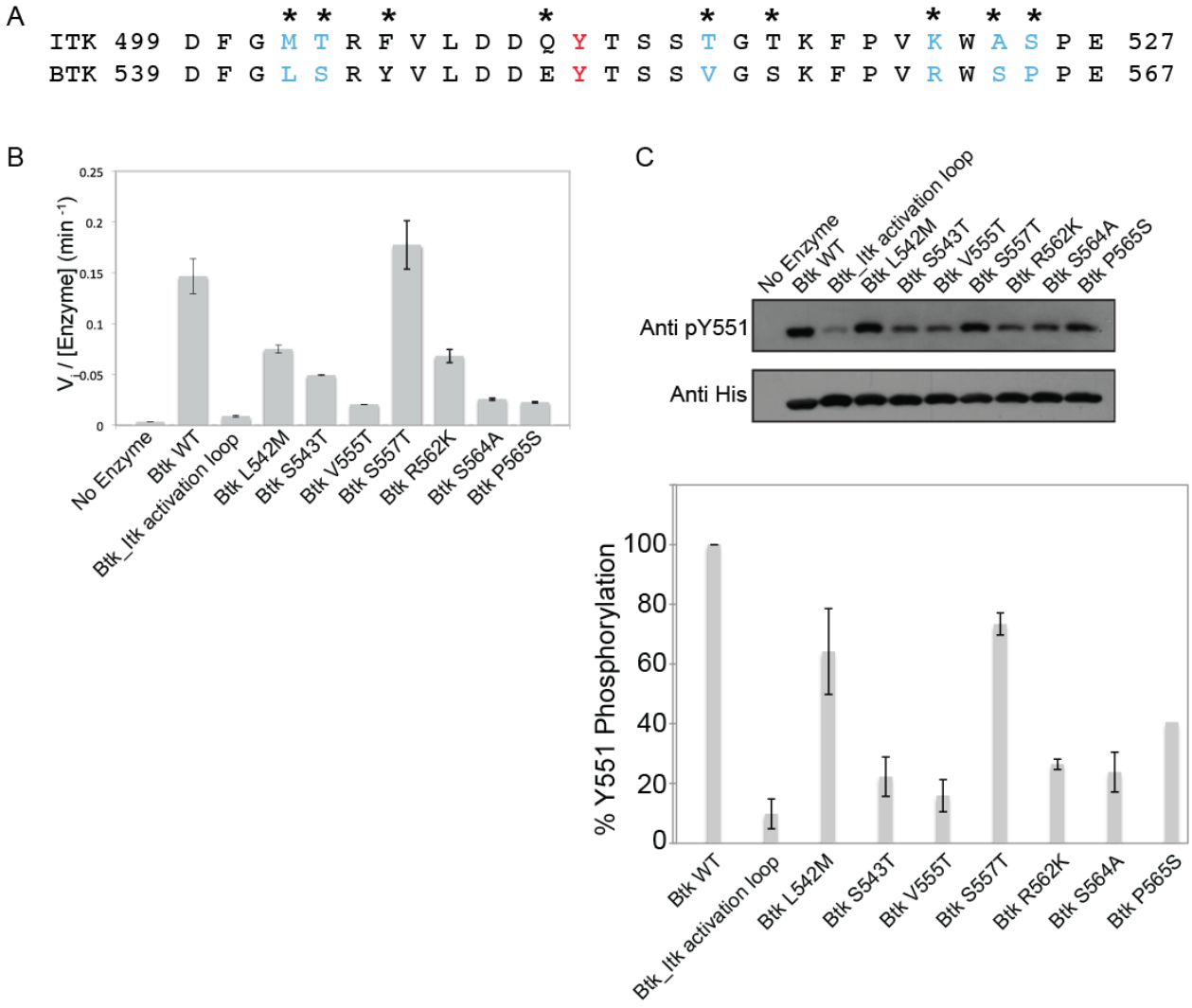


Fig. S1. Mutational analysis of the kinase activation segment of Btk. (**A**) Alignment of the sequences of the activation loops of Itk and Btk. (**B** and **C**) The isolated kinase domain of WT Btk was mutated so that it contained the corresponding activation segment residues of Itk. The activities of the Btk activation segment mutants were tested by (B) an in vitro kinase assay with ^{32}P-ATP in which phosphorylation of a peptide substrate (Peptide B) was monitored or (C) by Western blotting analysis of the extent of phosphorylation of the activation loop (Btk Y551). Data in (B) are means ± SD from two independent experiments. The intensities of the pY551 bands in two independent experiments [represented by blots from a single experiment in (B)] were analyzed by densitometry and quantified by setting the abundance of pY551 in samples with WT Btk at 100% and reporting the relative amounts of pY551 for all other proteins as percentages. Error bars represent ± SD from the mean.

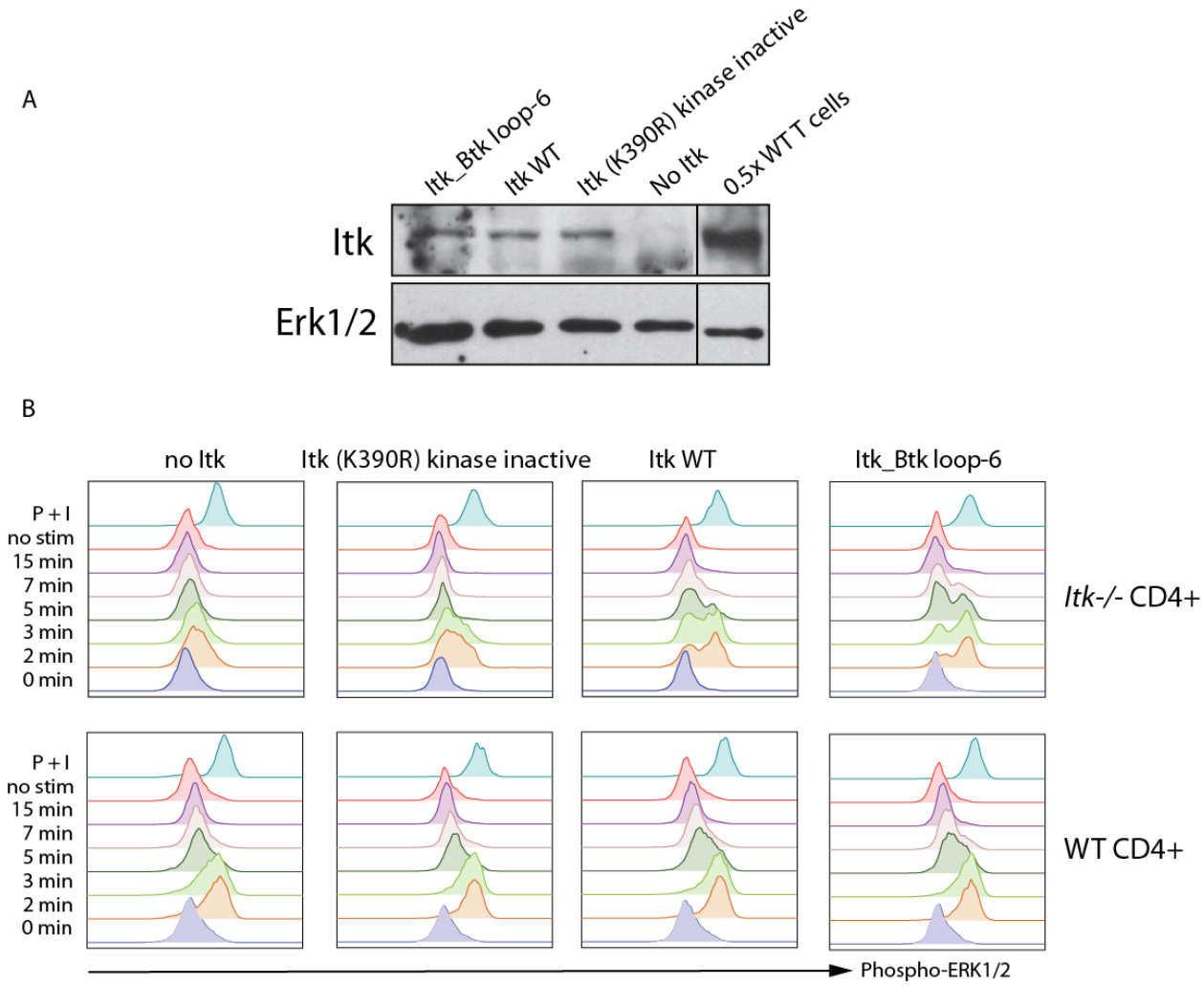


Fig. S2. Analysis of pERK1/2 abundance in primary T cells transduced with retroviruses encoding Itk. (**A**) Analysis of Itk protein abundance in retrovirally transduced *Itk*$^{-/-}$ T cells.*Itk*$^{-/-}$ OT-II TCR transgenic CD4^{+} T cells were infected with control retrovirus (No Itk) or were infected with retroviruses expressing the indicated Itk proteins. Equivalent numbers of Thy1.1^{+} cells were isolated by flow cytometry and lysed in RIPA buffer. As a control for Itk protein expression, one-half the number of wild-type T cells was analyzed (0.5× WT T cells). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes, which were then incubated with antibodies specific for Itk (top) and total ERK1/2 (bottom). Itk protein amounts in the transduced cells were ~10-fold less abundant that that of Itk in WT T cells. Data are representative of three independent experiments. (**B**) *Itk*$^{-/-}$or wild-type (WT) OT-II TCR transgenic CD4^{+} T cells were infected with control retrovirus (no Itk) or with retroviruses expressing the indicated ITK proteins. Thy1.1^{+} cells were isolated by flow cytometry and were left untreated or were stimulated with antibody against CD3(to activate the TCR) for 2, 3, 5, 7, or 15 min. Cells were fixed, permeabilized, and incubated with antibodies specific for CD4, Thy1.1, and pERK1/2. Histograms show pERK1/2 staining for gated CD4^{+} Thy1.1^{+} cells. Non-stimulated cells (no stim) did not receive any stimulatory anti-CD3 antibody. As a positive control, cells were stimulated with PMA and ionomycin (P + I) for 4 min.

Fig. S3. HDX-MS analysis of Btk and Btk_Itk loop-6. (**A**) Peptide map created with MSTools(*45*) for the online digestion of WT Btk and the Btk_Itk loop-6 mutant. The light blue bars below the sequence indicate the individual peptide fragments presented in the rest of the figure. (**B** to **E**) Comparison of the relative percentages of deuterium incorporation for peptide fragments from WT Btk and the Btk_Itk loop-6 mutant. Numbers above each plot corresponds to the length of the peptide fragment analyzed (full-length Btk numbering). The x-axis represents the time (min) for which the deuterium exchange reaction has been carried out. The y-axis in each graph represents the percentage of deuterium incorporated relative to the maximum possible exchangeable amides for Btk (blue diamonds) and the Btk_Itk loop-6 mutant (red squares). For the current analysis, we focused on data derived from the activation segments of the different kinases, and so complete analysis of differences throughout the kinase domains will be provided in a separate manuscript.

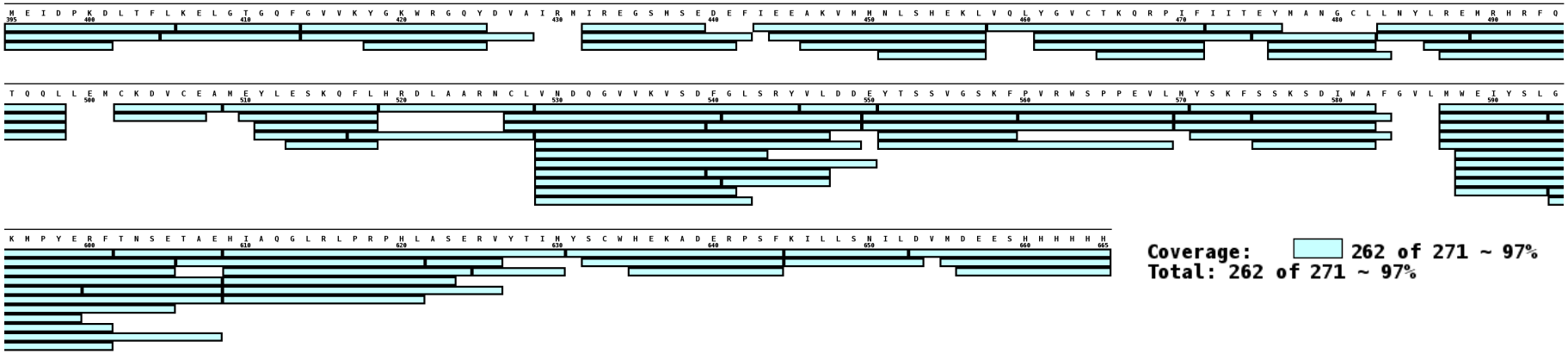
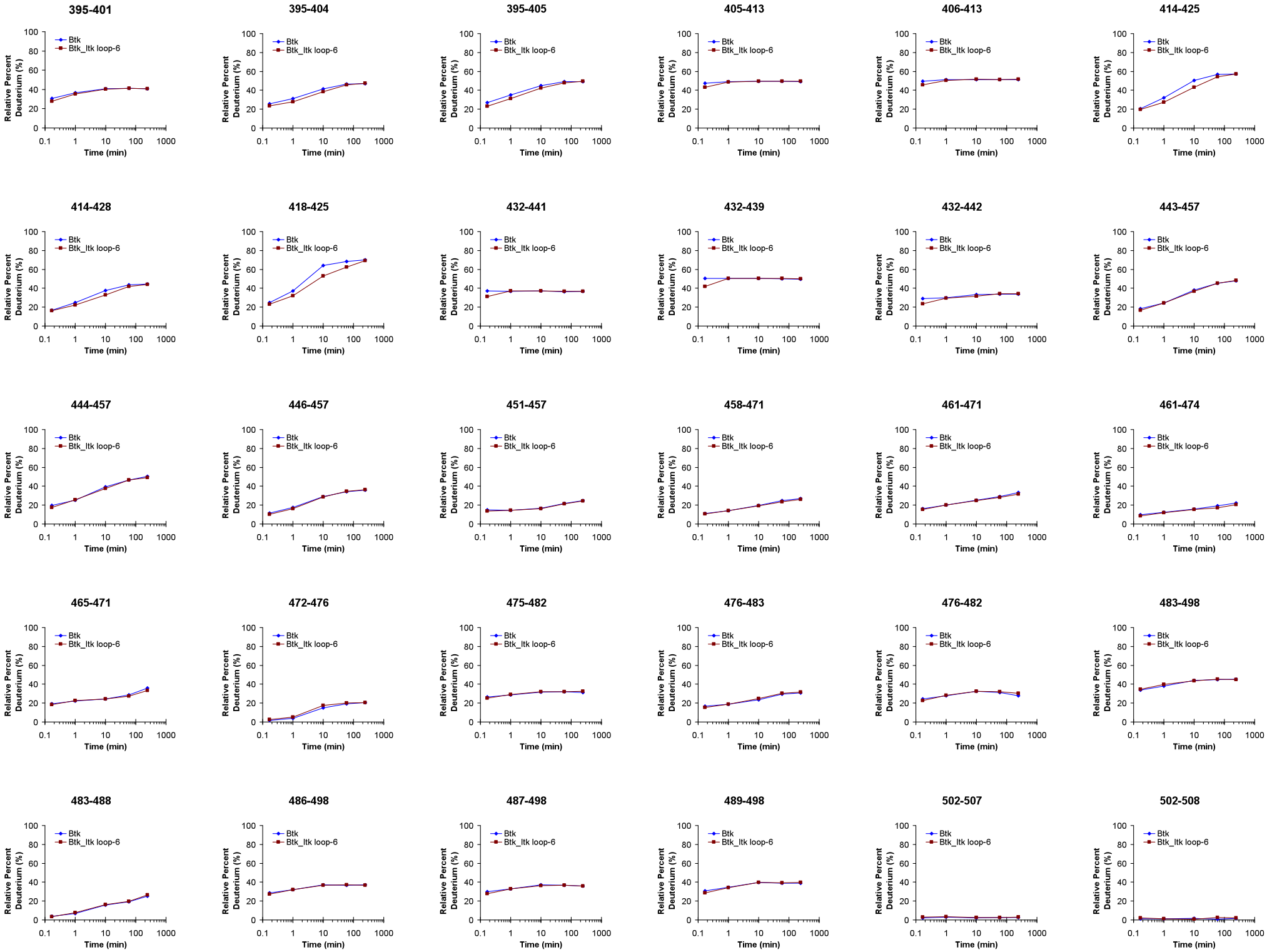


Figure S3 A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461-471
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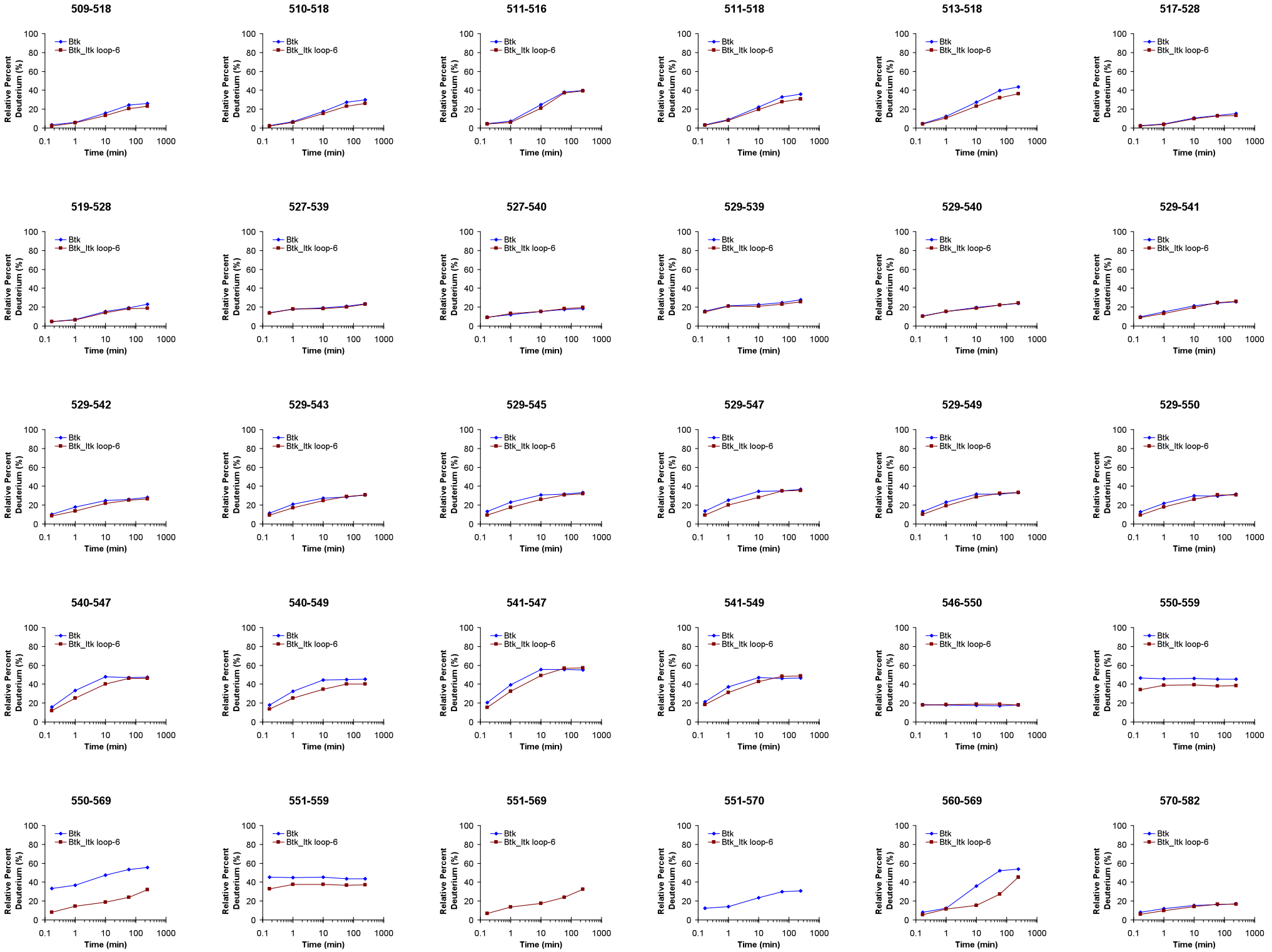
465-471
472-476
475-482
476-483
476-482
483-498
483-488
486-498
487-498
489-498
502-507
502-508
Btk
Btk_Itk loop-6
Relative Percent Deuterium (%)
Time (min)
0.1
1
10
100
1000
0
20
40
60
80
100

Figure S3 B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546-550
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550-569
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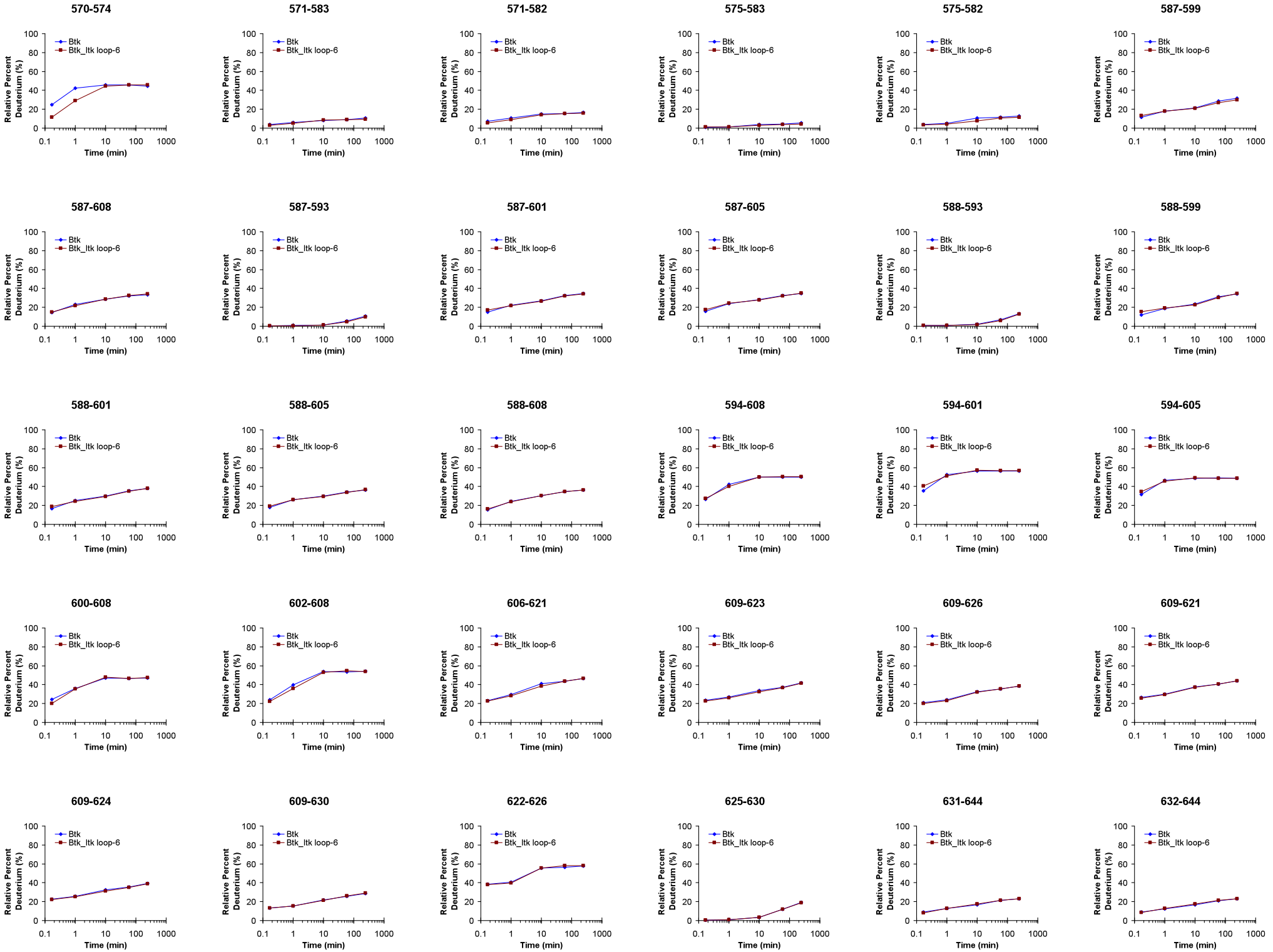
551-569
551-570
560-569
570-582
Btk
Btk_Itk loop-6
Relative Percent Deuterium (%)
Time (min)

Figure S3 C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602-608
606-621
609-623
609-626
609-621
609-624
609-630
622-626
625-630
631-644
632-644
Btk
Btk_Itk loop-6
Relative Percent Deuterium (%)
Time (min)

Figure S3 D

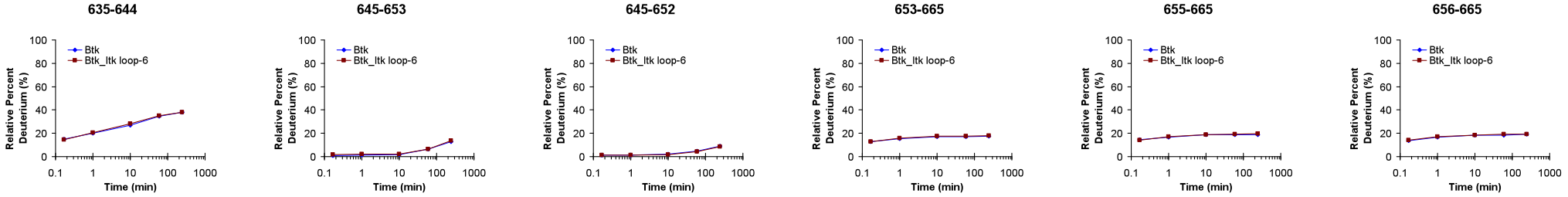
635-644
645-653
645-652
653-665
655-665
656-665
Btk
Btk_Itk loop-6
Relative Percent Deuterium (%)
Time (min)
0
20
40
60
80
100
0.1
1
10
100
1000

Figure S3 E

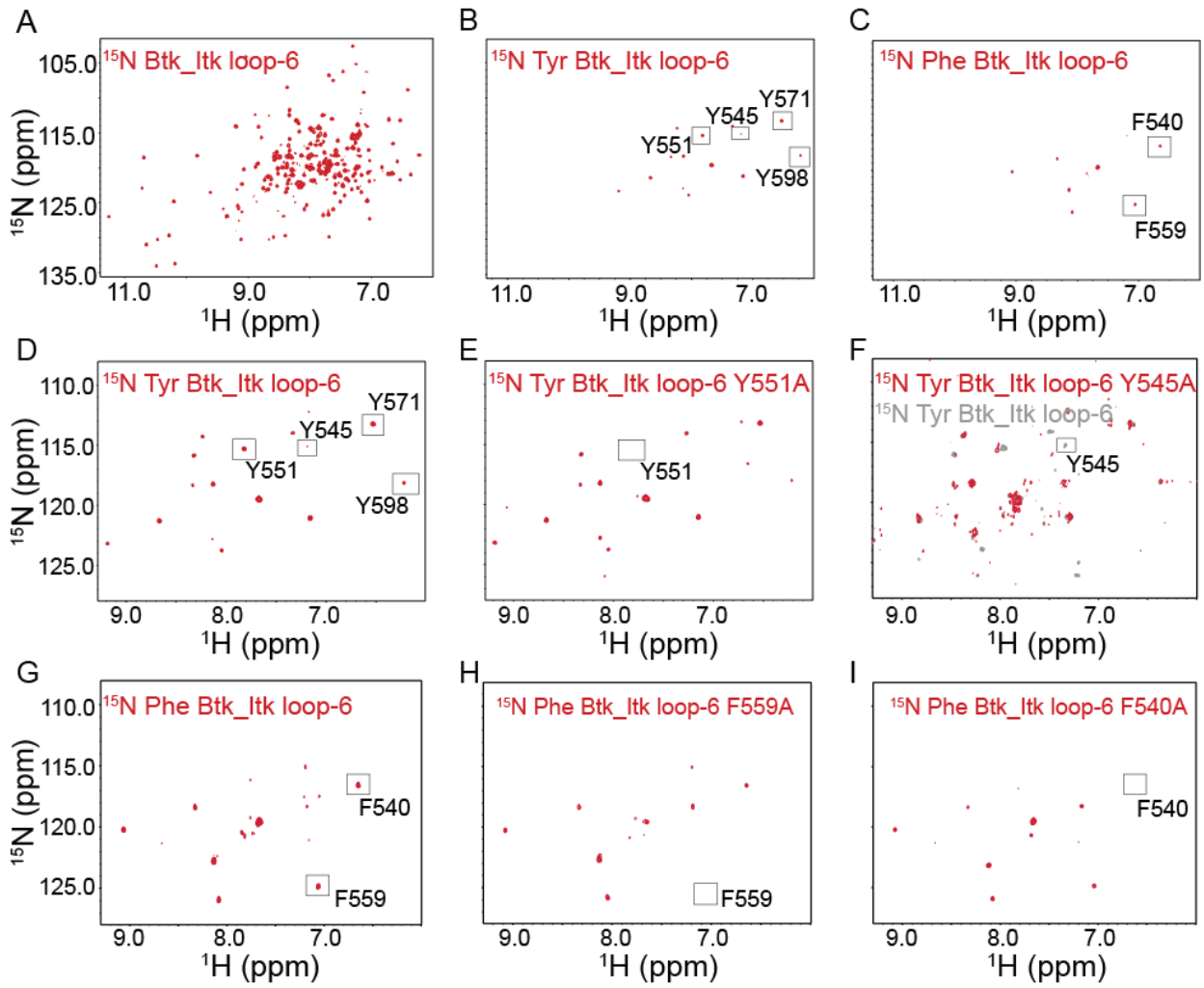

Fig. S4. Chemical shift assignment of the residues in the Btk_Itk loop-6 mutant. (**A** to **C**) ^{1}H-^{15}N TROSY HSQC spectra of the (A) ^{15}N uniformly labeled, (B)^{15}N Tyrosine selectively labeled, and (C)^{15}N Phenylalanine selectively labeled Btk_Itk loop-6 mutant. The peaks corresponding to Tyr^{551}, Tyr^{545}, Tyr^{571}, Tyr^{598}, Phe^{540}, and Phe^{559} are boxed and labeled. (**D** to **I**) Expanded views of the ^{1}H-^{15}N TROSY HSQC spectra of the Btk_Itk loop-6mutant in (B) or (C) with the contour level adjusted for maximum signal-to-noise.^{15}N Tyrosine selectively labeled ^{1}H-^{15}N TROSY HSQC spectra of (D) the Btk_Itk loop-6 mutant or (E) the Btk_Itk loop-6 Y551A mutant. Lower contour levels of the spectrum in (E) show the presence of the peak corresponding to Tyr^{545}, but not Tyr^{551}. (F) Overlay of the ^{1}H-^{15}N HSQC spectra of ^{15}N Tyrosine selectively labeled Btk_Itk loop-6 (gray) and Btk_Itk loop-6 Y545A (red). The peak corresponding to Tyr^{545} (boxed peak) is missing in the spectrum of the Btk_Itk loop-6 Y545A mutant. ^{15}N Phenylalanine selectively labeled ^{1}H-^{15}N TROSY HSQC spectra of (G) Btk_Itk loop-6, (H) Btk_Itk loop-6 F559A, and (I) Btk_Itk loop-6 F540A. The boxed peaks corresponding to Phe^{559}and Phe^{540} are missing in the spectra in (H) and (I), respectively. Each experiment was carried out at least twice.

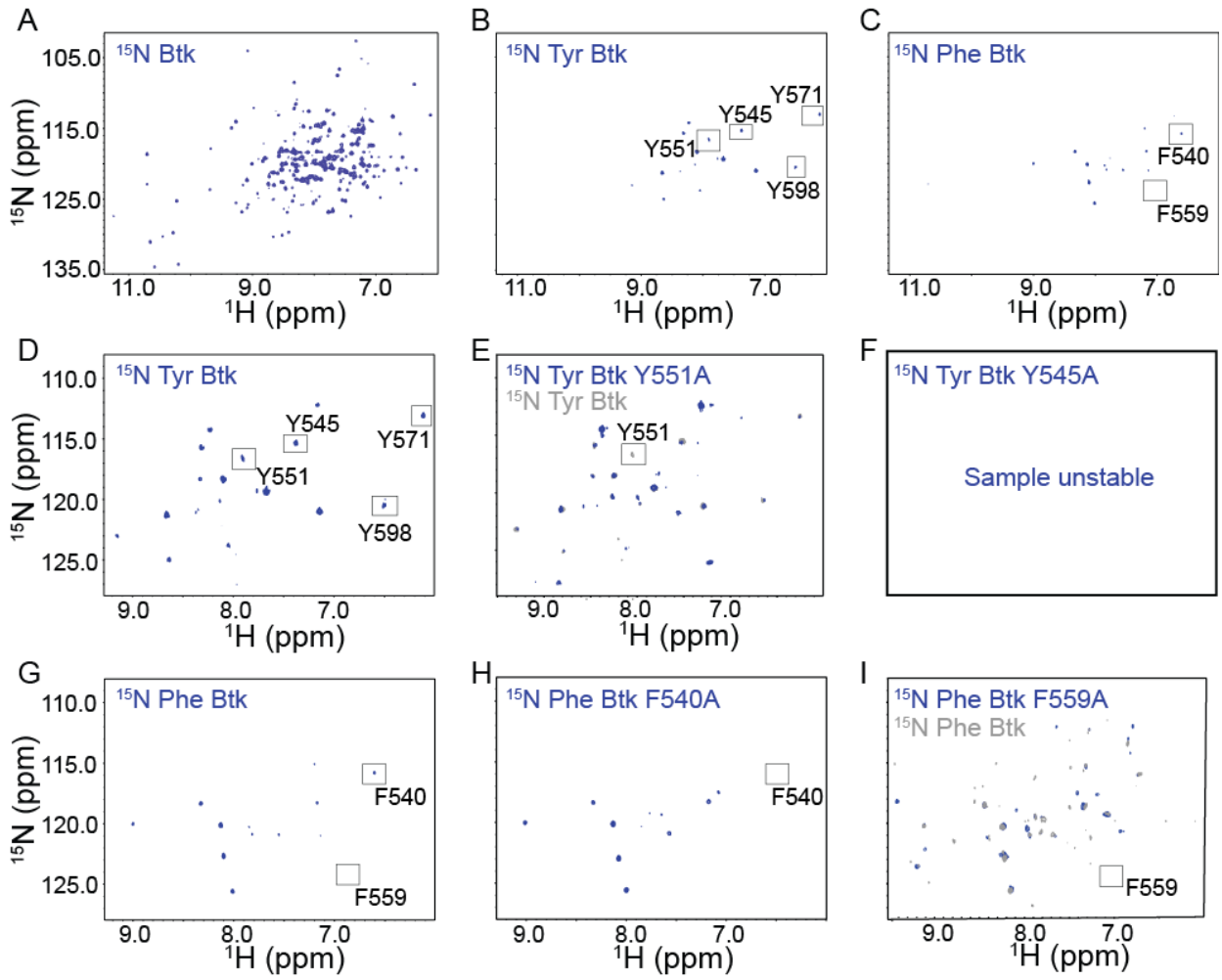

Fig. S5. Chemical shift assignment of the residues of WT Btk. (**A** to **C**) ^{1}H-^{15}N TROSY HSQC spectra of the (A) ^{15}N uniformly labeled, (B) ^{15}N Tyrosine selectively labeled, and (C) ^{15}N Phenylalanine selectively labeled WT Btk protein. The peaks corresponding to Tyr^{551}, Tyr^{545}, Tyr^{571}, Tyr^{598}, Phe^{540}, and Phe^{559} are boxed and labeled. (**D** to **I**) Expanded views of the ^{1}H-^{15}N TROSY HSQC spectrum of WT Btk in (B) or (C), with the contour level adjusted for maximum signal-to-noise ratio. (D) ^{15}N Tyrosine selectively labeled ^{1}H-^{15}N TROSY HSQC spectra of WT Btk. (E) Overlay of the ^{1}H-^{15}N HSQC spectra of ^{15}N Tyrosine selectively labeled WT Btk (gray) and the Btk Y551A mutant (blue). The peak corresponding to Tyr^{551} (boxed peak) is missing in the spectrum of the Btk Y551A mutant. (F) The ^{15}N Tyrosine selectively labeled spectrum of the Btk Y545A mutant could not be acquired because of poor sample stability. Assignment of Tyr^{545} peak within the spectrum of WT Btk was done based on its proximity to Tyr^{545} in the spectrum of the Btk_Itk loop mutant (Fig.6F). ^{15}N Phenylalanine selectively labeled ^{1}H-^{15}N TROSY HSQC spectra of (G) WT Btk and (H) the Btk F540A mutant. The boxed peak corresponding to Phe^{540} is missing in the spectrum in (H). (I) Overlay of the ^{1}H-^{15}N HSQC spectra of ^{15}N Phenylalanine selectively labeled WT Btk (gray) and the Btk F559A mutant (blue). The peak corresponding to Phe^{559} shows extensive line-broadening and is visible at lower contour levels (Fig.6H) of the spectrum in (A).