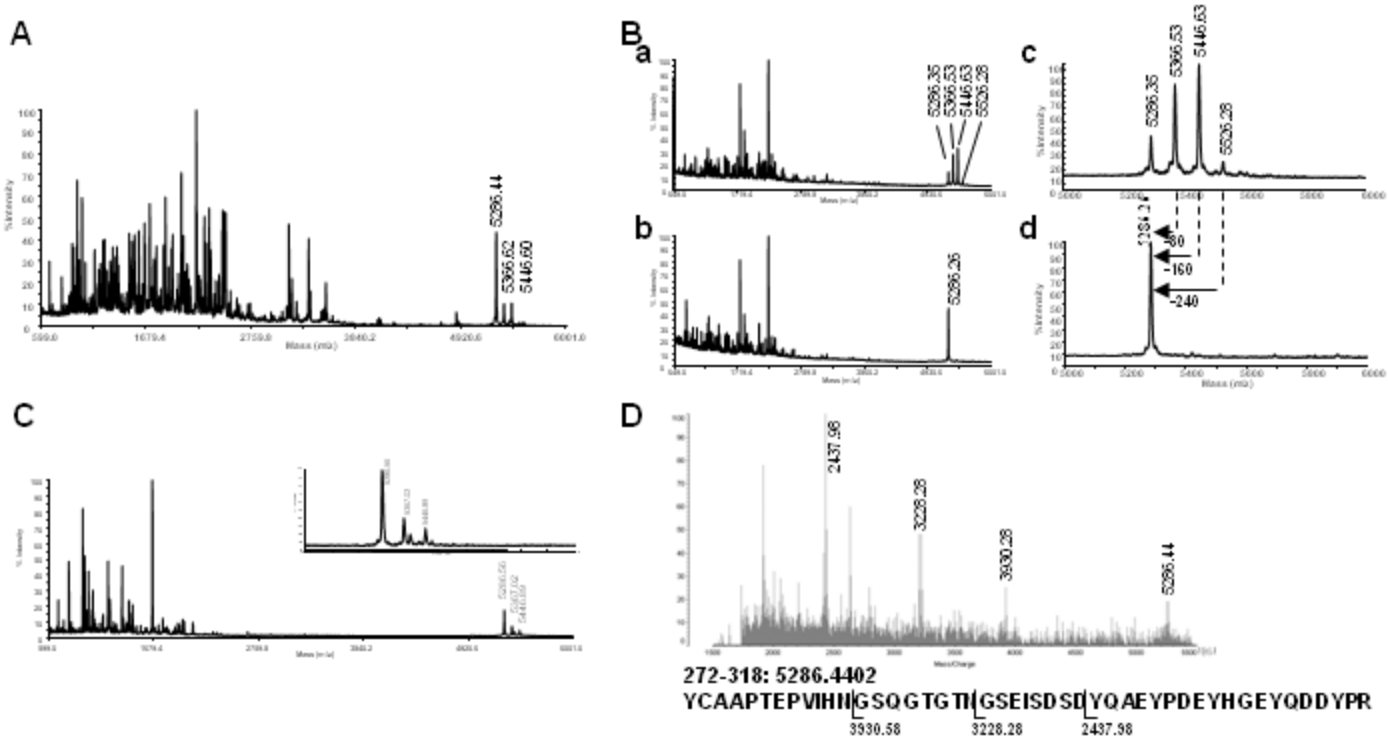
Sugiyama, N., Masuda, T., Shinoda, K., Nakamura, A., Tomita, M., and Ishihama, Y. (2007). Phosphopeptide enrichment by aliphatic hydroxy acid-modified metal oxide chromatography for nano-LC-MS/MS in proteomics applications. Mol Cell Proteomics *6*, 1103-1109.

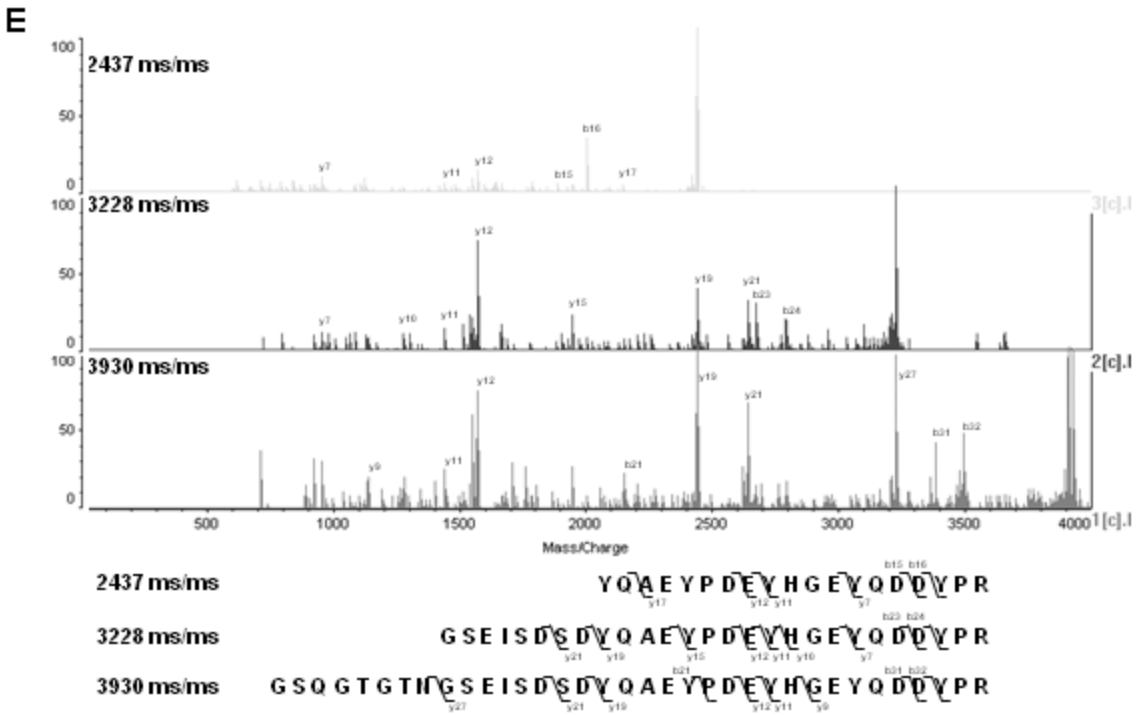
Thingholm, T.E., Jorgensen, T.J., Jensen, O.N., and Larsen, M.R. (2006). Highly selective enrichment of phosphorylated peptides using titanium dioxide. Nat Protoc. *1*, 1929-1935.

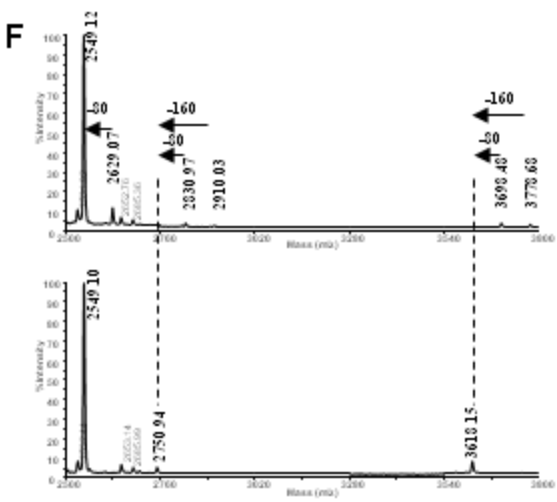
Supplemental Figure S1

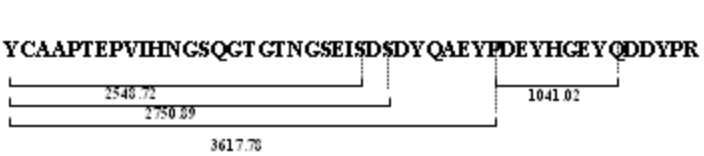


Supplemental Figure S2









Supplemental Figure S2 (cont.)

H

272 277 285 288 290 293 296 298

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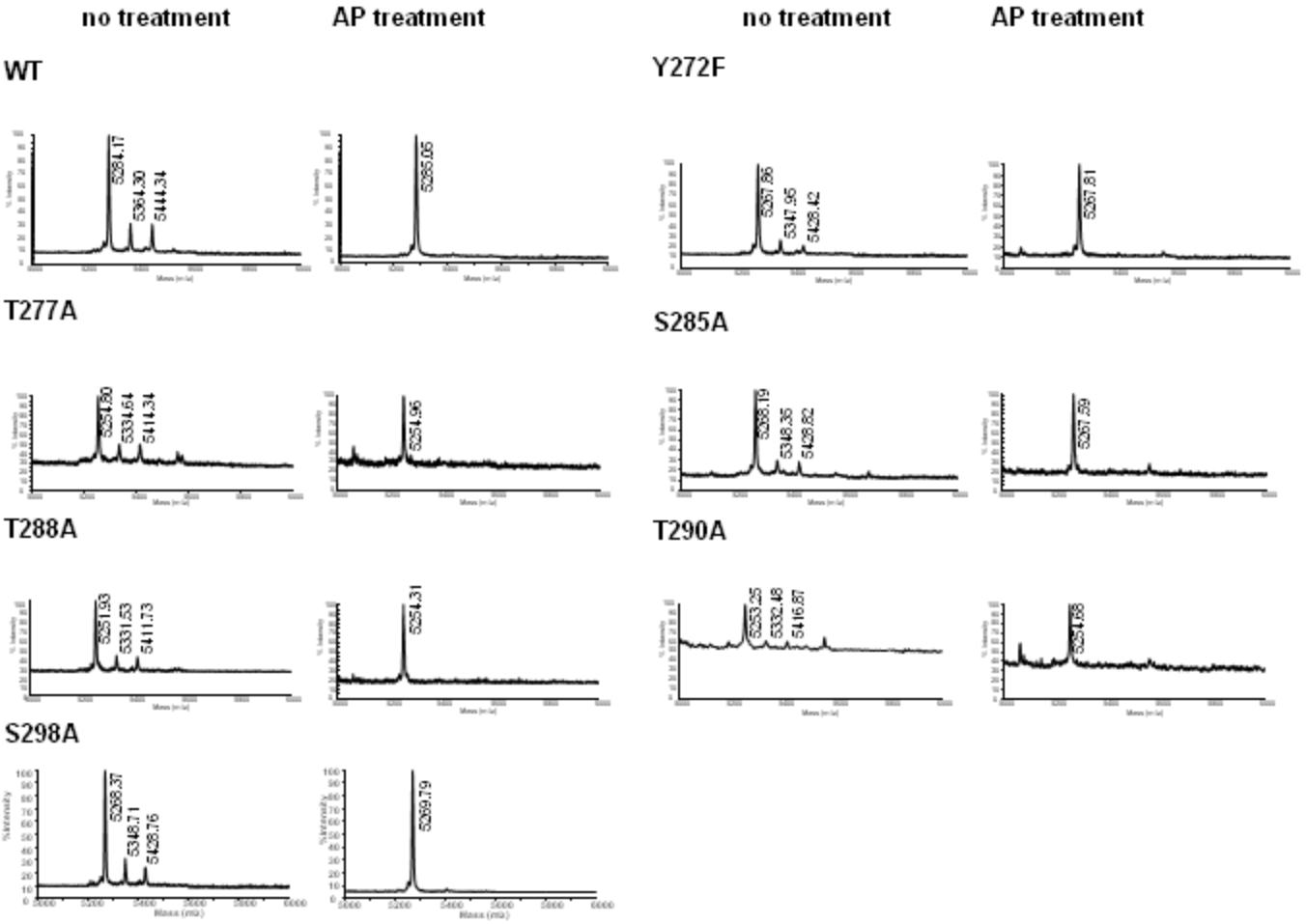
I



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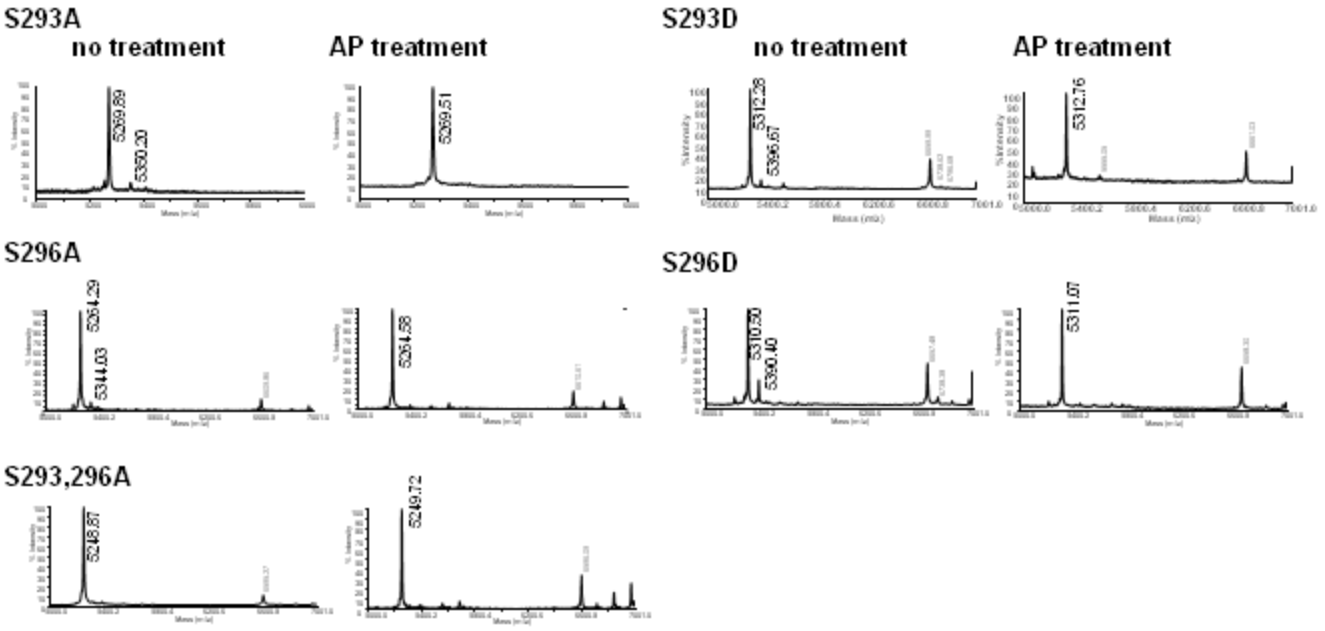
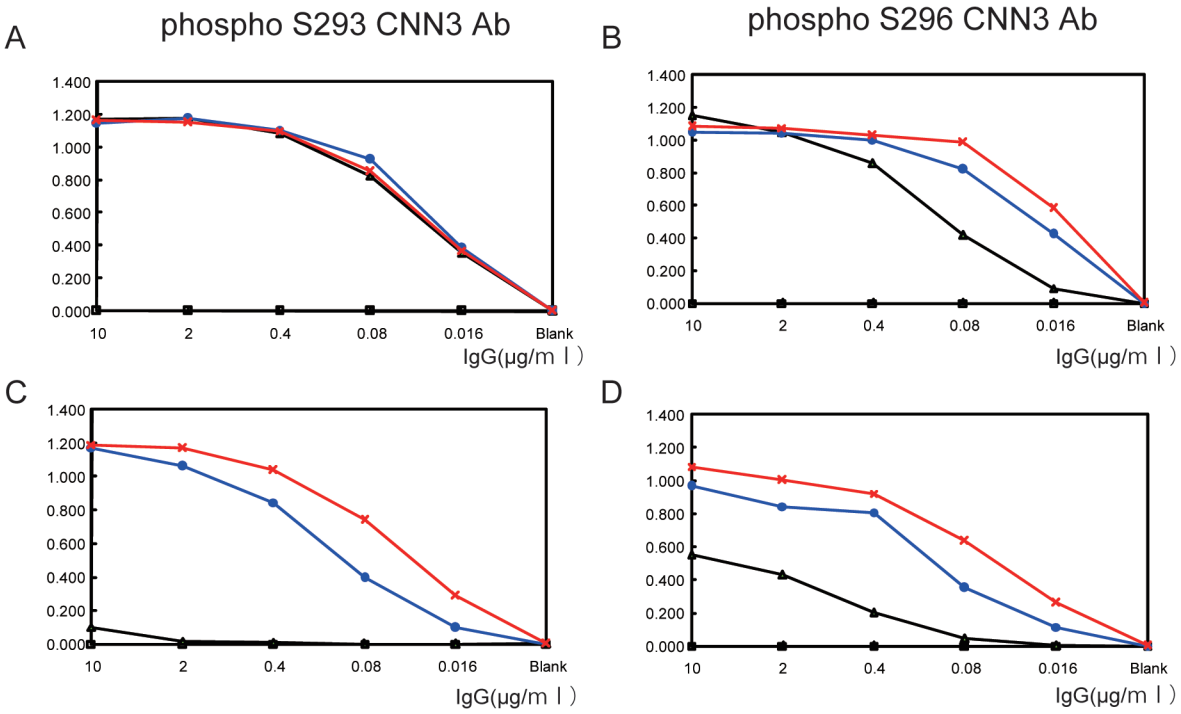
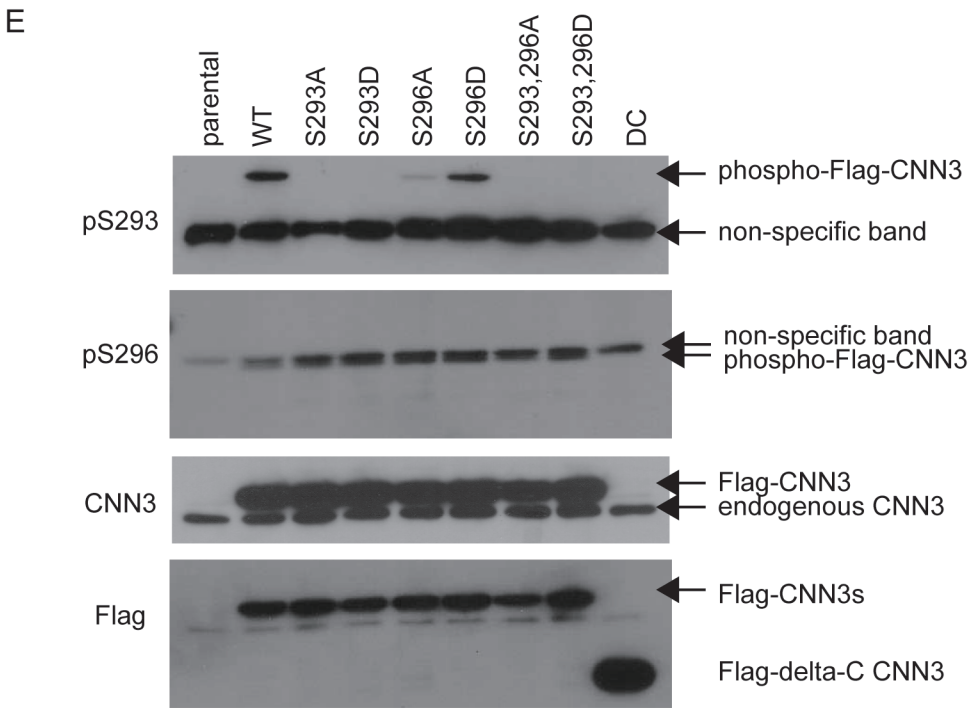
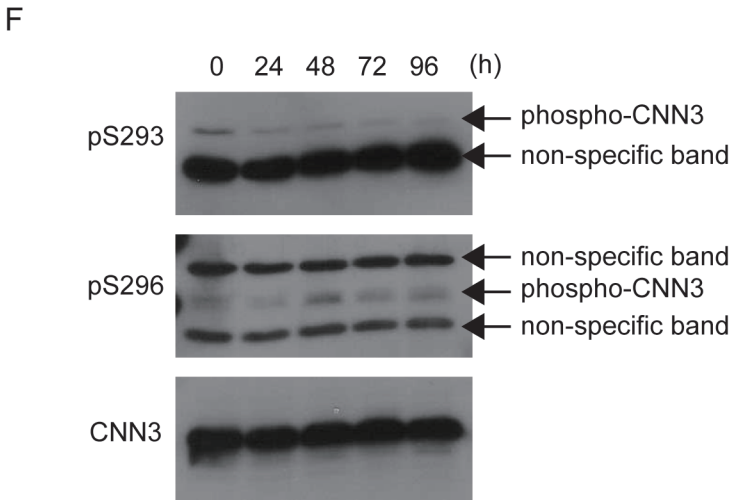


Supplemental Figure 3







Supplemental Figure 4