

When do you need a PRM assay? A case study in chromatin proteomics

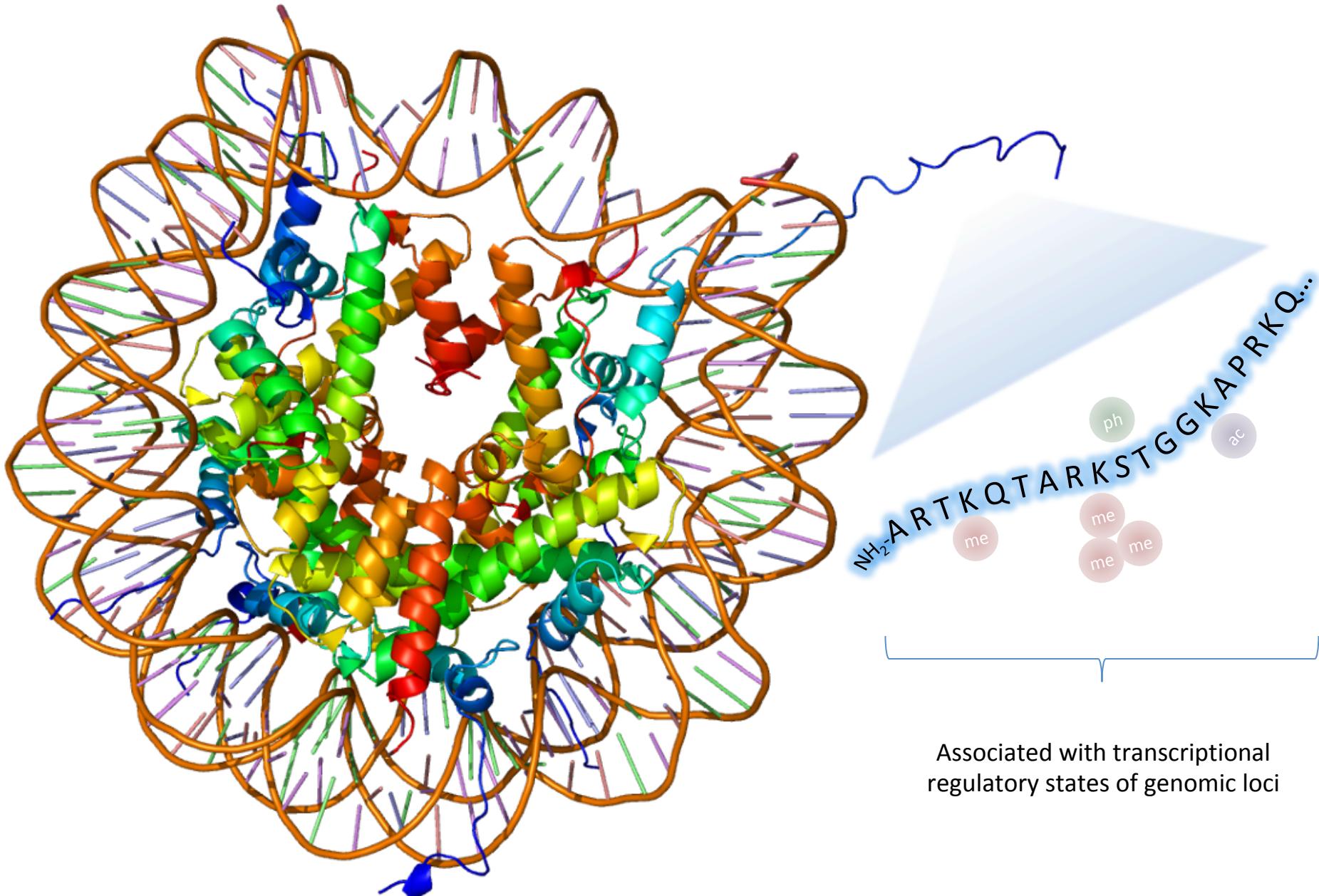
Jake Jaffe
ASMS Short Course 2018

Outline

- Chromatin and its “issues”
- Definitions
- When do PRM assays make sense?
- Considerations for PRM method development
- Examples of “Research Grade” PRM assays

PLEASE, PLEASE INTERRUPT AND ASK QUESTIONS!

Histones and their post-translational modifications



Histone Peptides: Isobars Galore!

Base peptide Res 27-40: KSAPATGGVKKPHR

A modified variant: K_{me3}SAPATGGVKKPHR (+42)

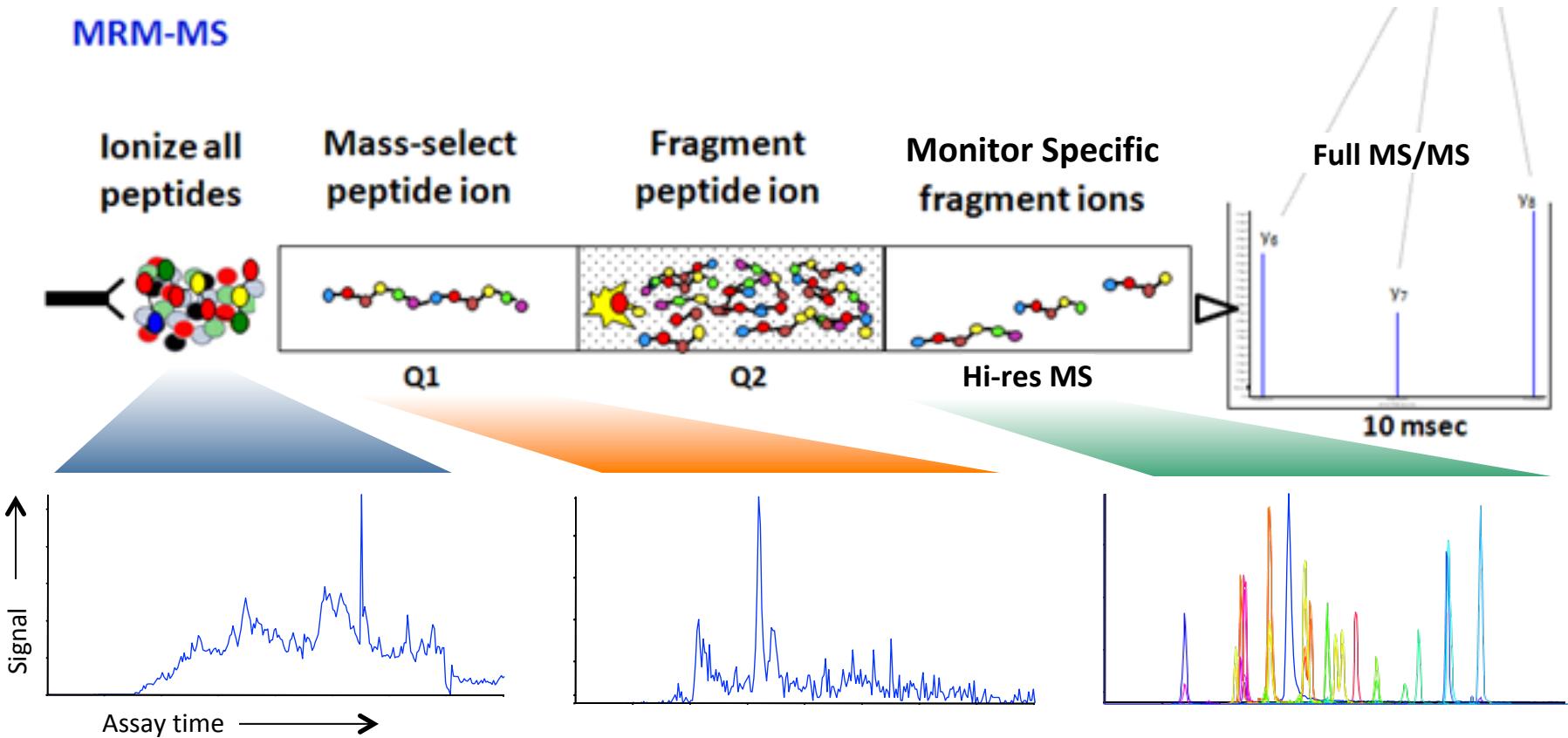
A modified variant: KSAPATGGVK_{me3}KPHR (+42)

A modified variant: K_{ac}SAPATGGVKKPHR (+42)

~50 variants of this peptide alone
Many isobars and near isobars

Hi-Res Targeted MS Analysis for Histone Modifications

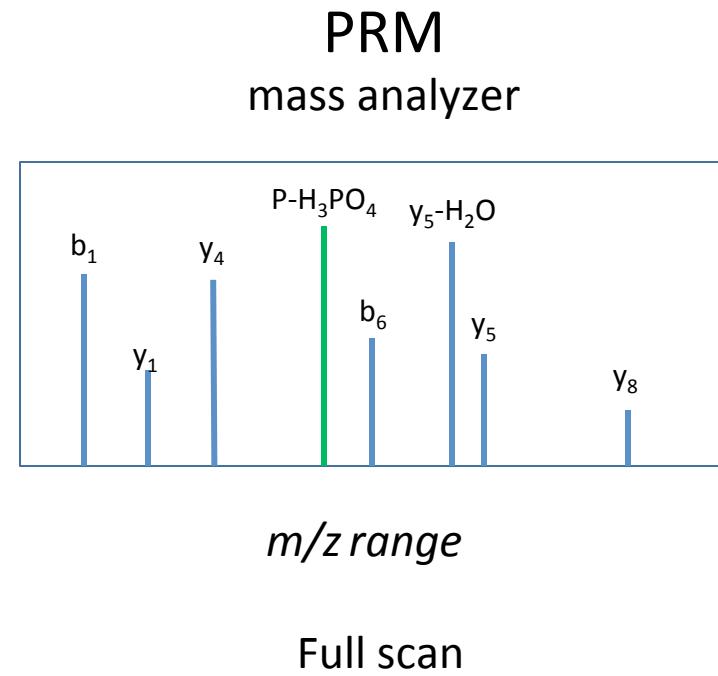
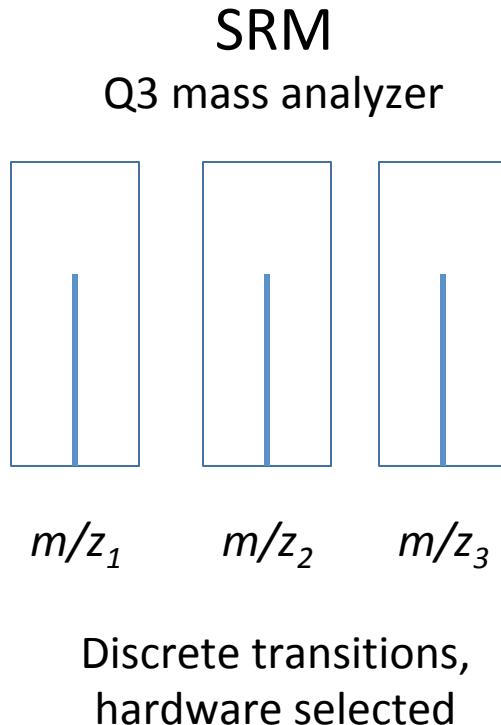
MRM-MS



- High resolution required for tricky histone analytes!
- Can use synthetic peptide internal standards for better quantification and proof of ID
- Can determine relative changes (cell labeling) or absolute amounts (synthetics)
- ***When you want to guarantee you measure it each and every time!***

Definition of PRM

- PRM = MRM-HR = HR-MRM = Targeted Full Scan MS/MS
- Closest spiritual cousin is triple-quad based MRM/SRM, but:

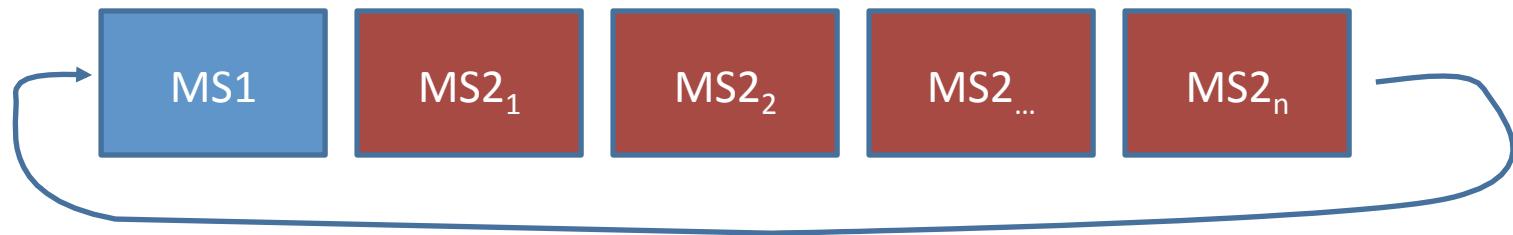
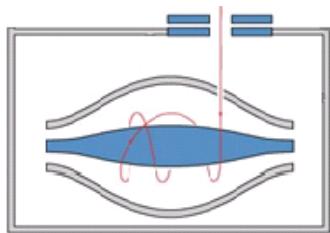


Definition, continued

- Assay is completely deterministic
- Precursor m/z (list) is specified
 - Possibly scheduled
 - Quadrupole or ion trap selection/isolation
- Fragmentation is performed
 - Any kind is OK
- Full MS/MS spectrum is recorded
 - Any analyzer: Orbitrap, TOF, scanning quad, ion trap, etc.
- Usually a full scan MS spectrum is also periodically recorded
 - Two chances to verify and quantify!

Common configuration: high resolution mass analyzer

- Orbitrap or TOF



- Precursor cycle vs. Acquisition loop cycle

- Precursor cycle: time it takes to loop through precursor list
 - May vary during method
 - Governs points across peak
- Acquisition loop cycle: Time from full scan to full scan with intervening # of MS/MS
 - May affect instrument performance, full scan points across peak

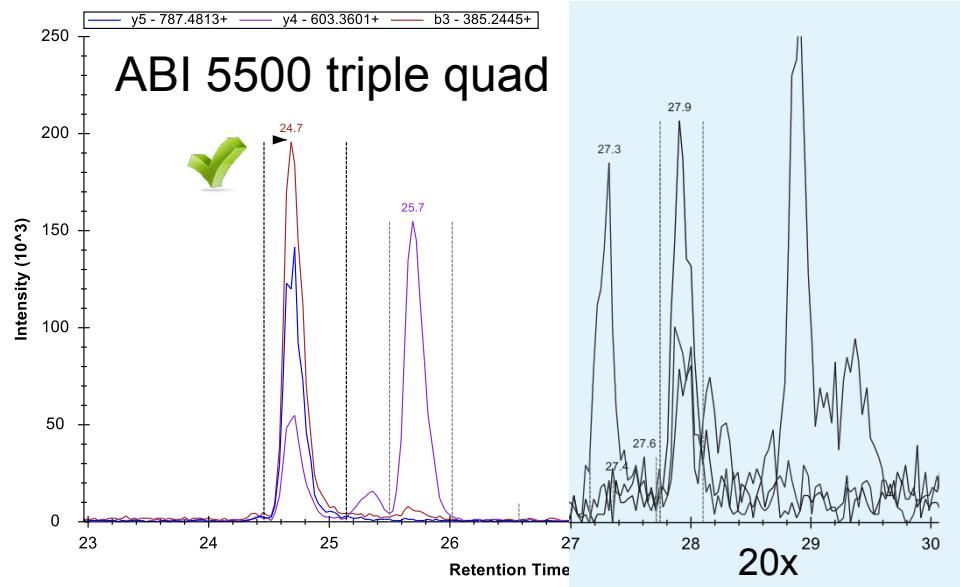
When do PRMs make sense?

- Exquisite selectivity required
 - Unit (quadrupole) vs. ppm (hi-res)
- Post-translational modification localization is required
 - GVDQ(pS)PLTPAGGK vs. GVDQSPL(pT)PAGGK
- Rapidly convert discovery data to targeted assay
 - Stay within platform
- You don't have a triple quad!
 - But still want the benefits of targeted proteomics

High resolution adds value to selectivity

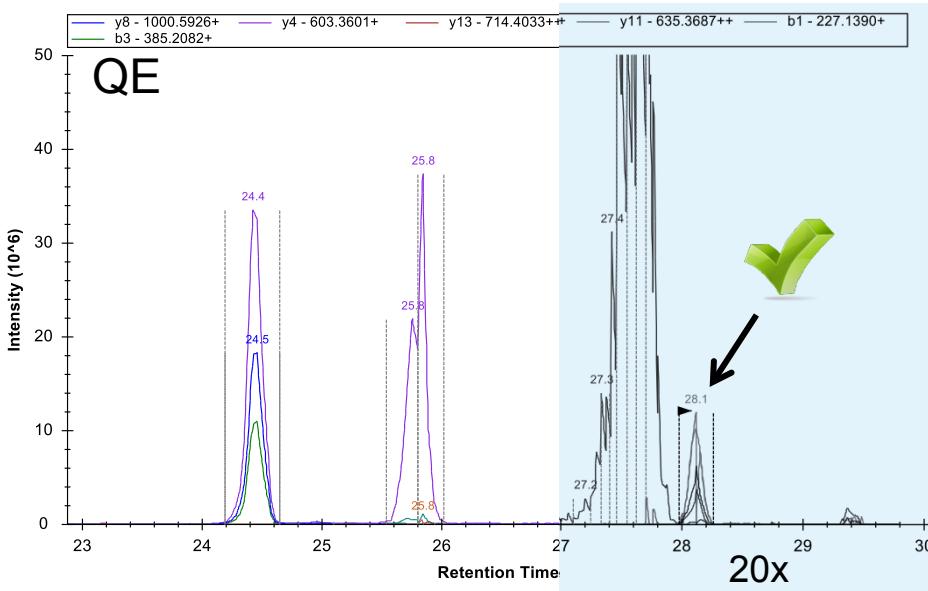
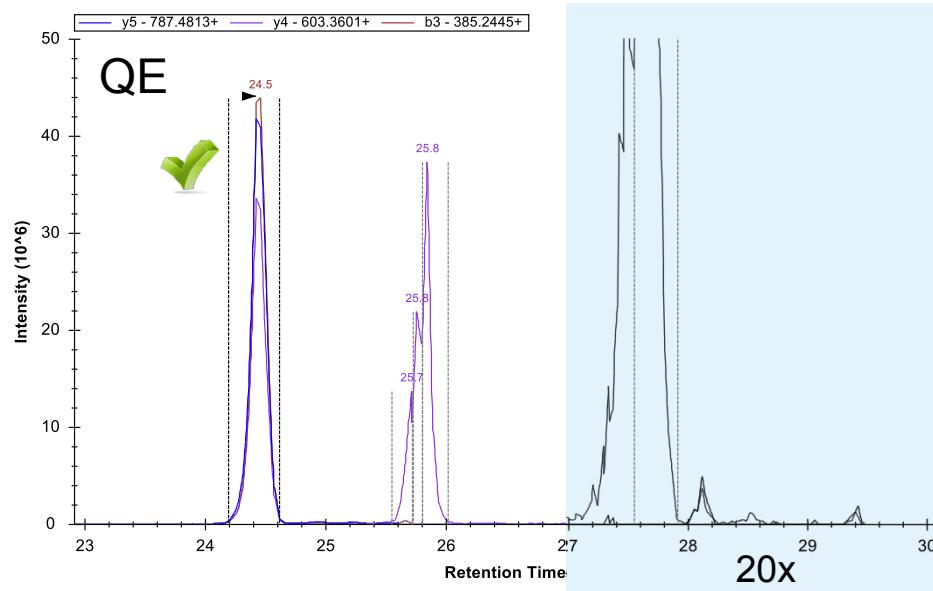
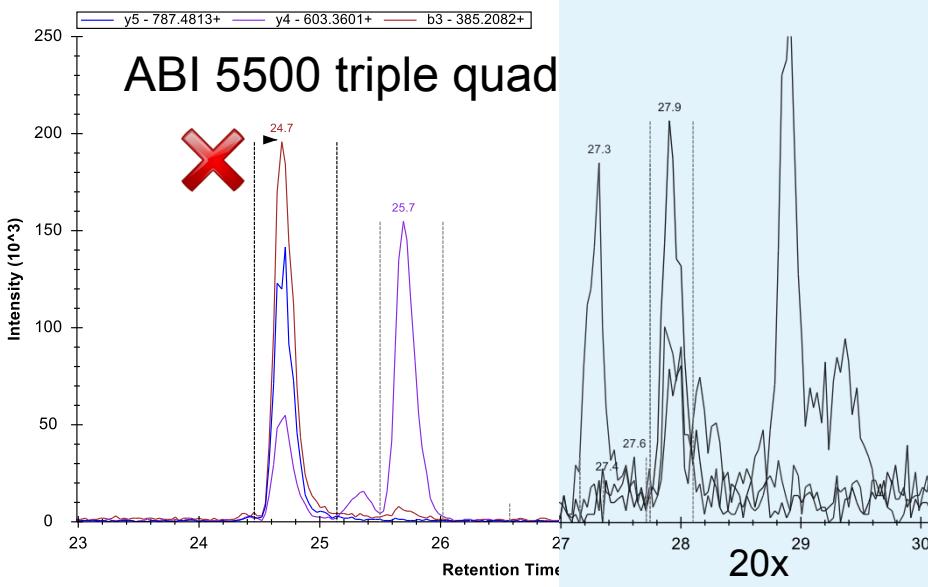
K_{me3}SAPATGGVK_{pr}K_{pr}PHR₁₀

m/z 551.9940 z=3



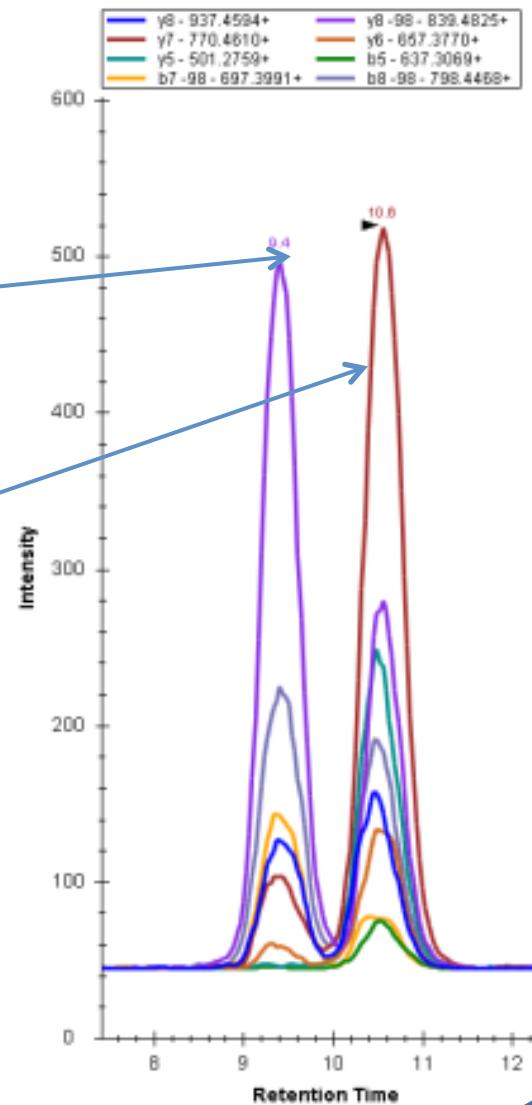
K_{ac}SAPATGGVK_{pr}K_{pr}PHR₁₀

m/z 551.9819 z=3



PTM Localization – shared ions, differential ions

Differentially Phosphorylated Peptides
With Same Base Sequence

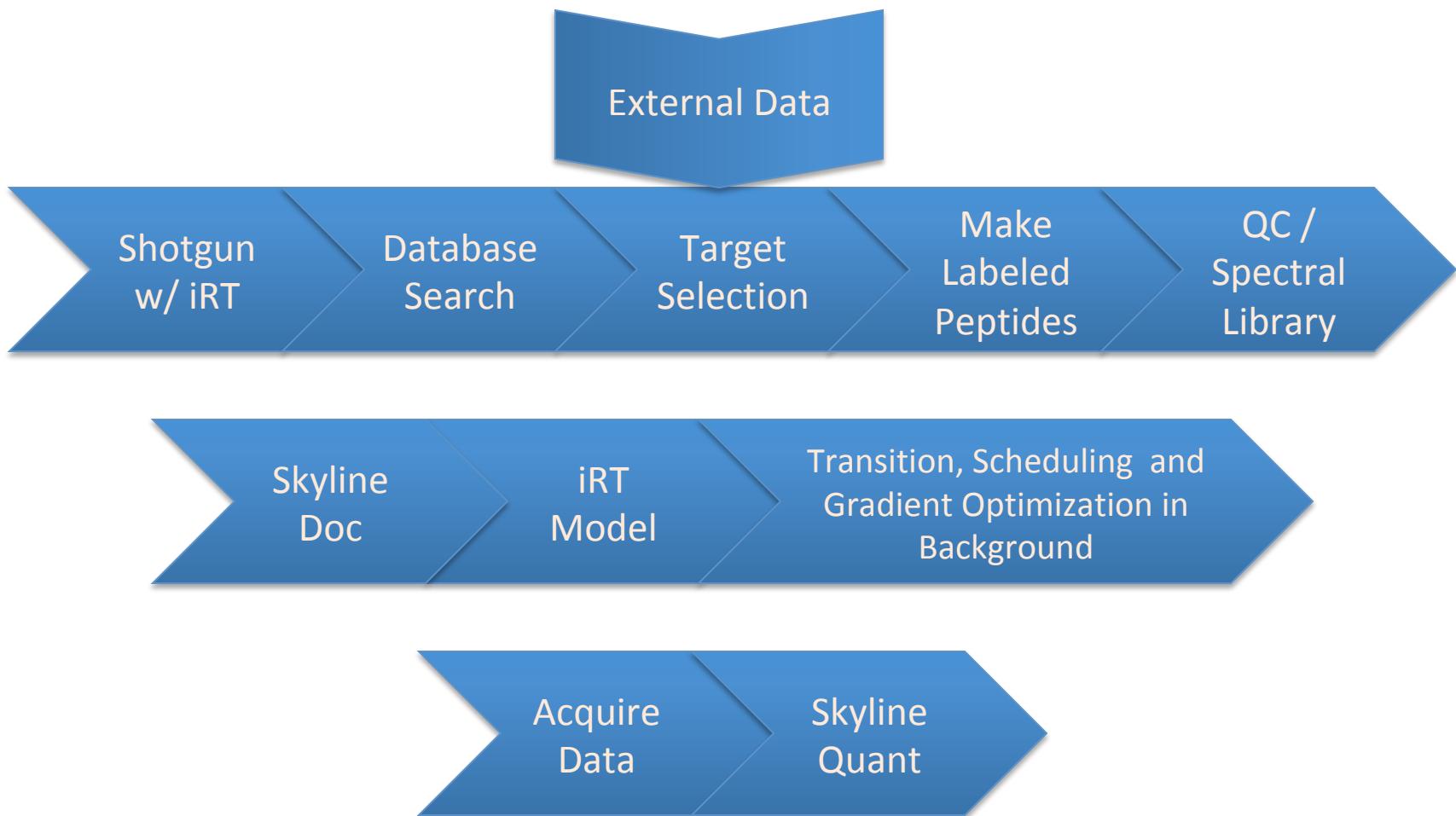


Discovery Proteomics to PRM – Short version / Label Free



* iRT peptides recommended

Discovery Proteomics to PRM – Long version



Planning ahead for success

- Strongly consider including iRT peptides in every single sample you run in your lab
 - Diverse retention times, well spaced
 - High enough levels to trigger MS/MS
 - Or, include targeted scans
 - Or, determine RTs with precursor quant in Skyline
 - This can also be very beneficial for scheduling tight windows
- Use a search engine supported by Skyline spectral library import
- Set up your funky PTMs in advance in your document
- Learn about Skyline's PTM notation for import

Skyline notation shorthand example: phosphorylation

- Enable the phosphorylation mod in your document
 - Make sure it's "variable"
- Now import a peptide through the dialog box

APEPT[+80]IDEK
APEPT[ph]IDEK

- Voila!
- There is also a way to make spectral libraries by hand using **BiblioSpec / Skyline**:

https://skyline.gs.washington.edu/labkey/announcements/home/support/thread.view?entityId=86be1b94-d328-102e-a8bb-da20258202b3&_anchor=716#row:716

Document refinement

- Keep a lot of transitions around initially
 - You can always get rid of them later
 - You can take them from the spectral libraries
 - In theory: the more transitions, the more signal-to-noise
 - Also in theory more sensitive than MRM, but generally not in practice
- Take advantage of the raw data spectrum viewer functionalities
 - Helpful for both MS and MS/MS inspection
- Use that high res!
 - Narrow your import m/z tolerances
 - Inspect the ppm errors

The all important dotp

- dotp = dot product
 - Metric observed transition relative intensities in comparison with spectral library example
- Better than a search engine score!
 - Expect > 0.9 under most circumstances
- Extremely useful in differentiating among similar analytes
- Spectral library quality important
 - Garbage in, garbage out

Standardization Considerations

- Label free
 - Requires high degree of system reproducibility
 - Hard to compare samples longitudinally
- Synthetic peptides
 - Highest degree of rigor
 - Highest cost in time, \$
 - More optimization required
- SILAC
 - Increases complexity, chance for interference
 - Standard is “prenormalized”
 - Consider growing up a vat of standard for longitudinal performance
- Chemical labels? (+ standards?)

Data analysis considerations

- Be patient, use all metrics at your disposal
- Consider time window import limits
 - But relies on RT or other indicators in spectral library / RT models
- Consider further minimizing your document when happy with data
 - Hi-res data, skyd files get big

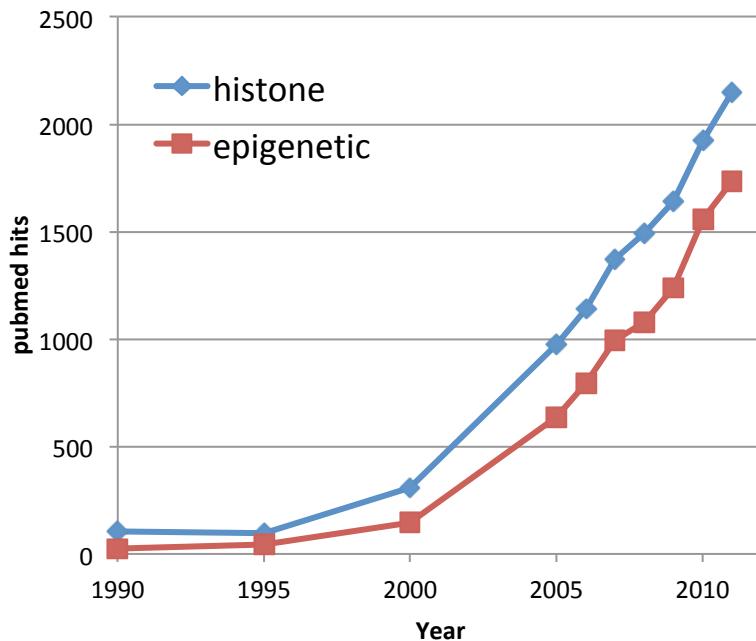
“Research Grade” PRM Concept

- A quantitative, targeted proteomics assay suitable for “everyday” use
- Ideally standardized with synthetic peptides (or SILAC)
- Rapid design cycle using discovery data/platform
- Enables longitudinal comparisons across days, months, years
- Output useful for rapidly guiding biology
- NOT:
 - Obsessed with LOD/LOQ
 - Suitable for clinical deployment

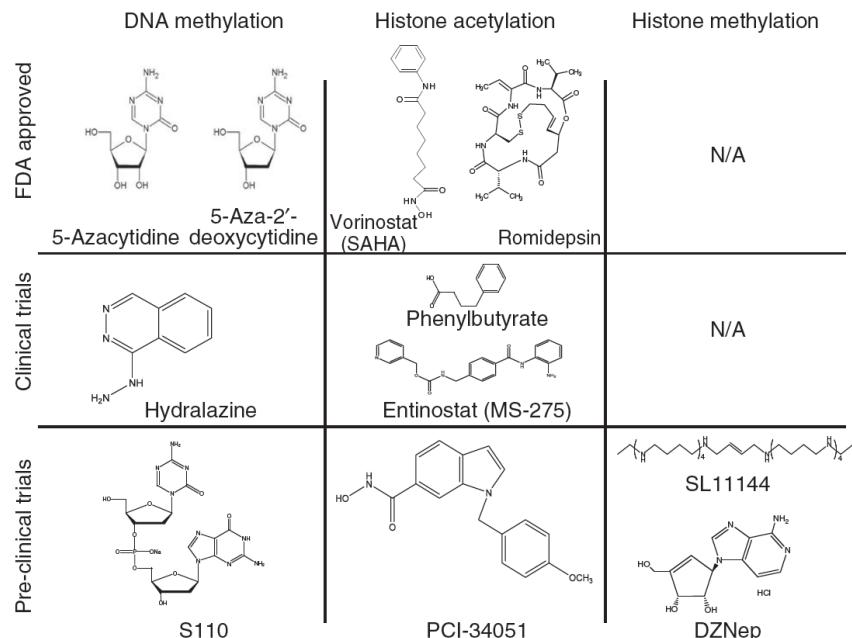
EXAMPLE OF A RESEARCH GRADE CHROMATIN PRM ASSAY

Growing interest in histone marks

Pubmed search:
cancer AND...

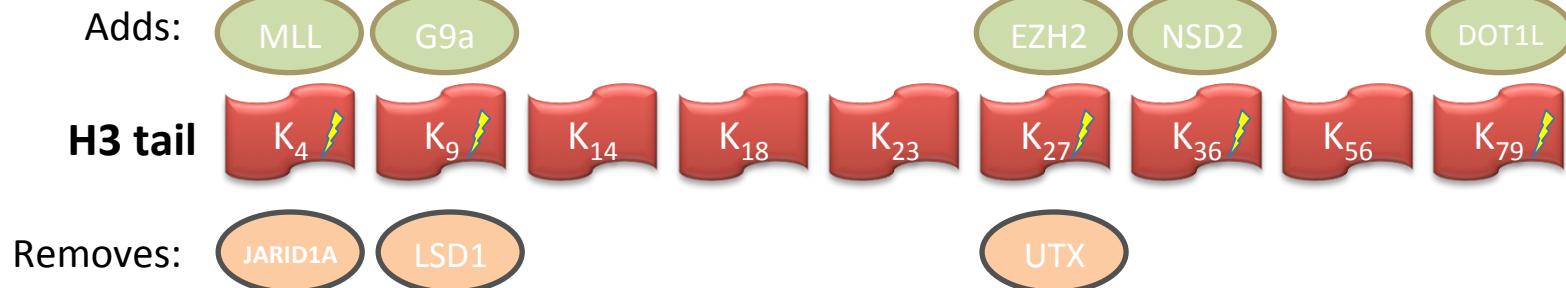


Epigenetic-directed therapeutics

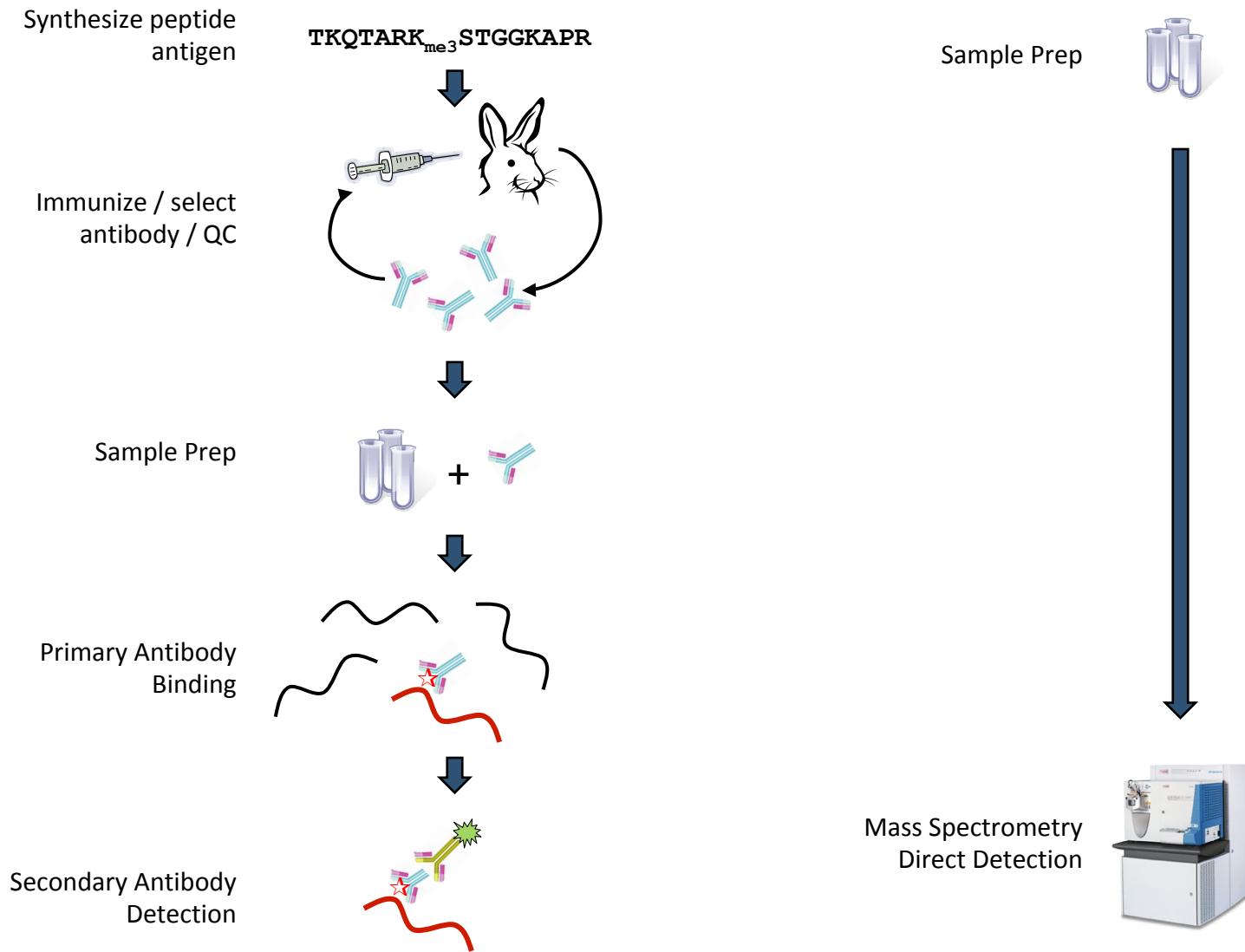


from Kelly et al., *Nat. Biotech.* 2010 28:1069

Histone methylation dysregulation in cancer:



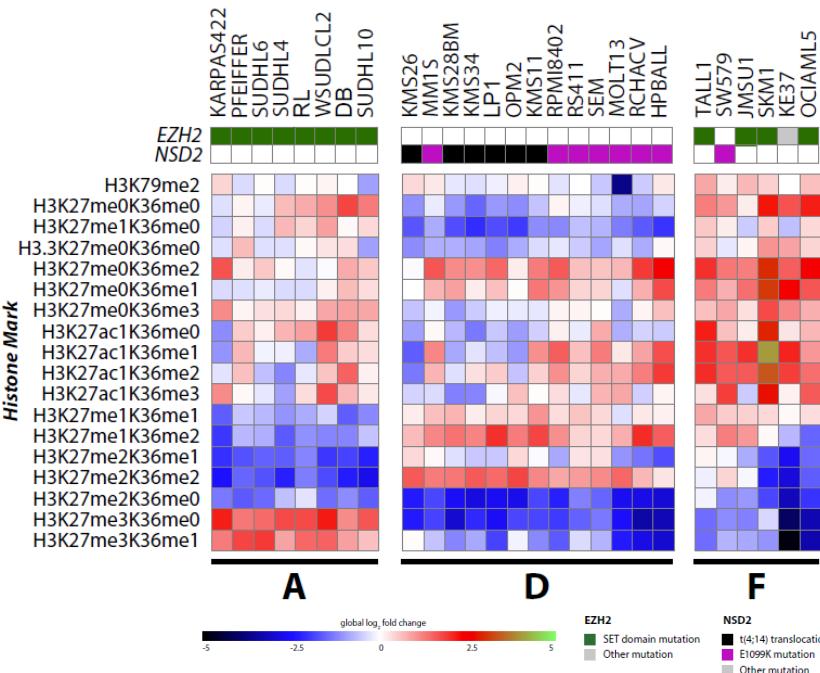
Indirect vs. Direct Observation of Histone Modifications



GCP – Research tool for epigenetics

Global chromatin profiling reveals NSD2 mutations in pediatric acute lymphoblastic leukemia

Jacob D Jaffe^{1,11}, Yan Wang^{2,11}, Ho Man Chan^{3,11}, Jinghui Zhang^{4,5}, Robert Huether^{4,5}, Gregory V Kryukov¹, Hyo-eun C Bhang³, Jordan E Taylor¹, Min Hu⁷, Nathan P Englund², Feng Yan², Zhaofu Wang⁷, E Robert McDonald III³, Lei Wei^{4,5}, Jing Ma^{5,8}, John Easton^{5,8}, Zhengtian Yu⁷, Rosalie deBeaumont³, Veronica Gibaja³, Kavitha Venkatesan³, Robert Schlegel³, William R Sellers³, Nicholas Keen³, Jun Liu², Giordano Caponigro³, Jordi Barretina³, Vesselina G Cooke³, Charles Mullighan^{5,8}, Steven A Carr¹, James R Downing^{5,8,11}, Levi A Garraway^{1,9–11} & Frank Stegmeier^{3,11}

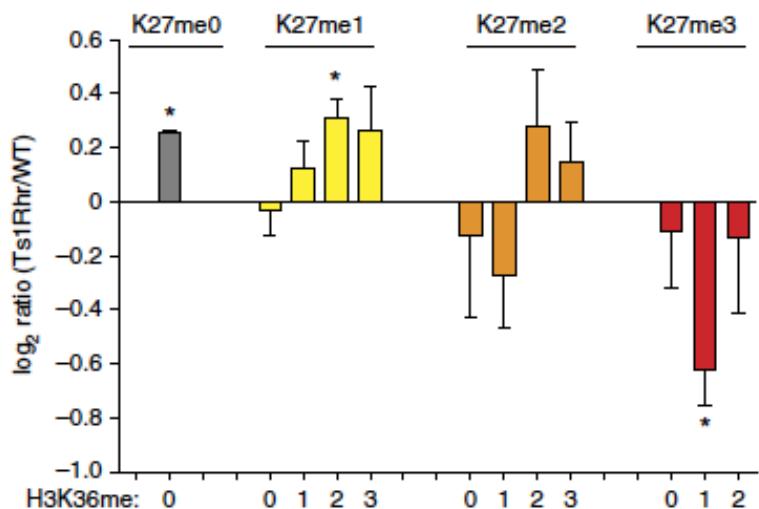


ASXL1 Mutations Promote Myeloid Transformation through Loss of PRC2-Mediated Gene Repression

Omar Abdel-Wahab,^{1,12} Mazhar Adli,^{2,12} Lindsay M. LaFave,^{1,3,12} Jie Gao,⁵ Todd Hricik,¹ Alan H. Shih,¹ Suveg Pandey,¹ Jay P. Patel,¹ Young Rock Chung,¹ Richard Koche,² Fabiana Perna,⁴ Xinyang Zhao,⁶ Jordan E. Taylor,⁷ Christopher Y. Park,¹ Martin Carroll,⁸ Ari Melnick,⁹ Stephen D. Nimer,¹¹ Jacob D. Jaffe,⁷ Iannis Aifantis,⁴ Bradley E. Bernstein,^{2,*} and Ross L. Levine^{1,10,*}

Triplification of a 21q22 region contributes to B cell transformation through HMGN1 overexpression and loss of histone H3 Lys27 trimethylation

Andrew A Lane¹, Bjoern Chapuy¹, Charles Y Lin¹, Trevor Tivey¹, Hubo Li², Elizabeth C Townsend¹, Diederik van Bodegom¹, Tovah A Day¹, Shuo-Chieh Wu¹, Huiyun Liu¹, Akinori Yoda¹, Gabriela Alexe², Anna C Schinzel^{1,3}, Timothy J Sullivan⁴, Sébastien Malange⁵, Jordan E Taylor³, Kimberly Stegmaier^{2,3}, Jacob D Jaffe³, Michael Bustin⁶, Geertruy te Kronnie⁷, Shai Izraeli^{8,9}, Marian H Harris¹⁰, Kristen E Stevenson¹¹, Donna Neuberg¹¹, Lewis B Silverman², Stephen E Sallan², James E Bradner¹, William C Hahn^{1,3}, John D Crispino¹², David Pellman^{2,13} & David M Weinstock^{1,3}



Production Pipeline Supports Chromatin Profiling

Day 1

Cell Lysis
 2×10^6 cells
30 min

Histone Extraction

Histone Crash

Gel

Protein Assay

QC

SILAC mix

Day 2

Protein Propionylation
25-50 ug input

Protein Cleanup

Protein Digestion
1 ug trypsin/well
Over night

Day 3

Peptide Propionylation

Peptide Cleanup

Peptide Resus/
Dilution

Targeted MS

● Agilent Bravo LH – fully automated

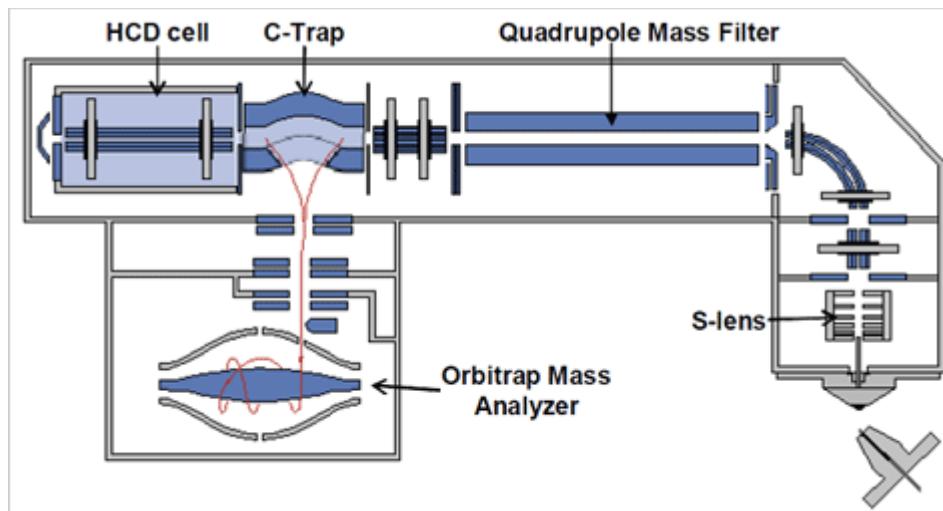
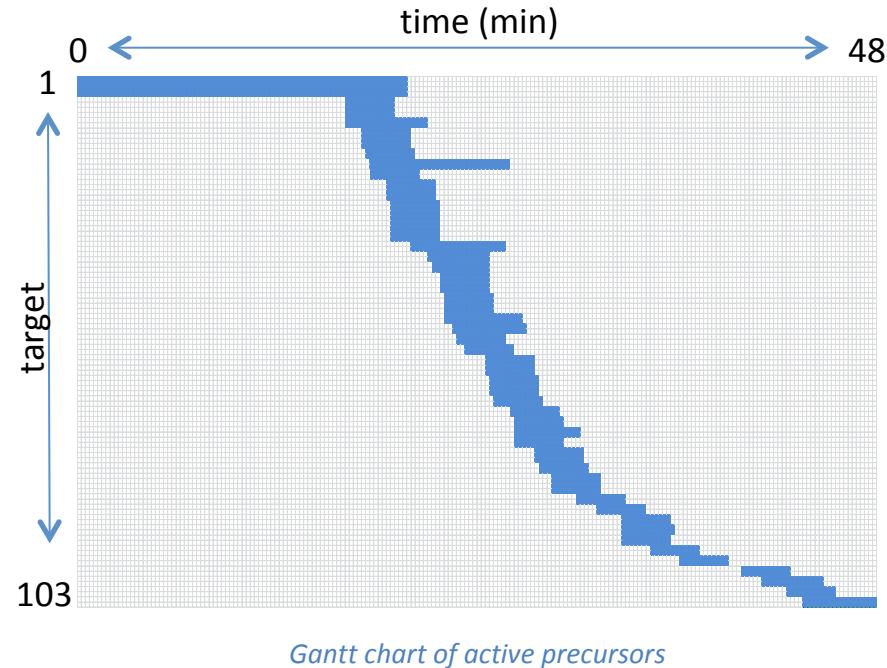
● 96-well SPE – semi-automated

Std spike

Targeted HiRes LC-MS assay configuration

■ Q-Exactive MS

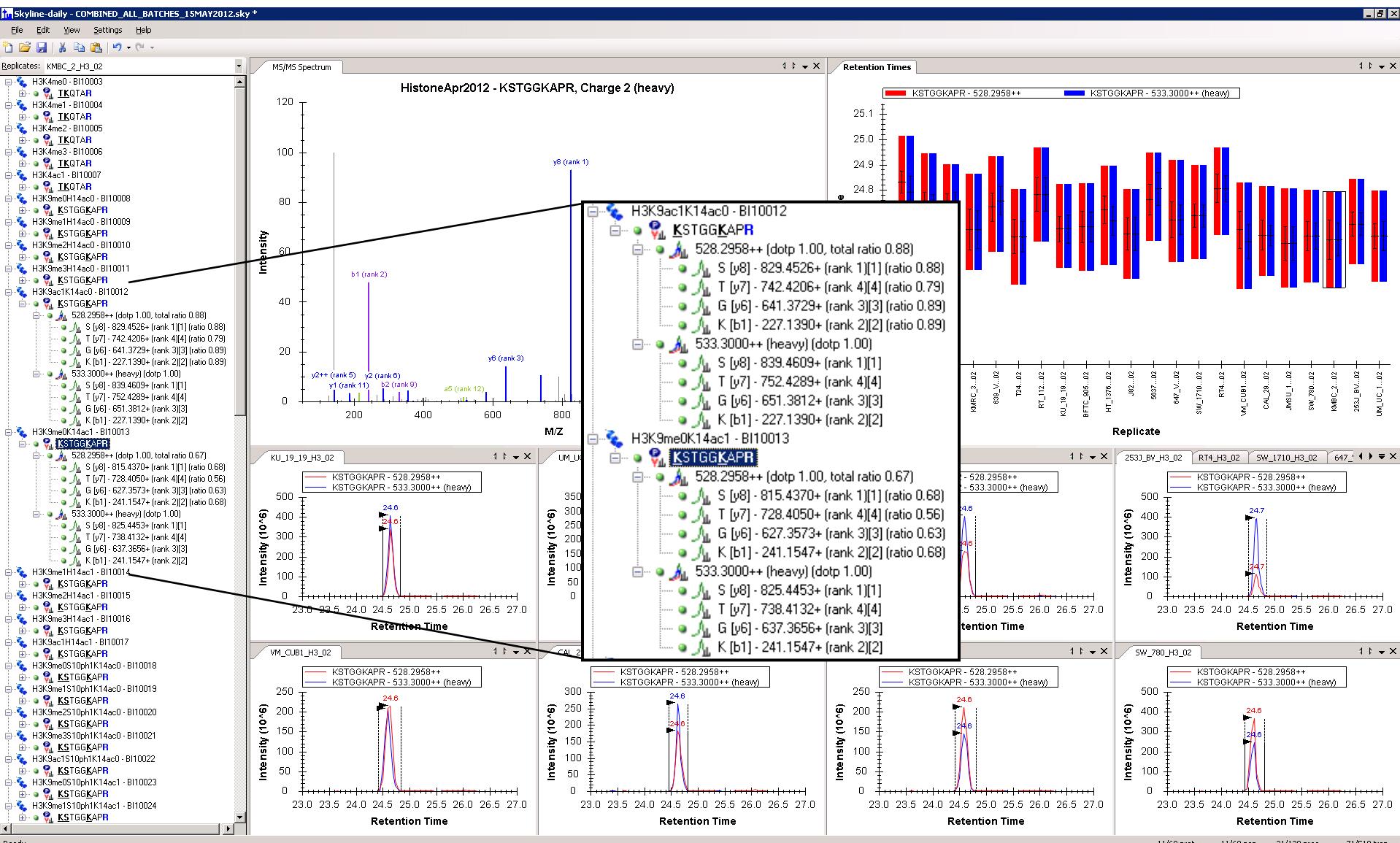
- MS1 @ res 35,000
- Targeted MS2 @ res 17,500
- Fully scheduled
- (1) 103 H3, (2) 73 non-H3 targets
 - Some cover multiple
- Proxeon NanoLC
- Homemade C18 nanospray columns



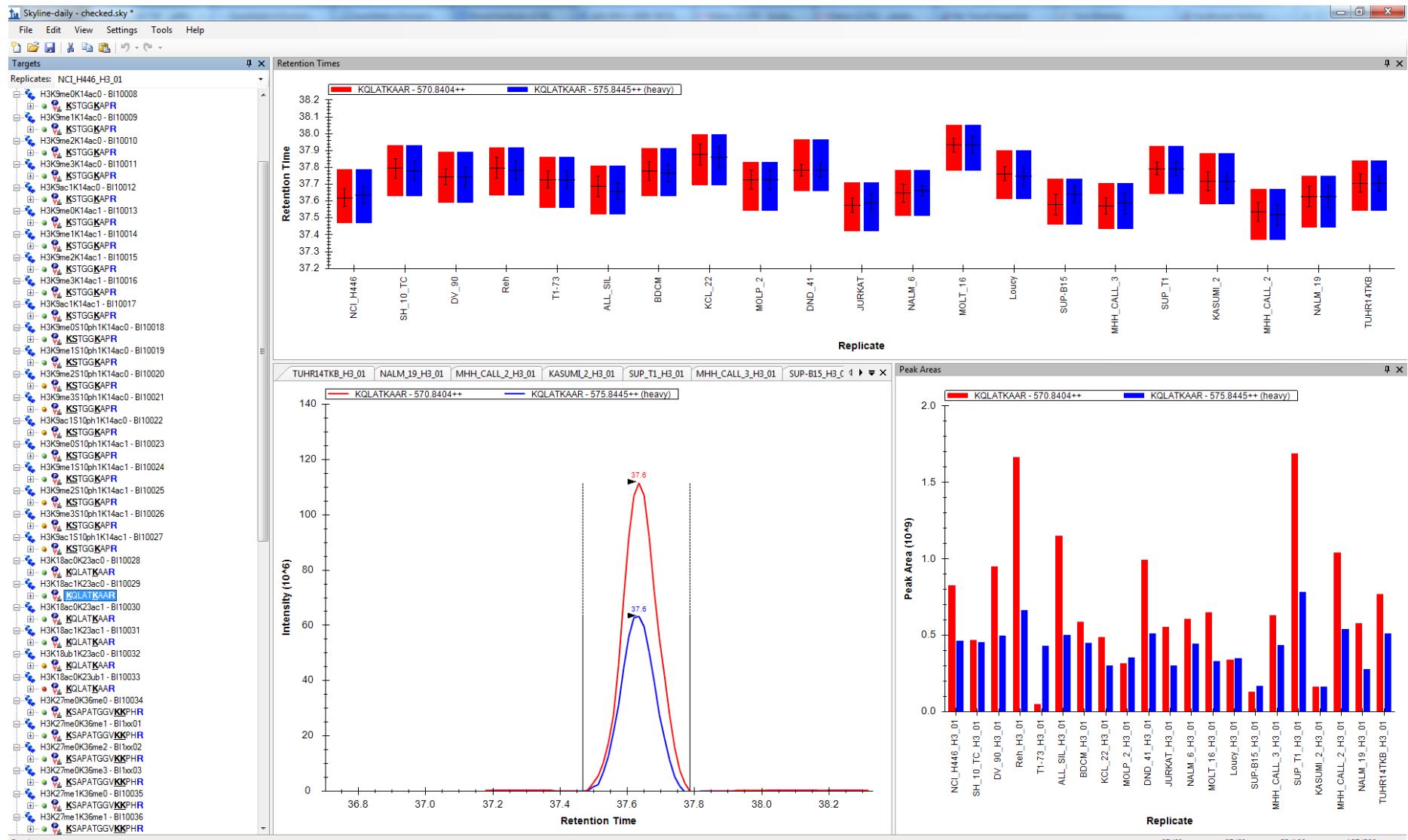
“Research Grade”

Targeted MS Assays

Skyline eases targeted quantification workflow



Robust Quantitative Platforms



Ready

27/60 prot 27/60 pep 53/120 prec 197/522 tran

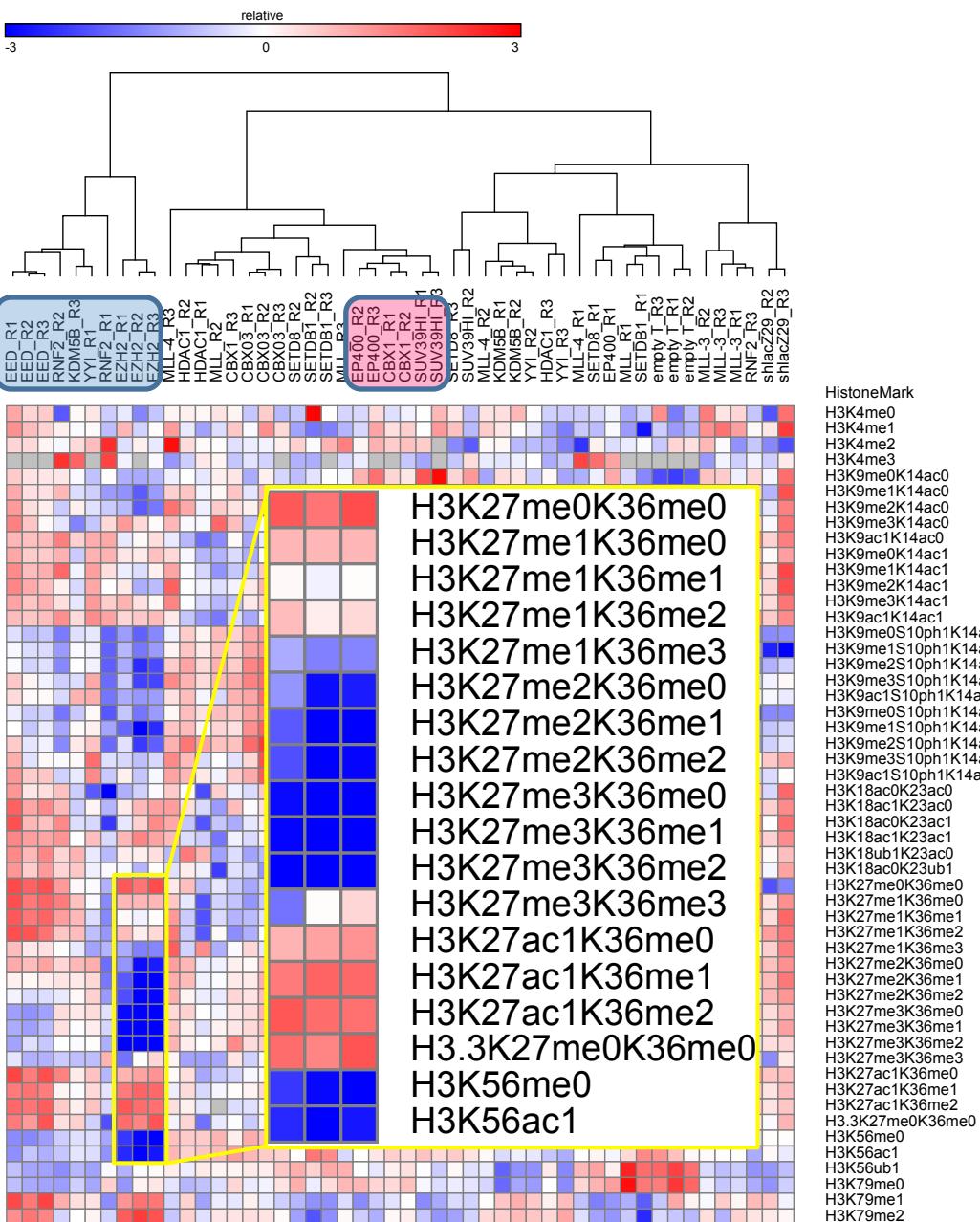
Experimental perturbation of chromatin proteins

- Measure effect of knock-downs of chromatin modifiers and other associated proteins

HDAC1	Histone Deacetylase	SETD8	H4K20 methyltransferase
MLL	H3K4 methyltransferase	KDM5B	H3K4 demethylase; JARID1B
CBX03	H3K9me binder; repression	EP400	Part of NuA4 acetyltransferase complex; targets H2A and H4
EZH2	H3K27 methyltransferase	MLL-4	H3K4 methyltransferase
CBX1	H3K9me binder; repression	MLL-3	H3K4 methyltransferase
SETDB1	H3K9 methyltransferase	RNF2	Histone Ubiquityltransferase
SUV39H1	H3K9 methyltransferase (K9me1->K9me3)	empty T	control
YY1	DNA binding, recruits PRC2; repression	shlacZ29	control
EED	PRC2 complex member; no catalytic activity		

- shRNA knockdown in biological triplicate
- 293T cells
- Use synthetic peptides to standardize this assay

Molecular Chromatin Signatures of knockdowns in 293T



EHZ2 knockdown signature
K27me3 down
K27ac1 up
K79me1,me2 up

Groups:

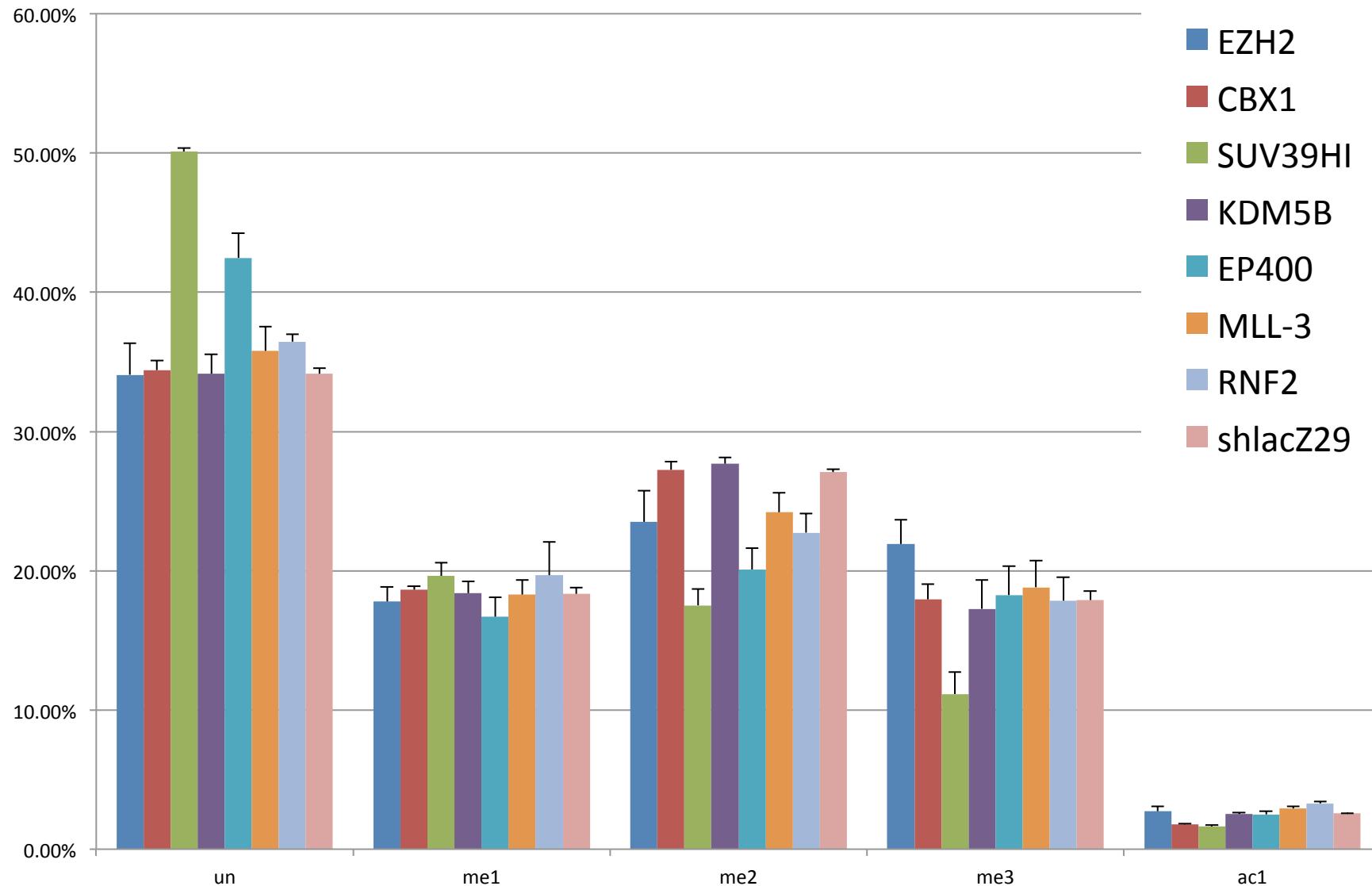
- EZH2, EED, RNF2, YY1

SUV39H1 knockdown
signature
K9me2,me3 down
S10ph up

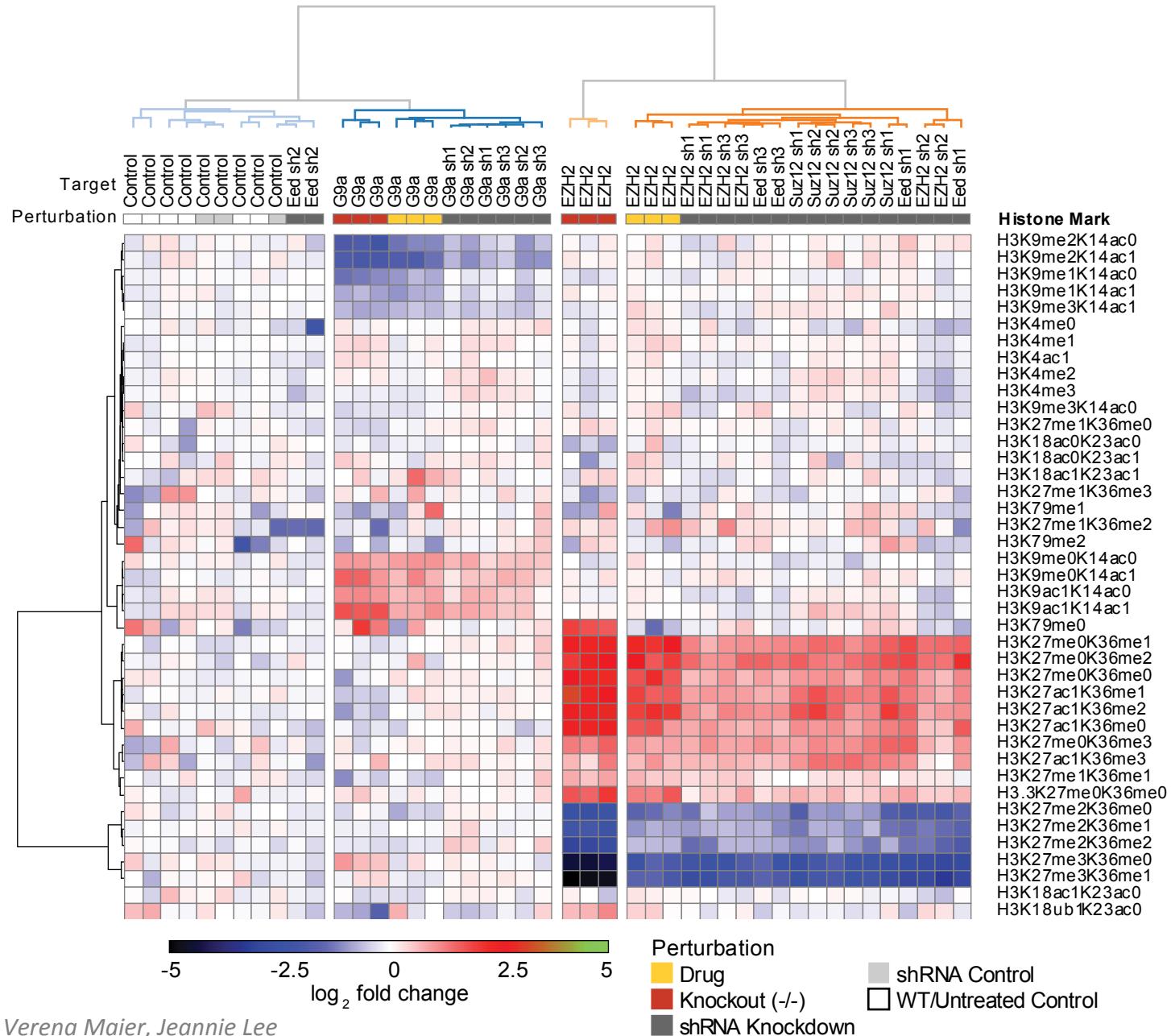
Groups:

Column cluster complete linkage by Pearson correlation

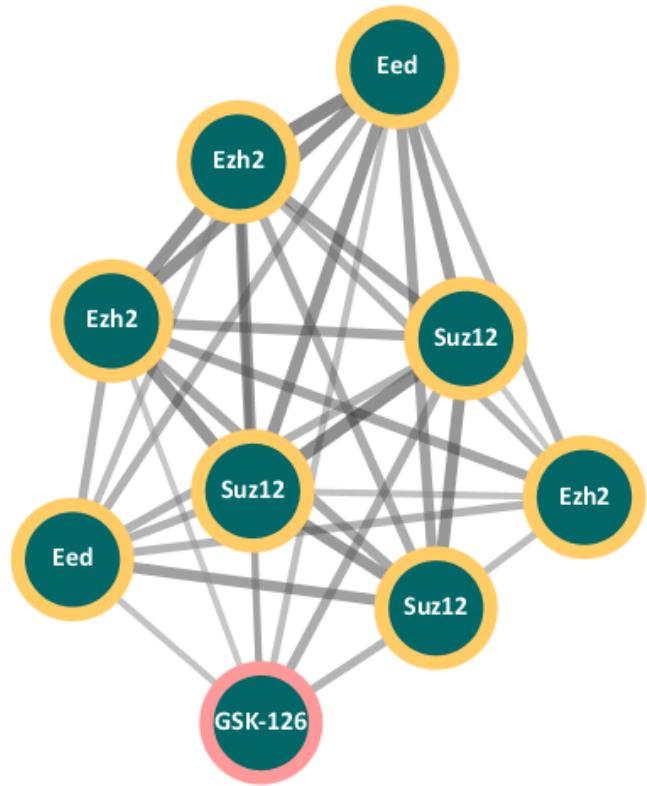
Site occupancy at H3K9 with selected knockdowns



GCP Signature Data



Connectivity Maps through Chromatin Signatures

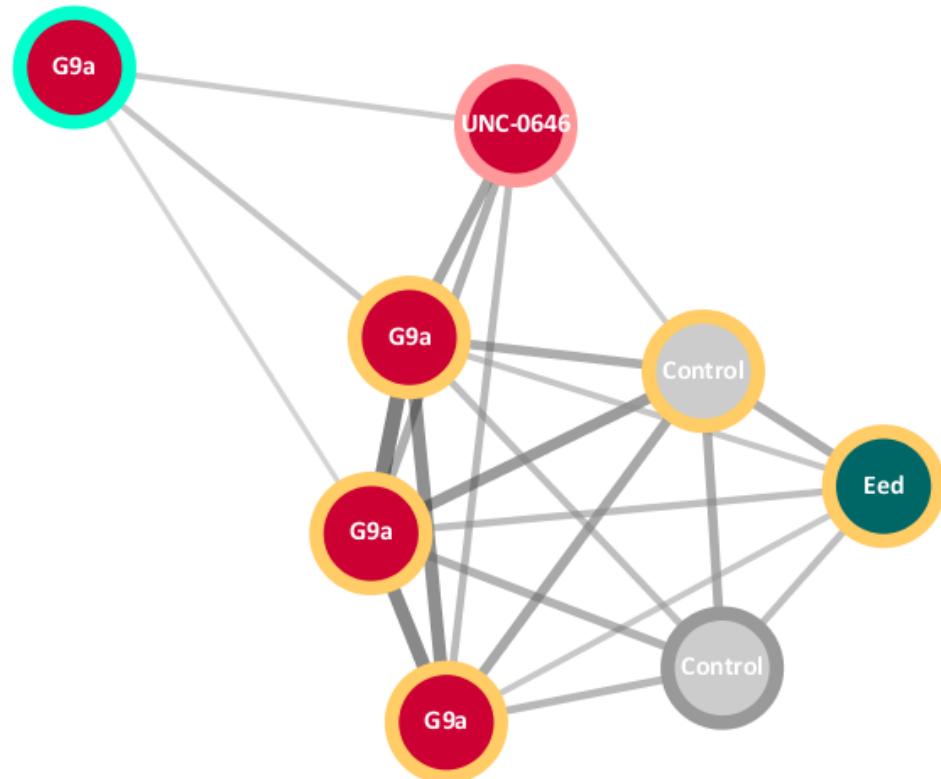


Target Complex

PRC2

G9a-GLP

None



Treatment Type

shRNA

drug

knockout

none

First effort at scale: Cancer Cell Line Encyclopedia

- Collection of ~1000 cancer cell lines with:

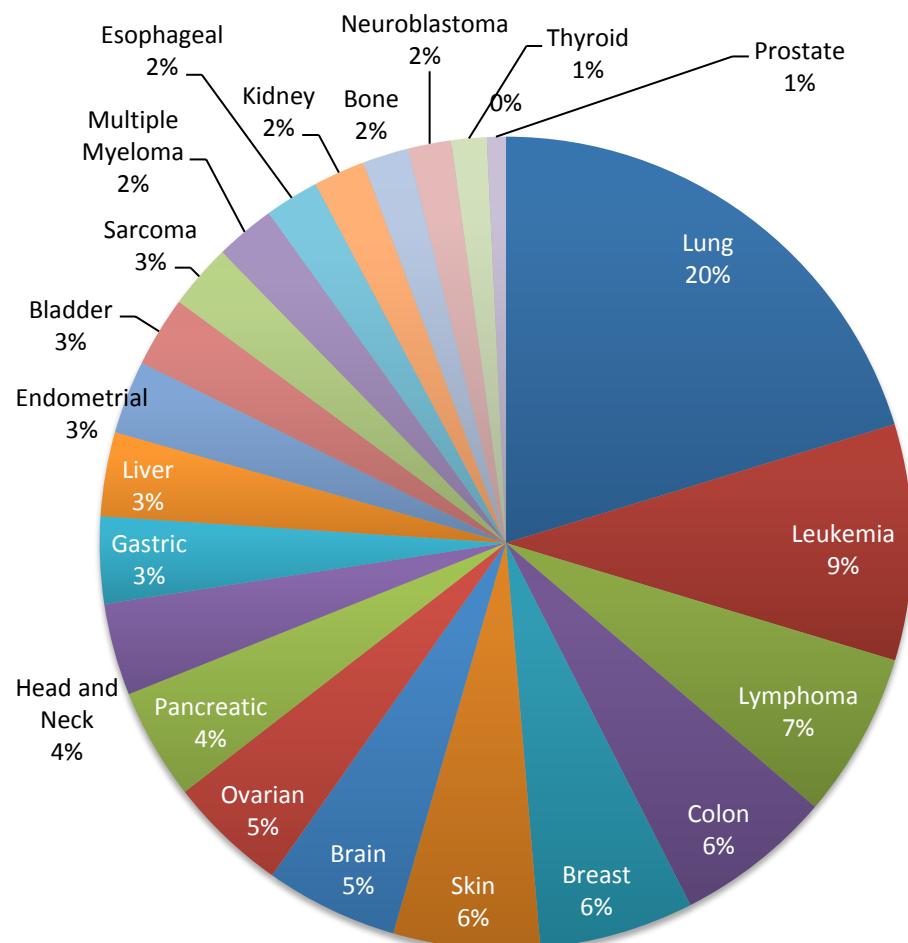
- Partial genome sequencing
- Expression analysis
- Copy number information

- Joint effort of Broad Institute and Novartis

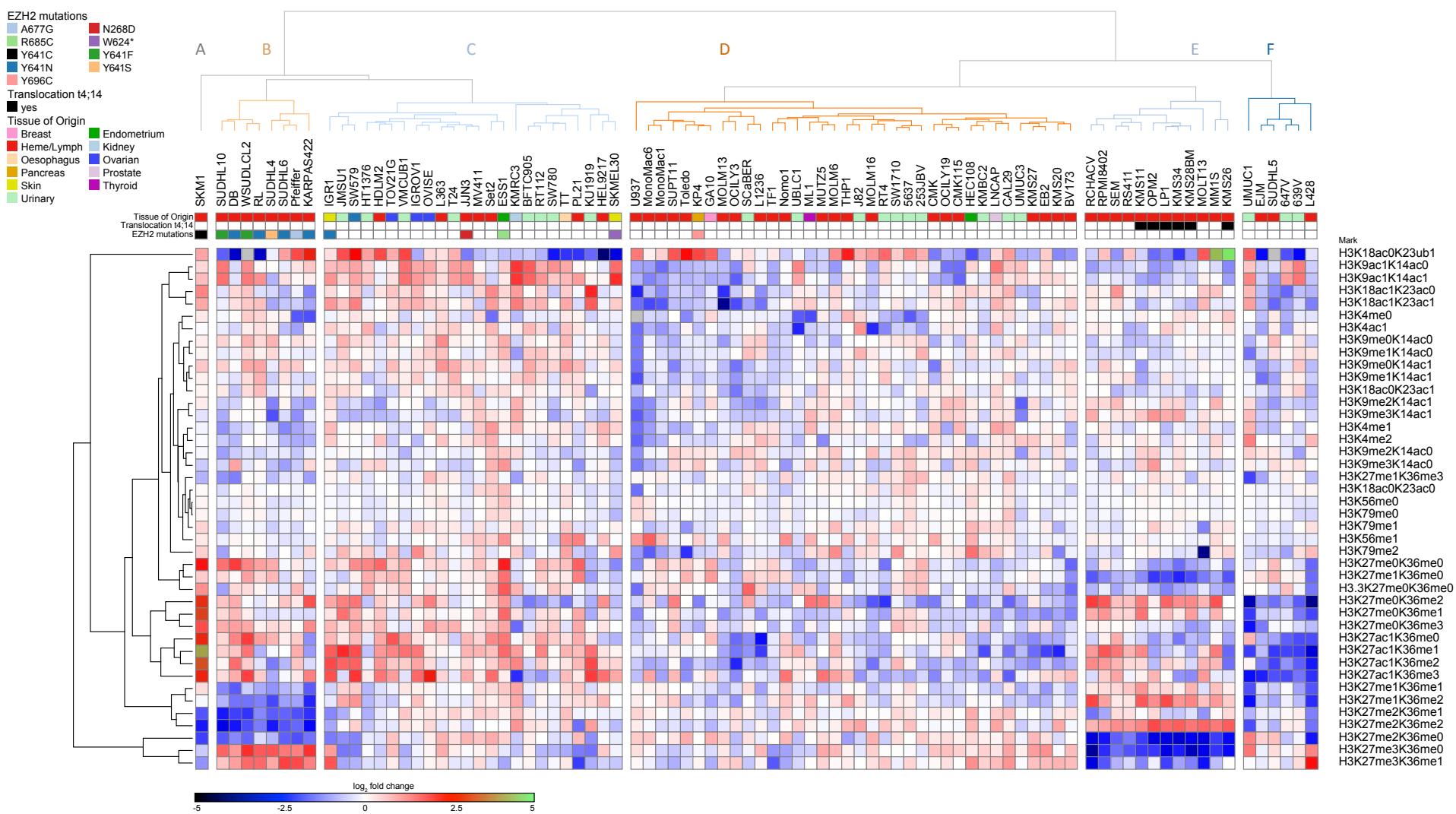
- Characterizing:

- Signaling
- Metabolites
- ***Chromatin profiling***
 - vs. *SILAC pool of 3 cell types*
 - » *MCF7, 293T, HeLa*

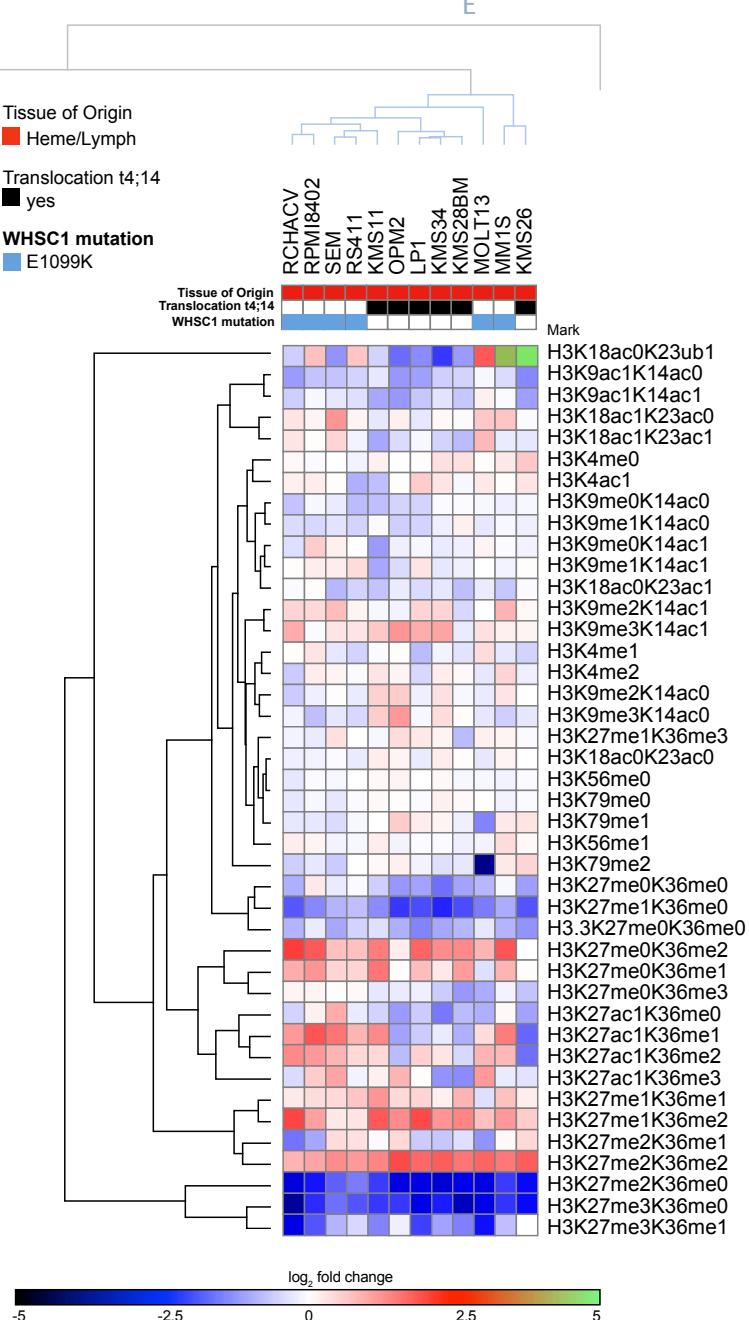
Composition of CCLE Collection



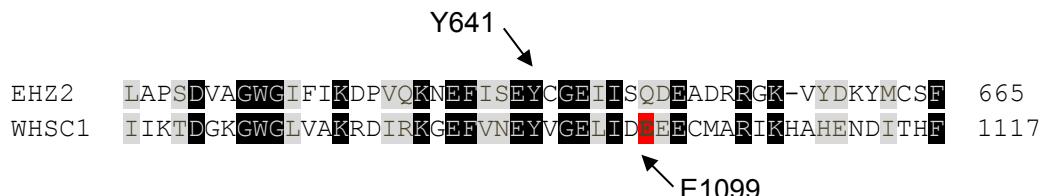
Chromatin Molecular Signatures in the CCLE



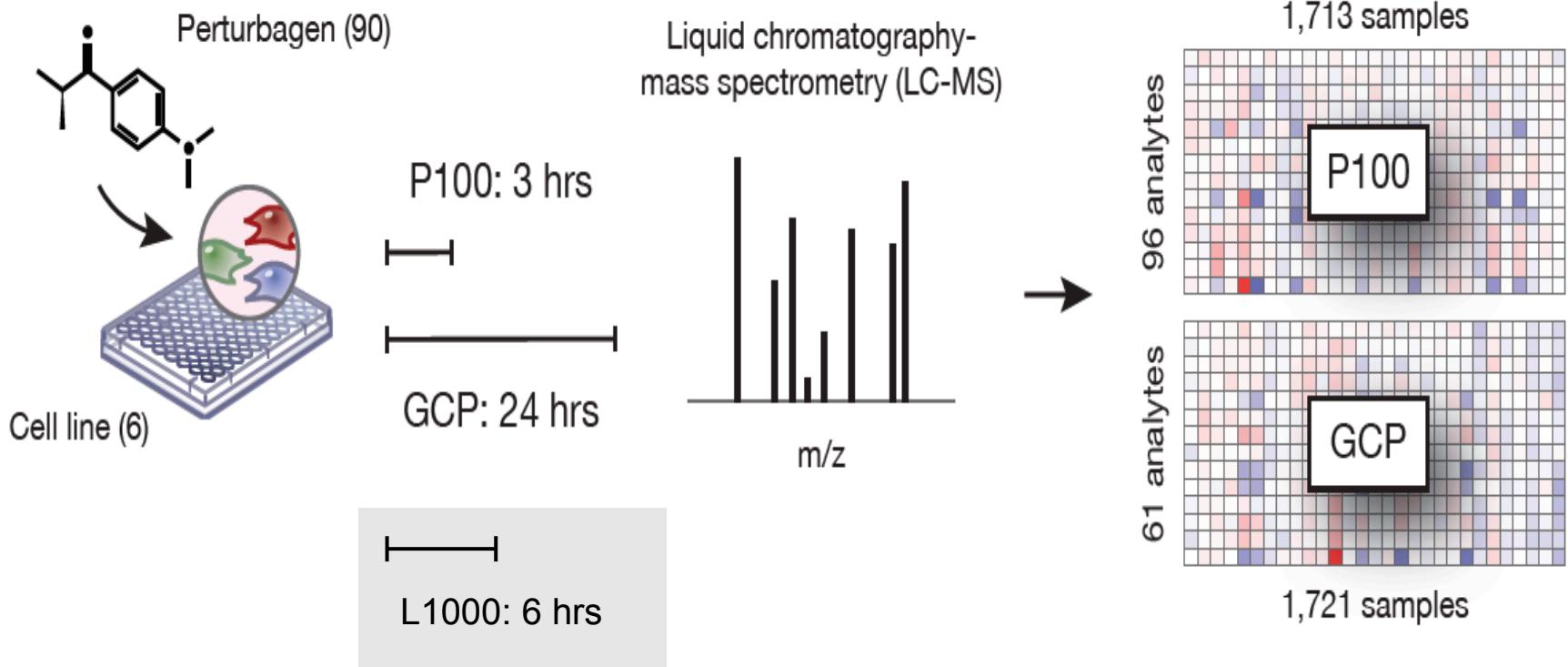
NSD2 Chromatin Molecular Signature



- Chromatin profiling suggests NSD2 E1099K is a GOF mutation functionally equivalent to t4;14
- NSD2 occurs in the SET domain proximal to other residues of importance



An even bigger scale: drug perturbations



Unprecedented amount of drug perturbation proteomics data

Cells	Provenance	Epigenetically Active	Neuroactive	Kinase/Pathway Inhibitors	Cardiotoxic
A375	Skin Cancer	●	●	●	●
YAPC	Pancreatic Cancer	●	●	●	●
A549	Lung Cancer	●	●	●	●
MCF7	Breast Cancer	●	●	●	●
PC3	Prostate Cancer	●	●	●	●
NPCs ^{H9}	H9 hESCs	●	●	●	●
Astrocytes ^{H9}	H9 hESCs	●	●	●	●

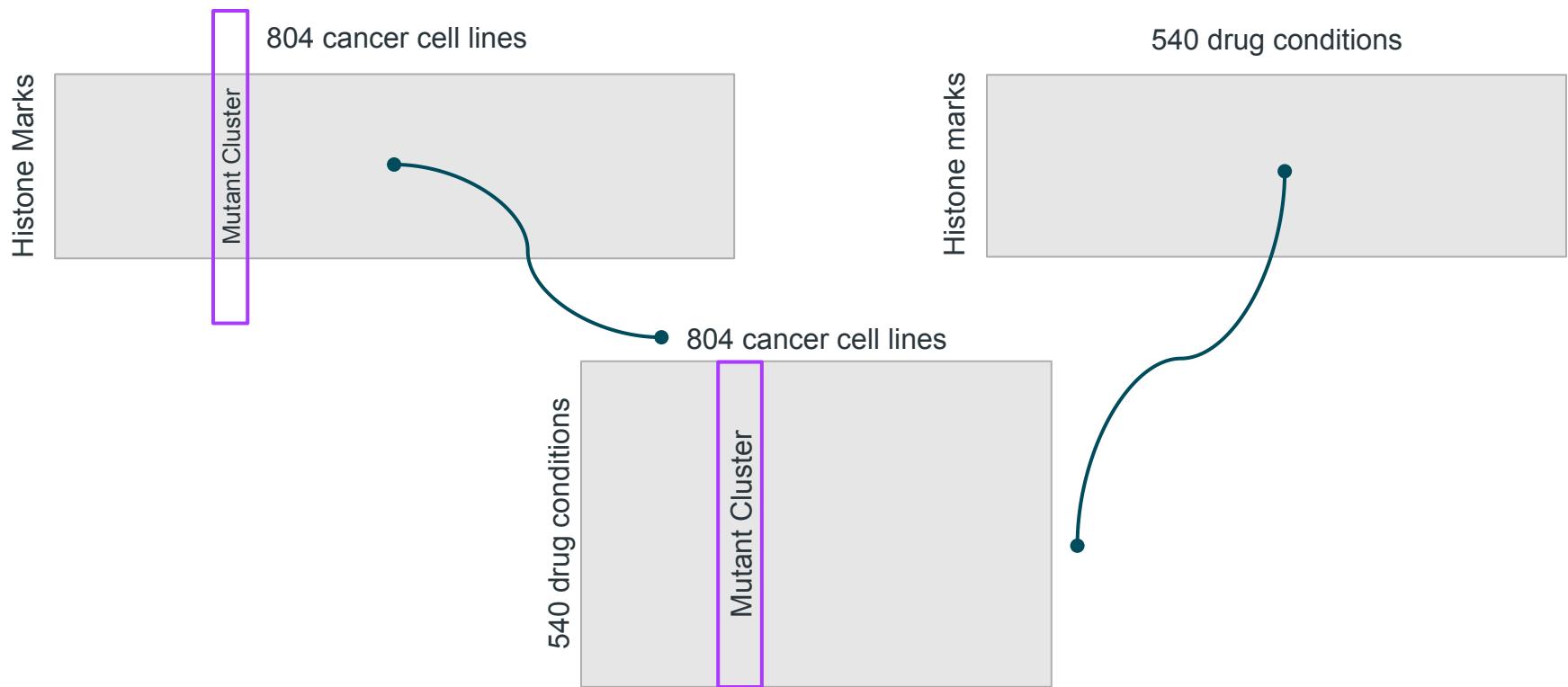
5300+

- Completed
- In Progress

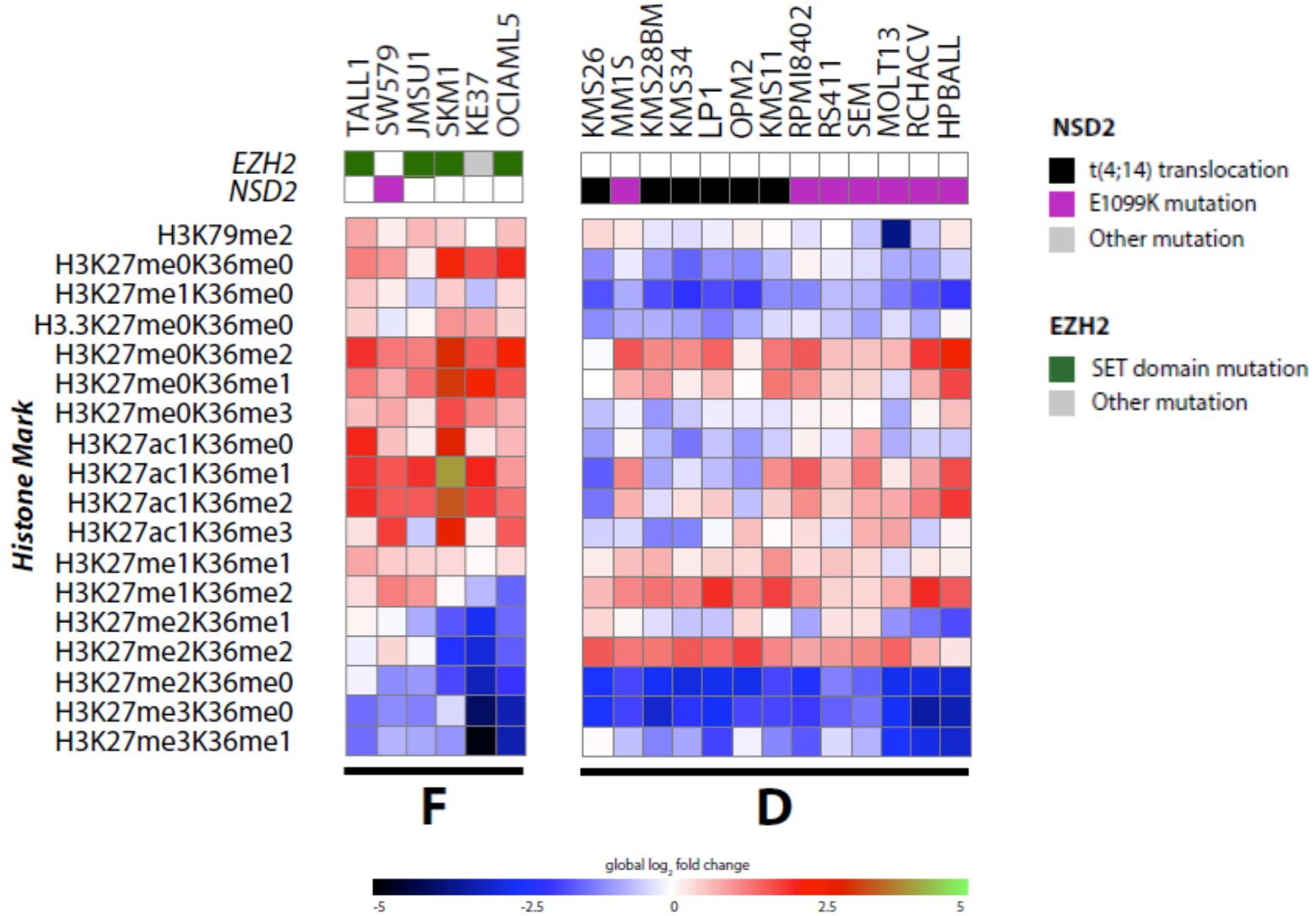
<https://panoramaweb.org/labkey/project/LINCS>



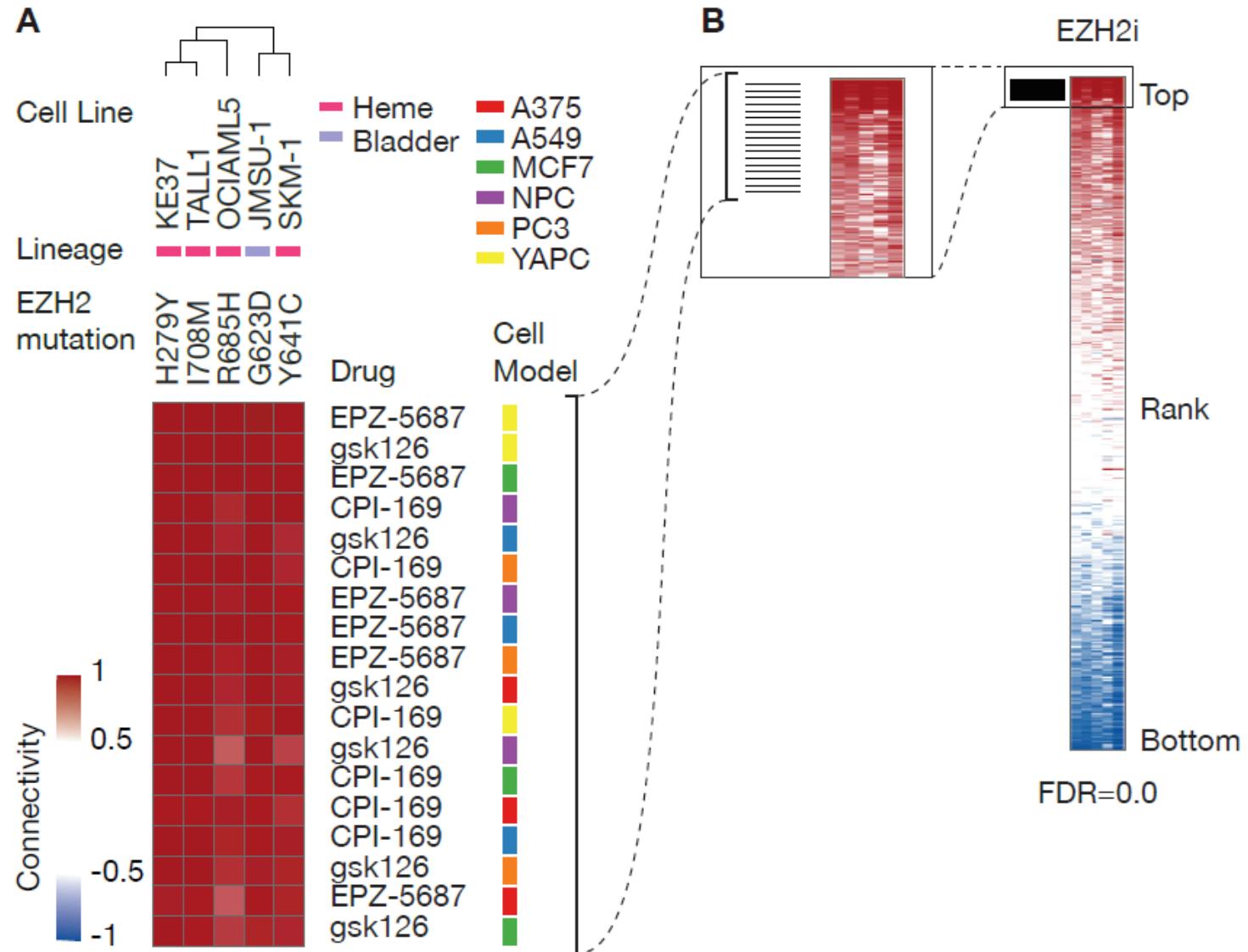
Comparing States of Nature to Drug Perturbations



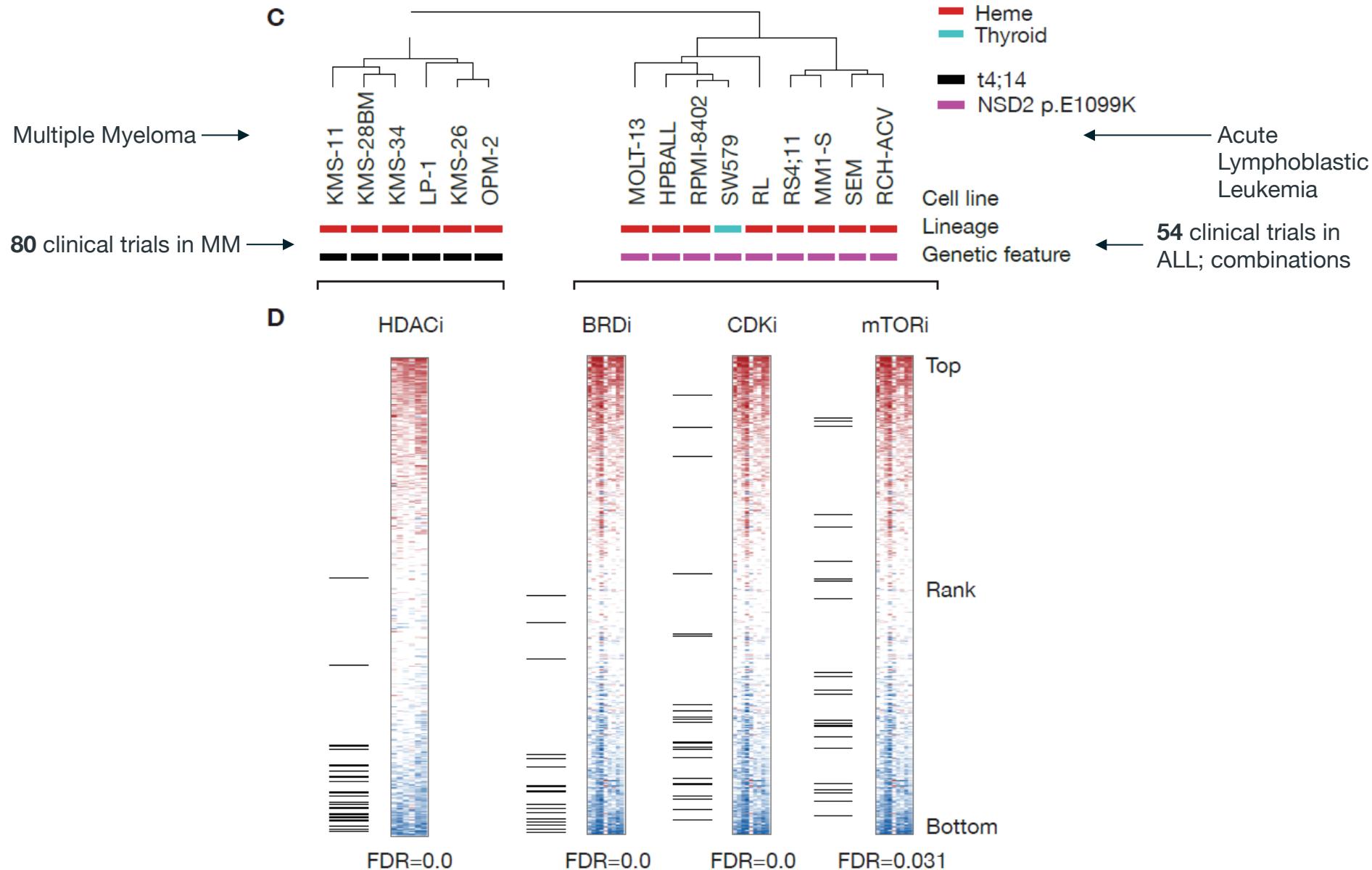
Revisiting selected CCLE profiles



EZH2 mutants connect to EZH2 inhibitors



NSD2 mutants can be segregated by drug connections



Summary

- PRM assays are suitable when MRM/SRM assays lack selectivity
- Configuration of PRM assays follows naturally from discovery proteomics
 - Most principles of analytical methods carry from MRM/SRM
- A research grade PRM assay for chromatin proteomics demonstrates an impactful use case
 - Could not have done with SRM
 - Longitudinal data collection / sample comparison
 - Functional and therapeutic insights demonstrated

Acknowledgements

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