CASE WESTERN RESERVE UNIVERSITY
SCHOOL OF MEDICINE

Mark R. Chance

Center For Proteomics & Bioinformatics
Case Western Reserve University

Chief Scientific Officer, Neo Proteomics.

Hacking Structural Biology for Drug Discovery Using Mass Spectrometry

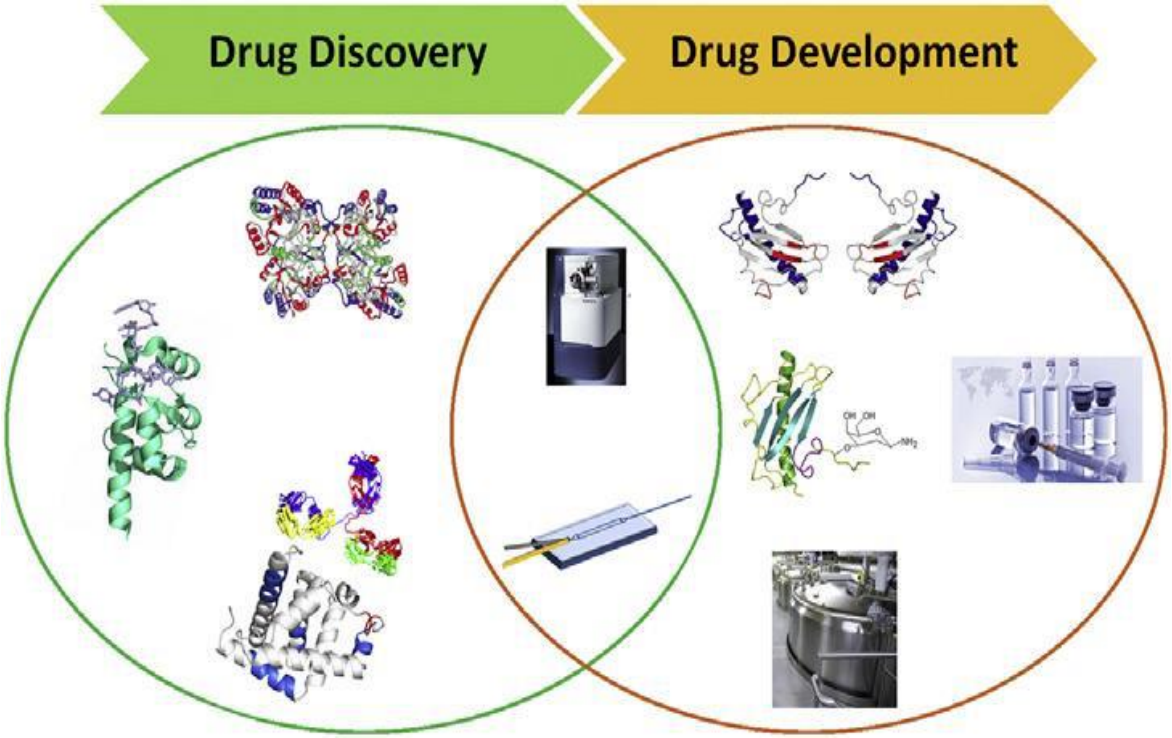
Declared Conflict: CWRU technologies for footprinting and systems biology are licensed to Neo Proteomics Inc. where MRC is a shareholder and officer. MRC also serves on the Science Advisory Board of GenNext Technologies

NeoProteomics

GenNext TECHNOLOGIES

Drug Discovery & Development

Drug Discovery
- Target ID
- Protein therapeutics (GPCR, mAbs)
- mAb-drug conjugates
- Protein-Ligand interaction
- Protein-Protein interaction

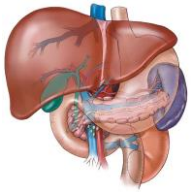


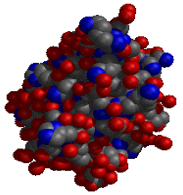
Drug Development
- Structural modifications
- Biosimilar antibodies
- Formulation
- Manufacturing

Bin Deng et al., Analytica Chemica Acta, 2016

Proteomics: a key enabling technology for structural & systems biology

mpvlsrprpw...



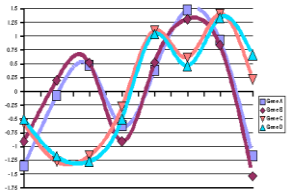


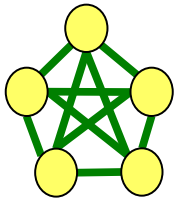
Where

What

When

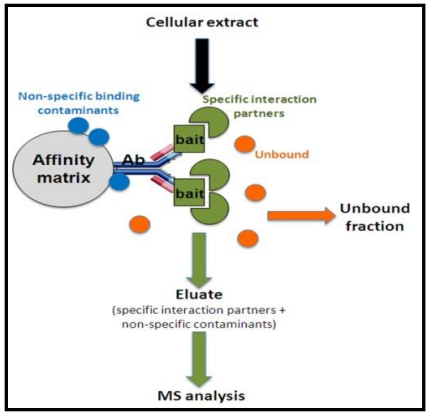
Relationships





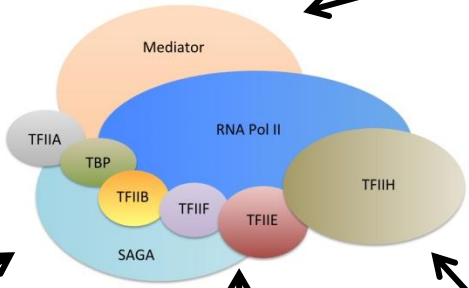
Proteomics From Molecules to Man

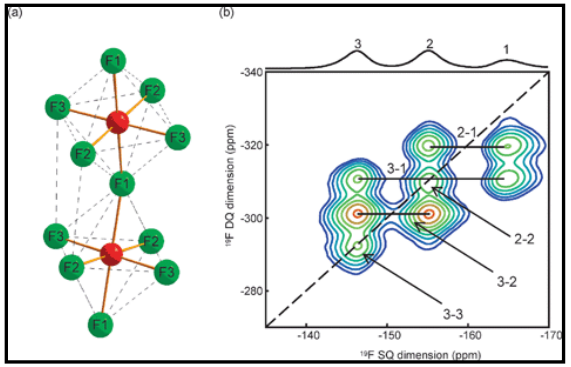
Structure & Dynamics of Macromolecular Complexes



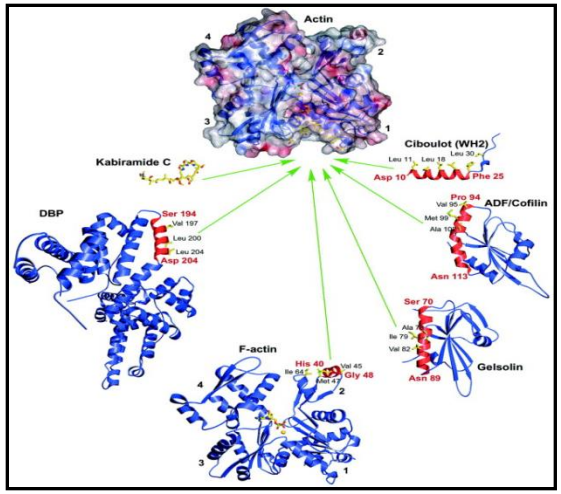
Molecular Biology/
Bottom up/Top down MS

Mega-Dalton Complexes

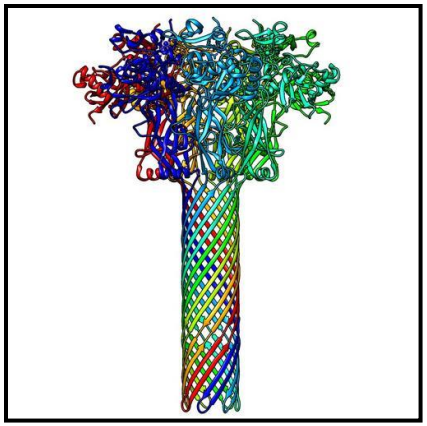




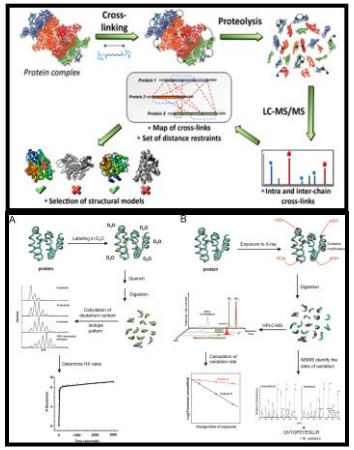
NMR



Protein Crystallography



Cryo EM



Cross-linking/HDX/FP

Gavin et al. Nature, 2002 (Cellzome)

Turn, Turn, Turn
Advantages and Limitations of Major Structural Biology Techniques

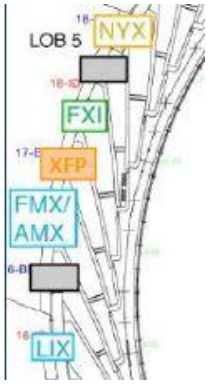
Resolution, size, and limits on amounts of material are approximate for 10-100K protein sizes.

Technique	**Size (sample state)**	**Resolut-ion Limits**	**Amounts**	**Notes**
NMR	<100 kD (solution)	~3-4 Å	µmoles/ milligrams	Requires labeled recombinant protein, disordered regions can be observed but may not be assigned.
Crystallo-graphy	Limited by crystal quality	< 1-3 Å	µmoles/ milligrams	Mutant constructs necessary for many membrane proteins, disordered regions invisible. Gold standard for structural water
Cryo-EM: Single particle	>100 kD (vitrified ice)	Mostly >3 Å	nanomoles/ µgrams	Resolution and size limits improving, best samples have symmetry, disordered regions invisible.
Cryo-EM: Tomo-graphy	Cells or tissues)	30-40 Å	thin sections/ individual cells	Resolution improving; captures large-scale spatial organization in cells.
SAXS	> 10 kD (solution)	> 20 Å;	nanomoles/ µgrams	Native material can usually be used, (similar to FP samples)
Footprinting: HRF-MS [and HDX-MS]	HRF-MS: No limit [<100K for HDX] (solution)	Peptide to single-residue (single base for NA)	picomoles/ nanograms	Native material can usually be used (both), absolute surface area can be estimated (HRF), disordered regions visible (HRF). Studies in cells/tissue possible (HRF).

Chance et al., *J. Mol. Bio*., 2020

CWRU at NSLS-II

A Structural Biology Village



MS-Footprinting/HDX
- Local conformation change
- Binding interfaces

Small Angle X-ray Scattering/EM/ Native MS
- Global Conformation
- Large Complexes
- Identity & Stoichiometry

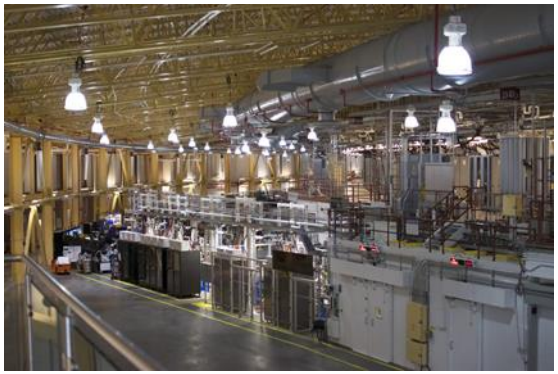
Other Biophysical Data
- Crosslinking, mutagenesis

Computational Approach
- Homology Modeling
- Docking
- Molecular Dynamics

High-Resolution Structure & Models (Cryo-EM, & MX)
- Medium to High resolution
- Local and global

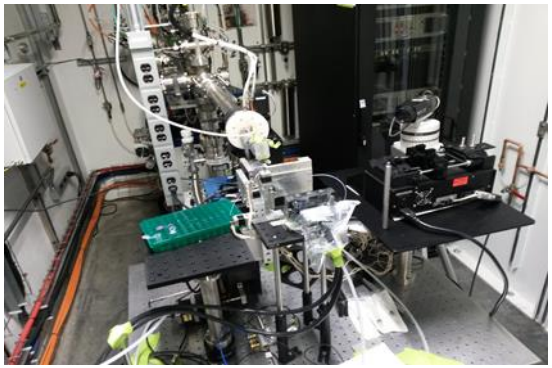
Integrative Approach to Structure Determination

CSB at National Synchrotron Light Source-II



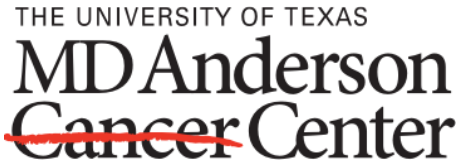
NSLS-II Floor Area

FMX, AMX Crystallography



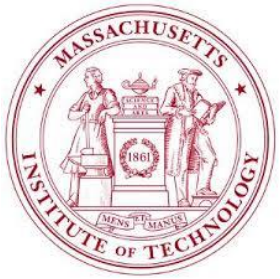
XFP Footprinting

Yale University
School of Medicine



Stanford
University

Perelman
School of Medicine
University of Pennsylvania



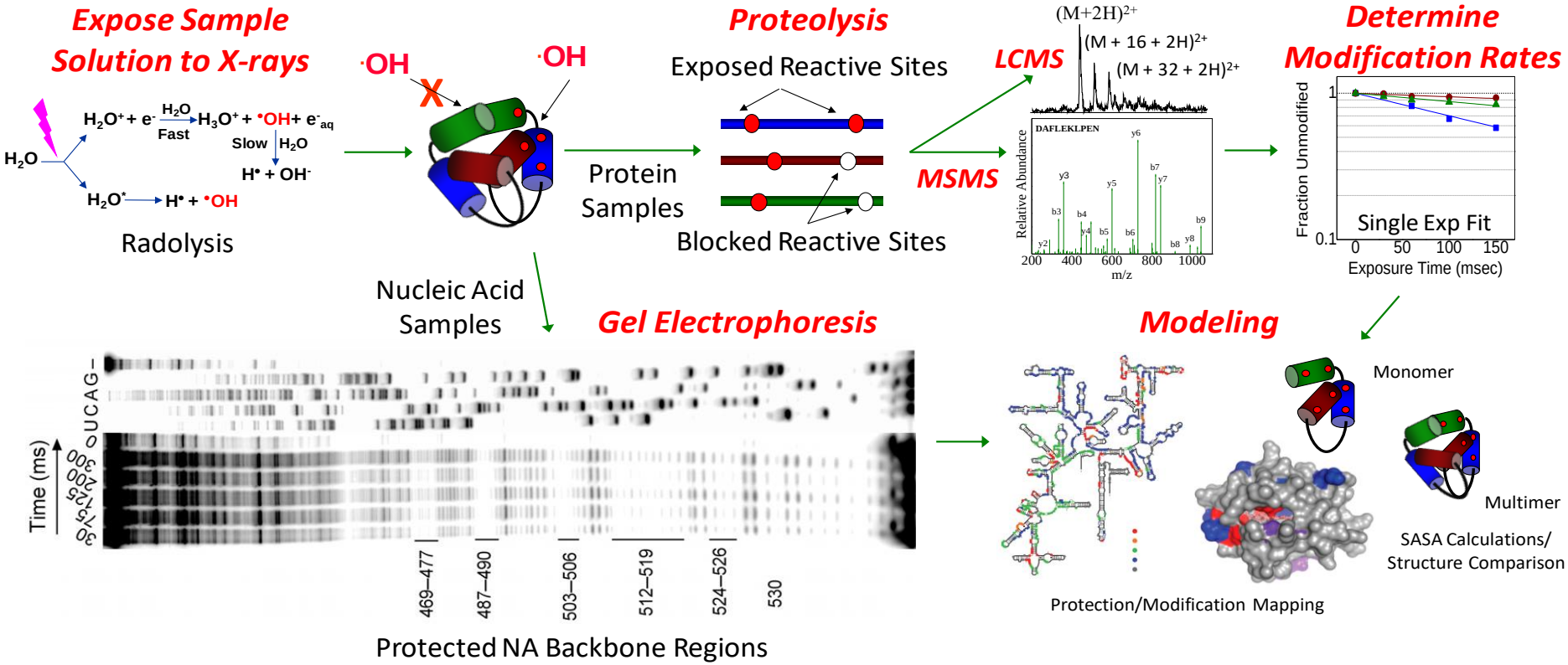
Sharing Resources
Award 2017

SCHOOL OF MEDICINE
CASE WESTERN RESERVE UNIVERSITY

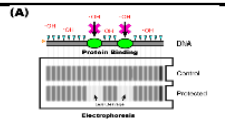
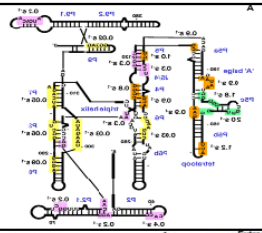
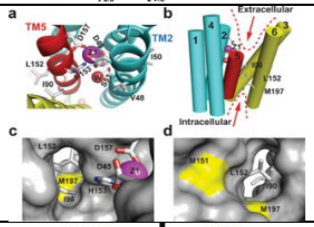
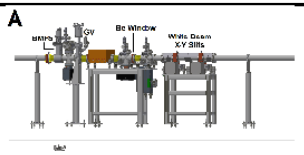
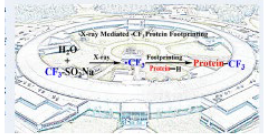
Center for Proteomics and Bioinformatics (2005)
Center for Synchrotron Biosciences (1994)

What is Footprinting?

- Chemical alteration of a macromolecule in solution
  - Sensitive to surface accessibility
    - Identifies binding sites
  - Nucleic Acids Footprinting:1970
    - Protein Footprinting: 2000s
    - Hydroxyl radical #1 reagent



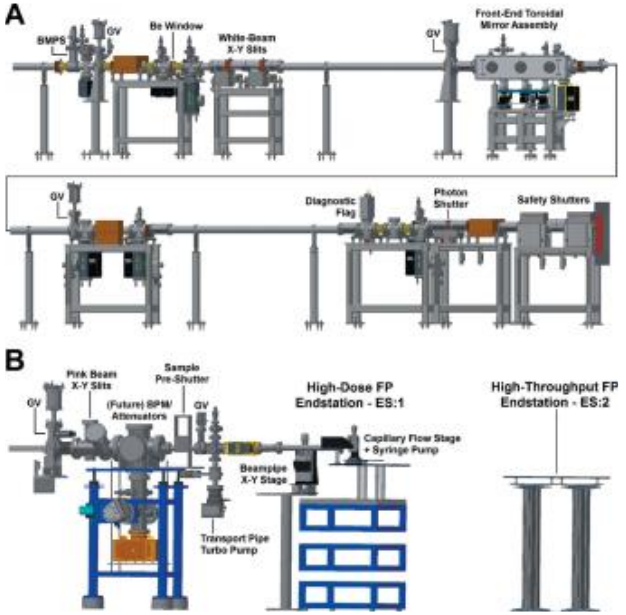
Thirty Years of Synchrotron Footprinting Development

Beamline	Years	Key Outcomes	Graphic/Notes	Key Papers
NSLS X19C	1995-1996	Proof of concept as General User		JMB 1997
NSLS X9A	1996-1999	Time-resolved RNA footprinting		Science 1998
NSLS X28C	2000-2014	Protein footprinting (steady-state and time-resolved). Focusing mirror innovation.		PNAS 2009 Nature 2014
ALS (5.3.1 /3.2.1)	2014-2016	Technology and equipment. Mirror and new continuous flow methods.		Comm. Biology 2022
NSLS-II 17-BM	2016-2024	Ultra-high flux density / high-throughput methods		Cell 2019
NSLS-II 17-BM	2024-2029 Proposed	Continued innovation (Multiplex chemistry/direct dosimetry/MS onsite)		

Generation of OH radicals from radiolysis

Beamline BM-17- X-ray Footprinting of Biological Materials Beamline

MAJOR RESEARCH INSTRUMENTATION



Radiolysis of water

100eV = 2.87 OH radicals

Ionizing Radiation

$$H_2O \rightarrow H_2O^{\bullet+} + e^- \xrightarrow[\text{Very fast}]{H_2O} H_3O^+ + {}^{\bullet}OH + e^-_{aq}$$

$$H_2O \rightarrow H_2O^* \rightarrow H^{\bullet} + {}^{\bullet}OH$$

slow, H_2O: $H^{\bullet} + {}^{-}OH$

OH radicals also produced by: Photolysis/Fenton/Plasma

https://www.bnl.gov/nsls2/beamlines/highlights.php?q=17-BM

GenNext FOX™ Flash Oxidation Technology

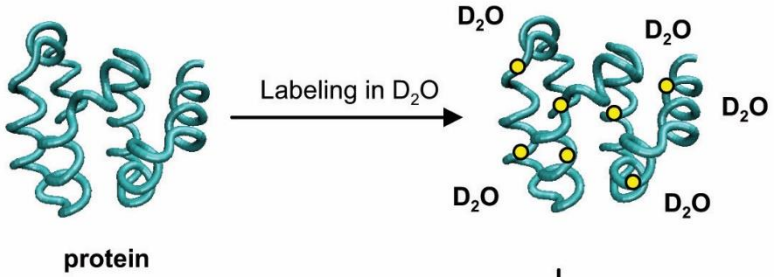
- FOX system has photolysis by high energy plasma lamp.
- Peroxide and sample mixing automatic
- Real-time radical dosimetry determines effective radical yield and enables adjustment for scavenging.
- Product collector provides for automated collection of labeled protein

Benchtop to Beamline supported ecosystem

https://gnxtech.com

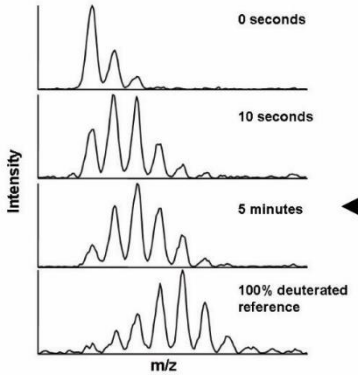
Molecular Footprinting of Proteins

HDXMS



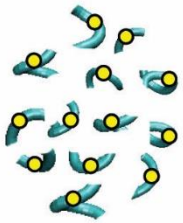
Quench

Digestion

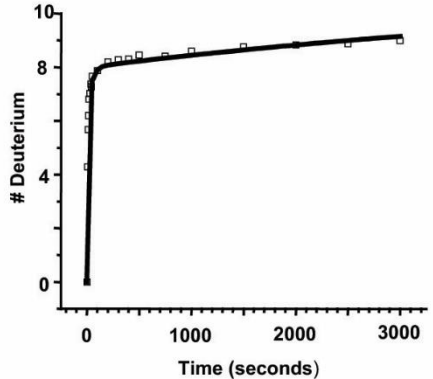


Calculation of deuterium content

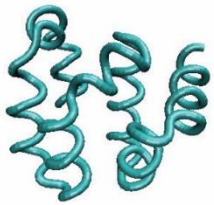
Isotope pattern



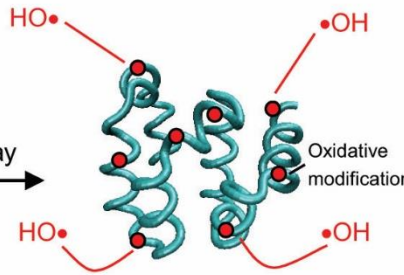
Determine HX rates



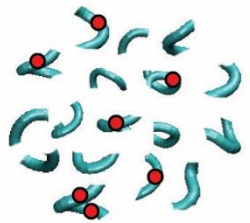
PFMS



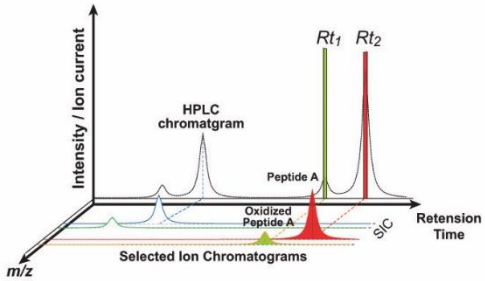
Exposure to X-ray



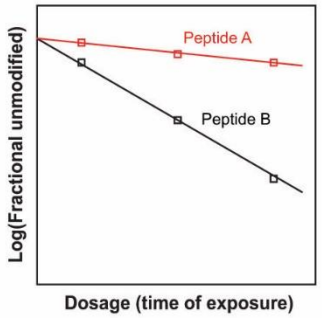
Digestion



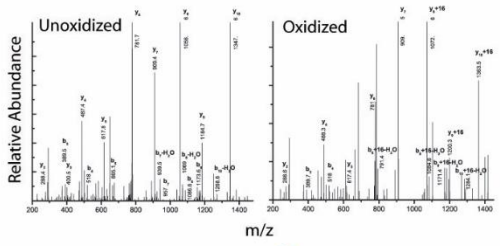
HPLC-MS



Calculation of oxidation rate



MSMS identify the sites of oxidation



QVYQFEYESLLR

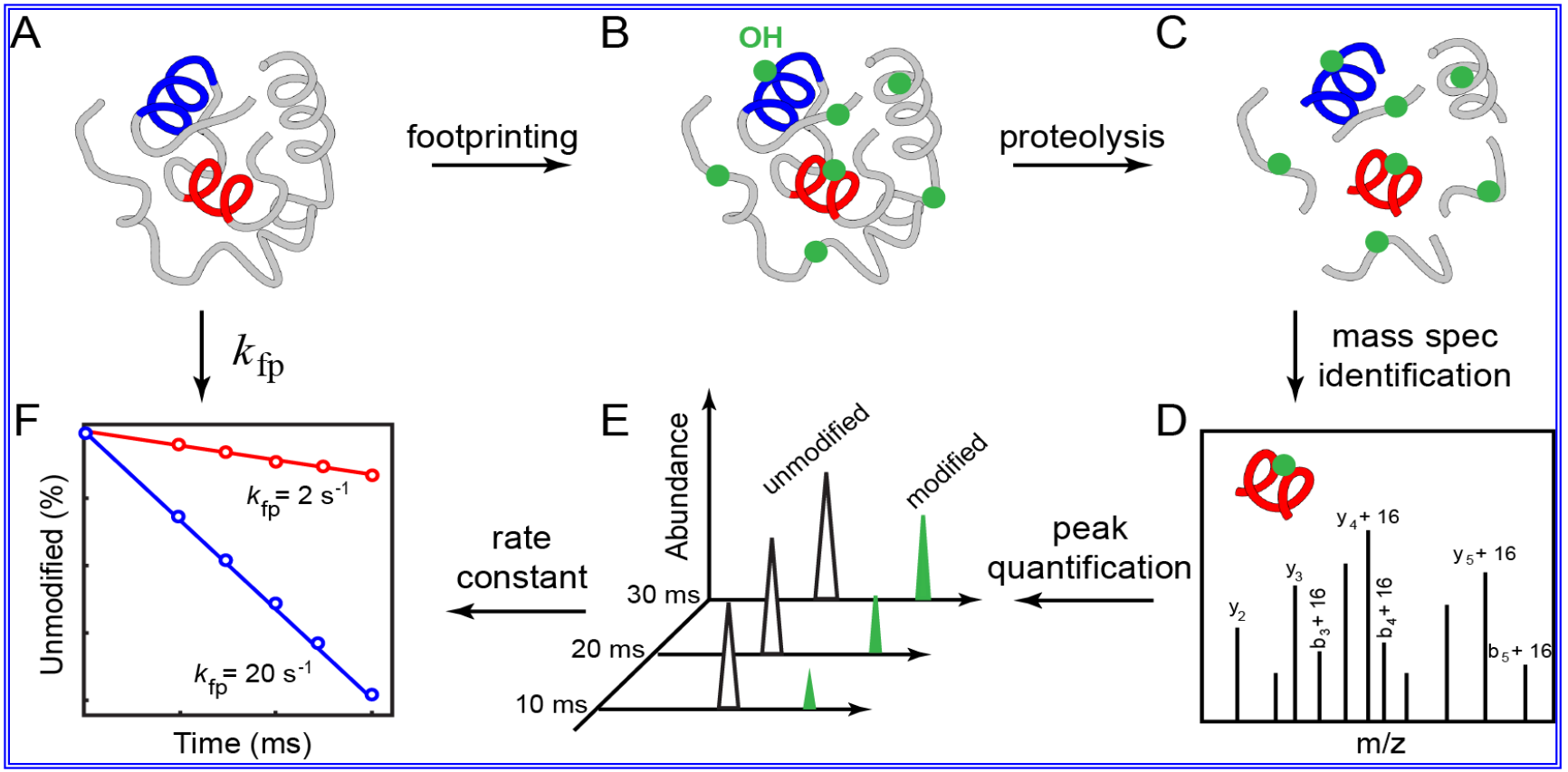
+16 oxidation

Complementarity of Footprinting Approaches

- HDX unsurpassed for assessing **protein backbone secondary structure** in solution (vs NMR). Drawbacks include size limitations due to need for pepsin cleavage, structural resolution at peptide level. Can use conventional ion-trap instruments.
- Covalent labeling reports **solvent accessibility** at **side chain** level resolution, large or complex macromolecules and membrane proteins feasible, many proteases and chromatographic methods. Requires Hi-res instruments.
- **Standard protocols are at peptide level**

MEDIUM- RESOLUTION FOOTPRINTING STRATEGY

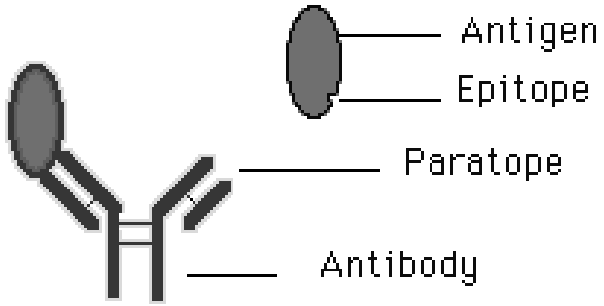
Peptide level analysis



Courtesy of Sichun Yang

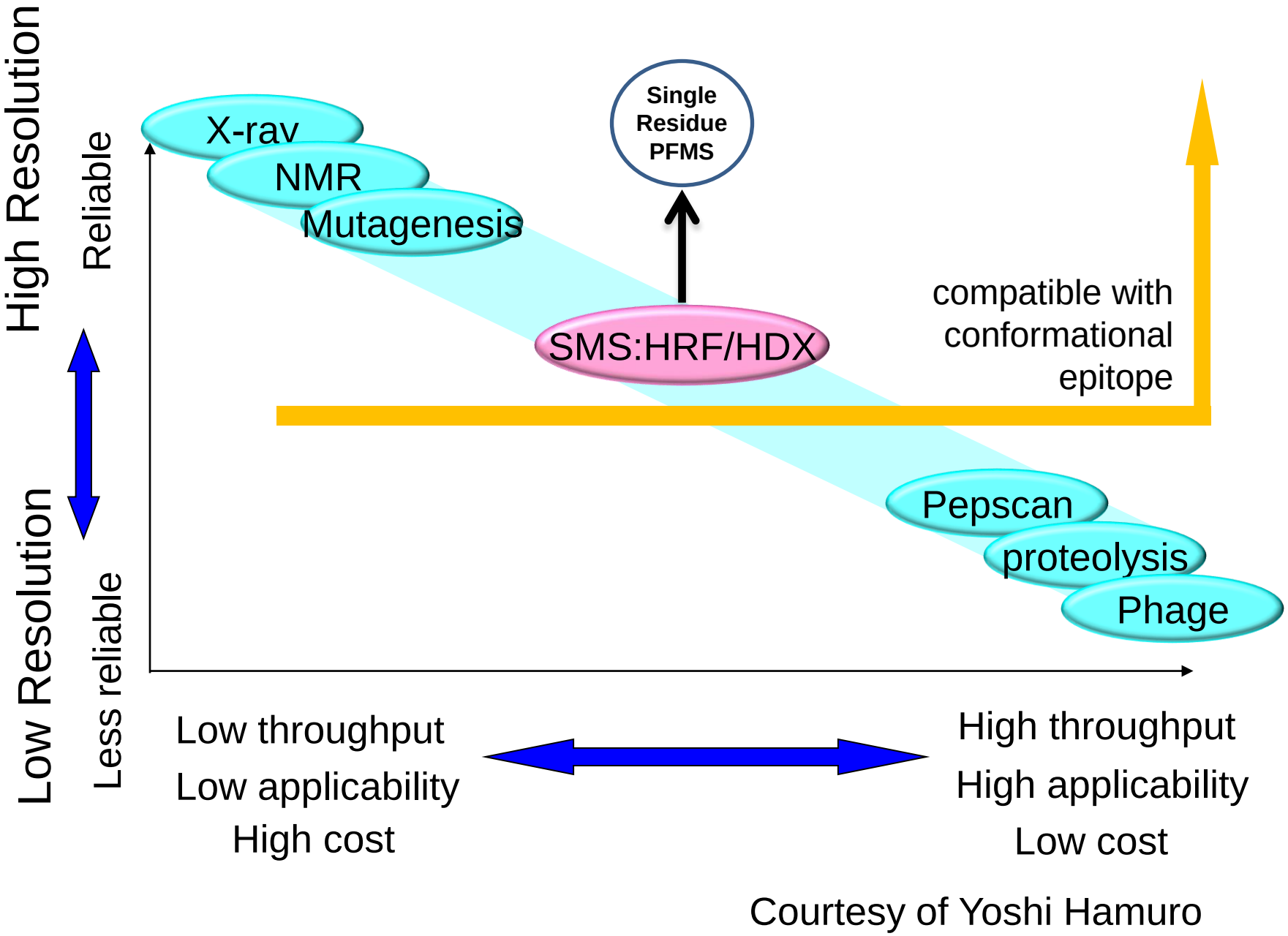
Mass Spectrometry for epitope mapping and structure assessment of mAbs

- **Antigen epitope mapping** by structural mass spectrometry provides novelty and patentability of for mAb substance.
- IP protection can both uniquely establish IP relevant to antigen binding motif and/or block competitors.
- **Paratope mapping** important for re-engineering antibody



Sandercock and Stroz, *Nat. Biotech*., 2012

Structure assessment/epitope mapping



Courtesy of Yoshi Hamuro

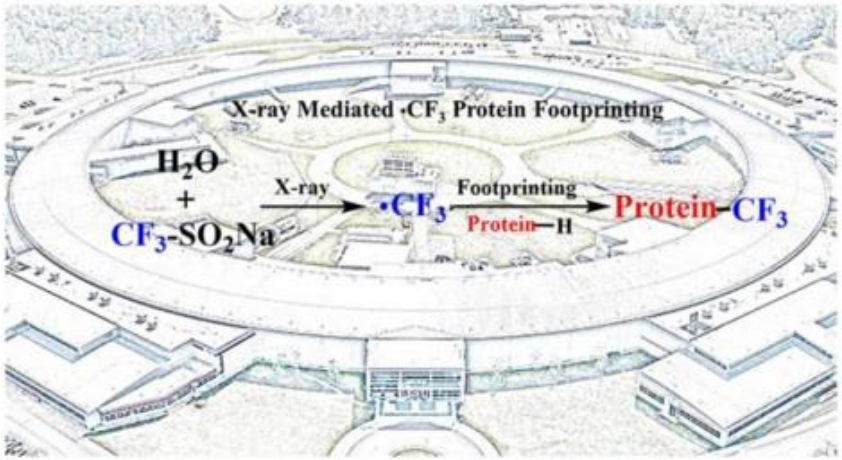
Enhancing Structural Resolution of PF with chemistry

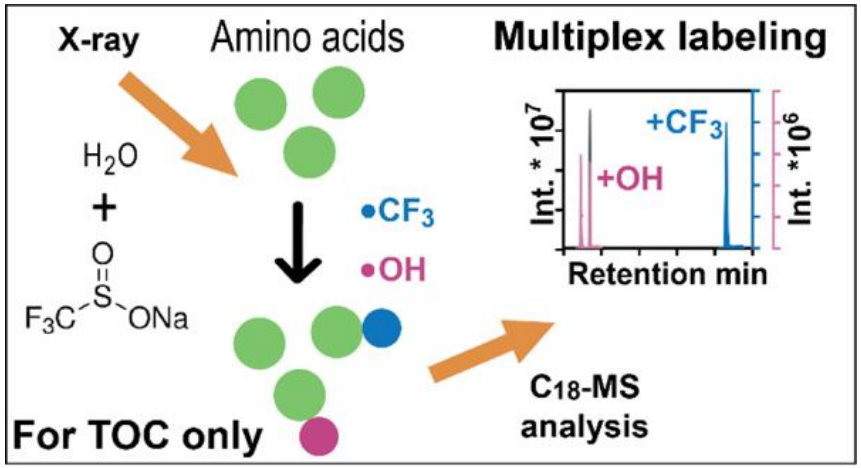
Reagent chemistry	Principal target residues	Mass shifts
OH radical	Aliphatic, aromatic, Met, Cys	Aliphatic: +16, +14 Aromatic/sulfur containing: +16, +32, +48 Arg: +16, −43; Asp, Glu: +16, −30; His: +16, +5, −22
EDC/GEE	Asp, Glu, C-terminus	+57, +85
Methylene carbene	All residues, minimal specificity	Variable (+14 methylene addition)
Carbethoxy	His, Lys, Tyr, Ser, Thr, Cys	+72

Typically Up to 20% of residues labeled

Kiselar & Chance, *Ann. Rev. Biophys*. (2018)

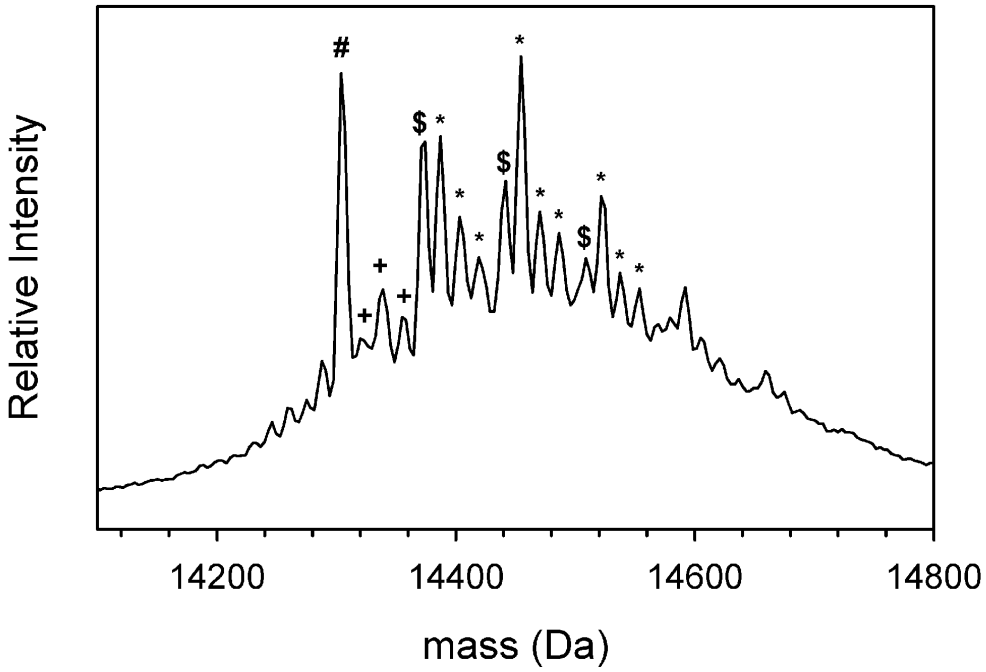
CF3-OH Multiplex Chemistry





Intact MS of lysozyme

Charge state +10; 5.7 OH (+) and 0.5 CF3 ($) per molecule average, *-mixed CF3-OH labels



Increases density of side chain labeling by 50-150% depending on peptide.

Cheng et al, *JASMS,* 2020; Jain et al, *Anal. Chem*. 2022; Farquhar, *BBRC*, 2023

HIGH-RESOLUTION FOOTPRINTING STRUTEGY

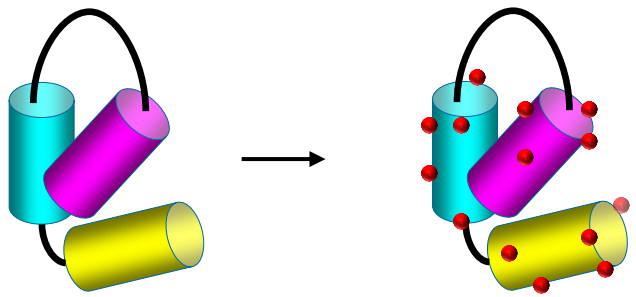
Residue level analysis

X-ray/Light/H2O2

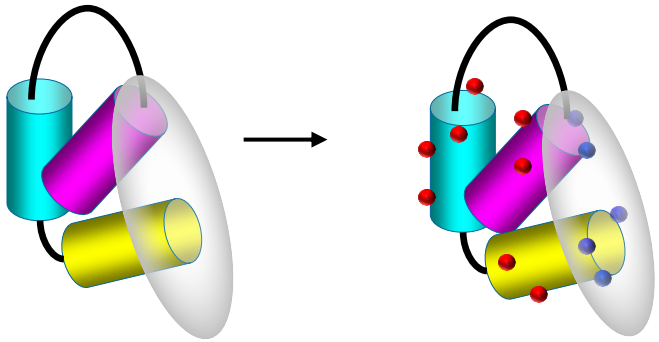
H_2O-CF3 ⟶ •OH

Flexible Proteolysis

UPLC-MS analysis



Protein (A state)

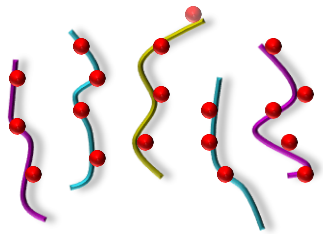


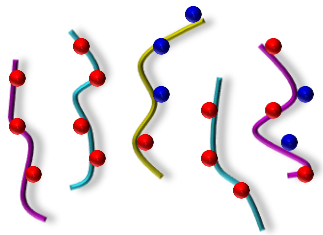
Protein + Ligand (B state)

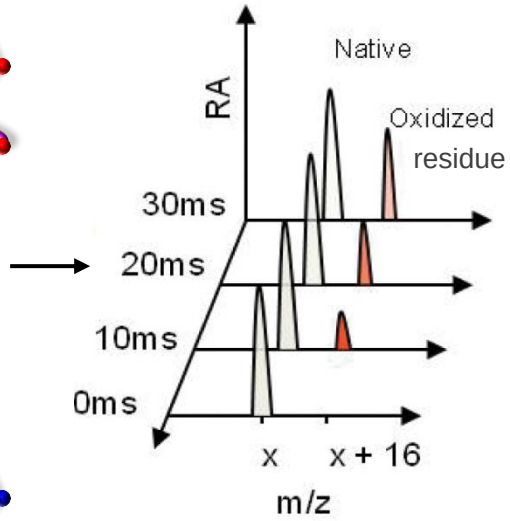
Trypsin

Asp-N

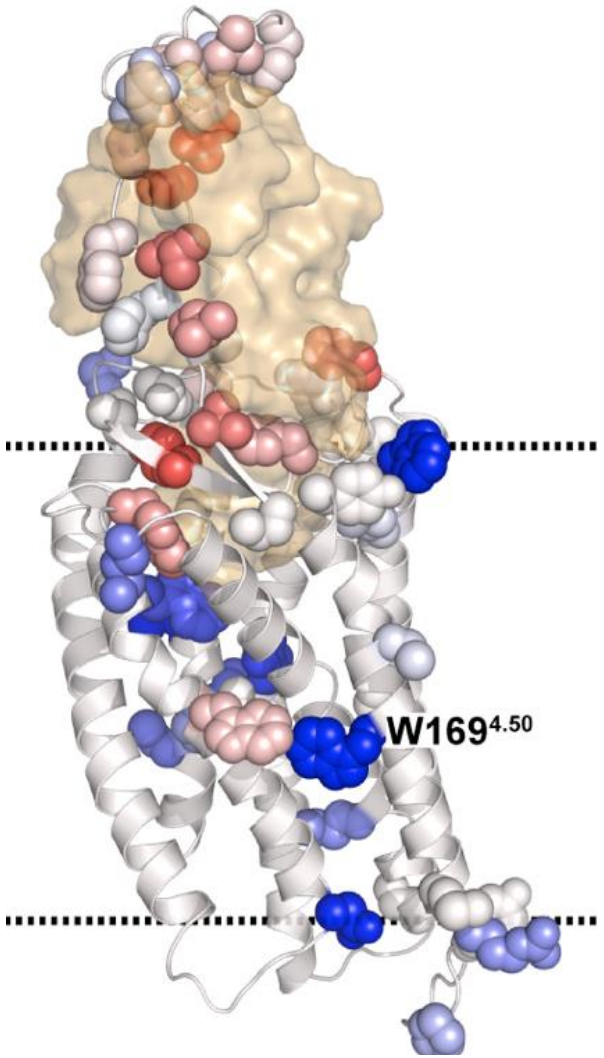
Pepsin

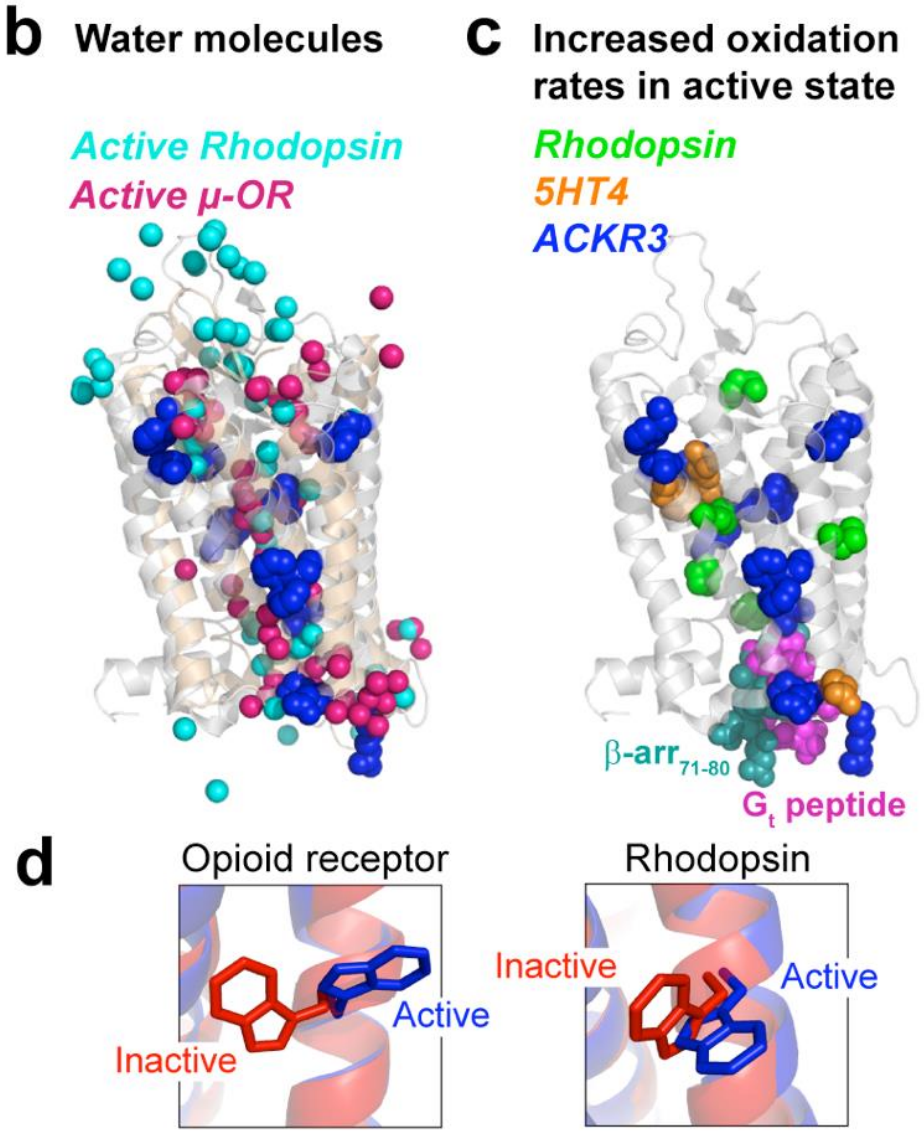






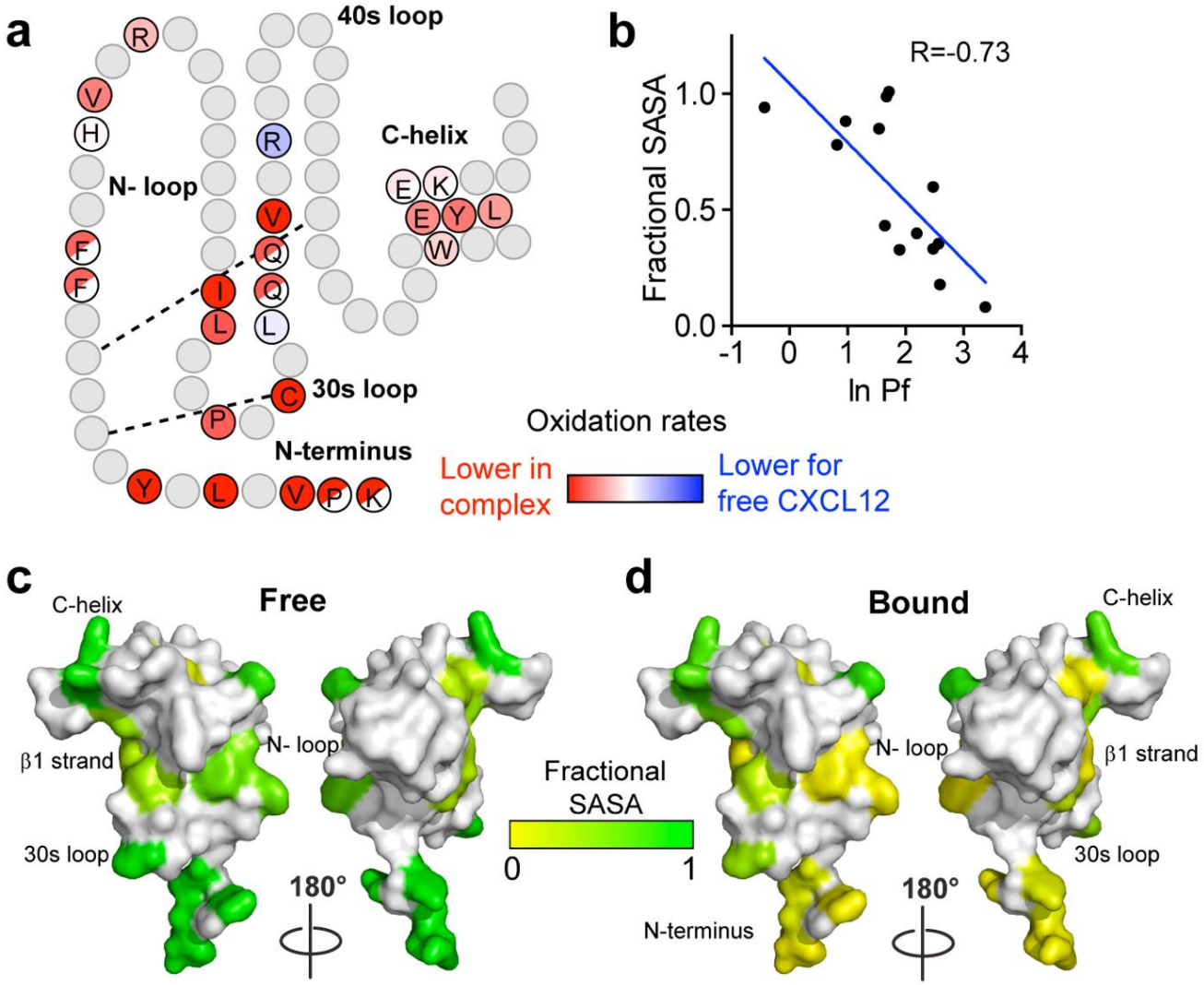
Chemokine (CXCL12) signaling in 7TMs (ACKR3) occurs through conserved paths mediated by structural waters





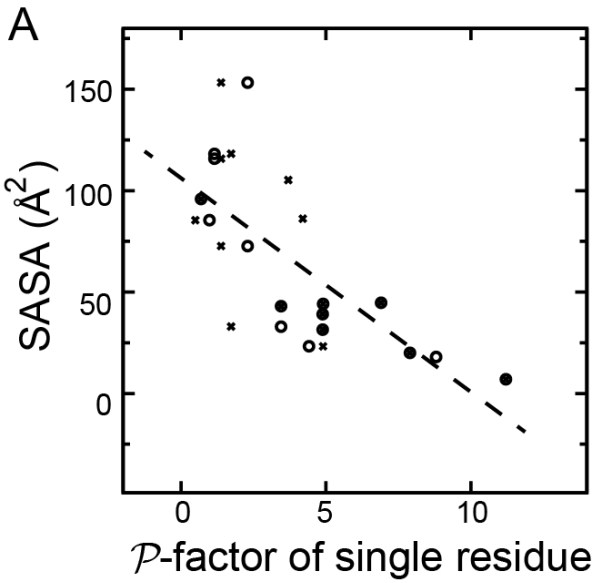
Gustavsson et al., (2017) *Nature Comm*.

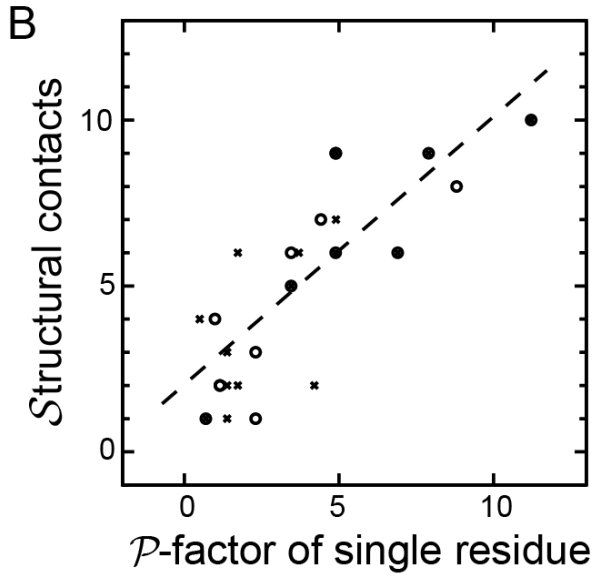
Binding Epitopes of Chemokine Signaling: SDF-1 and CXCR7

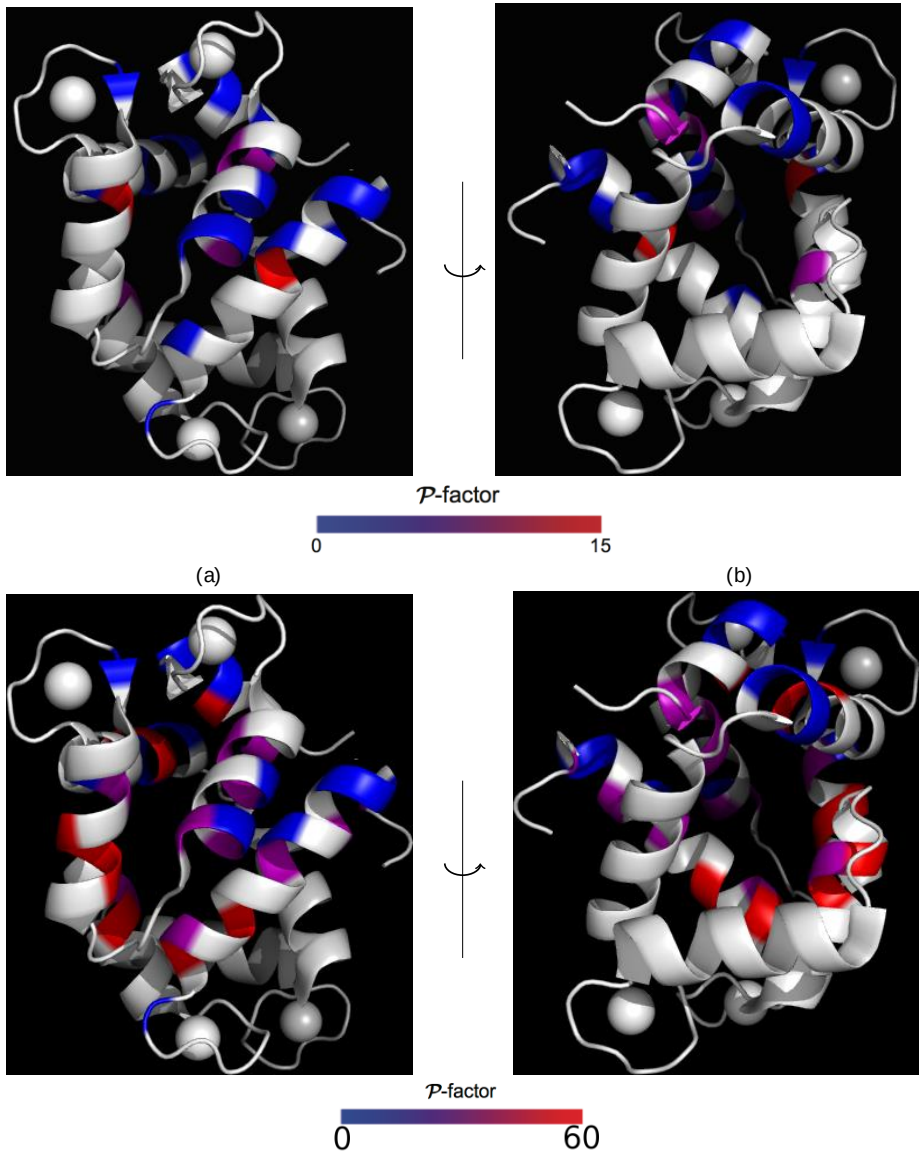


Salanga, et al. *J. Biol. Chem*., 2014, and Gustavsson et al., *Nat. Comm.*, 2017

Calmodulin Structure Assessment





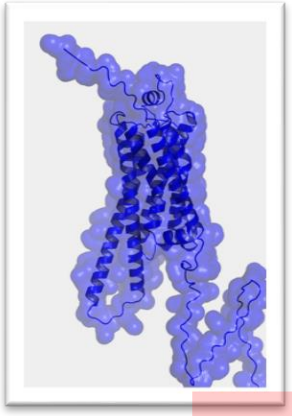


Kaur[(c)] et al., *Mol Cell Prot.*, 2015 [(d)]

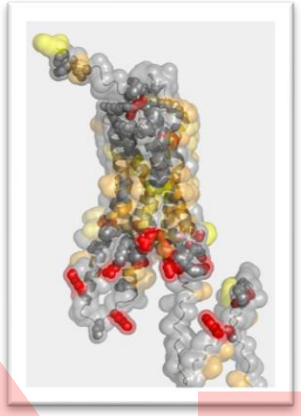
NeoProteomics

Structurally aware experimental design

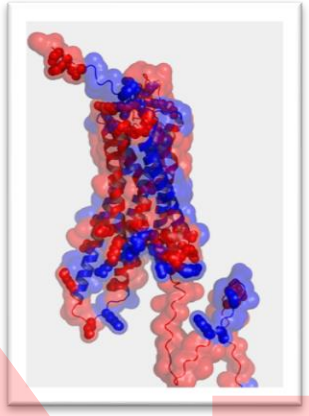
Model
(Alphafold or pdb)



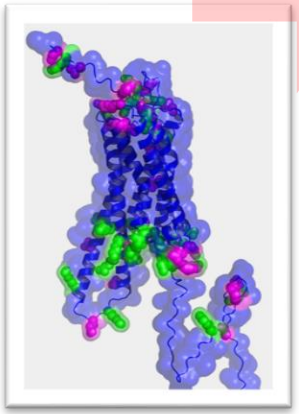
SASA



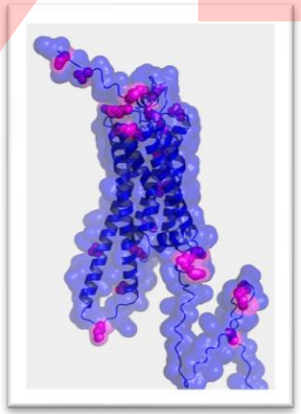
Intrinsic reactivity
OH



Pilot digest
coverage



Proteolytic
cleavage
sites



GEE sites

Additional
experimental
or biochemical
considerations

Maximized
value and
information
content of
footprinting
studies

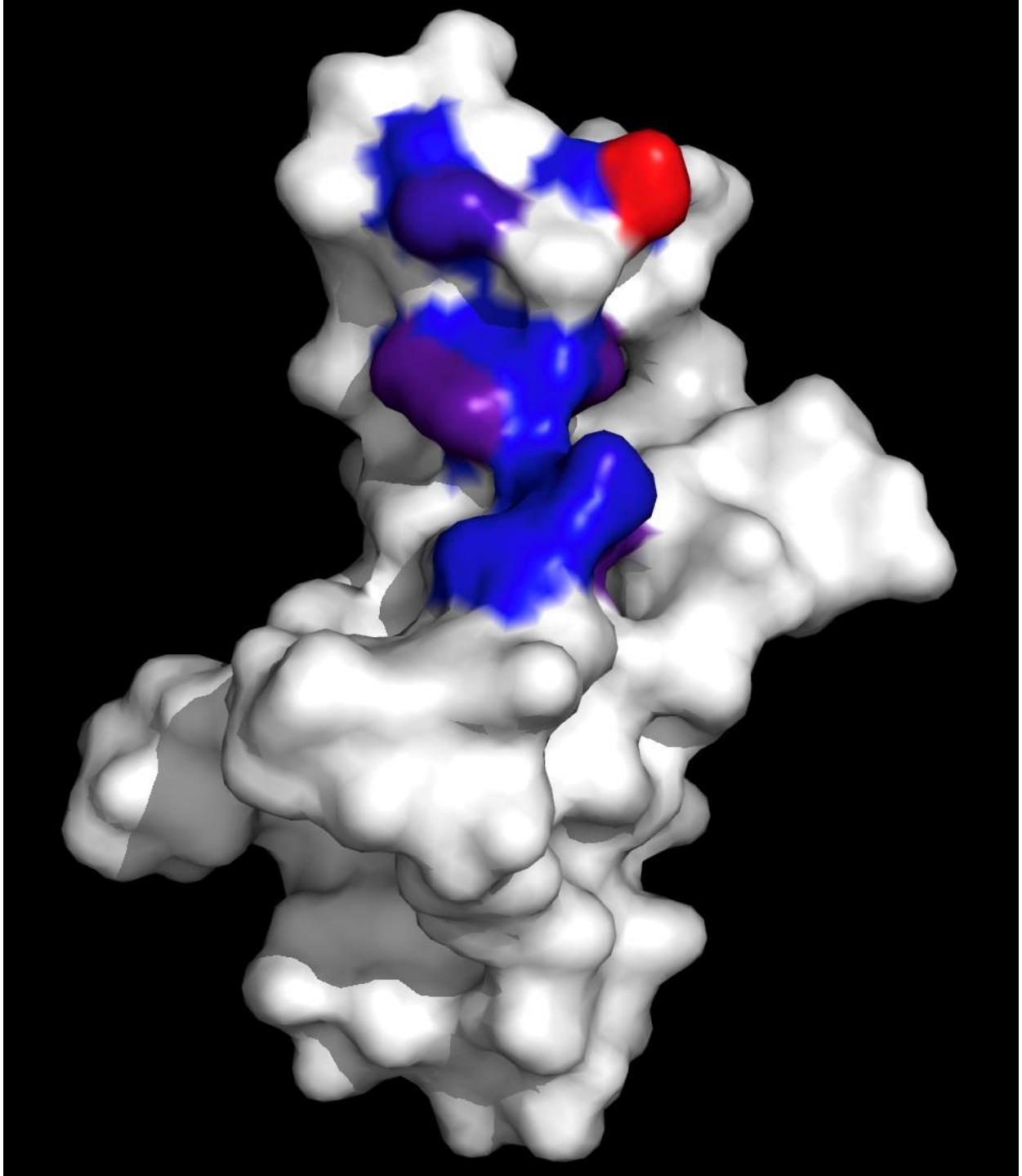
Slide credit D. Lodowski

Single residue resolution epitope protection map using HRPF

Antigen protein surface representation showing detected residues color coded by PR value.
PR of 1.2-1.4 (purple), PR 1.4-1.9 (blue) and PR ≥ 2.0 (red).

Residues in white have PR < 1.2.
The identified "patch" of protections reveals a conformational epitope

NeoProteomics



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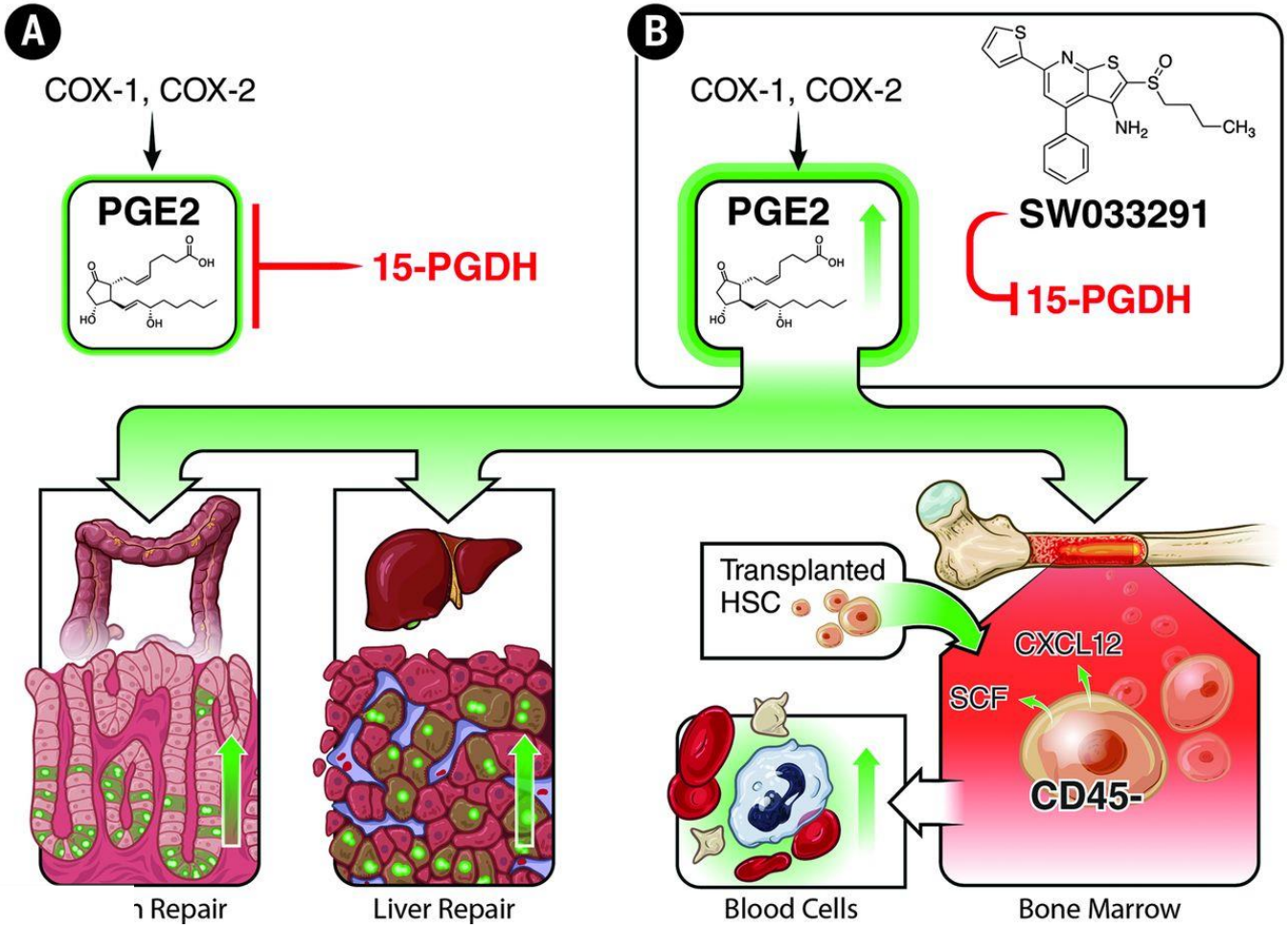
SOTA Applications of Footprinting: Inhibition of the prostaglandin-degrading enzyme 15-PGDH potentiates tissue regeneration

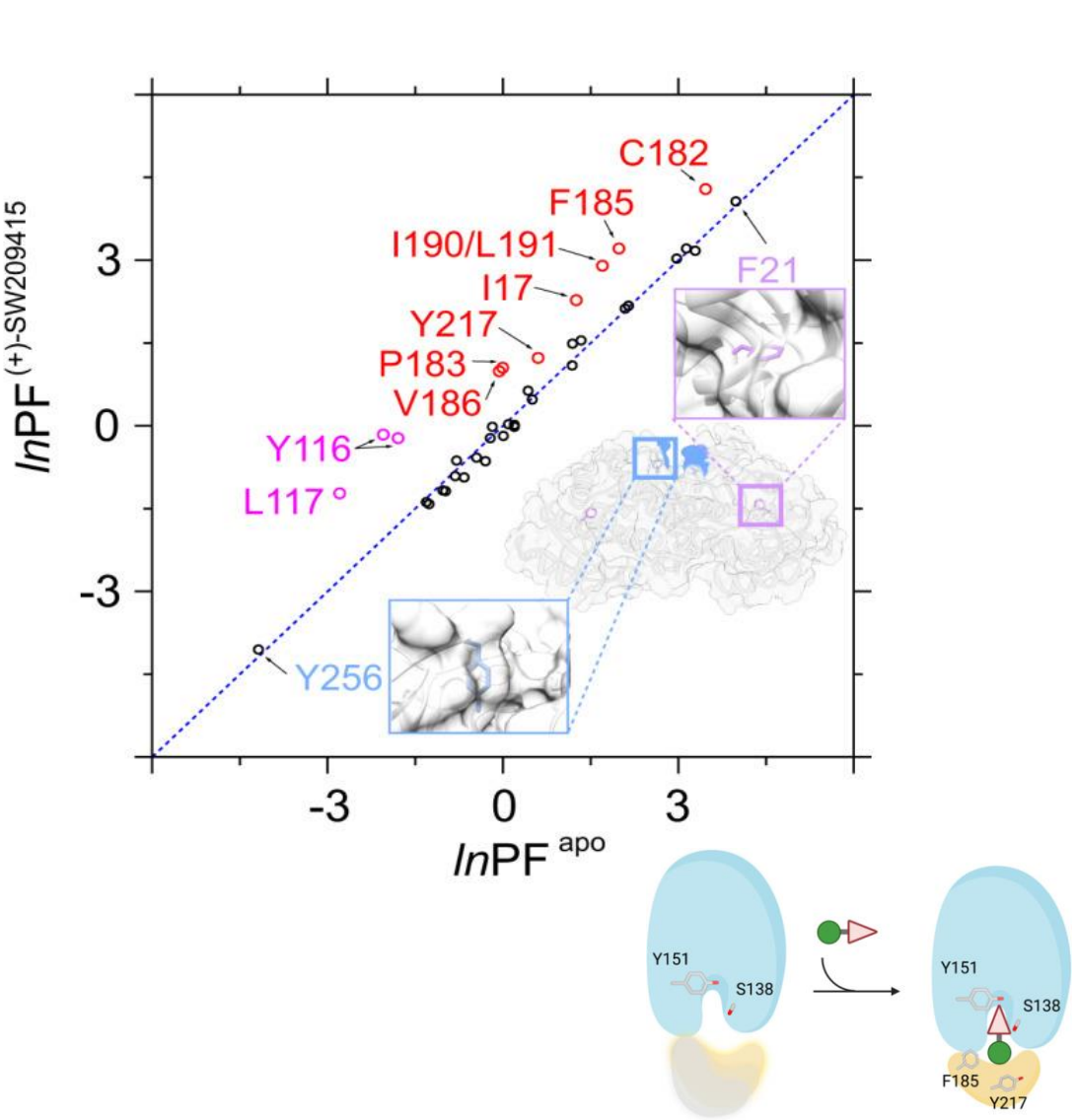


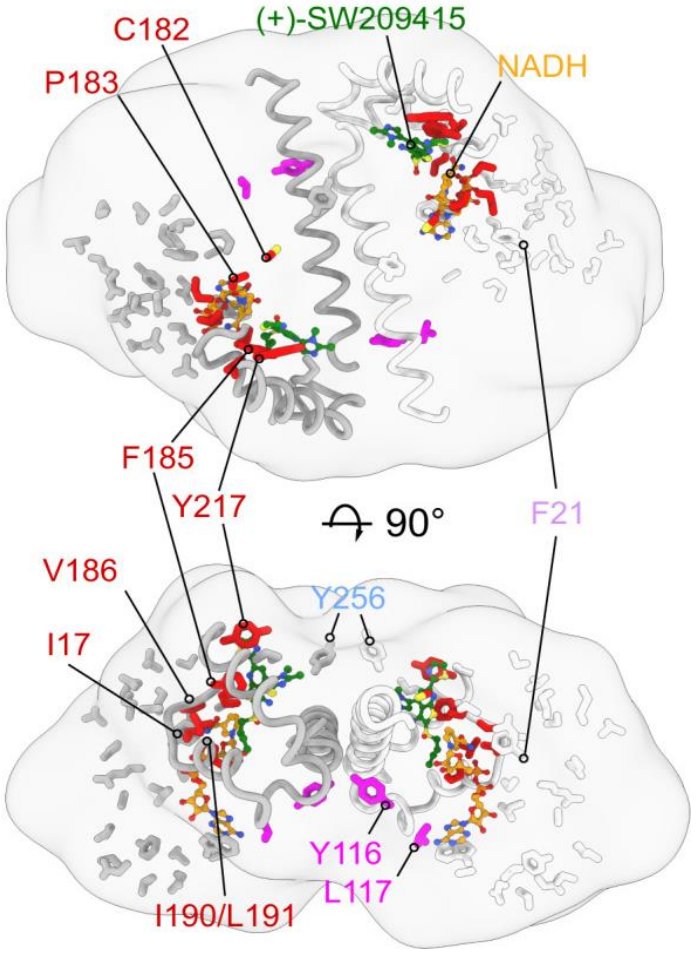
rodeo THERAPEUTICS

AMGEN

Zhang et al., *Science,* 2015

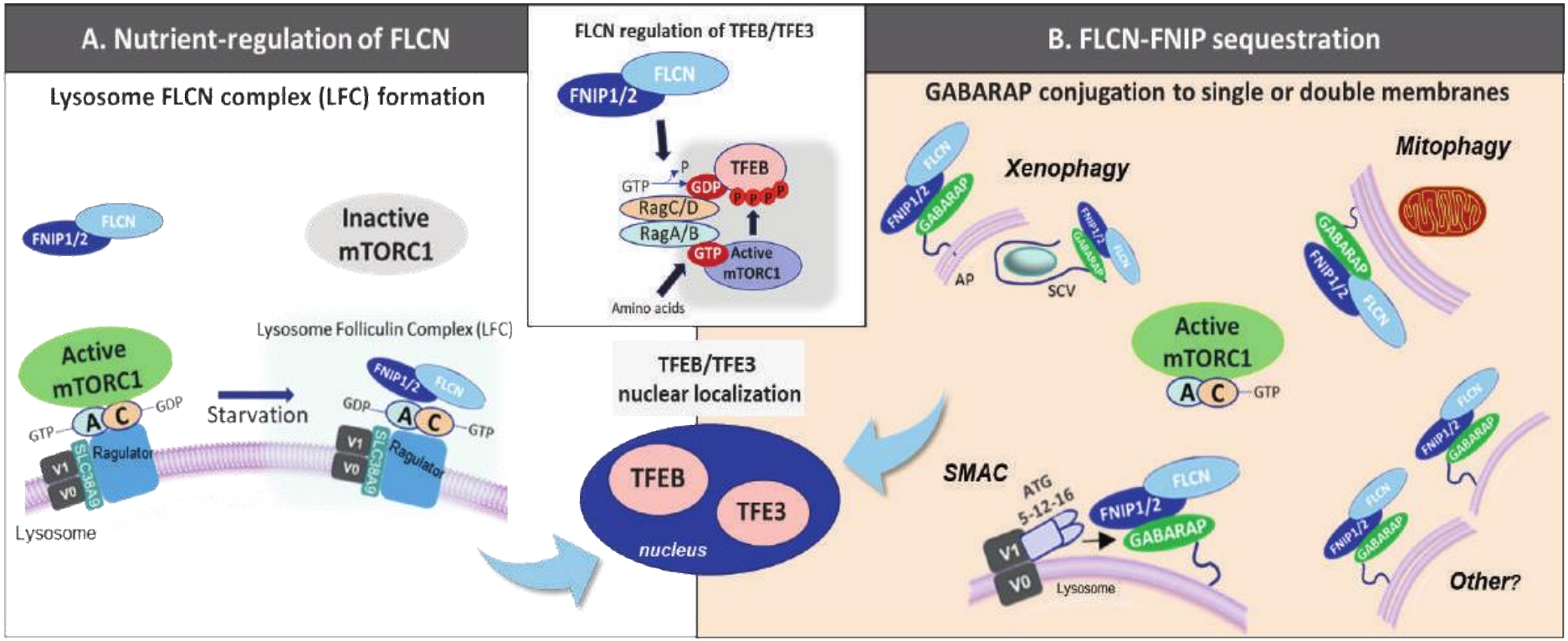
Understanding the basis for nanomolar binding of SW2109415 using footprinting driven structural analysis





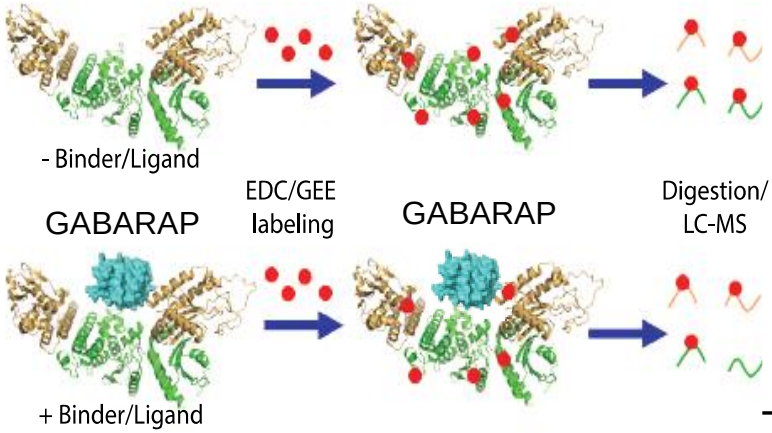
Huang W, et al. *Nat Commun*. 2023

SOTA Applications of Footprinting in Industry: Ternary complex formation of GABARAP binding to the FLCN/FNIP binary complex activates mTOR: Drug development in autophagy

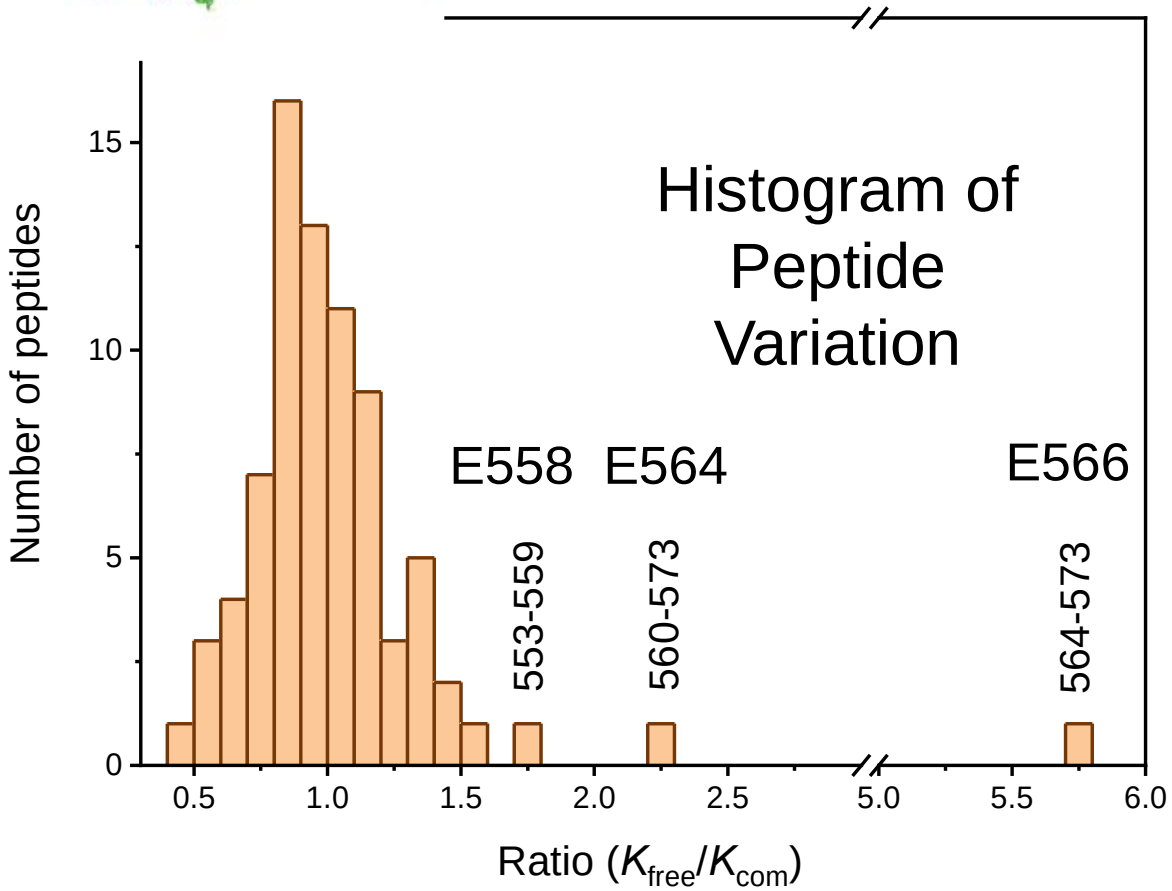


Goodwin et al.,
Sci. Advances, 2021

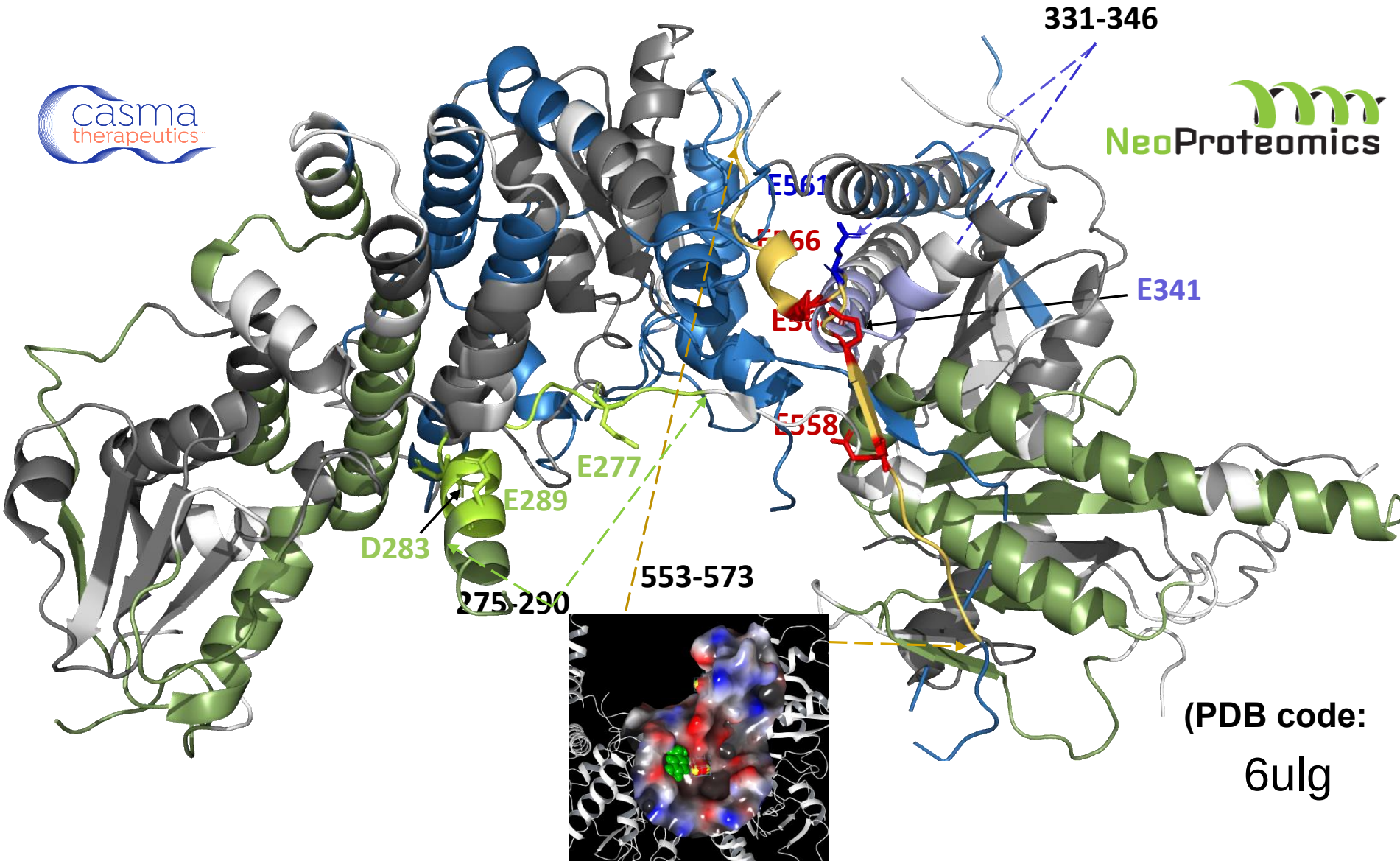
Mapping the GABARAP ternary complex interaction sites



~200 peptides detected (87% coverage)
~90 with reliable labeling
ProtMap-MS software automation



Modeling GABARAP protections on cryo-EM structure of FLCN-FNIP binary complex.



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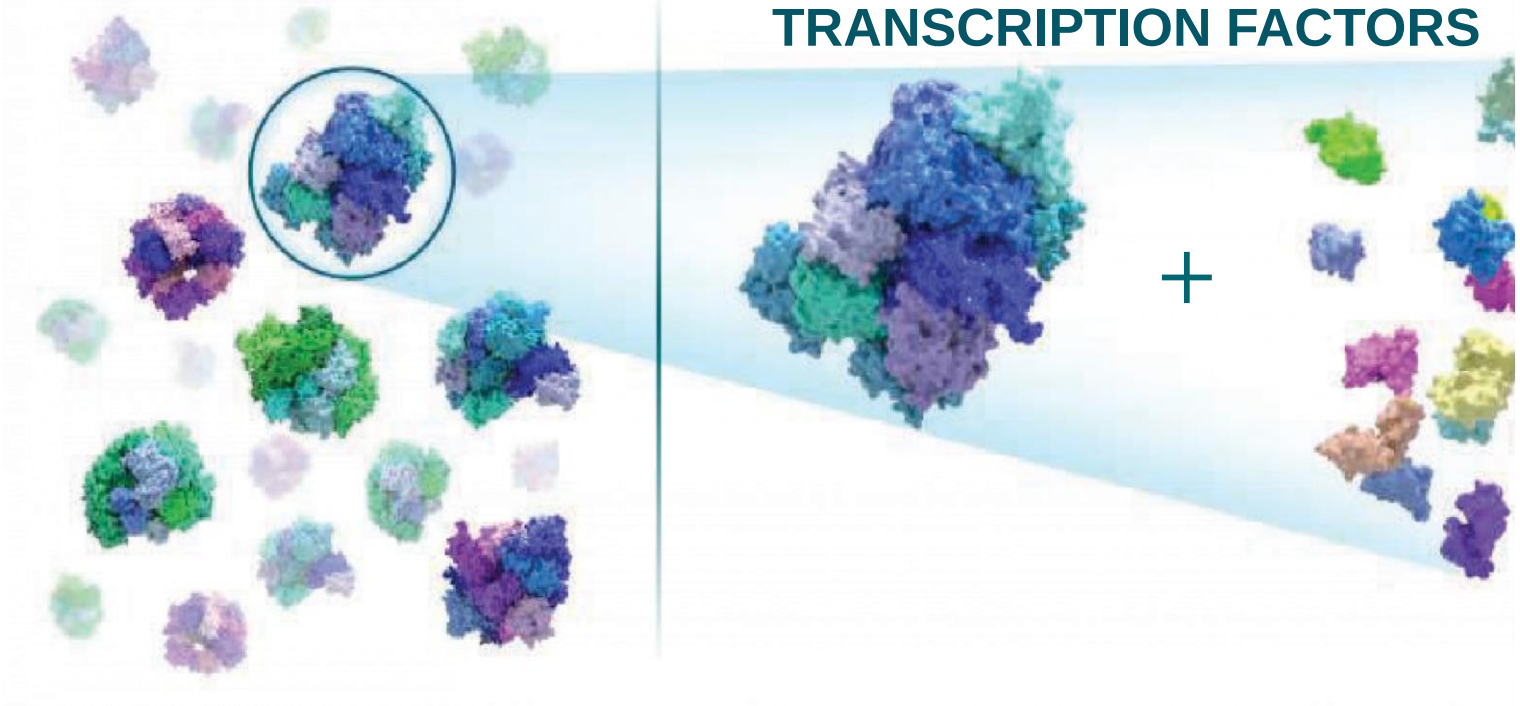
Drugging Undruggable Mega-Complexes

BAF COMPLEX AND ASSOCIATED TRANSCRIPTION FACTORS



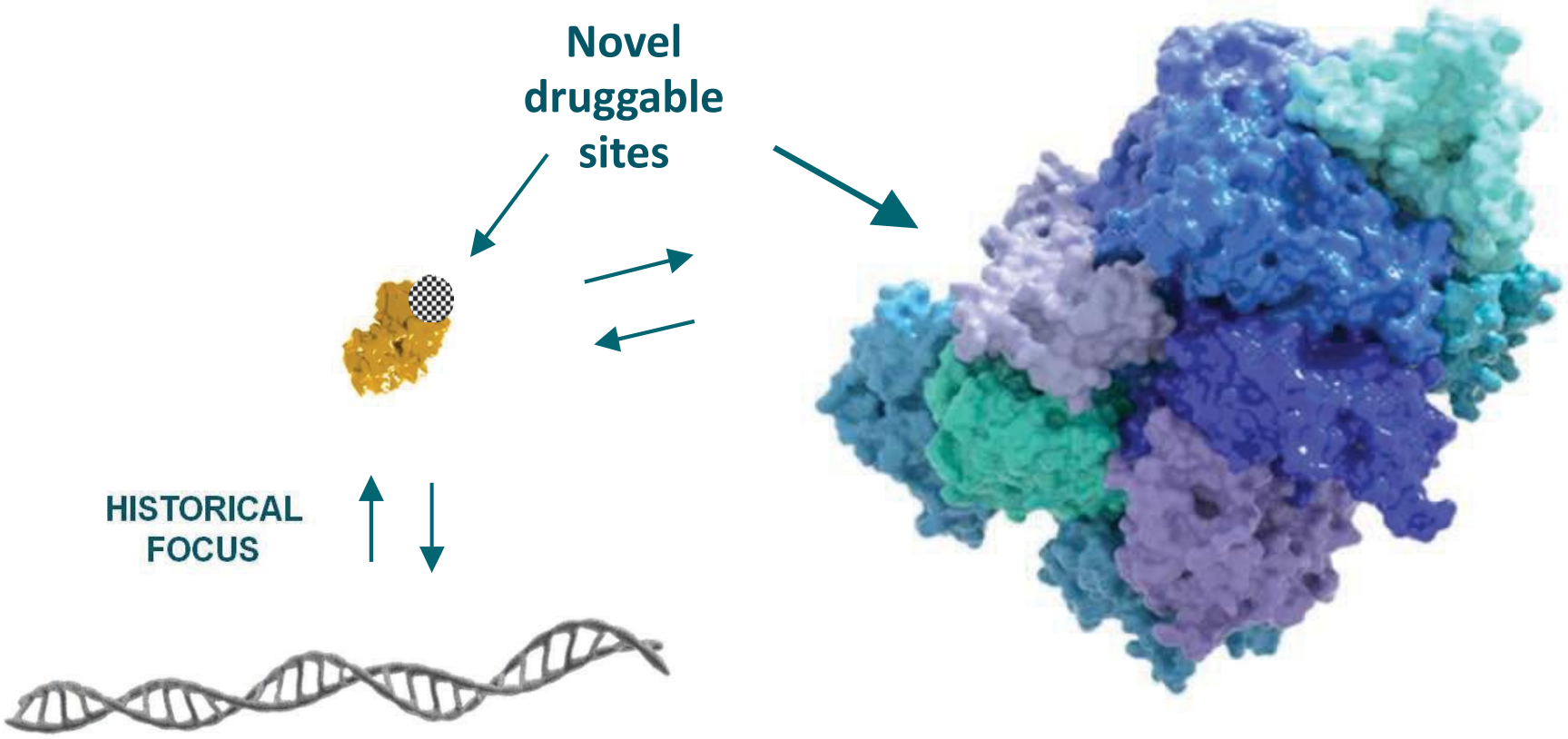
28 Chromatin Remodeling complexes and >1,000 TFs

BAF Complex Subunits Mutated and Dysregulated in Cancer

Estimate >100 Transcription Factors Associated with BAF Complex

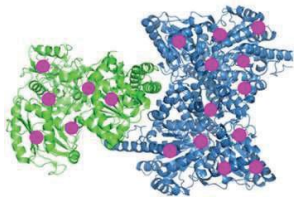
TF interactions with chromatin remodelers

TFs are implicated in a range of cancers and other diseases.
Historically difficult to target-featureless and tightly bound to DNA

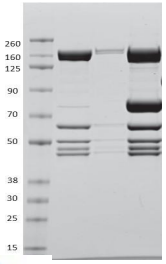


Novel approaches to drug transcription factors: including small molecules or degraders

Mass Spec Footprinting: Survey BAF and TF surfaces



+



Pull down with BAF-subcomplexes

NeoProteomics

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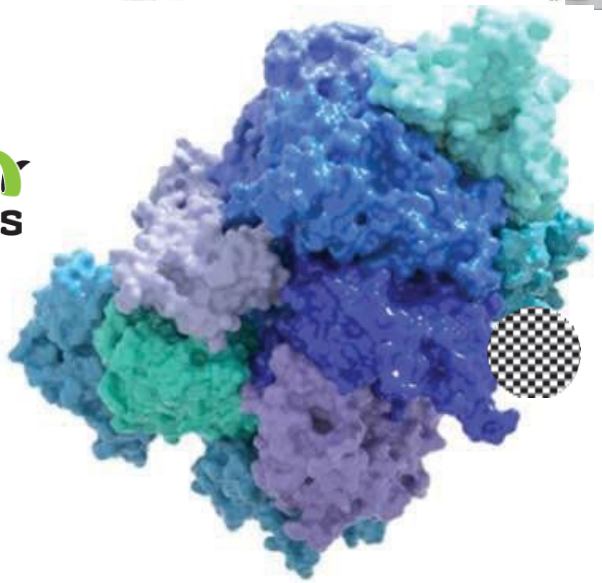
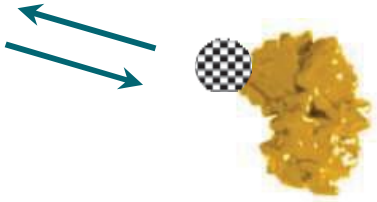
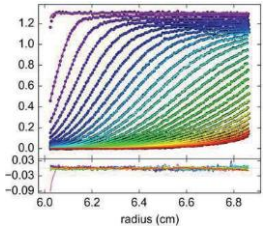


- >100 TFs linked to BAF
- Foghorn-Neo Proteomics pursuing multiple TFs

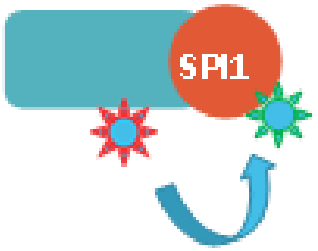


Biophysical, Structural and Biochemical confirmation of target interactions



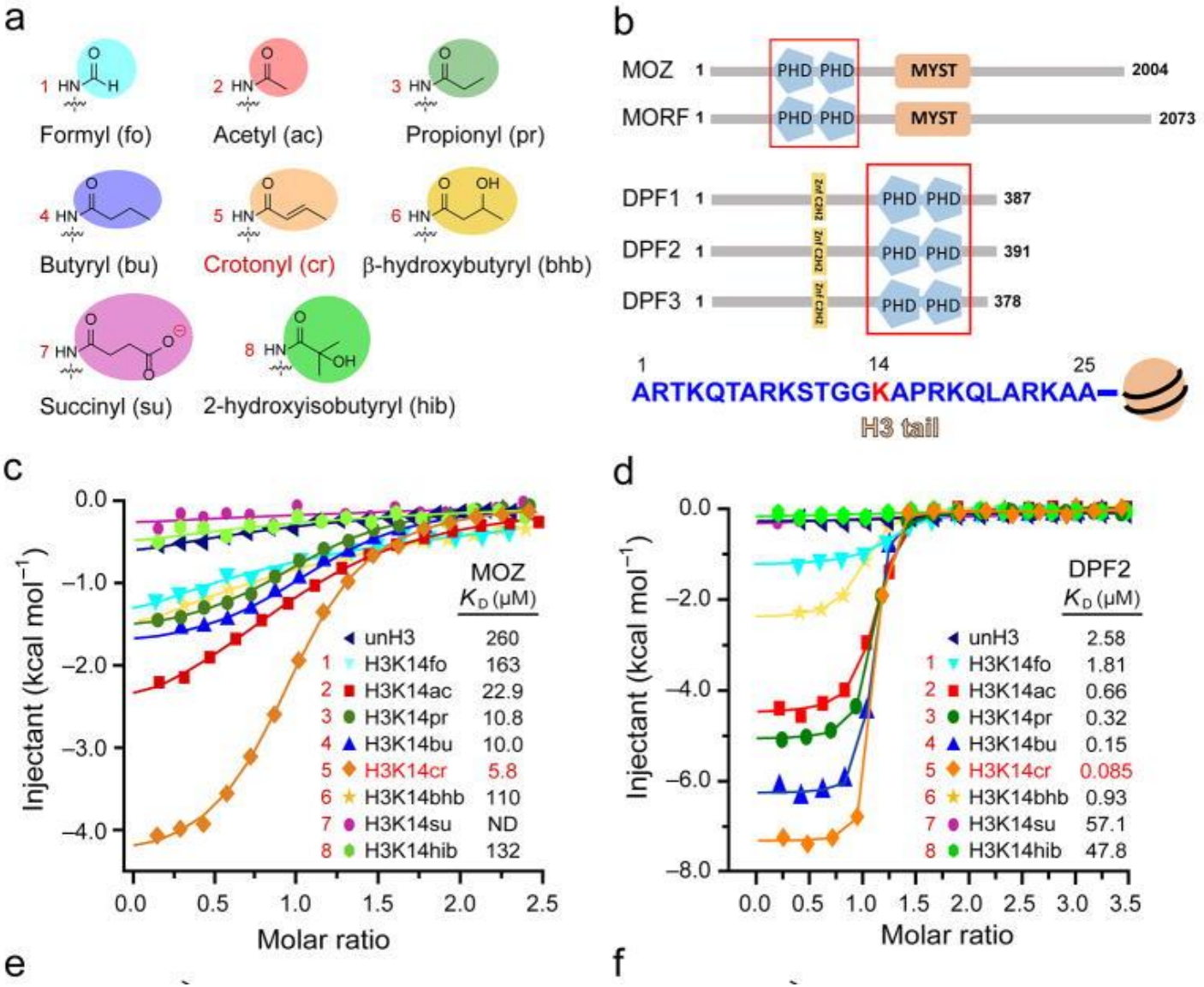
AUC/SPR/ITC

XTAL/NMR



TF-FRET

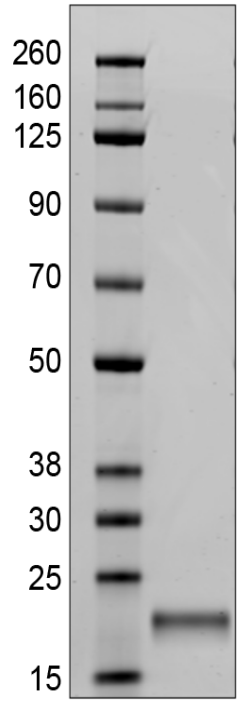
Crotonylation of histones is read specifically by Double PHD (DPF) fingers of MOZ and DPF2



Xiong et al., *Nat. Str. Biol.*, 2016)

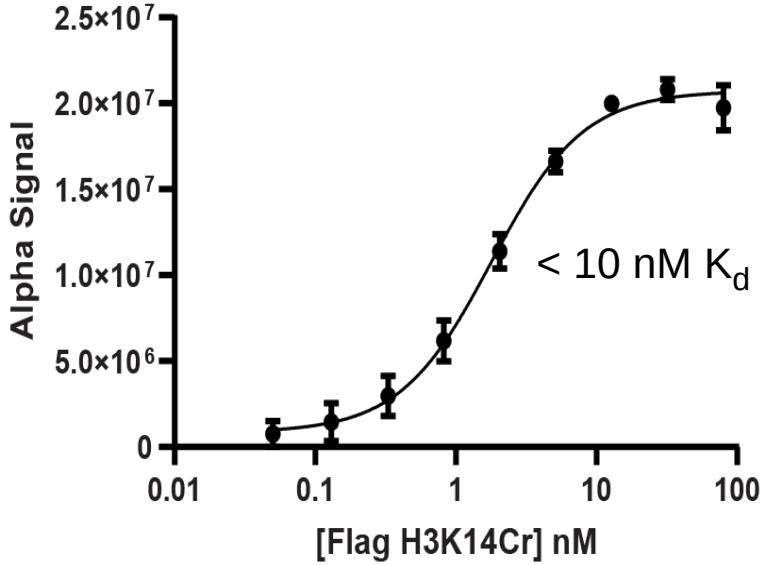
DPF domain of BAF45D binds to Histone H3(1-25) K14Cr

A.



Purified BAF45D vs MW markers

B.



Binding isotherm of peptide + BAF45D

C.

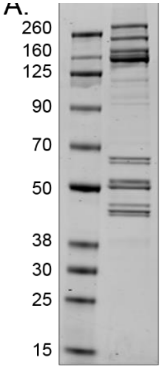
Location	Sequence BAF45D	Protection Ratio
242-253	EDSQPPTPVSQR	1.01
261-281	KGPDGLALPNNYCDFCLGDSK	1.19
286-300	**TGQPEELVSCSDCGR**	**1.40**
301-318	SGHPSCLQFTPVMMAAVK	NL
322-328	**WQCIECK**	**3.41**
329-350	**CCNICGTSENDDQLLFCDDCDR**	**4.34**
351-379	GYHMYCLTPSMSEPPEGSW SCHLCLDLLK	1.05

PHD1

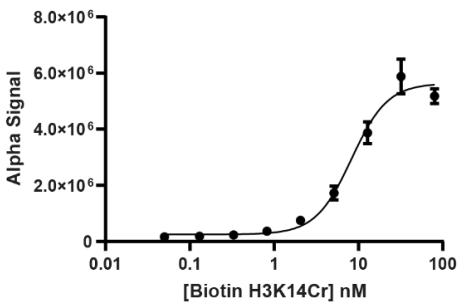
PHD2

Full BAF Binds H3K14Cr at BAF45D PHD 1 and PHD 2 sequences

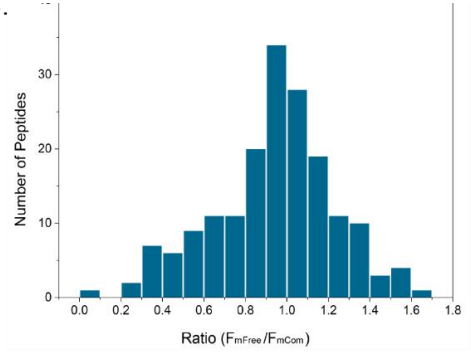
A.



B.



C.



D.

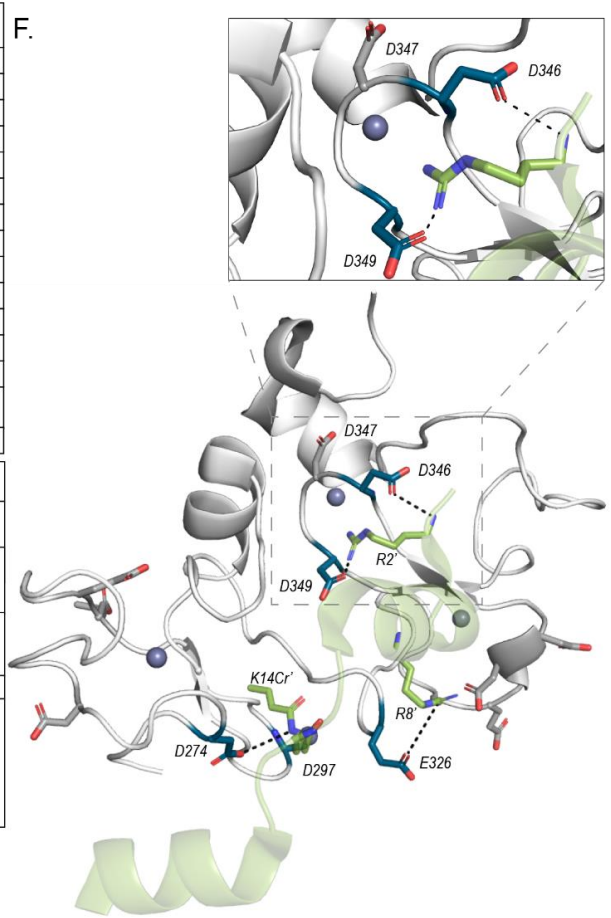
PHDs

Location	Sequence BAF45D within BAF	Protection Ratio
93-107	LSFPSIKPDTDQTLLK	0.44
109-124	EGLISQDGSSLEALLR	0.82
138-152	VDDDSLGEFPVTNSR	0.92
156-178	RILEPDDFLDDLDDEDYEEDTPK	0.98
197-205	LDASILEDR	1.13
206-216	DKPYACDICGK	1.05
220-241	NRPGLSYHYAHSHLAEEEGEDK	0.64
242-253	EDSQPPTPVSQR	0.99
261-281	KGPDGLALPNNYCDFCLGDSK	1.23
286-300	**TGQPEELVSCSDCGR**	**1.33**
301-318	SGHPSCLQFTPVMMAAVK	NL
322-328	**WQCIECK**	**1.24**
329-350	**CCNICGTSENDDQLLFCDDCDR**	**1.50**
351-379	GYHMYCLTPSMSEPPEGSWSCH LCLDLLK	0.89
410-423	DHDGDYKDHDIDYK	0.57

E.

Location	Residue level Modification	Protection Ratio	
		BAF45D within BAF	BAF45D
261-281	D264	1.02	0.86
	D274	ND	2.37
	D279	1.07	0.93
286-300	D290	0.87	ND
	D291	1.15	1.12
	D297	1.29	1.40
322-328	D326	1.24	3.41
329-350	D337	ND	1.28
	D339	ND	1.34
	D340	ND	1.28
	D346	1.76	5.22
	D347	0.90	1.19
	D349	1.41	6.51

F.



Histogram shows labeling of 177 peptides on BAF and complex out of 630 total detected (63% coverage)

Residues shown in contract with crotonly peptide modeled from MOZ domain homolog

Reveals Novel contacts with BRG/BRM

Martinez, AACR 2022

Conclusions: Hack Your Structural Biology Problems with SMS

Footprinting and Structural Mass Spectrometry Provides Actionable High-Resolution Information optimized for:

- Antibody-Antigen interactions
- Protein Degraders and Molecular Glues (modest binding affinity)
- Protein-small molecule interactions
- Mega Complexes in Gene Trafficking and Metabolism

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Thanks to all the team members!

casma therapeutics

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Janna Kiselar PhD., Associate Director

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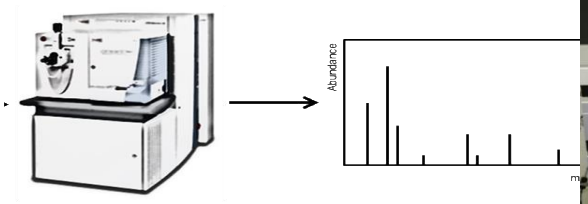

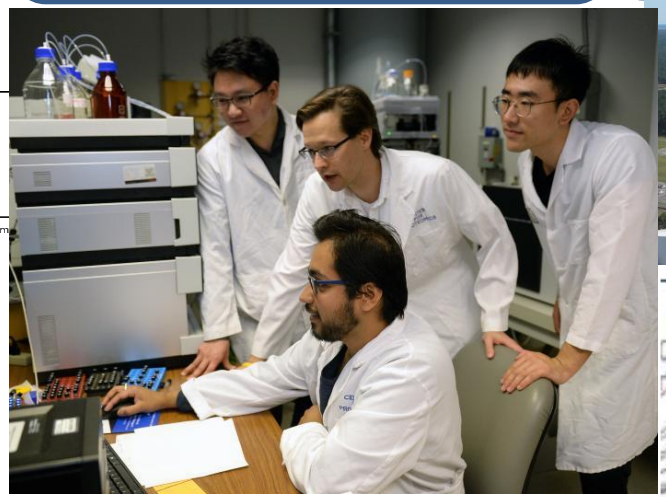

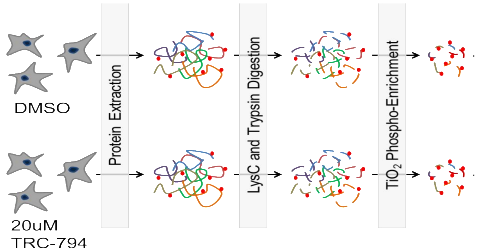

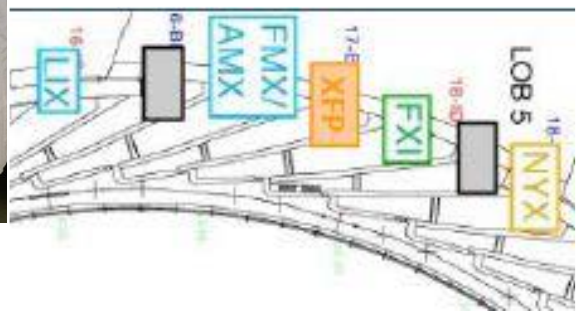

NeoProteomics

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