

Supplementary Information

Protonation- and substrate-regulated dimer opening couples brain-type creatine kinase to vesicular and actin-remodeling membranes

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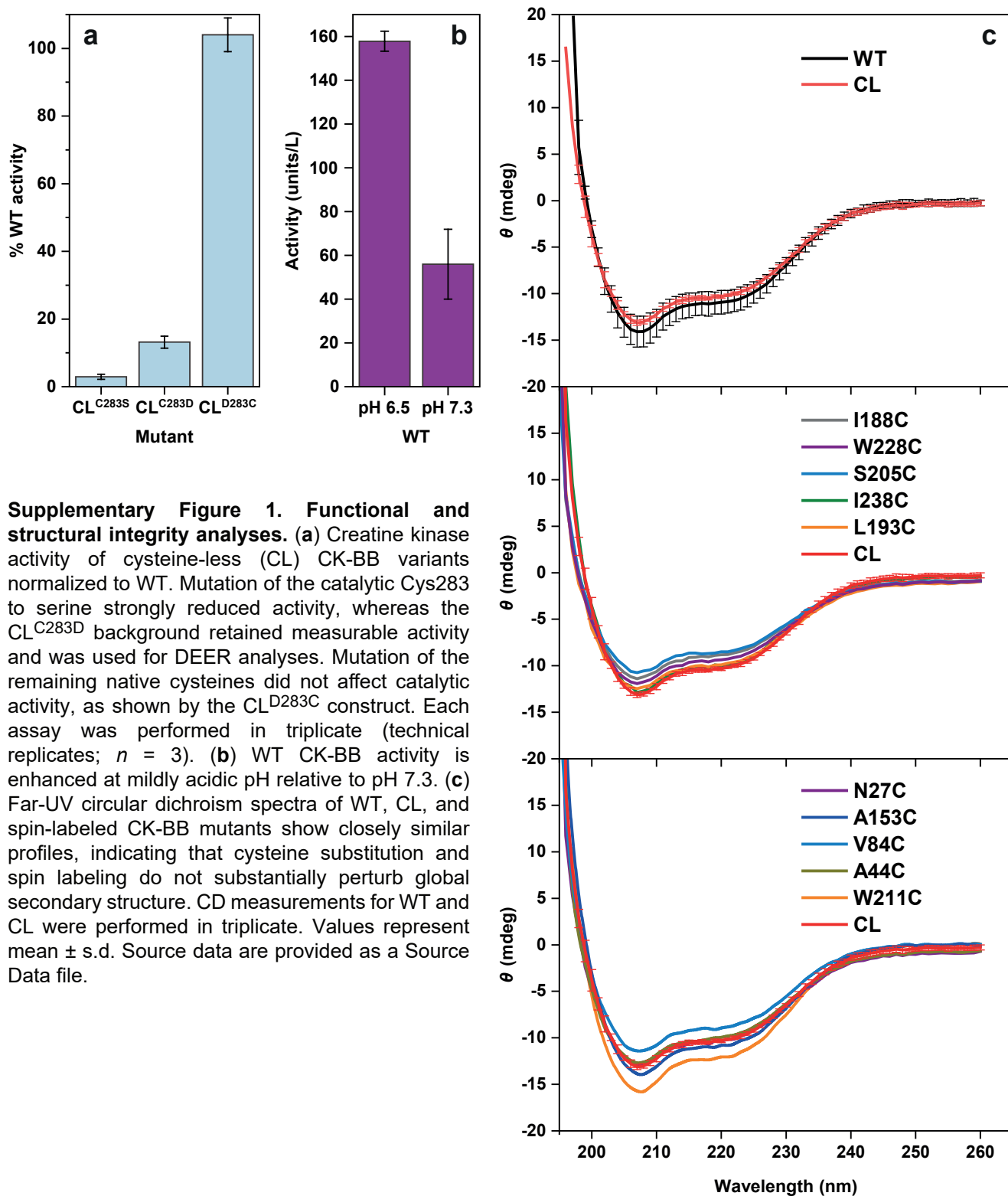
⁶ These authors contributed equally to this work.

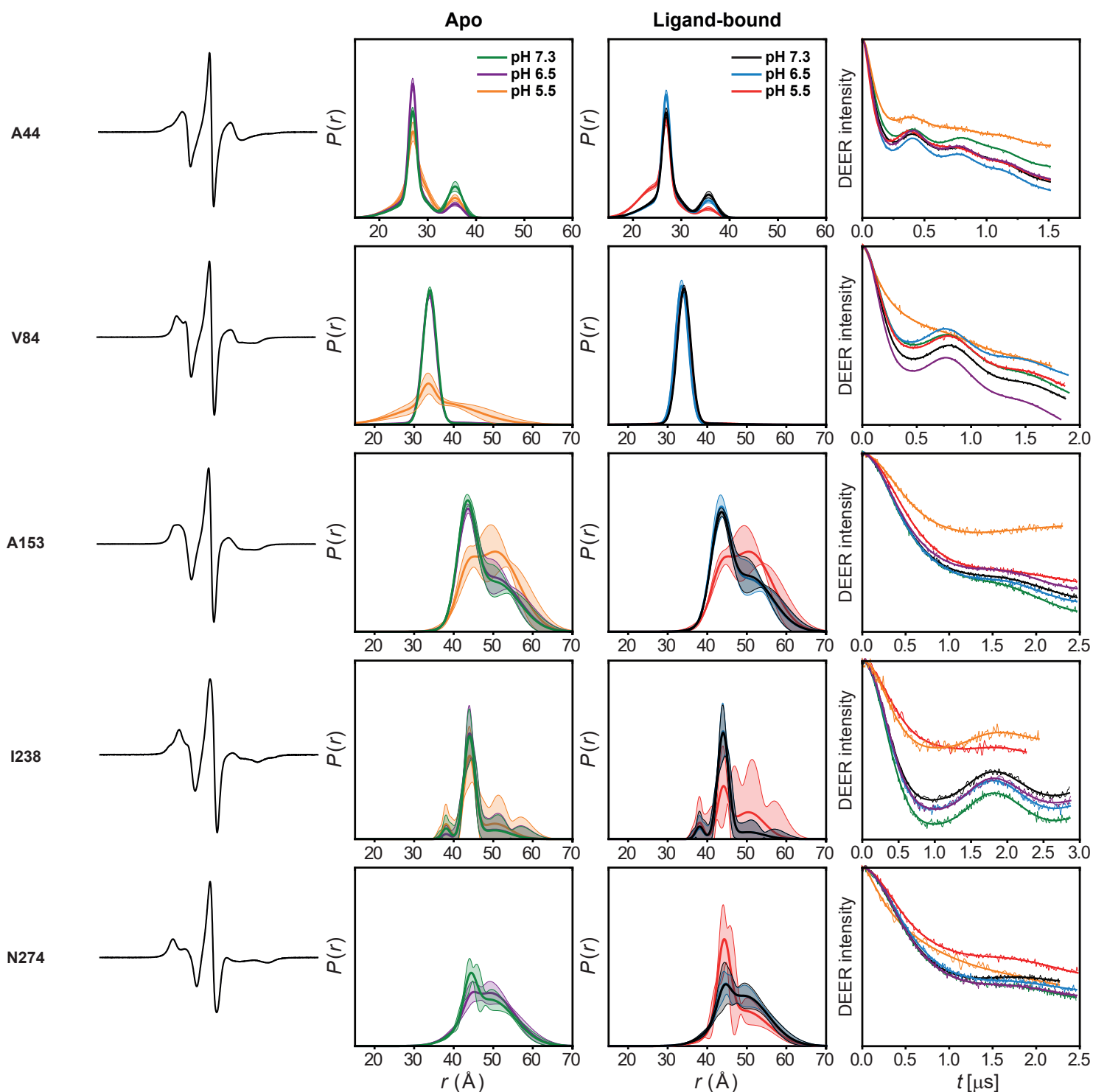
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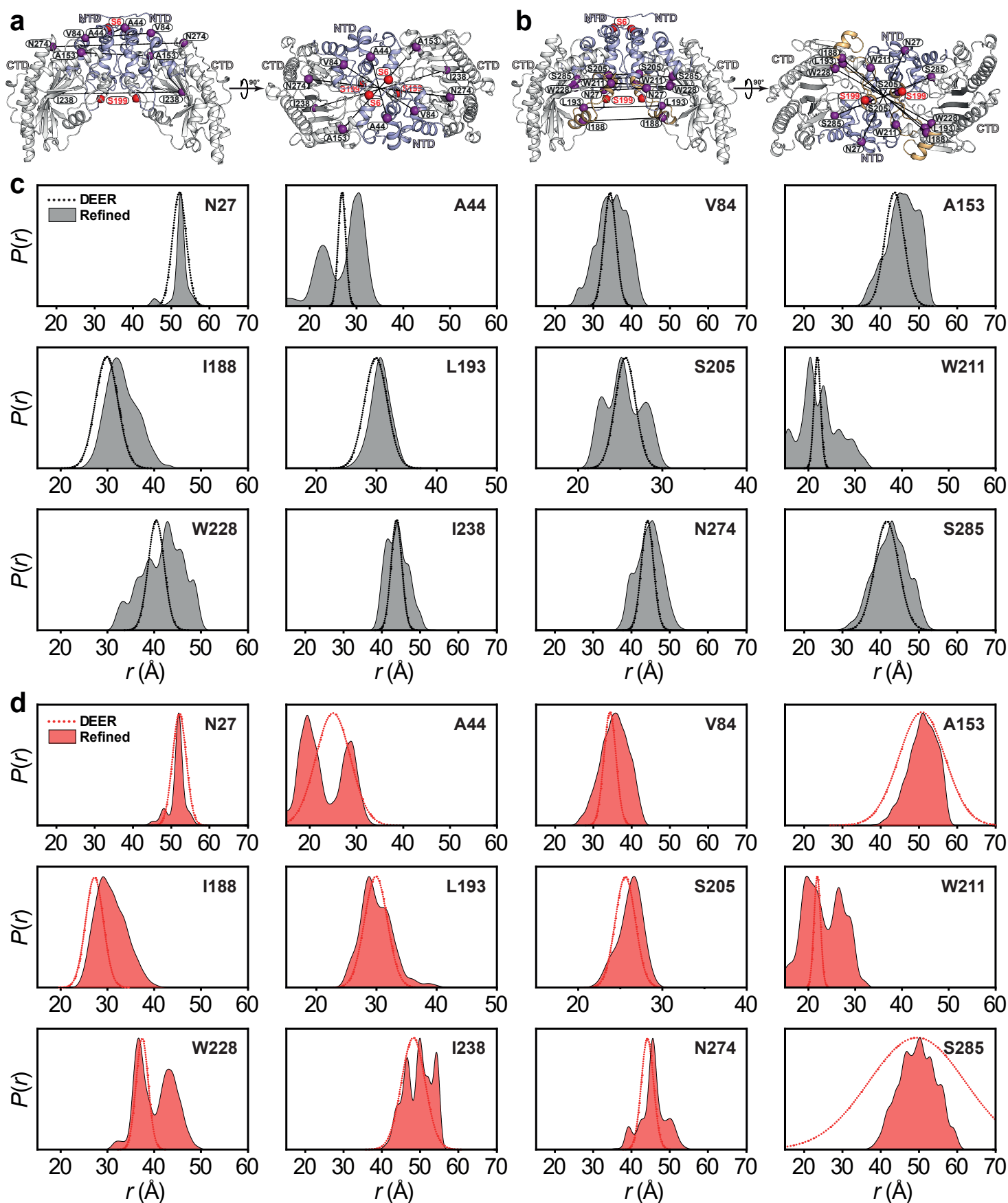
Supplementary Figs. 1-14

Supplementary Table 1

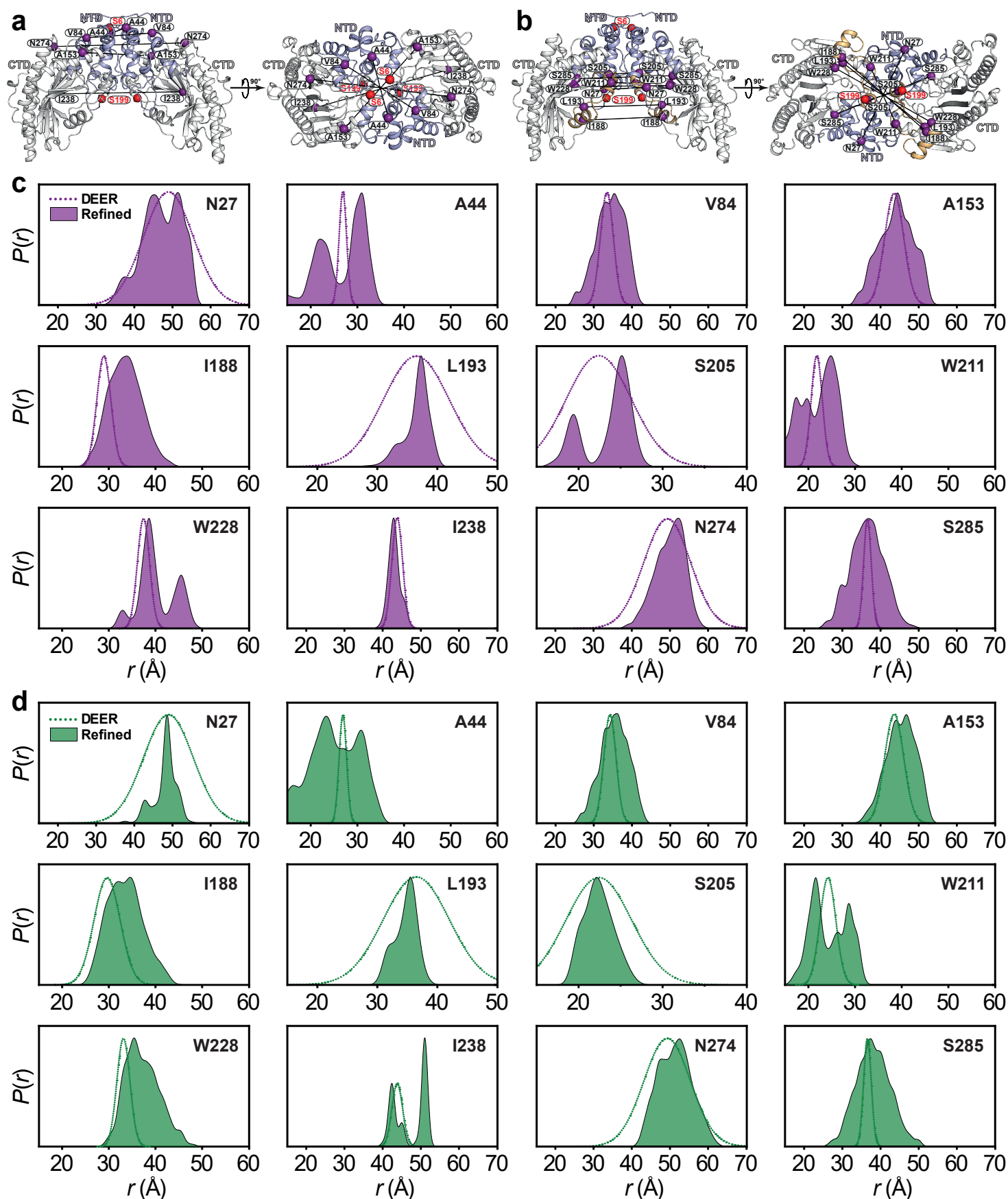




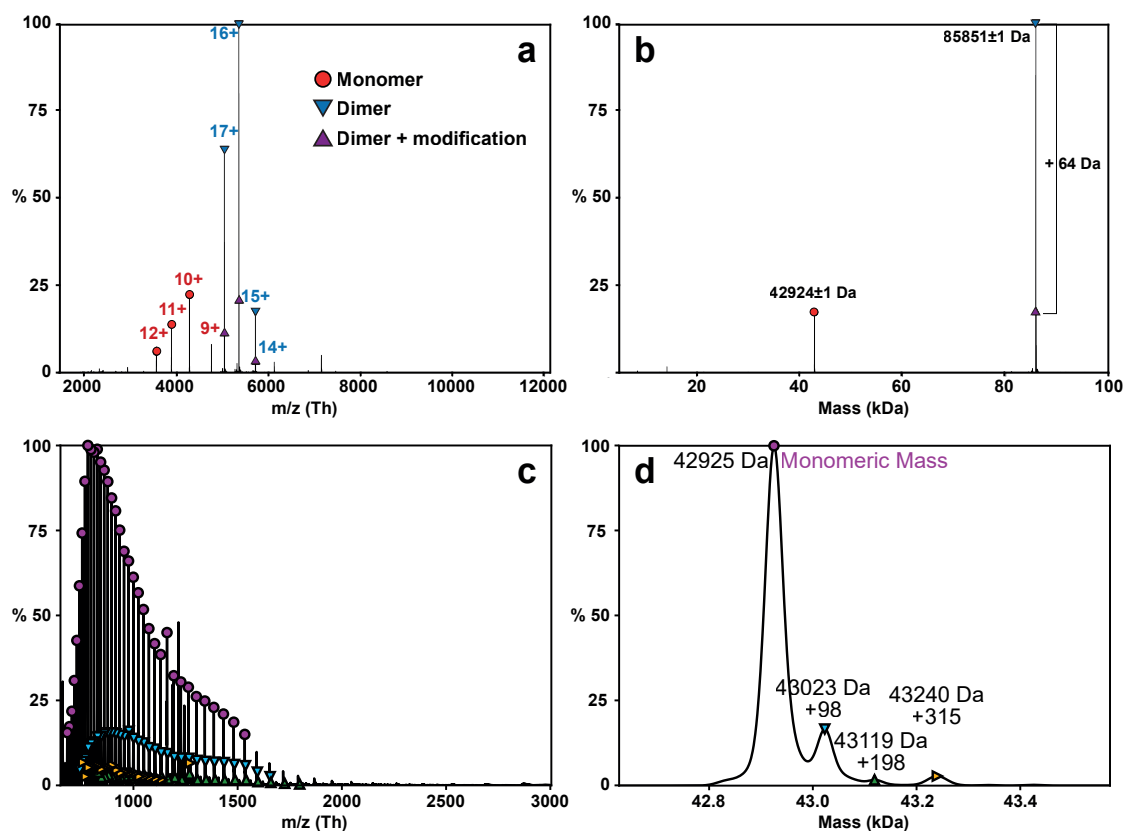
Supplementary Figure 2. DEER data analysis for inter-protomer spin-label pairs across the convex side of the NTD dimer interface and C-terminal beta-sheet core. For each mutant, from left to right, CW-EPR spectrum, DEER-derived distance distributions and primary DEER traces with corresponding fits are shown for apo and ligand-bound CK-BB at physiological and acidic pH. These reporters correspond to the convex-side measurements summarized in Fig. 2 and include sites spanning the NTD dimer interface and CTD beta-sheet core. Confidence bands indicate 2σ uncertainty from fitting of the primary DEER traces.



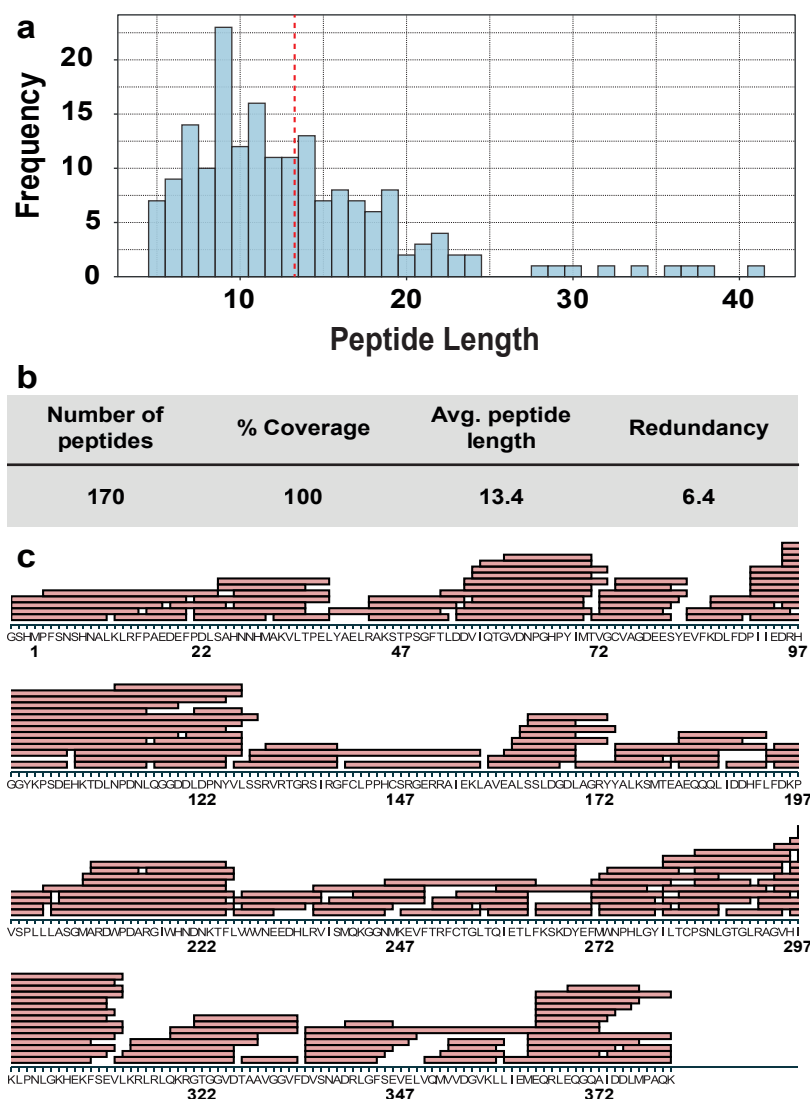
Supplementary Figure 4. DEER-guided refinement of ligand-bound CK-BB at physiological and acidic pH. (a,b) Inter-protomer spin-label pairs used for DEER measurements across the convex (a) and concave (b) sides of the CK-BB dimer. Spin-label sites are shown as purple spheres, and Ser6 and Ser199 phosphorylation sites are shown as red spheres. (c,d) Predicted distance distributions from DEER-refined ligand-bound CK-BB models at pH 7.3 (c, gray) and pH 5.5 (d, red), compared with the corresponding experimental DEER distance subpopulations shown as dotted lines. The refined models closely recapitulate the experimental distance subpopulations.



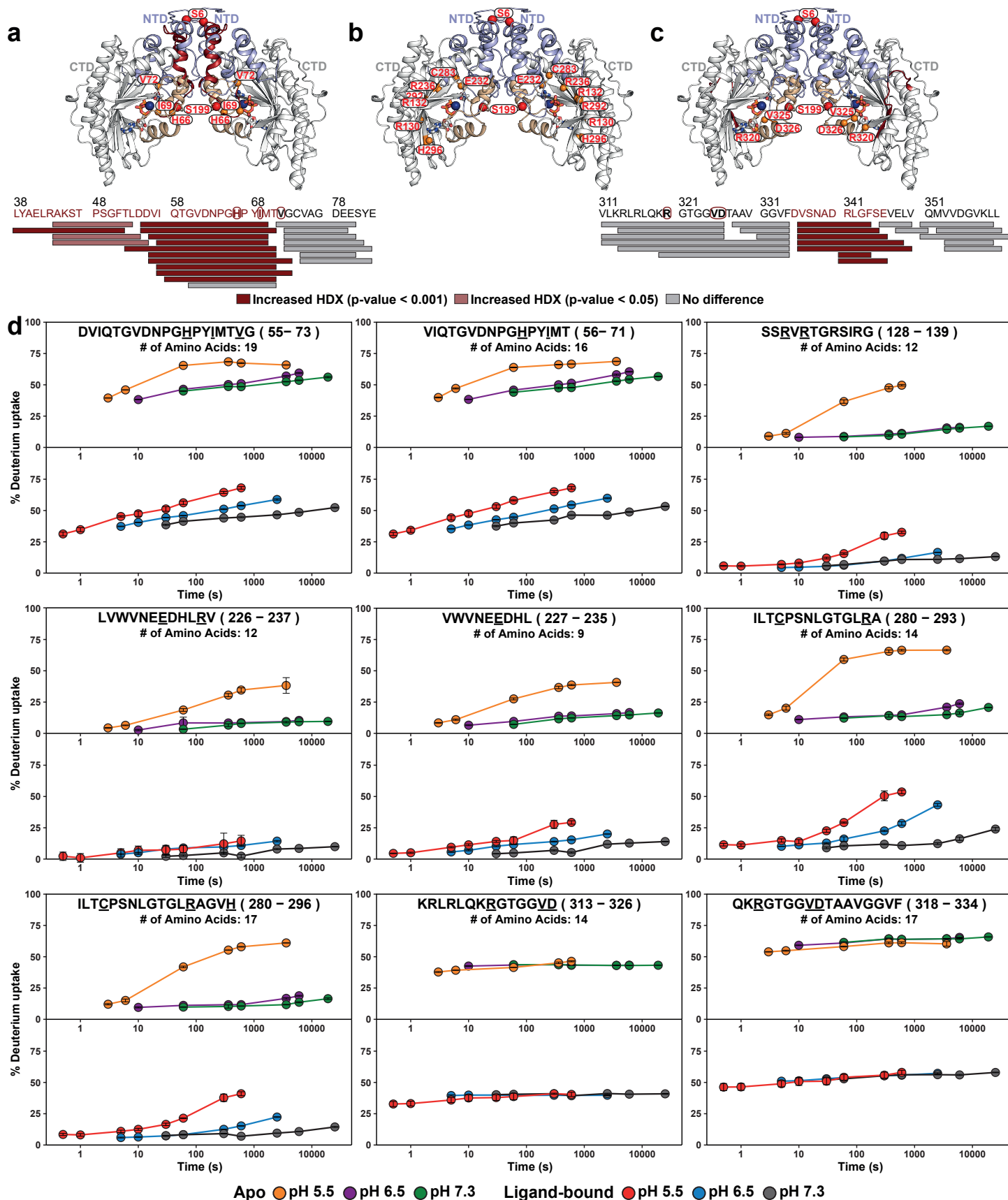
Supplementary Figure 5. DEER-guided refinement of apo CK-BB at physiological and acidic pH. (a,b) Inter-protomer spin-label pairs used for DEER measurements across the convex (a) and concave (b) sides of the CK-BB dimer. Spin-label sites are shown as purple spheres, and Ser6 and Ser199 phosphorylation sites are shown as red spheres. (c,d) Predicted distance distributions from DEER-refined apo CK-BB models at pH 6.5 (c, purple) and pH 7.3 (d, green), compared with the corresponding experimental DEER distance subpopulations shown as dotted lines. The refined apo models closely recapitulate the experimental distance subpopulations.



Supplementary Figure 6. Native MS confirms stable dimer formation by CK-BB. (a,b) Raw (a) and deconvoluted (b) native mass spectra of CK-BB. Charge-state series are labeled and color-coded for monomeric and dimeric species, with matching symbols linking raw and deconvoluted peaks. The dominant dimer peak is observed at $85,851 \pm 1$ Da, whereas the monomeric species likely reflects partial dissociation of the native dimer during ionization. A minor +64 Da species indicates an unidentified post-translational or chemical modification. (c,d) Raw (c) and deconvoluted (d) denaturing MS spectra showing a monomeric mass of 42,925 Da, in close agreement with the theoretical CK-BB mass of 42,925.5 Da; minor higher-mass species are also observed.

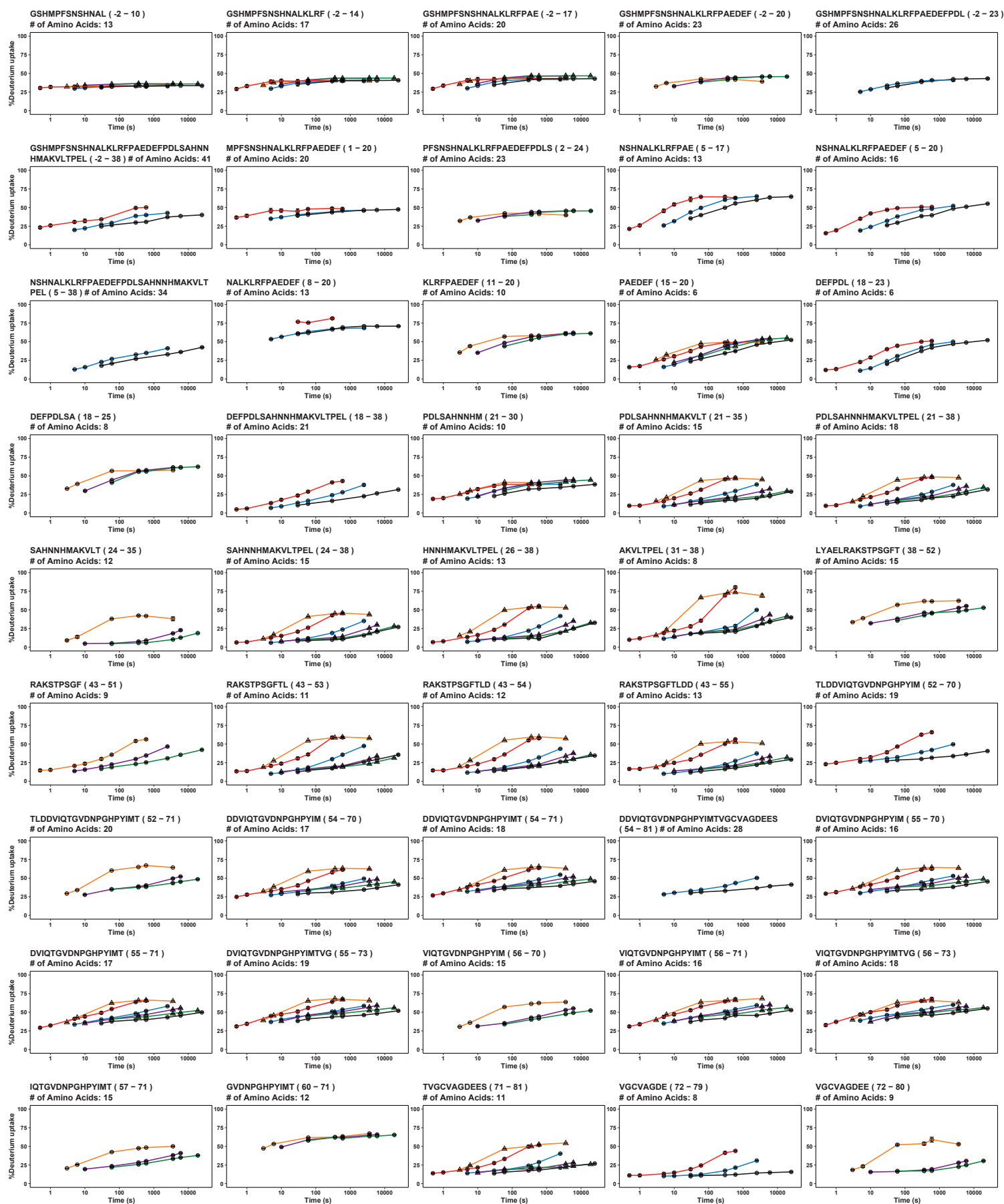


Supplementary Figure 7. HDX-MS peptide coverage and digestion quality for CK-BB. (a) Frequency distribution of identified peptides by length, with the dashed line indicating the average peptide length. (b) Summary of HDX-MS coverage metrics, including total number of peptides, sequence coverage, average peptide length and redundancy. (c) Peptide coverage map of CK-BB, with each red bar representing an MS/MS-verified peptide and its position along the protein sequence. The dataset provides complete sequence coverage with high redundancy, supporting robust peptide-level HDX analysis. Figures were generated using Kingfisher software.



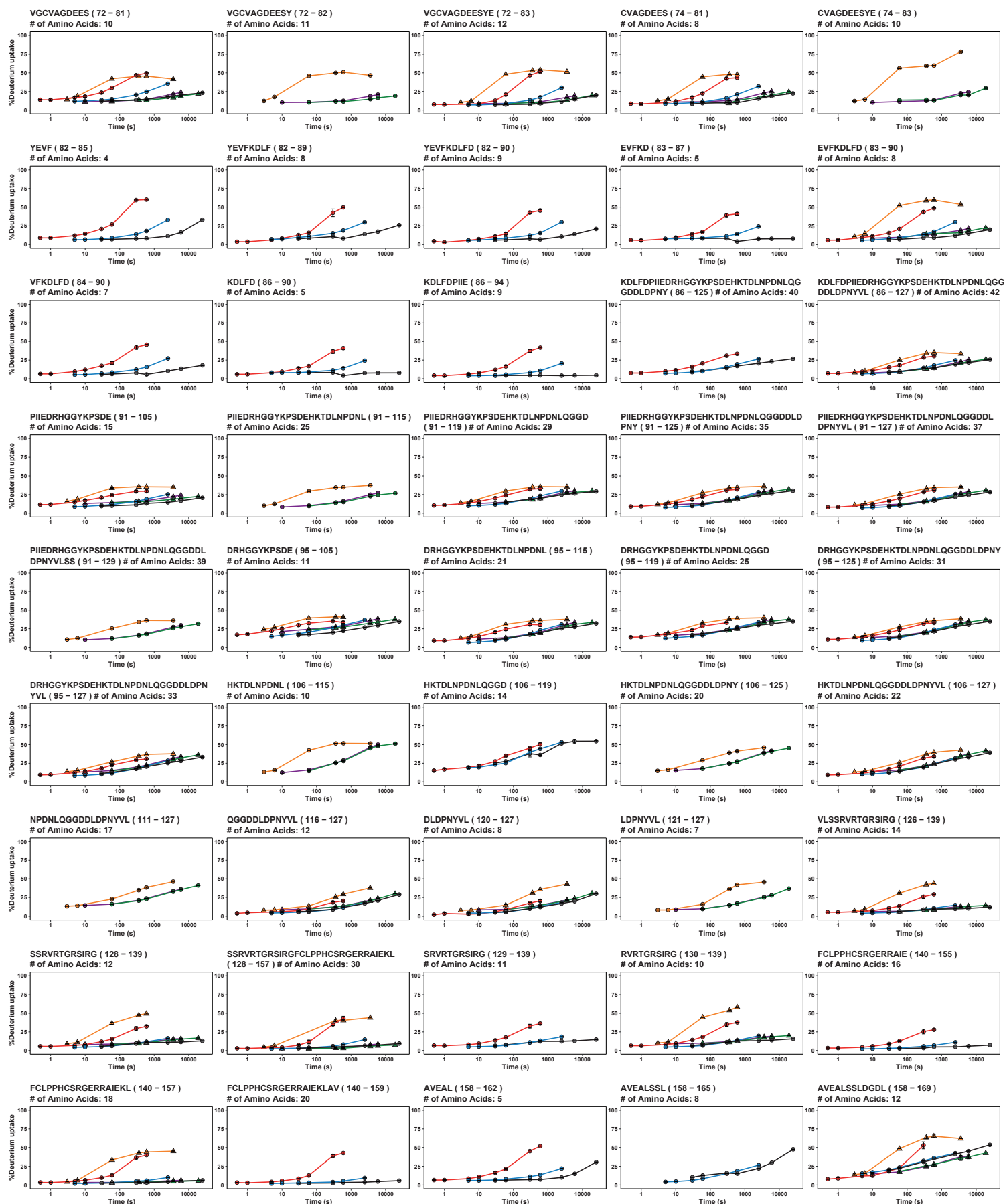
Supplementary Figure 8. Functional CK-BB regions show ligand-sensitized acid-dependent exchange. (a) CK-BB model highlighting the NTD interface and adjacent conserved flexible loop, including His66, Ile69 and Val72, which show increased deuterium uptake under acidic conditions. Functional residues in this region are shown as orange spheres. **(b)** Additional catalytic and substrate-binding residues on the concave surface. **(c)** CK-BB model highlighting a C-terminal beta-sheet core region that shows increased deuterium uptake in the ligand-bound state under acidic conditions. **(d)** HDX uptake plots for peptides spanning catalytic and substrate-binding regions. In apo CK-BB, peptides containing His66, Ile69, Val72, Arg130, Arg132, Glu232, Arg236, Cys283, Arg292 and His296 show little difference between pH 7.3 and 6.5, but markedly increased exchange at pH 5.5, consistent with a threshold-like acid-induced increase in local backbone dynamics and/or solvent exposure. In the ligand-bound state, most of these functional regions, including peptides containing His66, Ile69, Val72, Glu232, Cys283, Arg292 and His296, display graded pH-dependent increases in exchange, consistent with substrate-dependent sensitization of local dynamics. Global significance error, 0.32 Da; $p < 0.001$.

Apo ● pH 5.5 ● pH 6.5 ● pH 7.3 Ligand-bound ● pH 5.5 ● pH 6.5 ● pH 7.3



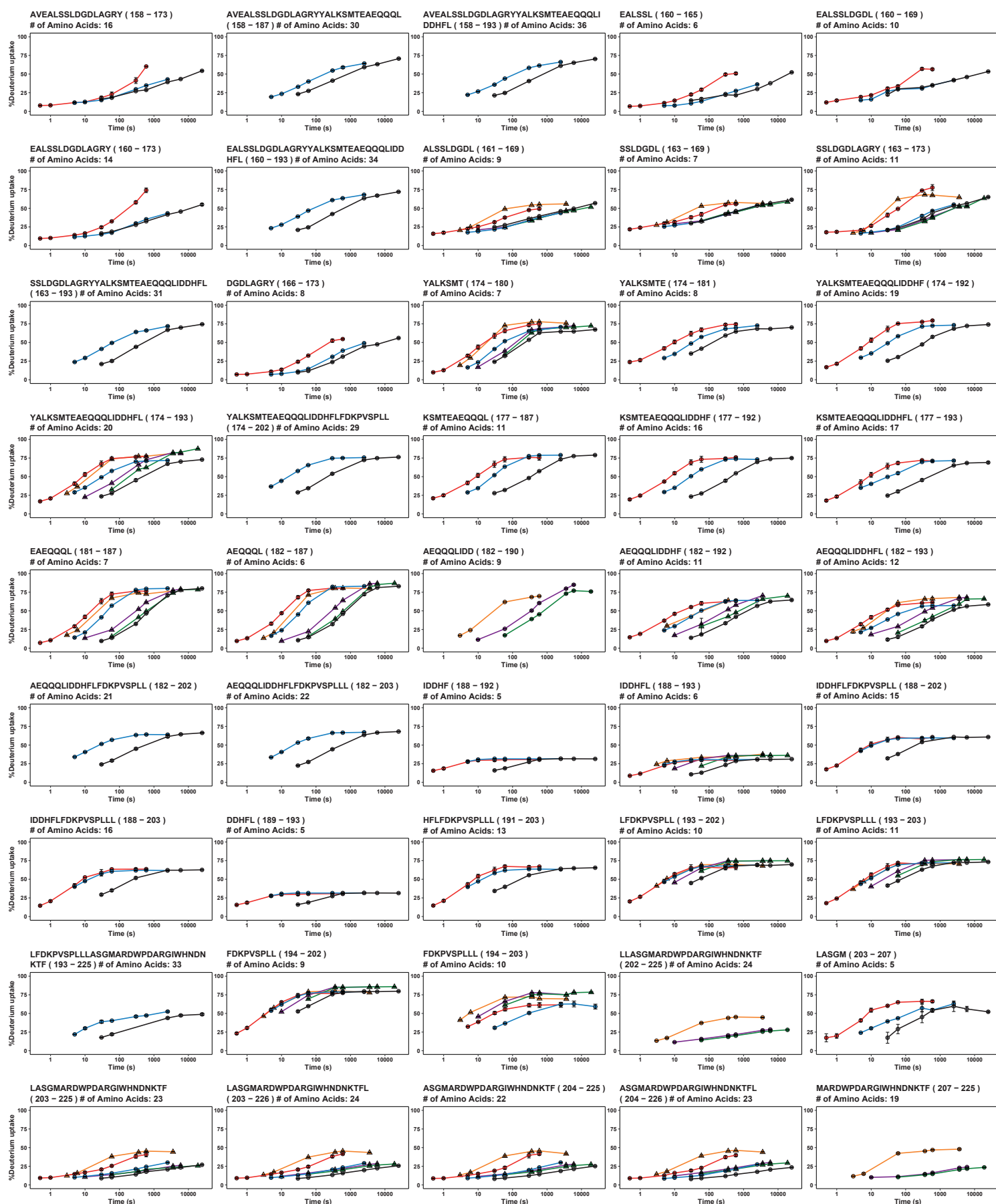
Supplementary Figure 9. HDX uptake kinetics across the CK-BB N-terminal region and flexible active-site loop. HDX uptake plots for overlapping peptides spanning residues -2 to 81, shown as percentage deuterium uptake over exchange time for apo and ligand-bound CK-BB at pH 7.3, 6.5 and 5.5. Peptides in the extreme N-terminal region show relatively modest pH- and ligand-dependent differences, whereas peptides spanning residues ~24–38 and the conserved flexible loop around residues ~52–73 exhibit pronounced acid-dependent increases in exchange. Several peptides containing the His66/Ile69 region show a threshold-like increase in the apo state, with stronger exchange at pH 5.5, while ligand-bound CK-BB displays more graded pH-dependent exchange, consistent with substrate-dependent sensitization of local backbone dynamics and/or solvent exposure.

Apo ● pH 5.5 ● pH 6.5 ● pH 7.3 Ligand-bound ● pH 5.5 ● pH 6.5 ● pH 7.3



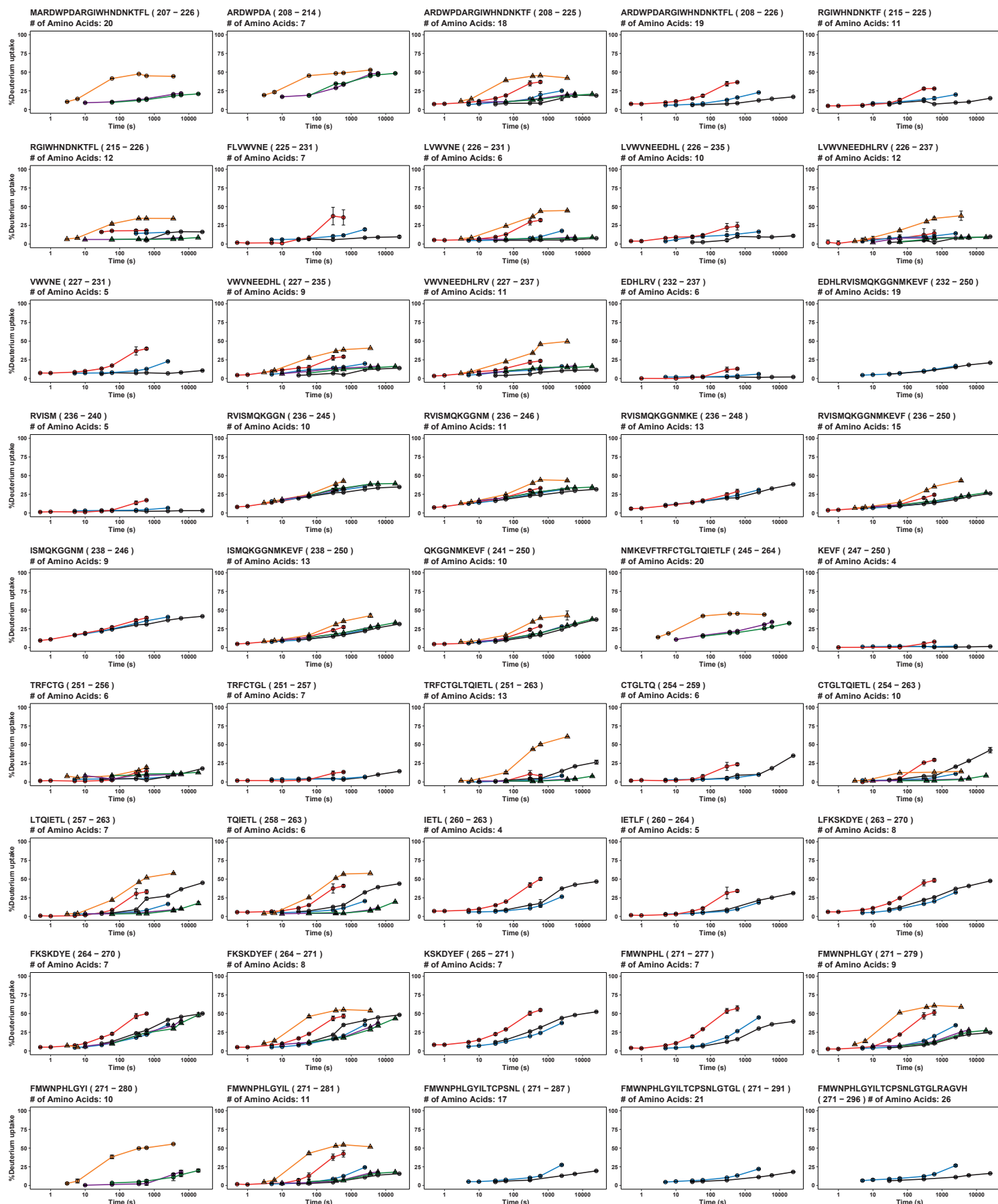
Supplementary Figure 10. HDX uptake kinetics across CK-BB residues 72–169. HDX uptake plots for overlapping peptides spanning residues 72–169, shown as percentage deuterium uptake over exchange time for apo and ligand-bound CK-BB at pH 7.3, 6.5 and 5.5. Peptides near the functional residue Val72, including residues 72–90, show pronounced acid-dependent increases in exchange, particularly at pH 5.5, consistent with enhanced local backbone dynamics and/or solvent exposure. In contrast, many longer peptides spanning residues 91–127 display comparatively modest pH-dependent changes, suggesting that this region remains relatively protected from acid-induced exchange. Peptides covering residues 126–139, near functional residues Arg130 and Arg132, and residues 140–159 show stronger acid sensitivity. Peptides spanning residues 158–169 also exhibit increased exchange under acidic conditions.

Apo ● pH 5.5 ● pH 6.5 ● pH 7.3 Ligand-bound ● pH 5.5 ● pH 6.5 ● pH 7.3



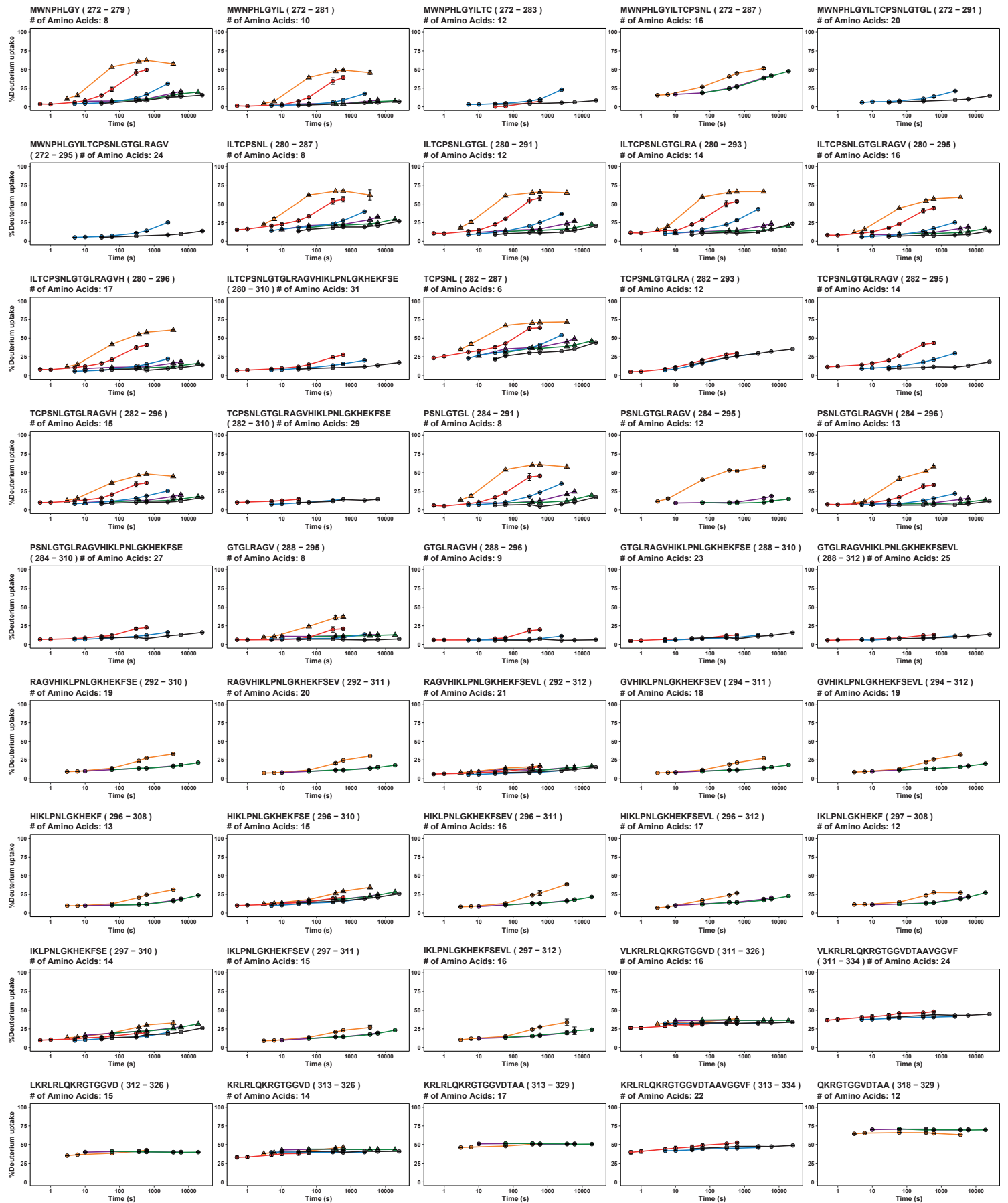
Supplementary Figure 11. HDX uptake kinetics across the nucleotide-binding His191 and Ser199 phosphorylation region of the CK-BB concave surface. HDX uptake plots for overlapping peptides spanning residues 158–226. Peptides spanning the His191/Ser199 region, particularly residues 174–193 and 181–193, show pronounced pH-dependent exchange, with acidic pH increasing uptake and ligand binding producing a more graded pH response across the three conditions. In contrast, short peptides centered on residues 188–193 show comparatively limited exchange changes, suggesting local protection within the His191-containing segment. Peptides flanking this region, including residues 158–173 and 203–226, also display acid-enhanced exchange, indicating that pH-dependent backbone dynamics extend beyond the central His191/Ser199 site across the surrounding concave-surface region.

Apo ● pH 5.5 ● pH 6.5 ● pH 7.3 Ligand-bound ● pH 5.5 ● pH 6.5 ● pH 7.3



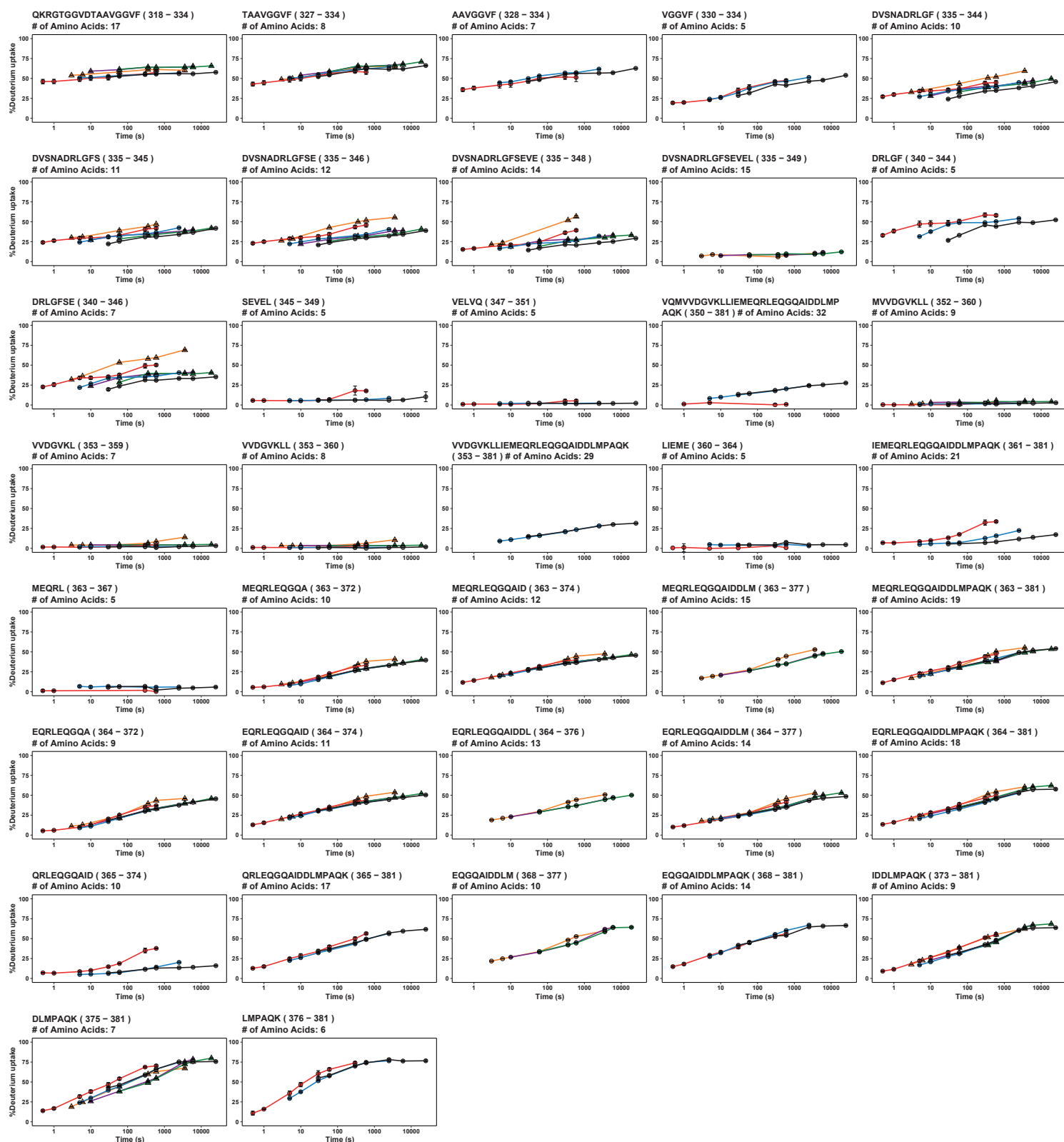
Supplementary Figure 12. HDX uptake kinetics across catalytic and ligand-binding regions of CK-BB. HDX uptake plots for overlapping peptides spanning residues 207–296, shown as percentage deuterium uptake over exchange time for apo and ligand-bound CK-BB at pH 7.3, 6.5 and 5.5. This region includes catalytic and ligand-binding residues Glu232 and Arg236. Peptides spanning residues 207–226 show acid-enhanced exchange, particularly at pH 5.5, whereas several peptides across residues 226–250, including the Glu232/Arg236 region, remain comparatively protected with modest pH-dependent changes. In contrast, peptides spanning residues 251–281 display stronger acid-dependent increases in exchange, especially in the apo state and in selected ligand-bound traces, consistent with enhanced local backbone dynamics and/or solvent exposure.

Apo ● pH 5.5 ● pH 6.5 ● pH 7.3 Ligand-bound ● pH 5.5 ● pH 6.5 ● pH 7.3



Supplementary Figure 13. HDX uptake kinetics across the CK-BB CTD catalytic core. HDX uptake plots for overlapping peptides spanning residues 272–334. This region includes catalytic and ligand-binding residues Cys283, Arg292 and His296. Peptides spanning residues 272–296 show pronounced acid-dependent increases in exchange, particularly at pH 5.5 in the apo state, consistent with enhanced local backbone dynamics and/or solvent exposure around the Cys283/Arg292/His296 region. Ligand binding produces a more graded pH response across several of these peptides. In contrast, peptides spanning residues 292–312 show lower overall exchange and limited pH dependence, suggesting a comparatively protected CTD-core segment. Peptides spanning residues 311–334 are largely pH- and ligand-invariant, despite relatively high baseline exchange for some peptides, indicating constitutive exposure or flexibility rather than acid-specific remodeling.

Apo ● pH 5.5 ● pH 6.5 ● pH 7.3 Ligand-bound ● pH 5.5 ● pH 6.5 ● pH 7.3



Supplementary Figure 14. HDX uptake kinetics across the C-terminal region of the CK-BB CTD. HDX uptake plots for overlapping peptides spanning residues 318–381, shown as percentage deuterium uptake over exchange time for apo and ligand-bound CK-BB at pH 7.3, 6.5 and 5.5. This region includes functional residues Arg320, Val325 and Asp326. Peptides spanning residues 318–334 show relatively high baseline exchange with minimal pH- or ligand-dependent changes, suggesting constitutive solvent exposure or flexibility. Peptides within residues 335–348 display modest acid-dependent increases in exchange, most evident at pH 5.5, whereas short peptides around residues 345–351 show little exchange and limited condition dependence. In contrast, the distal C-terminal peptides spanning residues 363–381 exhibit gradual exchange over time with largely overlapping pH and ligand conditions, indicating a comparatively stable and condition-invariant C-terminal segment.

Supplementary Table 1. pH-adjusted HDX labeling times. HDX labeling times were adjusted using theoretical pH correction factors to compensate for slower intrinsic backbone amide exchange at lower pH. Relative to pH 7.3, exchange times were extended by factors of 6.3, 20.0 and 63.1 at pH 6.5, 6.0 and 5.5, respectively.

pH	Time (factor)	Experimental labeling times (s)								
5.50	63.1	189	379	631	3786	22714	37857	227145	378574	1196737
6.00	20.0	60	120	200	1197	7183	11972	71829	119716	378441
6.50	6.3	19	38	63	379	2271	3786	22714	37857	119674
7.30	1.0	3	6	10	60	360	600	3600	6000	18967