Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information

Mass Spectrometry Reveals the Assembly Pathway of Encapsulated Ferritins and Highlights a Dynamic Ferroxidase Interface

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Materials and Methods

Cloning

Generation of pET28a-Hoch-H63A variant was conducted following the QuikChange® protocol (Stratagene) for site-directed mutagenesis using pET-28a-Hoch_3836 as template and primers shown in Table S1.

Protein expression and purification

Protein expression plasmids were transformed into *Escherichia coli* BL21 (DE3) and spread onto LB-agar plates supplemented with kanamycin (35 µg/mL). A flask containing 2*YT media (10 mL) supplemented with kanamycin (35 µg/mL) was inoculated with an individual colony of *Escherichia coli* BL21 (DE3) from the agar plates and grown overnight at 37 °C, with shaking 200 rpm, to form starter cultures. 2*YT media (1 L) with kanamycin (35 µg/mL) was inoculated with starter culture (1 mL) and shaken at 37 °C until an OD_{600} of 0.6 was reached. Cells were induced with IPTG (1 mM) and shaken overnight, 200 rpm, at 18 °C. Cells were harvested by centrifugation, 5,000 x g at 4 °C, and stored at -20 °C.

Cell pellets were resuspended in Wash Buffer (100 mM Tris.HCl pH 8.0, 150 mM NaCl) and sonicated on ice for 6 minutes (cycles of 30 seconds on/off) to produce cell lysate (MSE Soniprep 150). The lysate was clarified by centrifugation at 12,000 x g and filtered using a .22 µm syringe filter. Filtered lysate was loaded onto a Strep-Trap HP column (5 mL; GE Healthcare) and unbound proteins were washed off with 5 column volumes of Wash Buffer. Hoch-WT/Hoch-H63A was eluted by 6 column volumes of Elution Buffer (100 mM Tris.HCl pH 8.0, 150 mM NaCl, 2.5 mM desthiobiotin) and buffer-exchanged into SEC buffer (20 mM HEPES pH 8.0, and 150 mM NaCl) to remove the desthiobiotin using a centrifugal concentrator (buffer exchanged four times according to the manufacturer's instructions) (Sartorius, 5 kDa MWCO).

Mass Spectrometry (LC-MS, Native and Native Ion Mobility MS)

LC-MS, native MS (Figure 2 and Figure S4) and ion mobility experiments were performed on a Synapt G2 ion-mobility equipped Q-ToF instrument (Waters Corp., Manchester, UK). LC-MS experiments were performed using an Acquity UPLC equipped with a reverse phase C4 Aeris Widepore 50 × 2.1 mm HPLC column (Phenomenex, CA, USA) and a gradient of 5–95%B (Mobile phases: A= water + 0.1% formic acid, B=acetonitrile + 0.1% formic acid) over 10 minutes was employed. For LC-MS, samples were typically analysed at 5 µM, and data analysis was performed using MassLynx v4.1 and MaxEnt deconvolution. For native MS analysis, all protein samples were buffer exchanged into ammonium acetate (100 mM; pH 8.0) using Micro Biospin Chromatography Columns (Bio-Rad, UK) prior to analysis and the resulting protein samples were analysed at a typical final concentration of ~5 µM (oligomer concentration). For native MS ionization, nano-ESI was employed using a Nanomate nanoelectrospray infusion robot (Advion Biosciences, Ithaca, NY). Instrument parameters were tuned to preserve non-covalent protein complexes and were consistent for the analysis of both Hoch-WT and Hoch-H63A. After native MS optimization, parameters were: nanoelectrospray voltage 1.60 kV; sample cone 100 V; extractor cone 0 V; trap collision voltage 4 V; source temperature 60 °C; and source backing pressure 6.0 mbar. For iron titrations, 50 µM, 100 µM or 150 µM of fresh $FeSO_4 \cdot 7H_2O$ in 0.1% (v/v) HCl was added to Hoch-WT prior to buffer exchange into ammonium acetate (100 mM; pH 8.0). Ion mobility mass spectrometry (IM-MS) was performed using the travelling-wave mobility cell and nitrogen drift gas. The typical conditions used for IM-MS were: wave velocity 300 m/s; wave height 19 V; and sampling cone of 75 V. Collision cross sections (CCS) were determined using Driftscope v2.5 and calibrated using denatured equine myoglobin (Sigma-Aldrich).

Native FT-ICR Mass Spectrometry

High resolution native mass spectrometry was performed on a 12T SolariX 2XR FT-ICR MS (Bruker Daltonics) equipped with an ESI source (Figure 3 and Figure S3). Protein samples were buffer exchanged into ammonium acetate (100 mM; pH 8.0; as described above) prior to direct infusion. Source conditions and ion optics were optimised to transmit native proteins ions and when required, Continual Accumulation of Selected Ions (CASI) was employed to isolate charge states of interest. Typically, 2 Megaword data was collected in QPD (2ϖ) mode to produce a 6 second FID, which resulted in a typical mass resolving power of ca. 300,000. The resulting data was processed using Data Analysis (Bruker Daltonics) and theoretical isotope patterns were calculated using IsotopePattern (Bruker Daltonics).

HDX Mass Spectrometry

Hydrogen–deuterium exchange mass spectrometry (HDX-MS) experiments were performed on a Synapt G2 HDMS system coupled to an ACQUITY M-Class UPLC with the HDX manager module (Waters Corporation, Manchester, UK). A custom-built Leap automated platform was utilised for all sample preparation and injections. For HDX-MS analysis, three buffer solutions were employed: Equilibration buffer (4.7 mM K_2HPO_4, 0.3 mM KH_2PO_4 and 100 mL H_2O, adjusted to pH 8.0 with formic acid). Labelling buffer (4.7 mM K_2HPO_4, 0.3 mM KH_2PO_4 and 90.55 mL D_2O, adjusted to pH 8.0 with DCl) and Quench buffer (50 mM K_2HPO_4, 50 mM KH_2PO_4 and 100 mL H_2O, adjusted to pH 2.3 with formic acid). For each timecourse six timepoints were performed: T0 (0 minute; undeuterated control), T1 (0.1 minutes), T2 (0.5 minutes), T3 (2 minutes), T4 (5 minutes) and T5 (30 minutes) with each timepoint being performed in triplicate.

For each timepoint, a 32 µM protein solution was prepared in equilibration buffer and 5 µL of this protein solution was added to 57 µL equilibrium buffer (T0) or labelling buffer (T1-6). The final concentration of deuterium during the labelling step was 91.2%. Exchange was allowed to proceed at 4 °C. To arrest the exchange reaction, 50 µL of quench buffer was added to this initial solution and 50 µL of this quenched solution was immediately injected into the HDX manager (held at 1°C). After injection, samples underwent proteolytic digestion on a 2.1 x 30 mm Enzymate BEH pepsin column (Waters) for 3 minutes at 200 µL/min. After digestion, the peptide digest was loaded on an Acquity BEH C18 VanGuard 1.7µm C18 Trapping column to pre-concentrate the sample for 3 minutes at 200 µL/min. Following trapping, the digests were separated through a 2.1 x 5.0 mm Acquity BEH 1.7 µm analytical column prior to MS/MS analysis via the Water Synapt G2 MS system equipped MassLynx v4.1 software (Waters Corporation, Manchester, UK). The separation gradient was 5-95% acetonitrile with 0.1% formic acid over 12 minutes at 40 µL/min. Both the trapping and LC separation were performed at 1°C to minimize back exchange. The MS parameters for this experiment were: capillary voltage 2.5 kV; sampling cone 30 V; source temperature 80 °C and desolvation temperature 250 °C. The Synapt G2 was calibrated with sodium formate using the Acquity's intellistart software. In order to apply mass accuracy correction, Leucine Enkephalin (LeuEnk 200 pg/µL, Sigma) was used as a lock mass flowing at 10 µL/min. Post-processing and HDX quantification was performed using Proteinlynx Global Server 3.0.3 and Dynamx 3.0 software.

Supplementary Tables

Table S1. Primers used in this study

Construct	Forward Primer	Reverse Primer	Comment
Hoch-WT	GGCTTGGTCTCAAATGGGCAGCAGCGAA CAGCTG	GCGTAGGTCTCTAAGCTTATTCTTCCAGT TCCAGAATCGGACGTTC	BsaI sites
Hoch-H63A	CGAAGAGGTGGAAGCTGCAATGATGACC CTGG	CCAGGGTCATCATTGCAGCTTCCACCTC TTCG	Quickchange primers

All primers were purchased from IDT. Underlined regions represent restriction enzyme recognition sites; highlighted regions represent type IIs restriction enzyme overhang sites.

Table S2. Constructs used in this study

Construct	Sequence	Amino acids	Average Molecular weight (Da)	pI	Extinction coefficient (M^{-1} cm^{-1})
Hoch-WT	MSSEQLHEPAELLSEETKNMHRALVTLIEELEAV DWYQQRADACSEPGLHDVLIHNKNEEVE**H**AM MTLEWIRRRSPVFDAHMRTYLFTERPILELEER SWSHPQFEK*	107	12798.39	4.97	19480
Hoch-H63A	MSSEQLHEPAELLSEETKNMHRALVTLIEELEAV DWYQQRADACSEPGLHDVLIHNKNEEVE**A**AM MTLEWIRRRSPVFDAHMRTYLFTERPILELEER SWSHPQFEK*	107	12732.33	4.88	19480

Table S3. Average molecular masses of encapsulated ferritins obtained by LC-MS.

Protein	Observed Mass (Da)	Assignment	Theoretical Mass (Da)
Hoch-WT	12666.19 ± 0.11	monomer without Met	12667.20
	25331.96 ± 0.47	dimer without Met	25334.40
Hoch-H63A	12600.90 ± 0.04	monomer without Met	12601.13

The starting Methionine residue is not always retained, and this has been indicated in "Assignment". Error values generated from MassLynx v4.1.

Table S4. Iron content of purified Hoch proteins from ICP-MS analysis.

Protein	[Fe]/[protein] %
Hoch-WT	29.1 ± 17.1
Hoch-H63A	5.6 ± 1.5

Fe content of protein samples was analysed by ICP-MS on Hoch-WT (results previously published[1]) and Hoch-H63A proteins (monomeric fractions). Proteins were prepared in 50 mM Tris-HCl pH 8.0, 150 mM NaCl. The analysis was performed on n = 3 technical replicates and values shown represent mean average of values and standard deviation.

Supplementary Figures

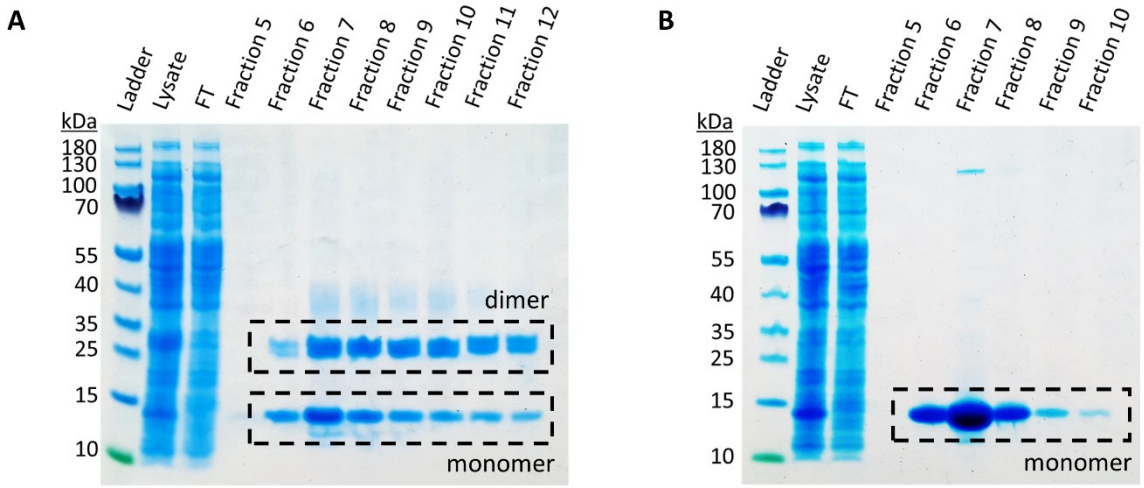


Figure S1. SDS-PAGE analysis of Hoch-WT and Hoch-H63A proteins after Strep-Trap purification

SDS-PAGE gels (NuPAGE® Novex® 12 well 4-12% Bis-Tris gels) of Strep-Trap purification fractions for Hoch-WT (**A**) and Hoch-H63A (**B**). Gels have been annotated with observed oligomerisation states. The appearance of bands corresponding to the dimeric form WT EncFtns on SDS-PAGE has been noted before (He et al, eLife, 2016;5:e18972) for Rru-EncFtn.

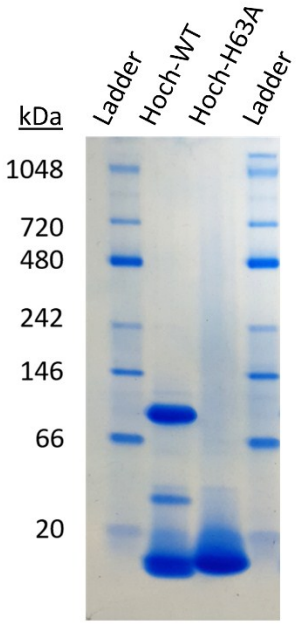


Figure S2. Native PAGE analysis of Hoch-EncFtn proteins after Strep-Trap purification at pH 8.0

Samples of purified Hoch-WT and Hoch-H63A were run on an Invitrogen NativePAGE Bis-Tris Native PAGE gel. The Hoch-WT protein displays multiple oligomeric states, which we tentatively assign as monomer, dimer and decamer oligomerisation states. In contrast the H63A displays a single major oligomerisation state.

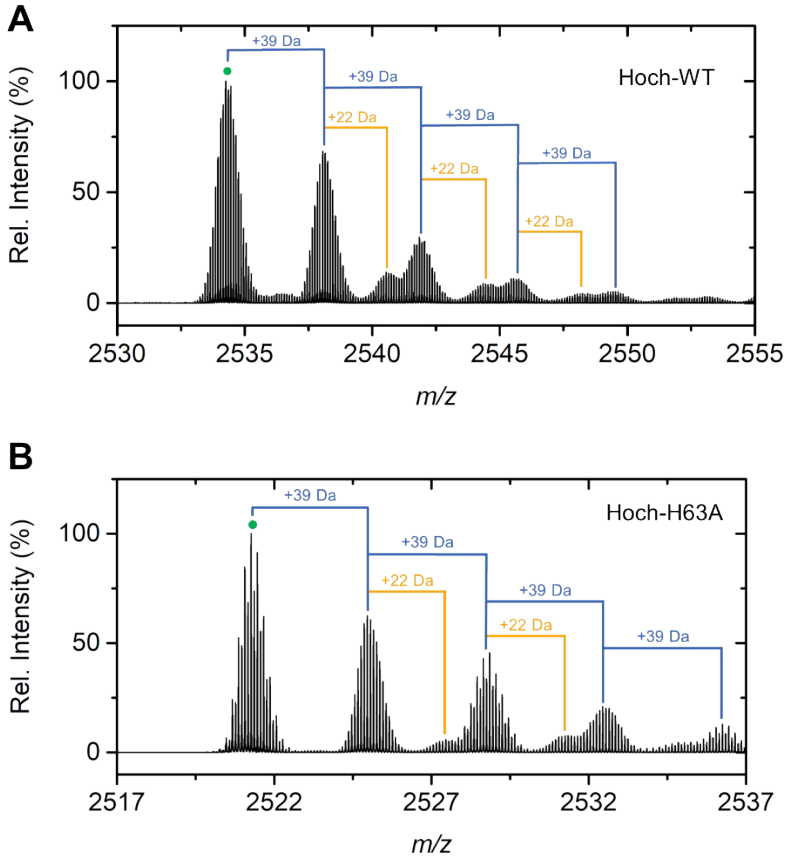


Figure S3. High Resolution Native FT-ICR Mass Spectra of Hoch-WT (A) and Hoch-H63A (B). The 10+ Dimer Charge State is shown for each.

High resolution MS spectra of the 10+ dimer charge state of Hoch-WT (A) and Hoch-H63A (B). The peaks corresponding to the theoretical mass of dimer with no adducts is highlighted by green circles. Potassium and potassium-sodium adduct series are shown by blue and orange lines respectively. No indication of Fe^{2+} binding (monoisotopic Δmass +54 Da) was observed in the dimer state of either variant.

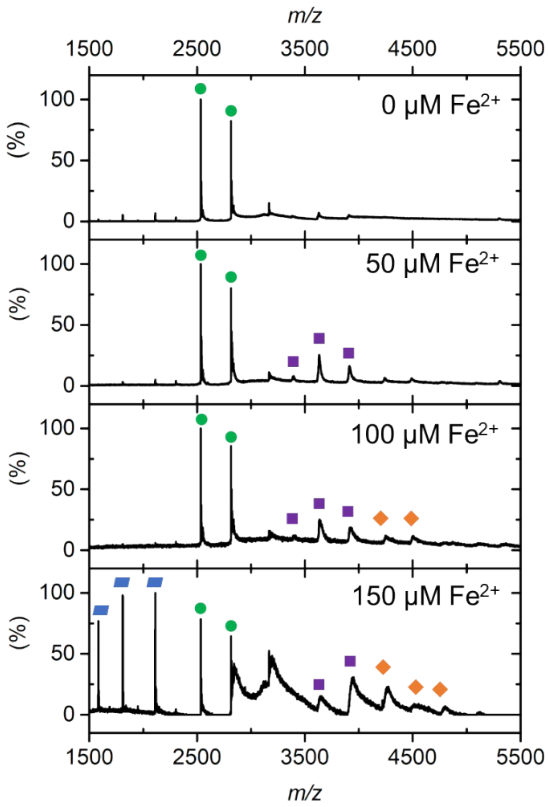
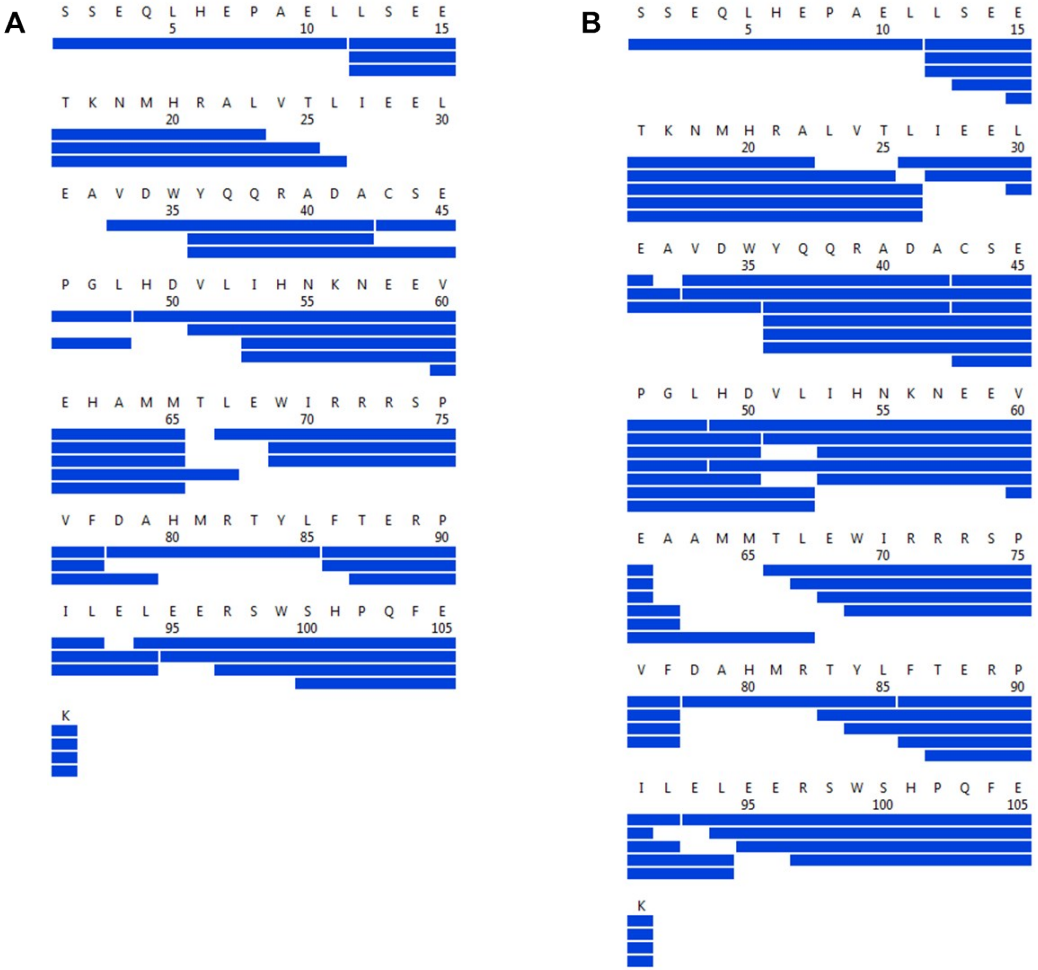


Figure S4. Iron Mediated Dimer Association of Hoch-WT

Hoch-WT was titrated with various iron concentrations before native nESI analysis. *Top panel*: native nESI spectrum of Hoch-WT displaying a charge state distribution consistent with dimer (green circles). *Lower panels:* Higher order assemblies – tetramer (charge states highlighted with purple squares) and hexamer (charge states highlighted with orange diamonds) appear with the addition of iron. Titration with 150 μM iron leads to the appearance of overlapping adducts, preventing accurate peak assignment. Consequently, higher activation energy was required, causing the monomer charge state distribution to be observed (blue parallelograms).

HDX MS Results



HDX S1. Peptide coverage maps for (A) Hoch-WT and (B) Hoch-H63A after HDX and pepsin digest.
Peptides observed by MS in all HDX timepoints are represented by blue bars. (A) Pepsin digestion of Hoch-WT resulted in 24 peptide providing 94% protein sequence coverage. (B) Pepsin digestion of Hoch-H63A resulted in 38 peptides, providing 100% protein sequence coverage.

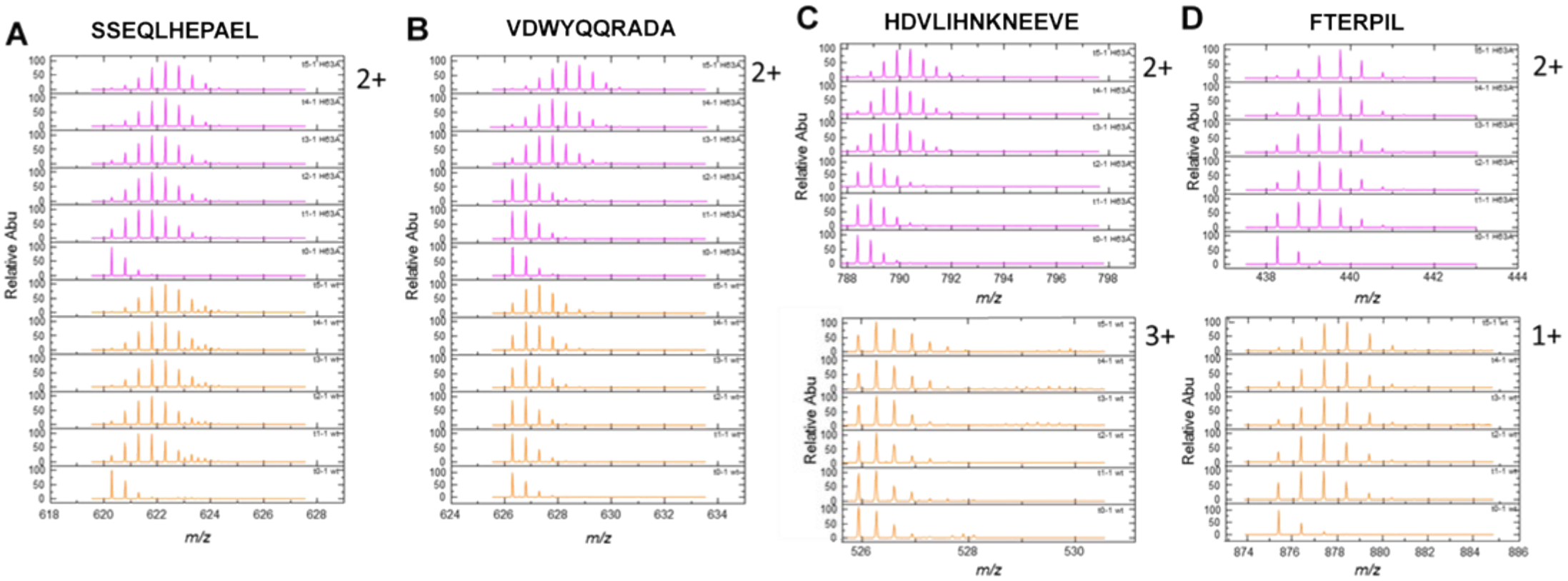
A

				WT Deuterium uptake (Da)									
Sequence	**Start (aa)**	**End (aa)**	**Exchangers**	**(10s)**	**(10s) SD**	**(30s)**	**(30s) SD**	**(120s)**	**(120s) SD**	**(500s)**	**(500s) SD**	**(1800s)**	**(1800s) SD**
SSEQLHEPAEL	1	11	9	2.41	0.08	2.60	0.09	2.88	0.13	3.15	0.07	3.38	0.29
LSEETKNMHRAL	12	23	11	0.88	0.09	0.97	0.08	1.23	0.09	1.43	0.07	1.67	0.31
LSEETKNMHRALVT	12	25	13	0.92	0.04	0.97	0.05	1.20	0.06	1.46	0.06	1.64	0.21
LSEETKNMHRALVTL	12	26	14	0.97	0.06	1.08	0.03	1.32	0.05	1.64	0.04	1.78	0.21
VDWYQQRADA	33	42	9	0.29	0.06	0.36	0.06	0.66	0.08	1.01	0.07	1.26	0.19
YQQRADA	36	42	6	0.26	0.06	0.36	0.04	0.64	0.05	0.95	0.03	1.19	0.19
YQQRADACSEPGL	36	48	11	1.56	0.07	1.86	0.05	2.37	0.11	2.77	0.04	3.07	0.36
CSEPGL	43	48	4	0.83	0.07	0.96	0.05	1.13	0.04	1.22	0.04	1.41	0.10
HDVLIHNKNEEVE	49	61	12	0.63	0.03	0.33	0.09	0.34	0.01	0.60	0.08	0.98	0.09
HDVLIHNKNEEVEHAMM	49	65	16	0.37	0.12	0.43	0.12	0.58	0.12	0.76	0.12	0.81	0.18
VLIHNKNEEVEHAMM	51	65	14	0.20	0.05	0.24	0.02	0.31	0.03	0.41	0.01	0.42	0.09
IHNKNEEVEHAMM	53	65	12	0.20	0.04	0.23	0.02	0.28	0.03	0.35	0.03	0.42	0.05
IHNKNEEVEHAMMTL	53	67	14	0.19	0.06	0.20	0.06	0.30	0.05	0.35	0.07	0.39	0.09
VEHAMM	60	65	5	0.00	0.03	0.02	0.03	0.03	0.03	0.02	0.03	0.02	0.04
LEWIRRRSPVF	67	77	9	0.52	0.06	0.58	0.06	0.63	0.05	0.67	0.06	0.75	0.09
WIRRRSPVF	69	77	7	0.46	0.02	0.50	0.02	0.56	0.03	0.62	0.02	0.73	0.08
WIRRRSPVFDA	69	79	9	0.41	0.06	0.50	0.06	0.62	0.16	0.57	0.04	0.62	0.08
DAHMRTYL	78	85	7	0.49	0.06	0.48	0.10	0.57	0.05	0.69	0.08	0.87	0.17
FTERPIL	86	92	5	1.34	0.07	1.48	0.05	1.72	0.07	1.88	0.01	2.12	0.15
FTERPILEL	86	94	7	2.02	0.10	2.27	0.07	2.62	0.10	2.92	0.07	3.18	0.27
TERPILEL	87	94	6	1.77	0.10	2.02	0.07	2.36	0.10	2.67	0.08	2.88	0.24
LEERSWSHPQFEK	94	106	11	2.27	0.10	2.55	0.08	2.90	0.14	3.16	0.03	3.43	0.28
EERSWSHPQFEK	95	106	10	1.98	0.11	2.26	0.08	2.56	0.14	2.79	0.05	3.00	0.23
RSWSHPQFEK	97	106	8	1.64	0.10	1.85	0.03	2.16	0.14	2.28	0.05	2.49	0.16
SHPQFEK	100	106	5	1.17	0.11	1.41	0.14	1.54	0.12	1.71	0.09	2.00	0.19

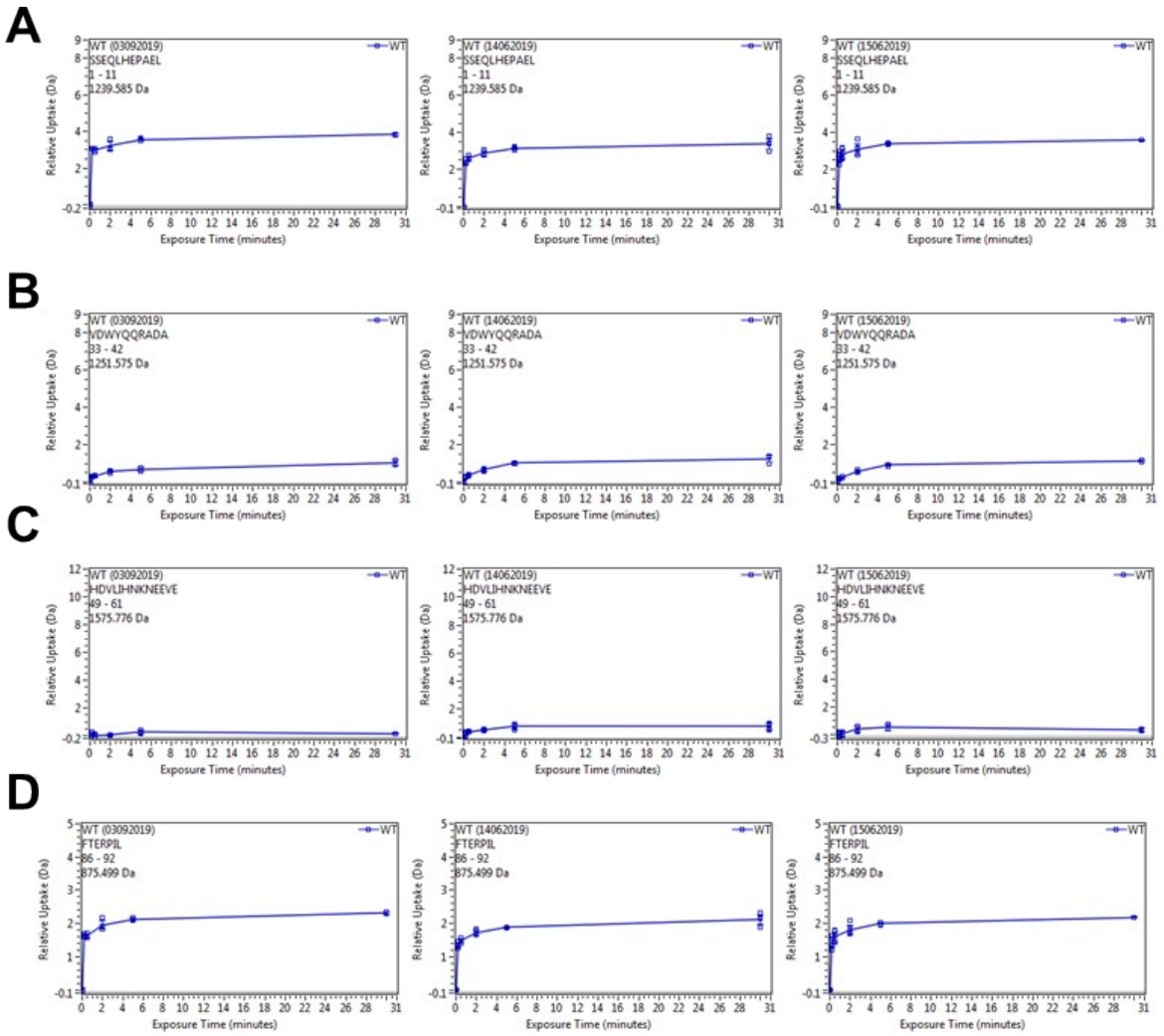
B

				H63A Deuterium uptake (Da)									
Sequence	**Start (aa)**	**End (aa)**	**Exchangers**	**(10s)**	**(10s) SD**	**(30s)**	**(30s) SD**	**(120s)**	**(120s) SD**	**(500s)**	**(500s) SD**	**(1800s)**	**(1800s) SD**
SSEQLHEPAEL	1	11	9	2.04	0.36	2.60	0.07	2.76	0.08	3.30	0.14	3.60	0.07
LSEETKNMHRA	12	22	10	1.10	0.26	1.47	0.04	2.03	0.04	2.55	0.07	3.14	0.03
LSEETKNMHRALVT	12	25	13	1.22	0.28	1.65	0.05	2.29	0.04	2.80	0.11	3.33	0.05
LSEETKNMHRALVTL	12	26	14	1.22	0.28	1.75	0.02	2.41	0.03	2.90	0.09	3.48	0.06
SEETKNMHRALVTL	13	26	13	1.12	0.25	1.54	0.08	2.24	0.03	2.69	0.17	3.14	0.20
ETKNMHRALVTL	15	26	11	0.45	0.10	0.57	0.04	0.89	0.06	1.27	0.07	1.55	0.04
LIEELE	26	31	5	0.05	0.07	0.19	0.09	0.22	0.04	0.28	0.04	0.29	0.02
IEELEA	27	32	5	0.12	0.01	0.12	0.01	0.13	0.01	0.13	0.01	0.13	0.01
LEAVDW	30	35	5	0.05	0.03	0.09	0.01	0.14	0.01	0.20	0.04	0.29	0.03
VDWYQQRADA	33	42	9	0.33	0.08	0.80	0.06	2.25	0.13	2.88	0.17	3.60	0.06
VDWYQQRADACSEPGLHD	33	50	16	2.68	0.34	3.33	0.16	3.53	0.16	4.07	0.22	4.33	0.17
YQQRADA	36	42	6	0.31	0.07	0.75	0.07	1.93	0.10	2.37	0.06	2.72	0.04
YQQRADACSEPGL	36	48	11	1.52	0.28	2.37	0.07	3.47	0.10	4.18	0.16	4.77	0.07
YQQRADACSEPGLHD	36	50	13	1.61	0.36	2.16	0.59	3.68	0.08	4.49	0.23	5.24	0.04
YQQRADACSEPGLHDVL	36	52	15	1.29	0.36	2.23	0.08	3.87	0.12	4.50	0.19	5.18	0.09
CSEPGL	43	48	4	0.73	0.16	1.05	0.04	1.14	0.02	1.36	0.08	1.47	0.02
CSEPGLHD	43	50	6	0.98	0.25	1.36	0.09	1.48	0.03	1.85	0.10	2.08	0.12
CSEPGLHDVL	43	52	8	0.78	0.19	1.20	0.08	1.81	0.04	2.34	0.09	2.79	0.10
HDVLIHNKNEEVE	49	61	12	0.46	0.04	0.89	0.08	2.27	0.10	2.52	0.16	3.10	0.17
HDVLIHNKNEEVEA	49	62	13	0.35	0.16	0.81	0.18	1.98	0.18	2.43	0.15	2.93	0.15
VLIHNKNEEVE	51	61	10	0.41	0.28	0.77	0.24	1.41	0.25	1.63	0.25	1.89	0.24
IHNKNEEVE	53	61	8	0.31	0.05	0.59	0.05	1.02	0.02	1.21	0.04	1.43	0.03
IHNKNEEVEA	53	62	9	0.24	0.06	0.55	0.09	0.91	0.02	1.08	0.08	1.28	0.11
VEAAMMTL	60	67	7	0.12	0.02	0.38	0.02	0.76	0.08	0.81	0.02	0.95	0.04
TLEWIRRRSPVF	66	77	10	0.35	0.09	0.54	0.09	0.58	0.06	0.64	0.07	0.97	0.14
LEWIRRRSPVF	67	77	9	0.50	0.12	0.66	0.02	0.72	0.03	0.81	0.02	1.05	0.03
EWIRRRSPVF	68	77	8	0.48	0.11	0.66	0.03	0.73	0.03	0.83	0.06	1.06	0.04
WIRRRSPVF	69	77	7	0.52	0.11	0.60	0.03	0.72	0.03	0.83	0.04	1.09	0.03
DAHMRTYL	78	85	7	1.04	0.03	1.10	0.14	1.42	0.03	1.71	0.08	1.91	0.02
TYLFTERPI	83	91	7	1.54	0.43	2.44	0.10	2.77	0.08	3.25	0.13	3.67	0.15
YLFTERPIL	84	92	7	1.22	0.27	1.80	0.14	2.19	0.02	2.39	0.05	3.06	0.07
FTERPIL	86	92	5	1.46	0.02	1.68	0.04	1.89	0.02	2.23	0.11	2.37	0.08
FTERPILEL	86	94	7	1.76	0.50	2.50	0.03	2.81	0.03	3.30	0.14	3.61	0.06
TERPILEL	87	94	6	1.72	0.01	2.06	0.02	2.25	0.07	2.94	0.12	3.19	0.09
ELEERSWSHPQFEK	93	106	12	2.35	0.31	2.97	0.03	3.24	0.06	3.78	0.15	4.03	0.06
LEERSWSHPQFEK	94	106	11	2.26	0.36	2.60	0.25	3.02	0.18	3.50	0.23	3.82	0.18
EERSWSHPQFEK	95	106	10	1.57	0.87	2.24	0.44	2.66	0.41	3.17	0.44	3.45	0.41
RSWSHPQFEK	97	106	8	1.52	0.20	1.93	0.04	2.10	0.03	2.51	0.07	3.00	0.14

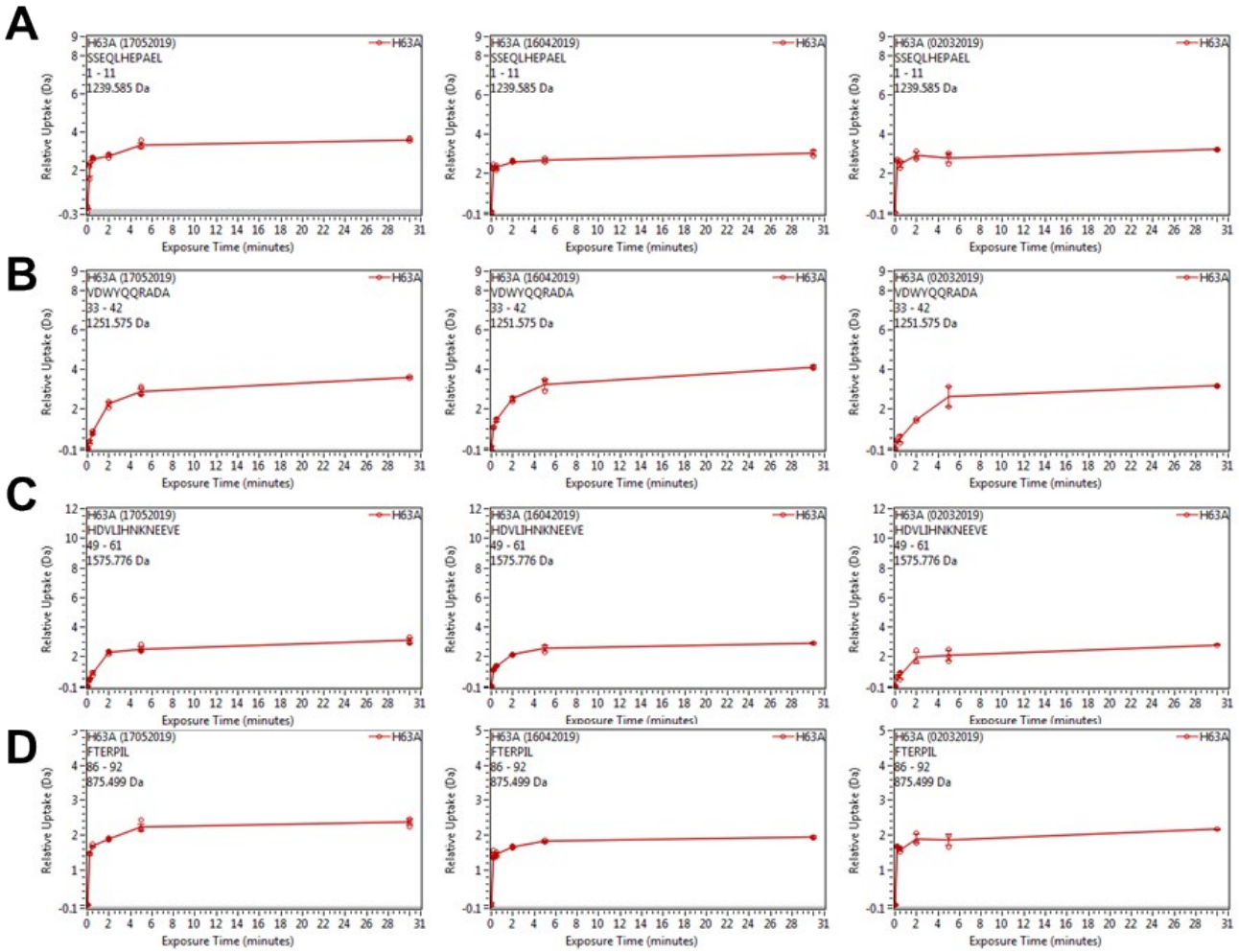
HDX S2. Recorded uptake of deuterium for each peptide and timepoint observed in HDX-MS of Hoch-WT(A) and Hoch-H63A (B). For each peptic peptide observed in the HDX MS analysis, the deuterium uptake (in Da) and standard deviation of triplicate data is shown for each timepoint (10s, 30s, 120s, 500s, 1800s) for each peptide. The number of exchangeable backbone amide hydrogens is also stated (Exchangers). Peptides highlighted in yellow are represented and discussed in the main text



HDX S3. Stacked mass spectral data displaying deuterium uptake over time for the four peptides highlighted in text. Hoch-WT (orange) and Hoch-H63A (magenta).
(**A**) aa1-11 SSEQLHEPAEL; (**B**) aa33-42 VDWYQQRADA; (**C**) aa49-61 HDVLIHNKNEEVE and (**D**) aa86-92 FTERPIL. Comparative analysis of the same peptic peptide obtain from Hoch-WT (orange) and Hoch-H63A (magenta). The isotope distribution recorded for each timepoint are displayed stacked plots (T0 (bottom) to T5 (top)).



*HDX S4. HDX uptake plots for the four peptides highlighted in the text from HDX-MS analysis for Hoch-WT. (**A**) aa1-11 SSEQLHEPAEL, (**B**) aa33-42 VDWYQQRADA; (**C**) HDVLIHNKNEEVE; (**D**) aa86-92 FTERPIL.* For each peptide, the three panels represent the deuterium uptake for a biological repeat. Each biological repreat was measured in technical triplicate (represented by the error bars in the plots)



HDX S5. HDX uptake plots for the four peptides highlighted in the text from HDX-MS analysis for Hoch-H63A. (**A**) aa1-11 SSEQLHEPAEL, (**B**) aa33-42 VDWYQQRADA; (**C**) HDVLIHNKNEEVE; (**D**) aa86-92 FTERPIL. *For each peptide, the three panels represent the deuterium uptake for a biological repeat. Each biological repreat was measured in technical triplicate (represented by the error bars in the plots)*

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References

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