

# Notch3 Epitope Mapping using Integrated Structural Proteomic Techniques

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## ABSTRACT

**Purpose:** Perform epitope mapping in solution using integrative structural proteomics tools including native, HDX and cross-linking mass spectrometry and compare with X-ray results.

**Methods:** HDX and crosslinking peptide analysis were performed on Orbitrap Eclipse platform with/out FAIMS Pro interface. QEXactive UHMR was used for Native LC/MS analysis. The raw files were analyzed using Thermo Scientific™ Proteome Discoverer™ 2.5 software with XlinkX 2.5 node or BioPharma Finder 4.1. HDX data analysis was performed with HDExaminer software.

**Results:** Similar to previous studies, we confirmed Notch3 epitopes in the LNR-HD linker and HD region residues, 1503-1515 and 1591-1600. However, we didn't observe any protection in LNR-B/C linker or LNR-B regions which may reflect different complex confirmation in solution vs solid state.

## INTRODUCTION

Epitope mapping is an important aspect of developing new therapeutic antibodies. Structural characterization of epitopes not only enables detailed understanding of mechanism of actions but also guides the design and selection of drug candidate molecules. There are multiple analytical methods used to identify protein antigen epitopes with X-ray crystallography being the most widely used. However, epitopes mapped using this method are derived from proteins in solid state and not in solution. In this study we applied an integrated mass spectrometry based structural proteomics approach: native mass spectrometry, hydrogen deuterium exchange (HDX) and cross-linking to identify the epitope of the Notch3 negative regulatory region (NRR) for MOR20350 Fab and compared it to previous results from X-Ray analysis<sup>1</sup>

## MATERIALS AND METHODS

### Sample Preparation

Recombinant Notch3 negative regulatory region (NRR, 1378-1640) and MOR20350 Fab were obtained from GenScript USA. For the HDX experiment, both Notch3 and Notch3/Fab proteins were mixed in 1:1 or 1:2 ratios, diluted (1 to 9 ratio) with labeling buffer, incubated at multiple time points, quenched with acid and subjected to online digestion in a fully automated manner with LEAP HDX robot (Trajan, Fig.1). For XL-MS experiments LC-SDA, DSG, EDC, DHSO and DSPP (PhoX<sup>2</sup>) were used to crosslink Ab:Ag(1.5:1) in PBS for 1hr at up to x100 molar excess of crosslinker to protein. After crosslinking, reactions were quenched with 0.5M ammonium bicarbonate and analyzed by SDS-PAGE or reduced, alkylated and digested with trypsin and lys-C for MS analysis. LC-SDA samples were UV activated to form XL and DSPP crosslinked peptides were enriched using Fe-NTA kit (Table 1). For native MS, Ab:Ag complex in different ratios was incubated 1hr or overnight at 4C.

### Liquid Chromatography and Mass Spectrometry

LC/MS bottom-up, HDX and cross-linking was performed using a Thermo Scientific™ UltiMate™ 3000 RSLCnano system and Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer. Crosslinked peptides were separated by RP-HPLC using a Thermo Scientific™ EASY-Spray™ column, 50 cm x 75 µm over a 45 min 2-28%, 12 min from 26-40 % gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at 300 nL/min flow rate. For HDX experiments, samples were digested using Pepsin/FPXIII 1:1 column(NovaBioAssays) and loaded on Acclaim PepMap™ 100, 1.0mm x 5cm trap column and analyzed on Thermo Scientific™ HyperSil™ Gold, 1mm x 5cm column using 15 min gradient at 45 µL/min flow rate. Native LC/MS was performed on a Thermo Scientific™ MAbPac™ SEC-1 column (2.1 mm ID x 150 mm L) using a Thermo Scientific™ Vanquish™ Flex UPLC system at a flow rate of 50 µL/min. Native proteins were detected by UV (280 nm) after chromatographic separation and mass spectra were acquired on a Thermo Scientific™ QEXactive™ UHMR mass spectrometer using full scan (MS1) mode at 25K@ m/z 400 resolution.

### Data Analysis

The data were analyzed with Thermo Scientific™ BioPharma Finder 4.1 and Proteome Discoverer™ 2.5 with XlinkX node 2.5. Deuterium calculation was performed using HDExaminer 3.2 (Sierra Analytics). Results visualization and distance restraints were performed using xINET<sup>3</sup> and Pymol v.2.3.3

Figure 1. HDX workflow set up



## RESULTS

Notch receptors regulate a diverse set of biological functions and have been implicated as oncogenic drivers in tumorigenesis. We used the negative regulatory region (NRR) domain of Notch3 to map the epitope for a Notch3 antibody (MOR20350). In 2016, a co-crystal structure of the Notch3 NRR/MOR20350 complex mapped the epitope for antibody binding to the LNR and HD regions of the antigen<sup>1</sup>. In this study as shown in Figure 2, we sought to map the epitope using orthogonal structural methods in solution to compare it to the previous crystal structure.

### Native LC/MS sample quality screening.

We initially performed native MS analysis to find optimal conditions to form recombinant Notch3 NRR/Fab complexes and verify sample quality. Significant Notch3 NRR degradation was observed due to presence of a furin-cleavage site (RARR) which resulted in multiple forms of Fab/NRR complexes and free, truncated forms of Notch3(Fig.3A upper panel). Changing this sequence to RASS, prevented protein degradation and enabled us to obtain a more homogenous complex of correct molecular weight(Fig 3A low panel). To evaluate complex formation, proteins were incubated for 1 hr at room temperature or overnight at 4 C in various molar excess ratios of antibodies to protein (Fig.4), molar ratio of Ab:Ag =1.5:1 was found optimal and used in all other experiments.

Figure 2. Epitope mapping using integrative structural proteomics approach

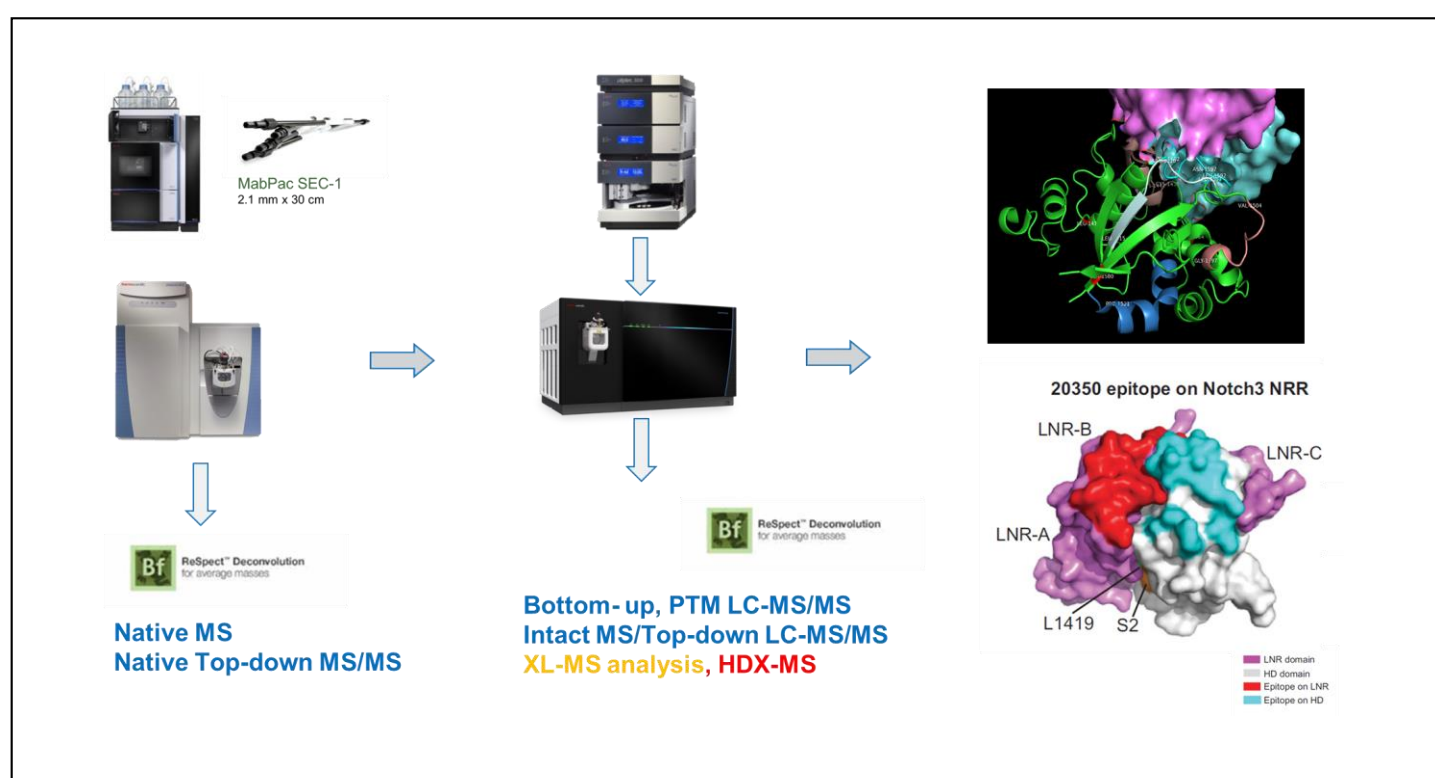


Figure 3. Notch3 NRR Quality Analysis by native LC/MS of original and modified samples(A) and its sequence (B). Glycosylation and mutation sites are highlighted.

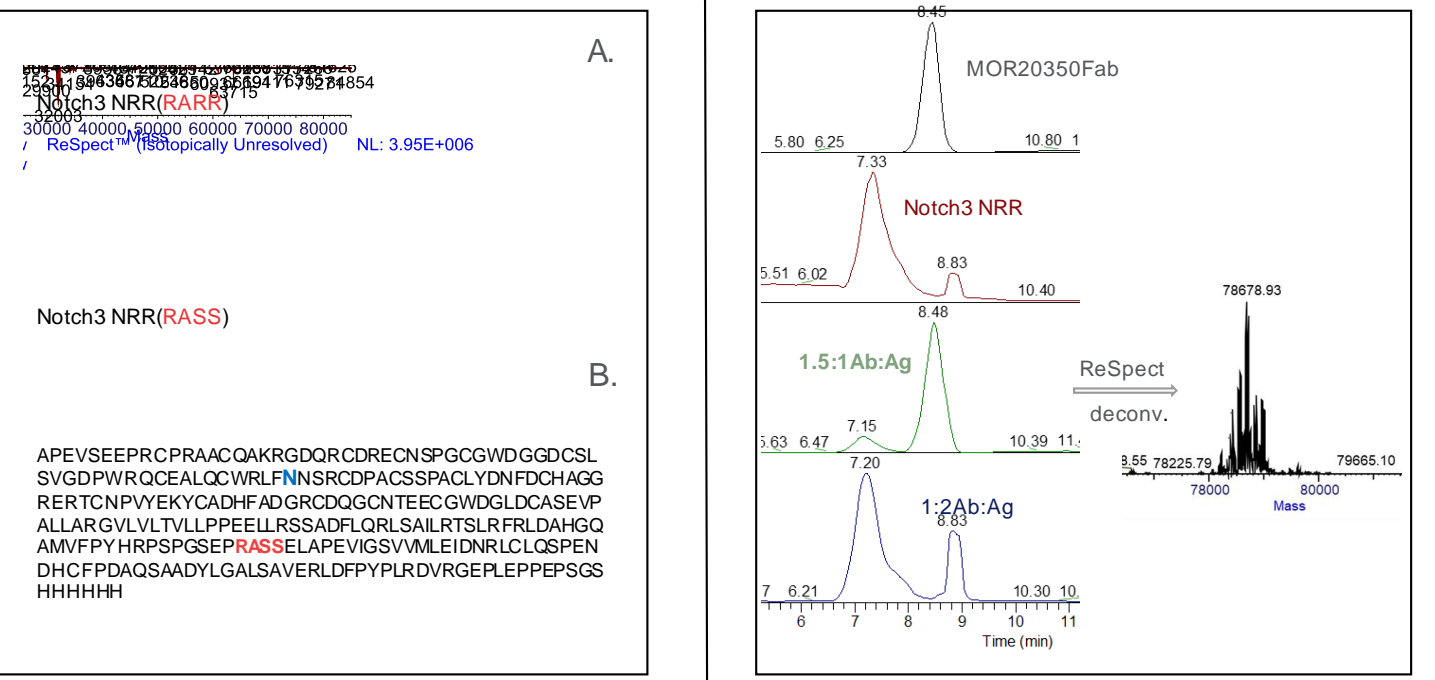
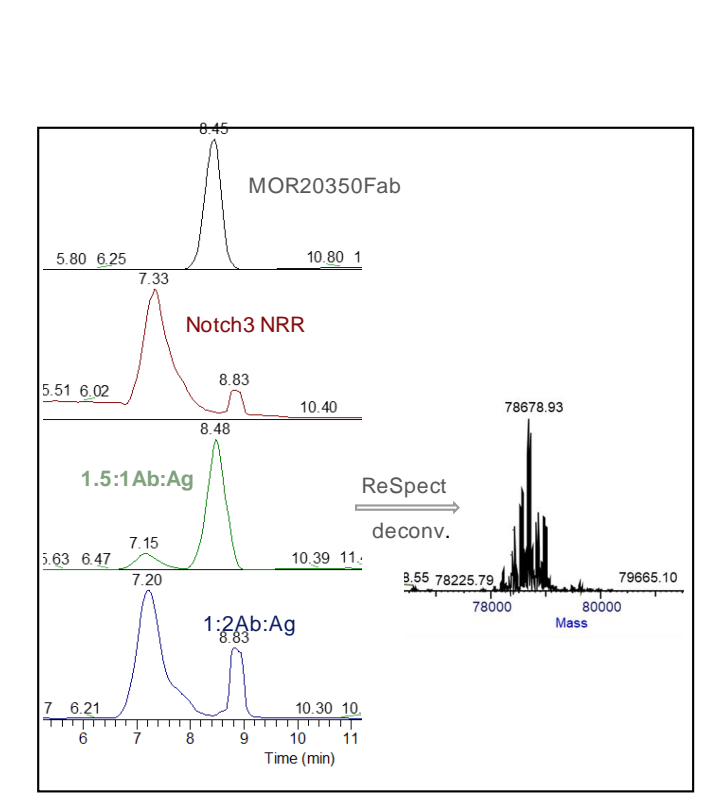


Figure 4. Optimization of complex formation using native LC SEC/MS analysis.



### Epitope/paratope mapping by XL-MS

For crosslinking experiments, we used reagents with different functionality including: DSG, LC-SDA, EDC, DSPP and DHSO (Fig.5A). Fab/Notch 3 NRR crosslinks using lysine specific crosslinkers such as DSG or DSPP were not observed, as the Notch3 NRR protein contains limited number of Lys residues (Fig 3B&5B). However, we identified most intra and inter crosslinked peptides in Fab using DSPP crosslinker (Fig.5B). We tried to use acid residue specific, MS cleavable crosslinker DSHO but didn't detect any inter crosslinked peptides (Fig.5B). Best results for epitope mapping were obtained with the zero linker EDC and heterobifunctional crosslinker LC-SDA (Fig.6, Table 1). We were able to confirm Notch3 NRR epitope in NMR-A/B linker and HD region and identified paratope residues in 20350 Fab at HC52, LC45&53 (Table 1, Fig.6&11)

Figure 5. Structures of non-cleavable and MS-cleavable crosslinkers used in the study(A) and Comparison of their crosslinking efficiency by SDS-PAGE(B). Different crosslinkers were incubated with Fab or Notch3 NRR alone or as complex at molar excess of crosslinker to protein (e.g. 20, 40 or 80-fold).

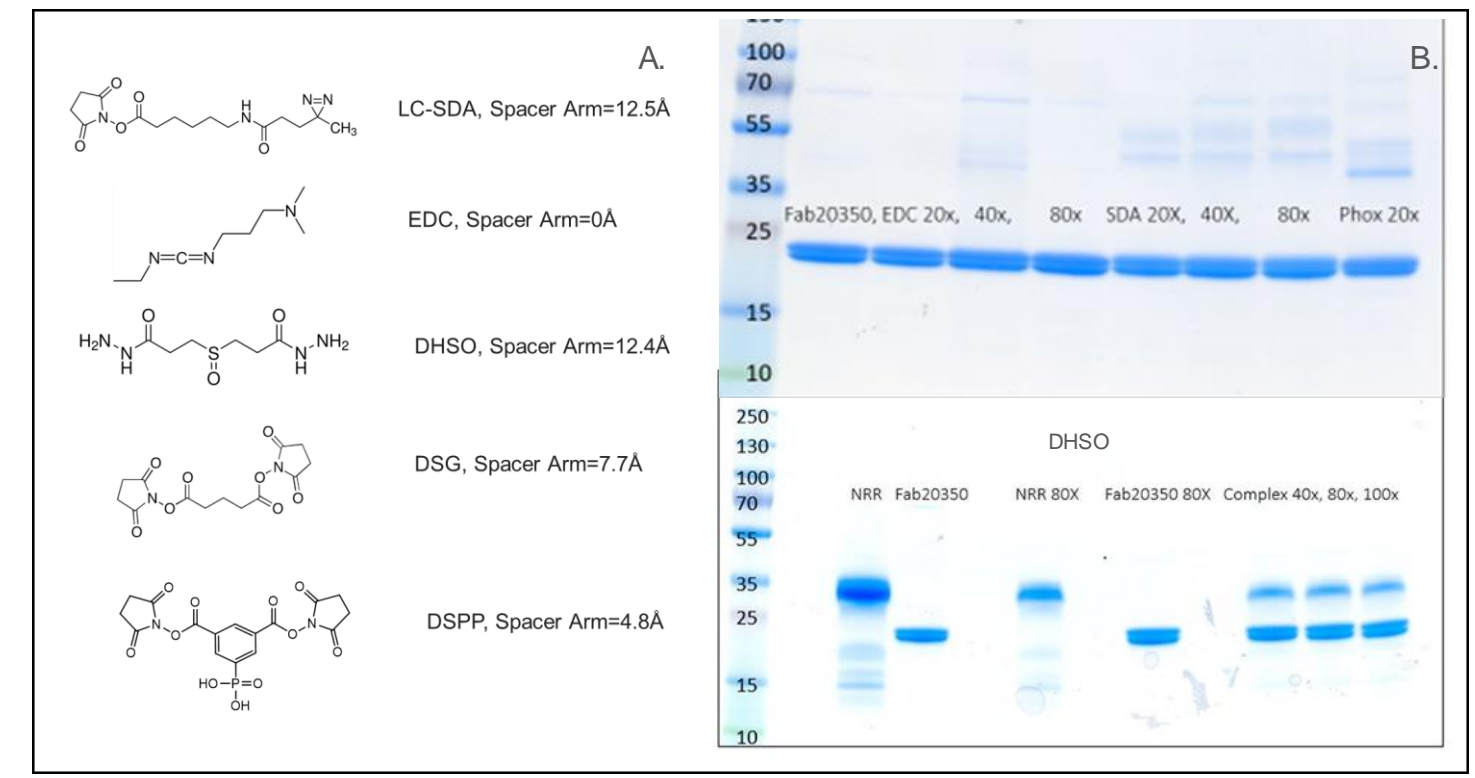


Figure 6. Paratope/Epitope Mapping using EDC and LC-SDA XL. SDS-PAGE (A) and Mapping Results into Crystal Structure (B).

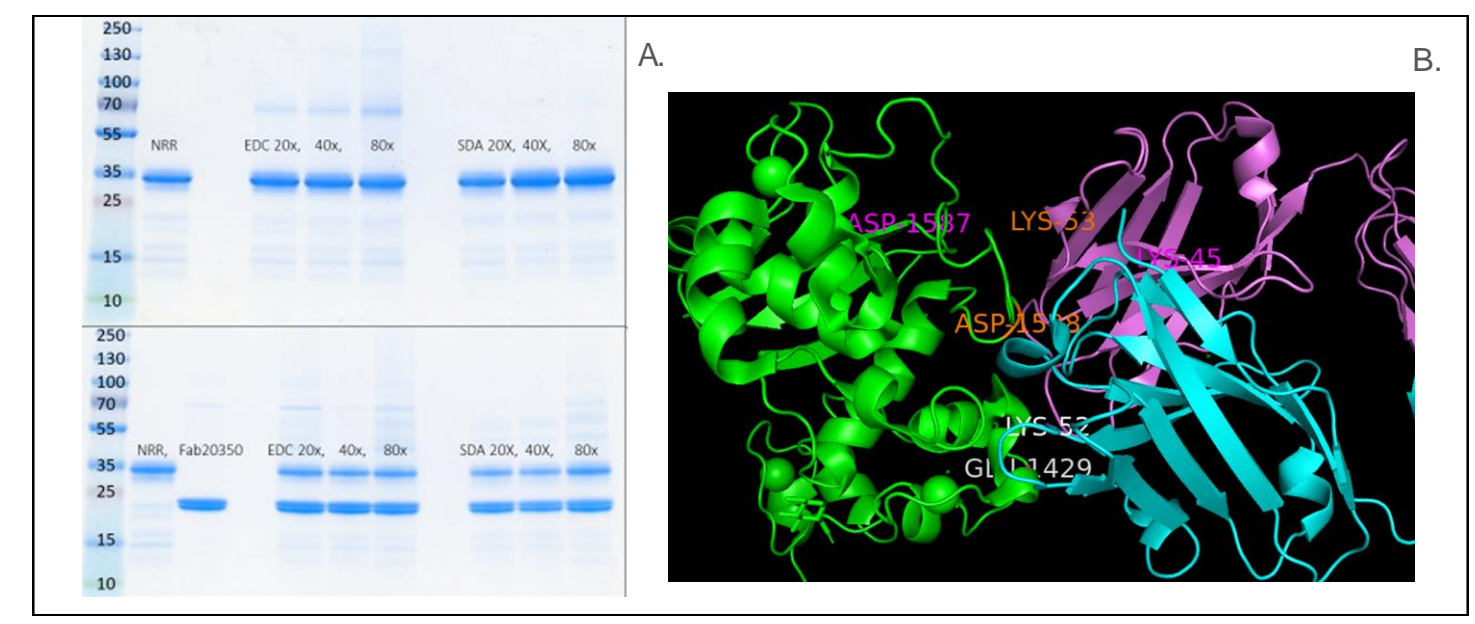


Table 1. Summary of Notch3 NRR/Fab crosslinked experiments. ND=not detected, NA= not applicable.

XL	Exper.conditions	#XL	# Inter XL	Paratope/Epitope
DSG	Ab:Ag=1.5:1, x 100, 16hrs	8	6	ND
DSPP	Ab, x 20, 1hr, FeNTA	73	35	NA
DHSO	Ab:Ag=1.5:1, x 100, 1hr	7	5	ND
EDC	Ab:Ag=1.5:1, x 80, 1hr	100	22	HC52-1429
LC-SDA	Ab:Ag-XL=1.5:1, x 80, 1hr	64	28	LC45-1587; LC53-1598
LC-SDA	Ab-XL:Ag=1.5:1, x 80, 16hrs	18	6	ND

### HDX Experiments

For the HDX analysis, over one hundred peptides were used for deuterium calculation which enabled us to achieve close to 99% sequence coverage, even in the presence of glycosylation and multiple disulfide bonds(Fig. 5&6A). Similar to previous studies<sup>1</sup>, we confirmed epitopes in the LNR-HD linker and HD region residues, 1503-1515 and 1591-1600. However, we didn't observe any protection in LNR-B/C linker or LNR-B regions which may reflect different complex confirmation in solution (Fig. 6B&C) vs solid state<sup>1</sup>. Consistent with this, we only observed inter-crosslinker peptides for LNR-A/B linker region (Fig. 7). Summary of all results is presented in Figure 8.

Figure 7. Notch3 Protein Deuterium Heat Map

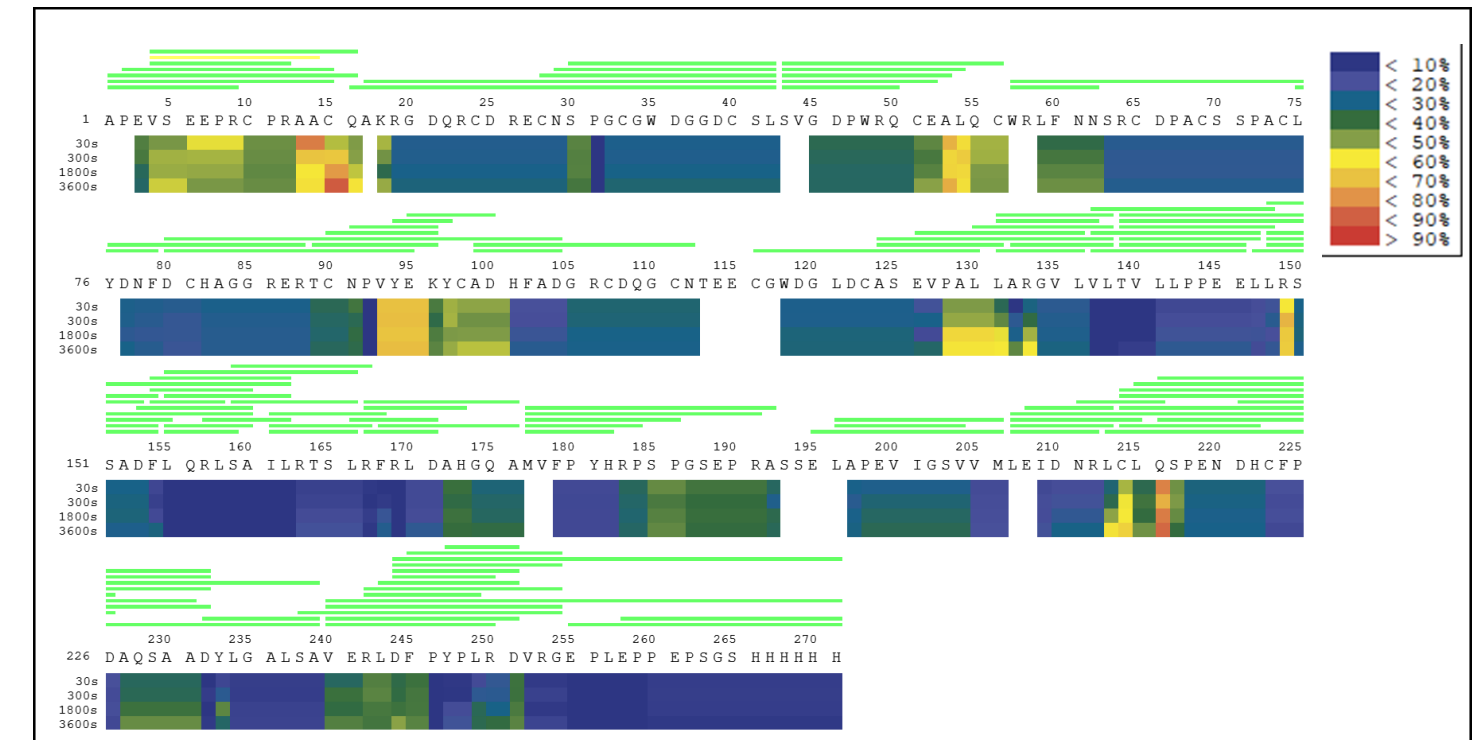


Figure 8. Notch3 Peptide Mapping(A) and Protection Factor Plots(B&C zoom in)

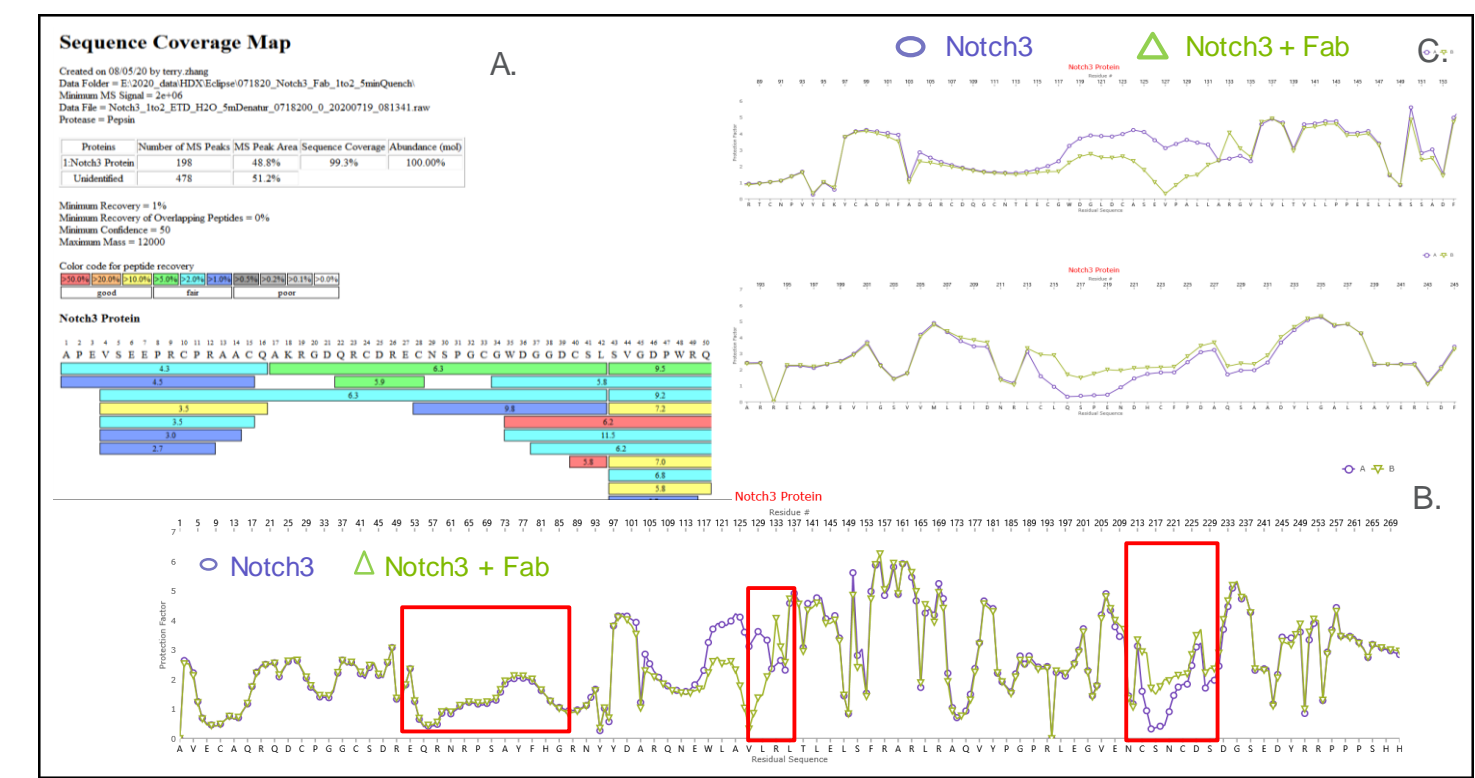


Figure 9. Mapping HDX-MS Data into Crystal Structure

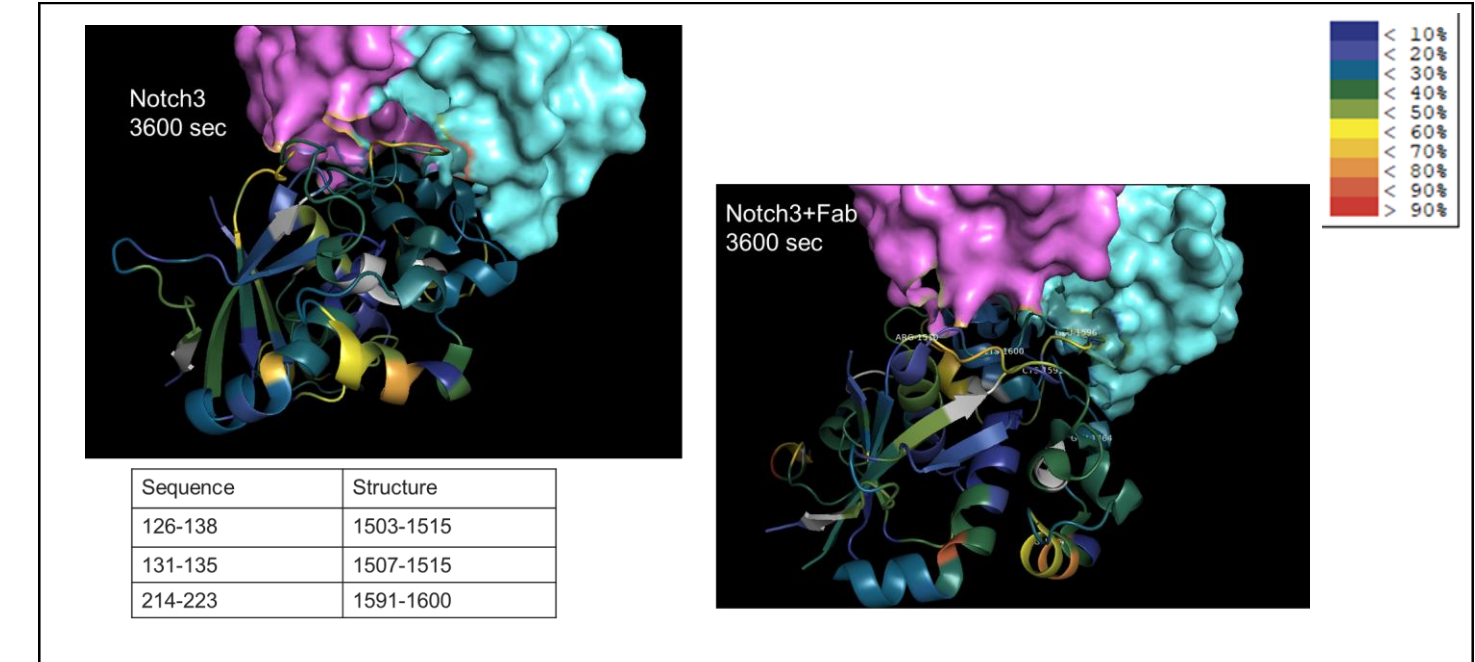


Figure 10. Summary of Results: 20350Fab Epitope on Notch3 NRR using HDX and XL-MS vs crystal structure<sup>1</sup>. Identified Notch3 NRR domains are highlighted in red or blue.

20350 Epitope on Notch3 NRR		
Domain	Residue	Number
LNR-A/B linker	GLN	1427
	CYS	1428
	GLU	1429
	CYS	1442
	PRO	1444
LNR-B	ALA	1445
	SER	1447
	SER	1448
	PRO	1449
	ALA	1450
	TYR	1453
	CYS	1458
	GLY	1461
	GLY	1462
	GLU	1464
LNR-B/C linker	LEU	1507
	LEU	1508
LNR-HD linker	ARG	1510
	LEU	1592
	SER	1594
HD β4-α3 linker	PRO	1595
	GLU	1596
HD α3 helix	ASN	1597
	ASP	1598
	HIS	1599
	PRO	1602
	SER	1606

### XL-MS Paratope/Epitope Mapping

HC-Notch3	LC-Notch3
52-1429	45-1587
	53-1598

### HDX Results

Sequence	Structure
126-138	1503-1515
131-135	1509-1513
214-223	1591-1600

## CONCLUSIONS

- Native LC/MS analysis is an essential step to verify sample quality and optimal conditions to form protein complex.
- DSPP, a trifunctional, enrichable Lys specific crosslinker, resulted in the most identified Fab crosslinked peptides, heterobifunctional UV activated LC-SDA and zero length EDC crosslinkers worked well for Lys limited Notch3 NRR epitope mapping vs traditional homobifunctional crosslinkers like DSG or DHSO.
- The fully automatic HDX workstation consisting of Trajan's LEAP HDX Extended Parallel System and Orbitrap Eclipse mass spectrometer demonstrated excellent HD exchange reproducibility and sensitivity and enabled epitope identification confirming co-crystal structure results.
- Epitope mapping using integrative structural proteomics tools: native, HDX and cross-linking mass spectrometry was developed and successfully applied for Notch3 NRR

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