Expanded View Figures

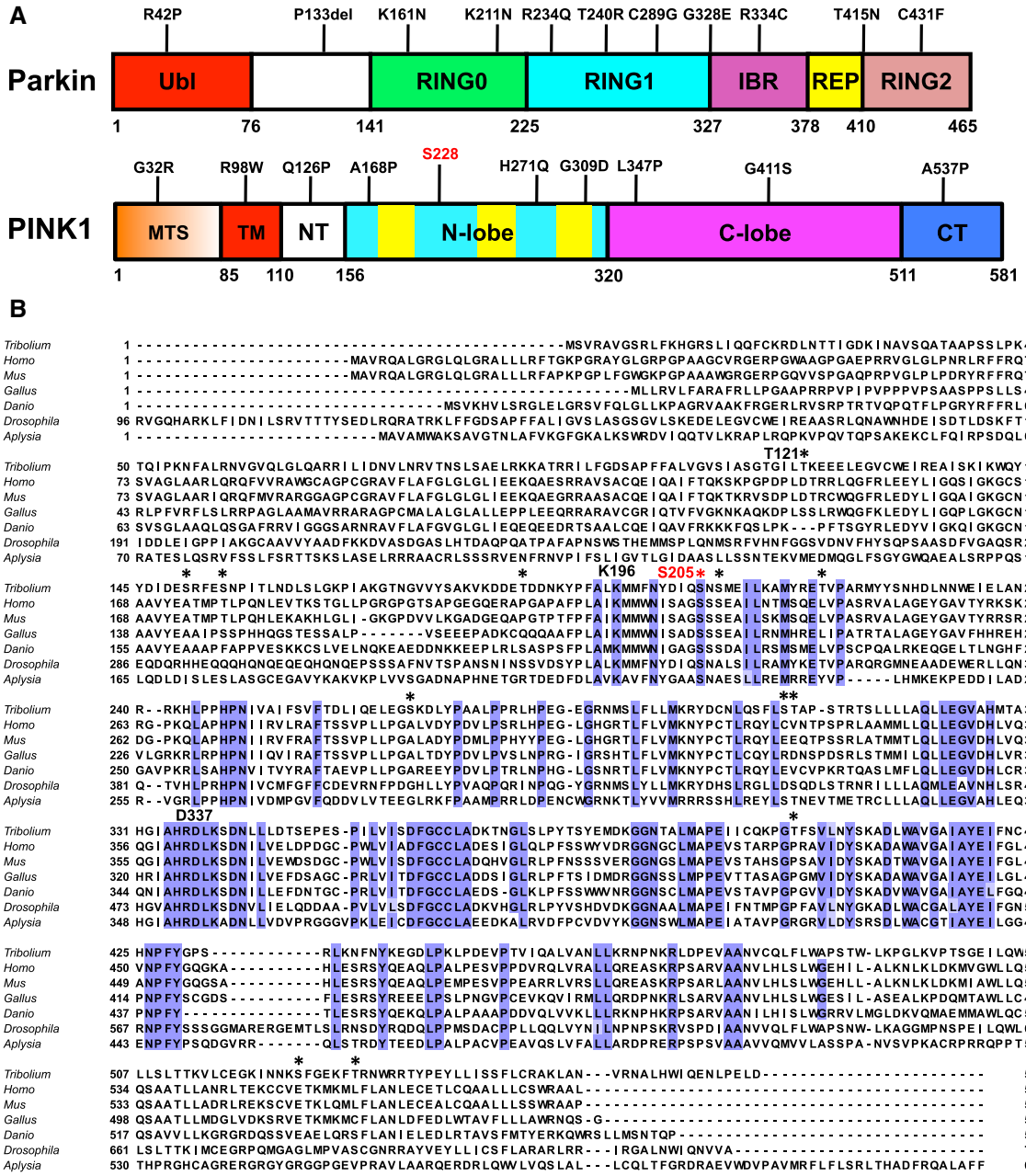

Figure EV1. Domain structure and sequence alignment of PINK1.

A Domain structure of human Parkin and PINK1. Selected PD mutations are shown on top, and residue numbers are shown below. The phosphorylation site Ser228 is highlighted in red.

B Multiple sequence alignment of PINK1 orthologs; *Tribolium castaneum* (Tc, red beetle), *Homo sapiens* (human), *Mus musculus* (mouse), *Gallus gallus* (chicken), *Danio rerio* (zebra fish), *Drosophila melanogaster* (fruit fly), and *Aplysia californica* (sea slug). The alignment shows residues conserved across all species (blue), sites of autophosphorylation found in purified TcPINK1 are indicated with *. Ser205 is colored in red, and active site residues Lys196 and Asp337 are indicated. The alignment was performed using the MUSCLE server (https://www.ebi.ac.uk/Tools/msa/muscle/) for the segment corresponding to TcPINK1$^{121–570}$. The N-terminal segments (corresponding to TcPINK1$^{1–120}$) have low conservation and were not aligned for better visual display of their lengths.

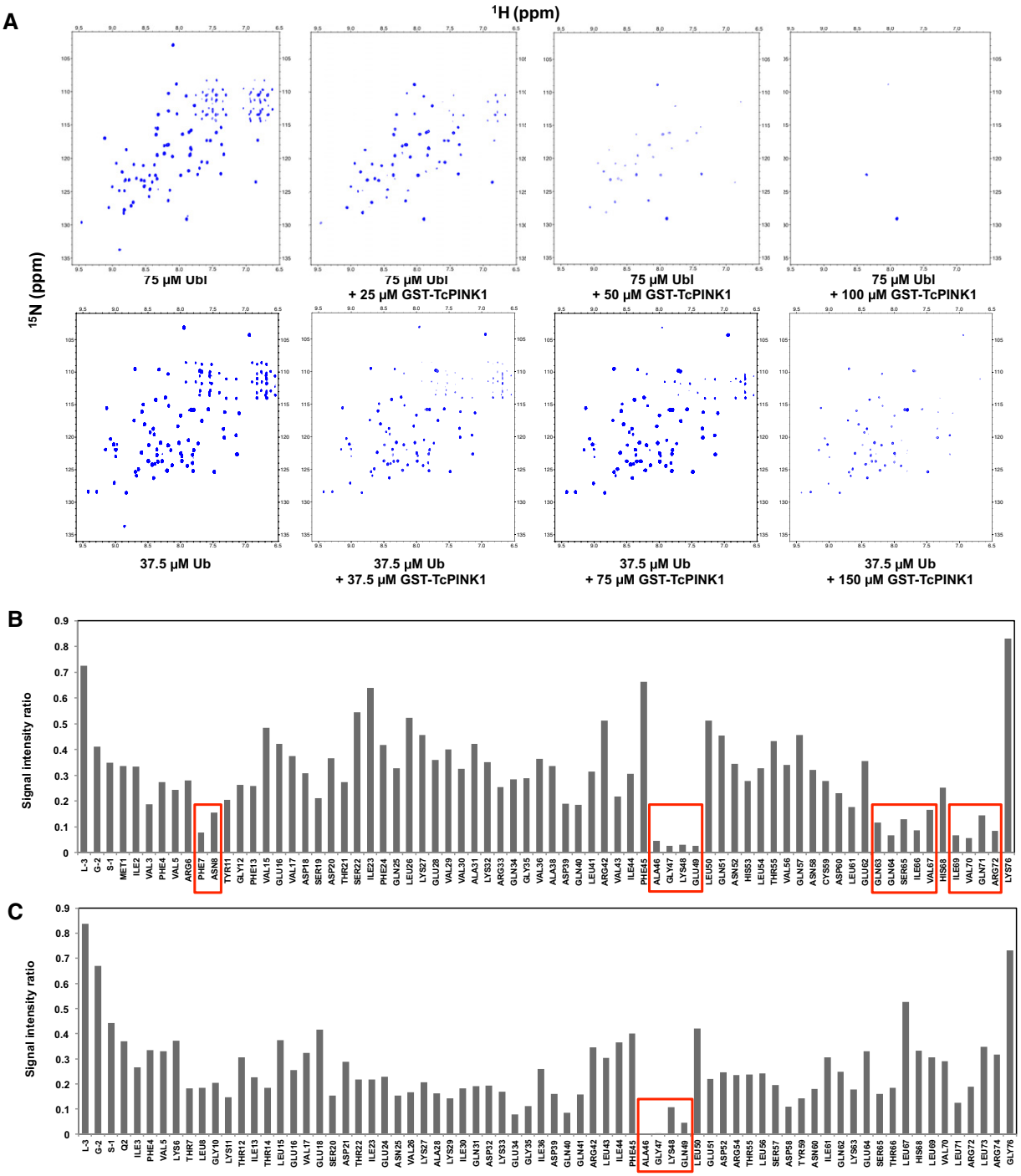

Figure EV2. TROSY NMR analysis of TcPINK1 binding to Ubl and Ub.

A Full ^{1}H-^{15}N TROSY NMR spectra of ^{2}H,^{15}N-labeled Ubl or Ub with different concentrations of GST-TcPINK1$^{121–570}$ (corresponding to Fig 1B).

B Bar graph of peak height ratios between the 75 μM Ubl + 25 μM GST TcPINK1$^{121–570}$ titration point and 75 μM Ubl for all the backbone amides from the H^{1}-N^{15} TROSY experiment shown in (A). Amides with the lowest value of peak height ratio are marked by red boxes.

C Bar graph of peak height ratios between the 37.5 μM Ub + 150 μM GST-TcPINK1$^{121–570}$ and 37.5 μM Ub for all the backbone amides from the H^{1}-N^{15} TROSY experiment shown in (A). Amides with the lowest value of peak height ratio are marked by red boxes.

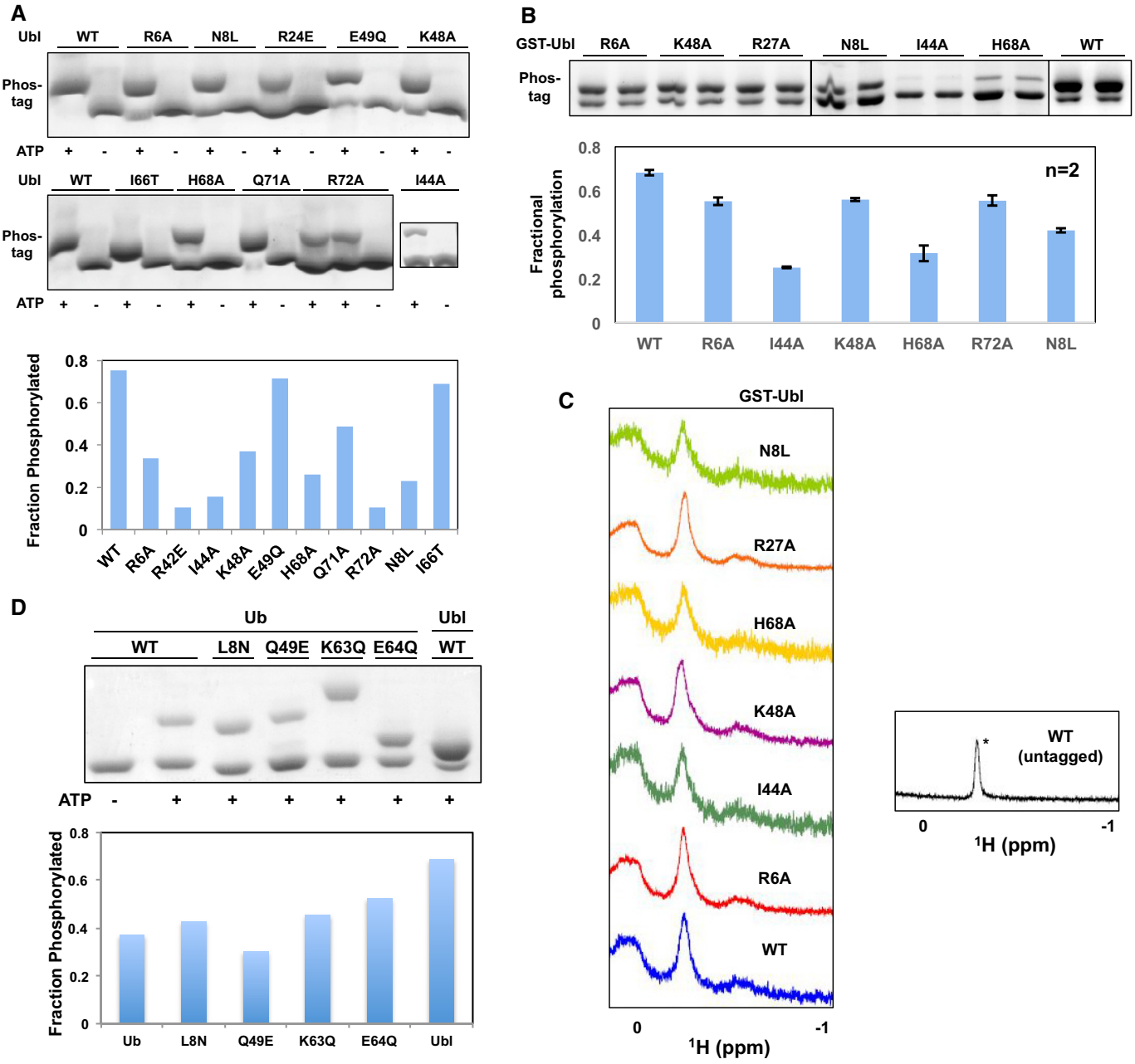

Figure EV3. Phosphorylation assays with Ubl and Ub mutants confirm the PINK1 binding site.

A (Left) Phos-tag gels of phosphorylation assays shown in Fig 1D. (Right) Fractional levels of phosphorylation of WT and point mutants of Ubl (left). The phosphorylation assays were performed with 30 μM of GST-Ubl and 2 μM GST-TcPINK1$^{143–570}$ and loaded on phos-tag gels followed by densitometry (n = 1).

B (Top) Phos-tag gels of phosphorylation assays and (bottom) fractional levels of phosphorylation of WT and point mutants of Ubl. The phosphorylation assays were performed with 12.5 μM of GST-Ubl and 2 μM GST-TcPINK1$^{121–570}$ and loaded on phos-tag gels followed by densitometry (n = 2). Error bars represent mean ± range of two independent phosphorylation experiments, loaded on the same gel.

C 1D proton NMR spectra of 30 μM GST-Ubl WT or mutants, or untagged Ubl showing the characteristic Leu61 peak at −0.2 ppm (*) observed in all the mutants shown.

D (Top) Phos-tag gels of phosphorylation assays and (bottom) fractional levels of phosphorylation of mutants of point of Ub made by mutating residues in Ub to corresponding residues in Ubl. The assays were conducted with 30 μM of GST-Ubl and 0.5 μM GST-TcPINK1$^{121–570}$ for 30 min (n = 1).

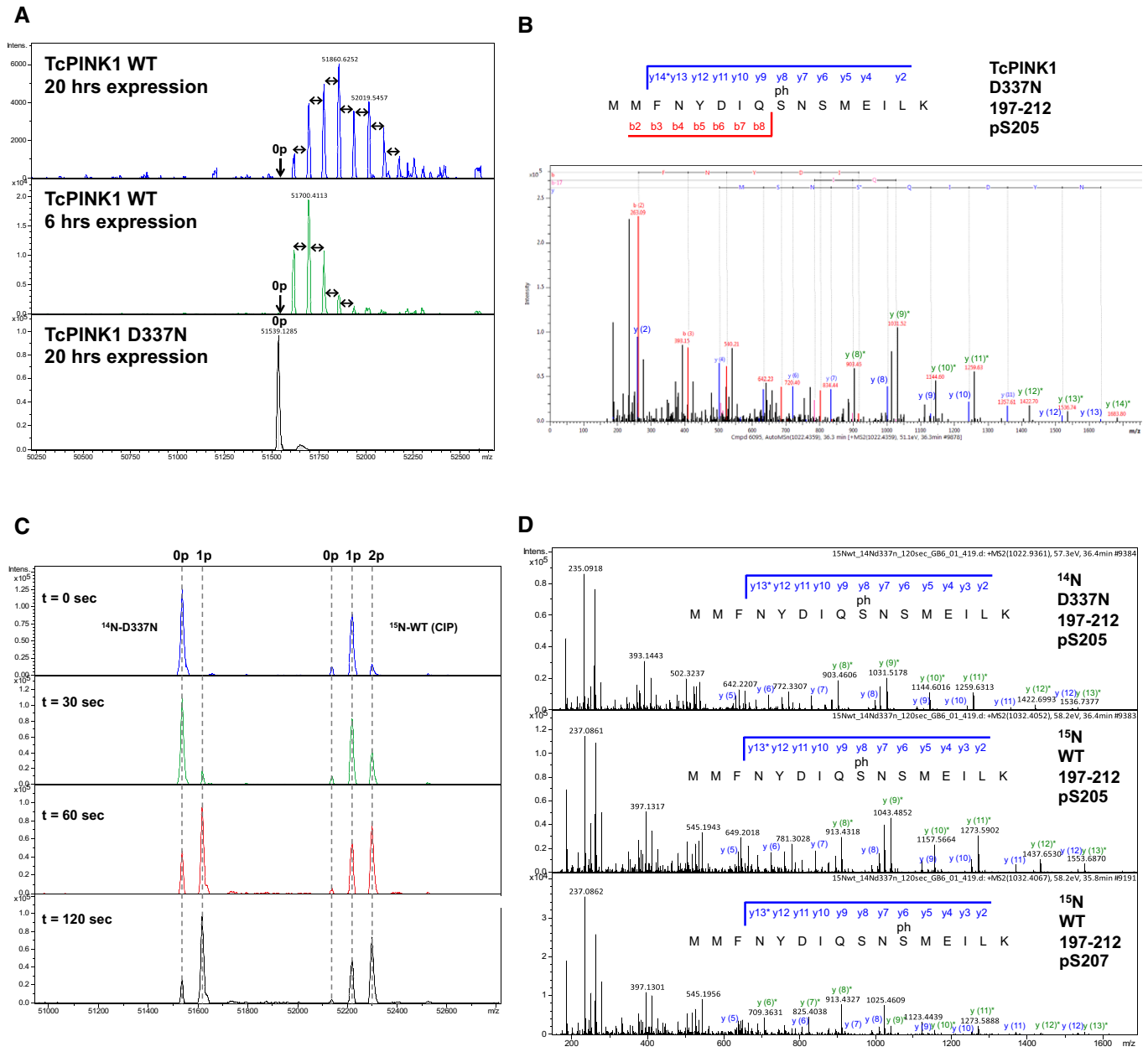

Figure EV4. Mass spectrometry analysis of TcPINK1 autophosphorylation.

A Intact mass spectra of TcPINK1$^{121–570}$ WT or D337N expressed for different times in *E. coli*. The double-edged arrows on the spectra are used to indicate a difference of 80 Da (1 phosphorylation) between peaks. "0p" indicates the theoretical mass of the unphosphorylated protein.

B MS/MS spectrum for the peptide 197-212 of TcPINK1$^{121–570}$ D337N, phosphorylated by GST-TcPINK1$^{121–570}$ WT (see Fig 3C). The b and y series indicate that phosphorylation takes place at Ser205. The y* series (green) show neutral loss of a phosphate group (H_3PO_4, −97.7 amu).

C Intact mass spectra of the time course phosphorylation assay of TcPINK1 D337N with CIP-treated ^{15}N-TcPINK1 WT (corresponding to the plot in Fig 3D).

D MS/MS spectrum for the peptide 197–212 of TcPINK1$^{121–570}$ ^{14}N-D337N and ^{15}N-WT, after 2-min phosphorylation (see Fig 3E). The b and y series indicate that phosphorylation takes place at Ser205 for the ^{14}N and ^{15}N-labeled peptides eluting at 36.5 min, and Ser207 for the ^{15}N-labeled peptide eluting earlier at 35.8 min. The y* series (green) show neutral loss of a phosphate group (H_3PO_4, −97.7 amu). The different phosphorylation sites give rise to y(6) and y(7) ions with different masses.

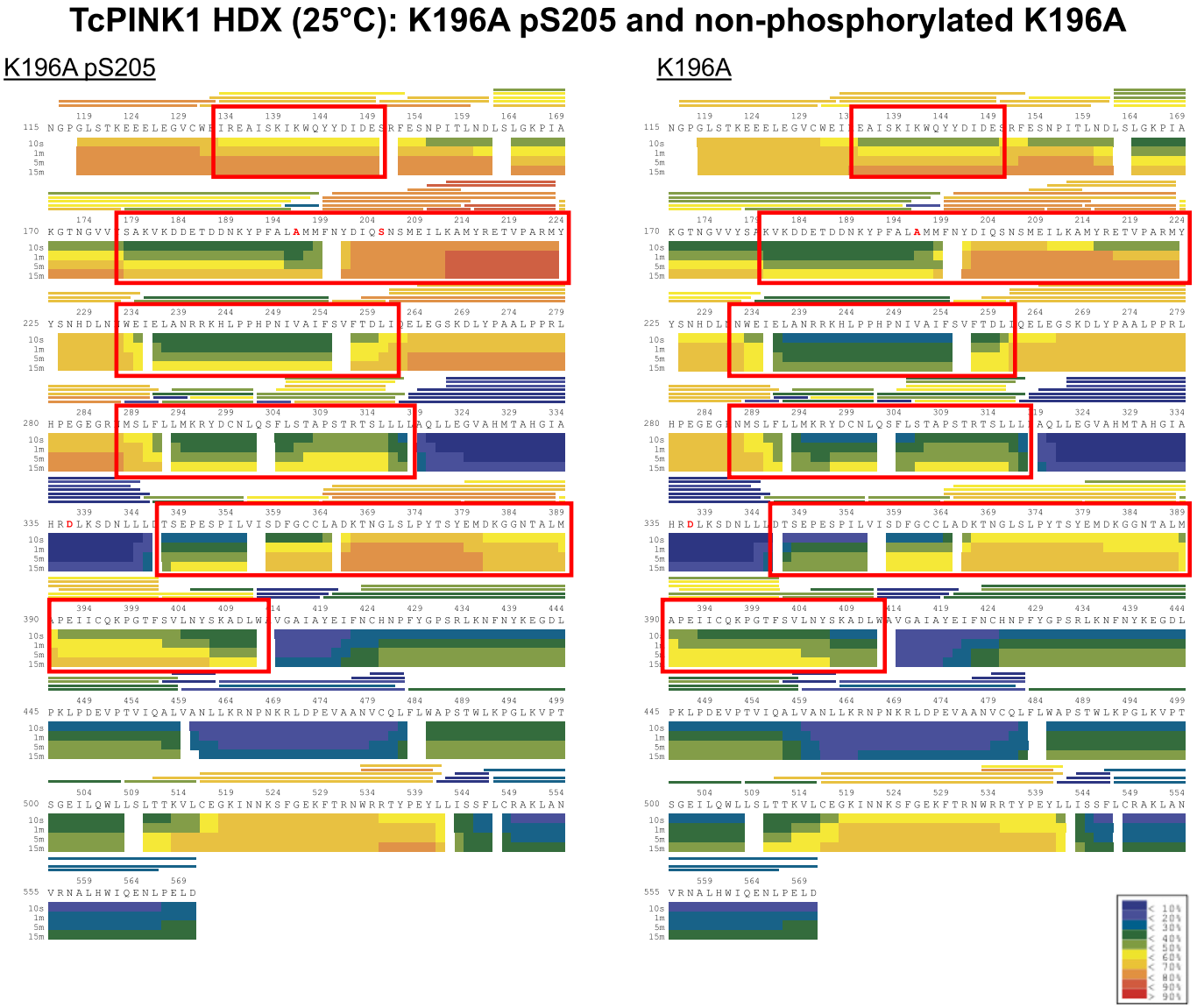

Figure EV5. HDX analysis of the effect of S205 phosphorylation on K196A.
Heat maps of K196A (phosphorylated at Ser205) before and after CIP treatment, representing the level of deuterium uptake over time by different regions of the proteins. Regions of the heat maps enclosed in red boxes show significant differences in deuterium uptake between the two proteins. The small inset on the bottom right contains the color-coding key for the heat map, with blue and red indicating low and high deuteration rates, respectively.