

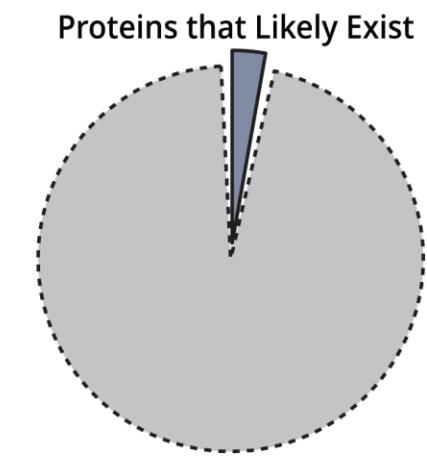
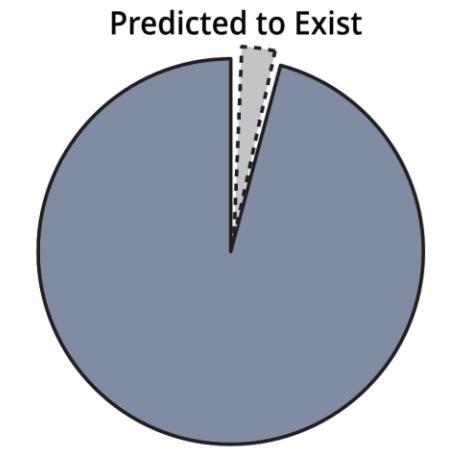


Standing in the Way of Proteome Profiling Depth

How the Selection of Chromatography and Mass Spectrometry Data Acquisition Limit Our View of the Proteome

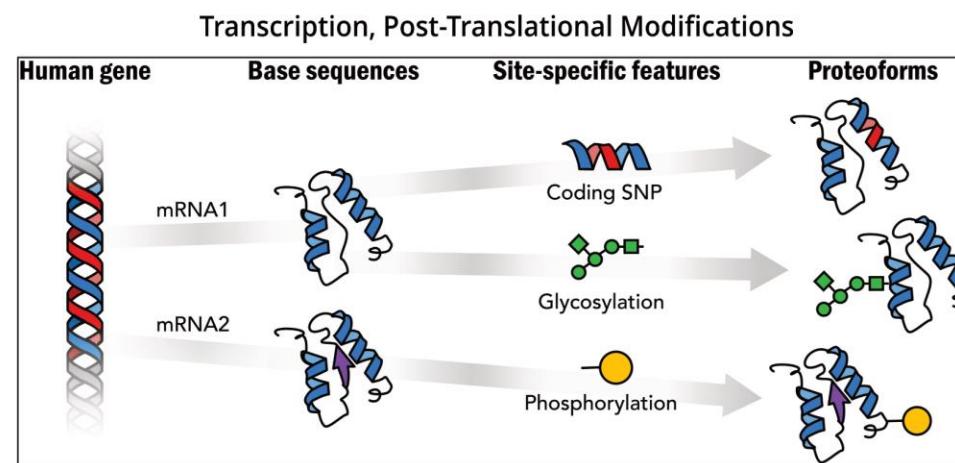
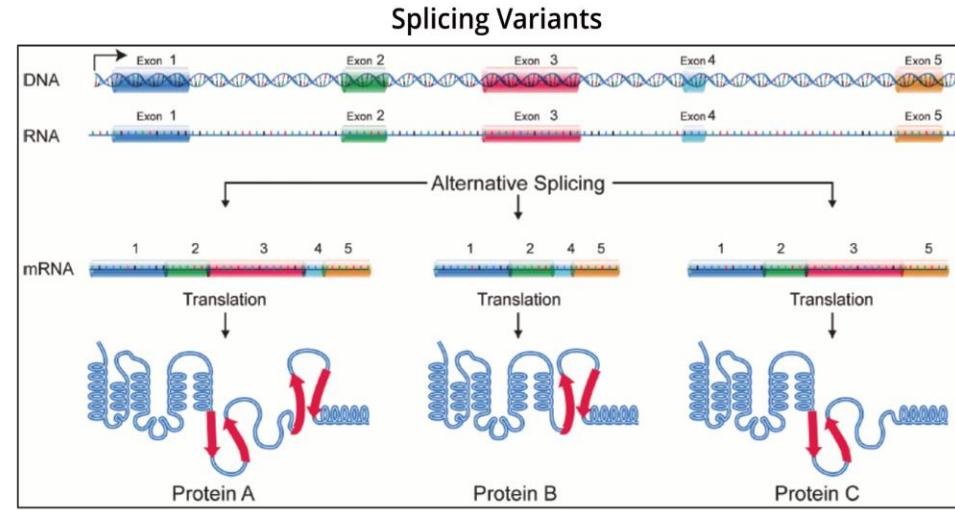
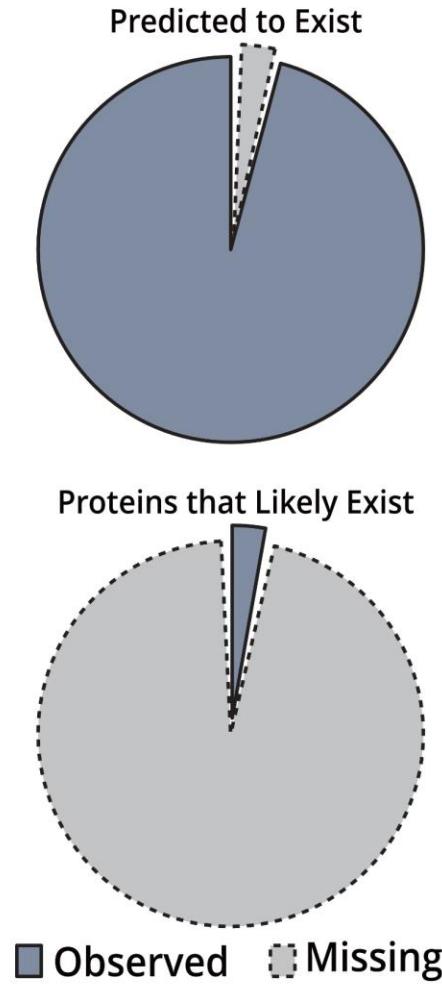
Graham Delafield
Ph.D. Thesis Defense
3.24.23

How Proteins Hide

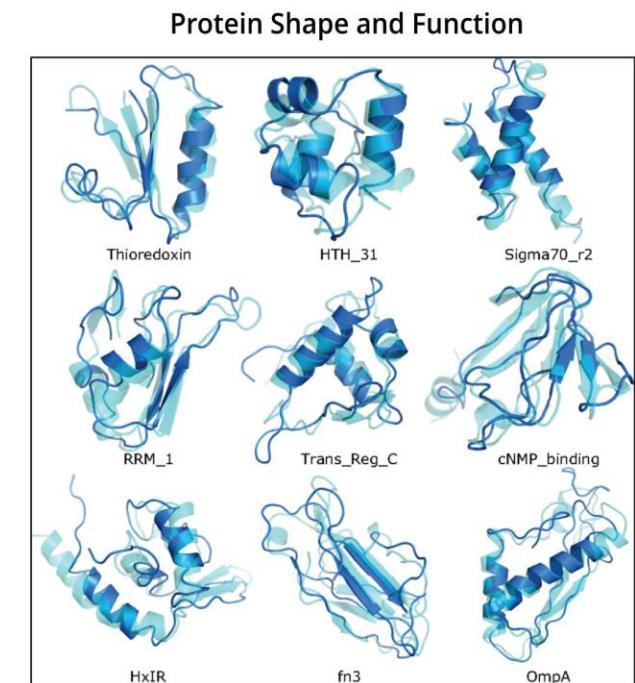
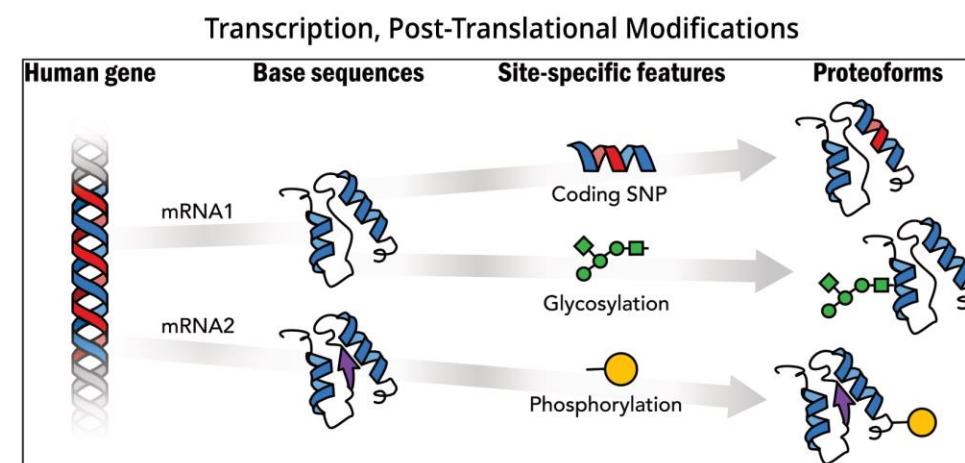
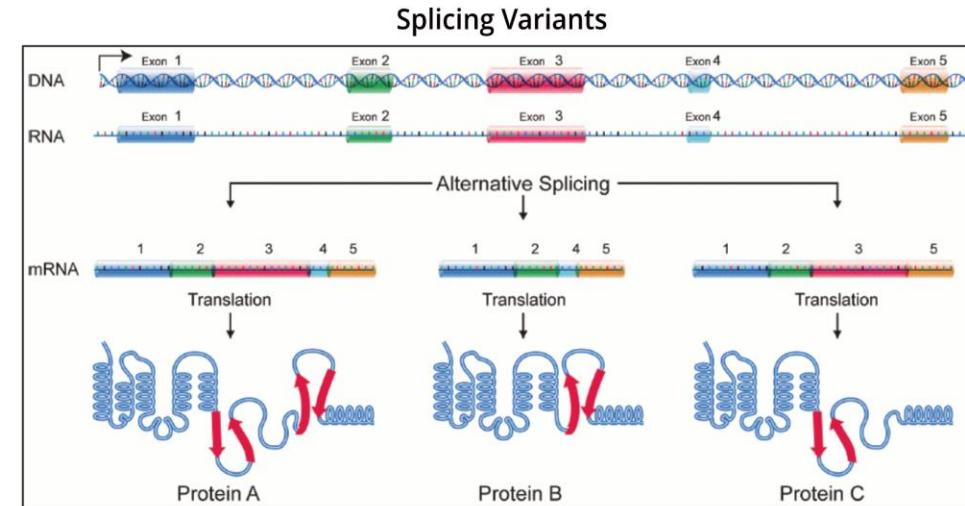
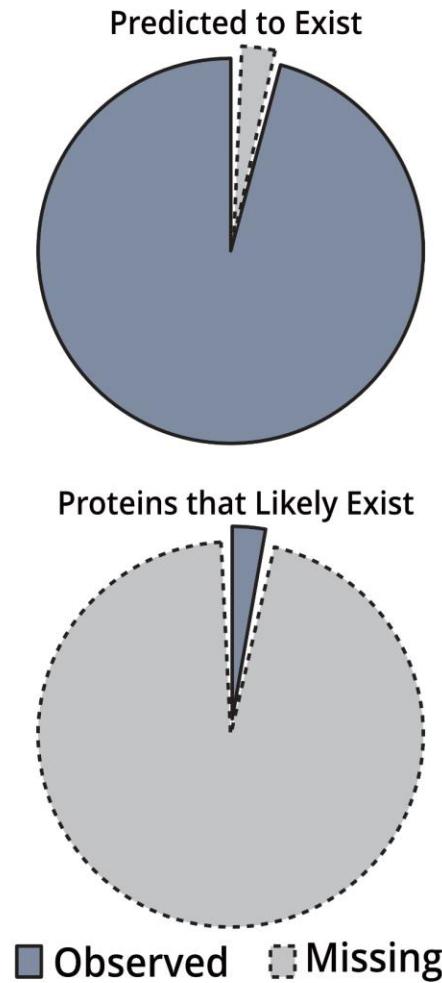


■ Observed □ Missing

How Proteins Hide

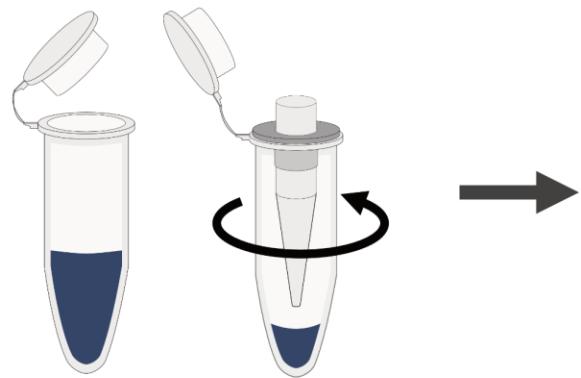


How Proteins Hide



Mass Spectrometry-Based Proteomics

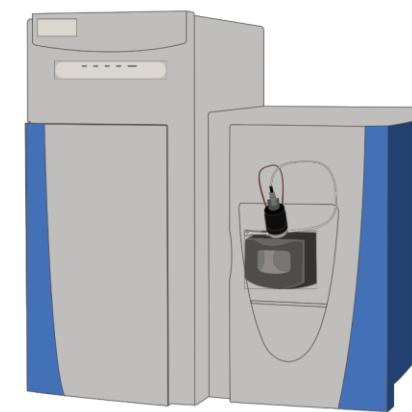
Preparation



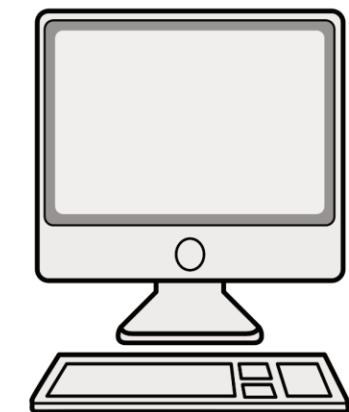
Separation



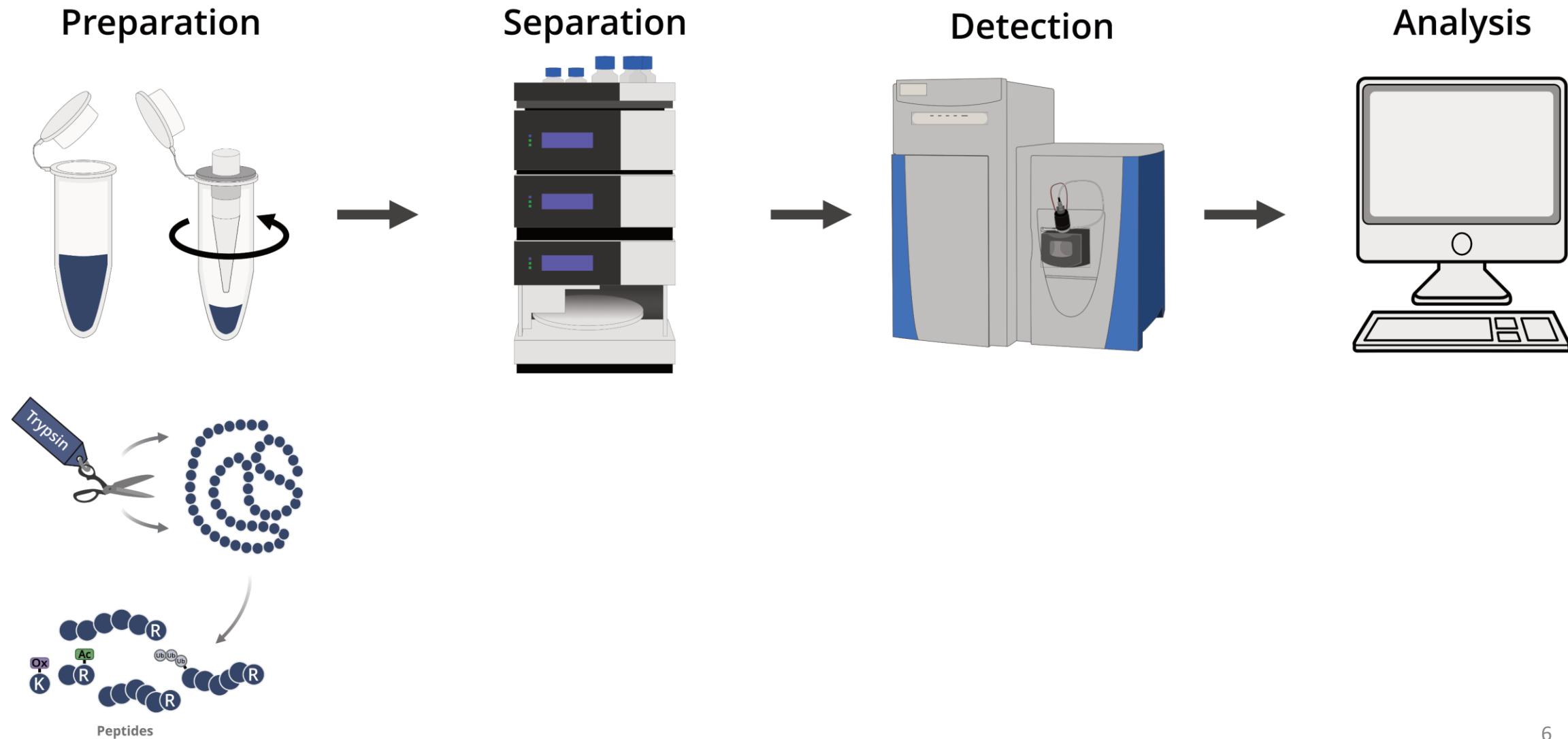
Detection



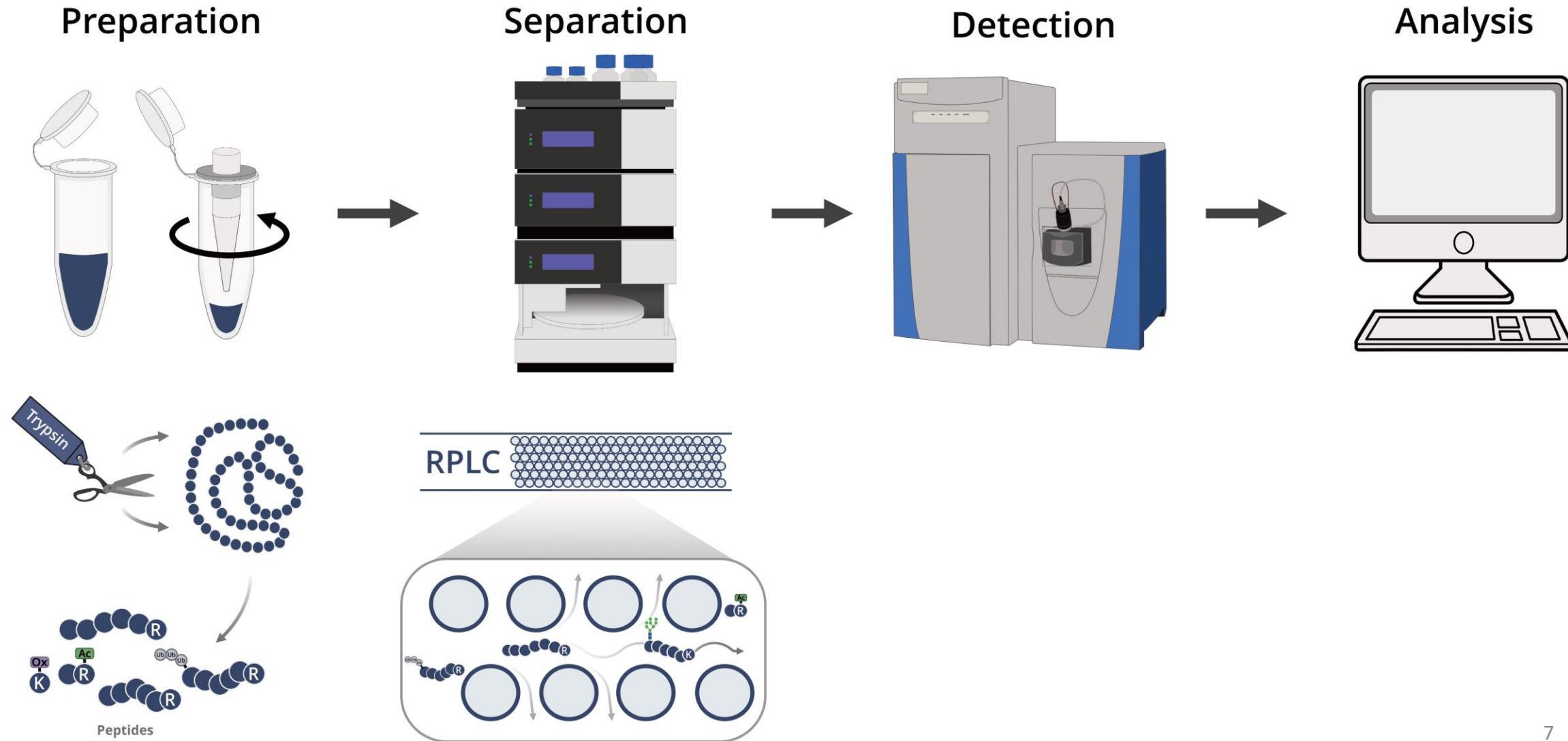
Analysis



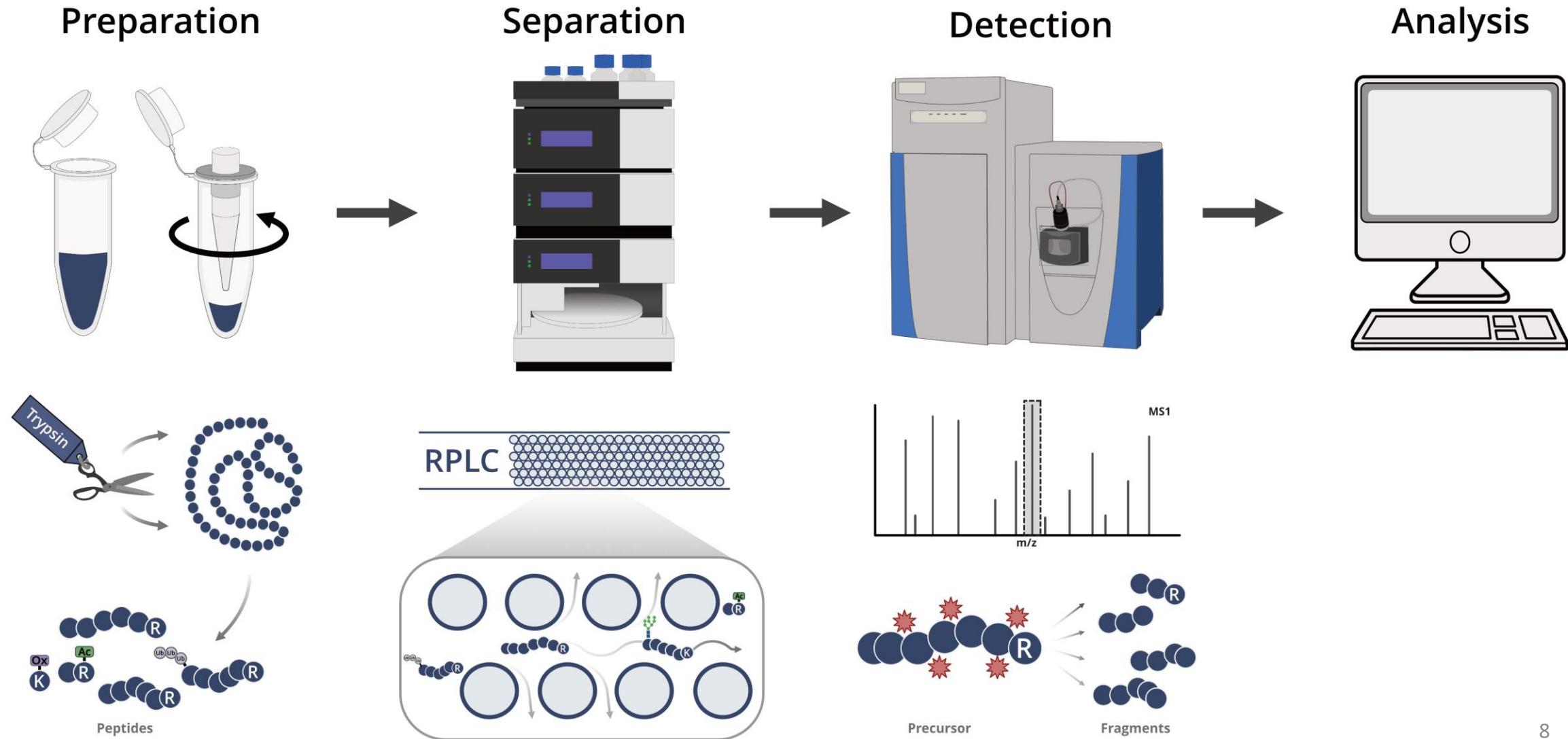
Mass Spectrometry-Based Proteomics



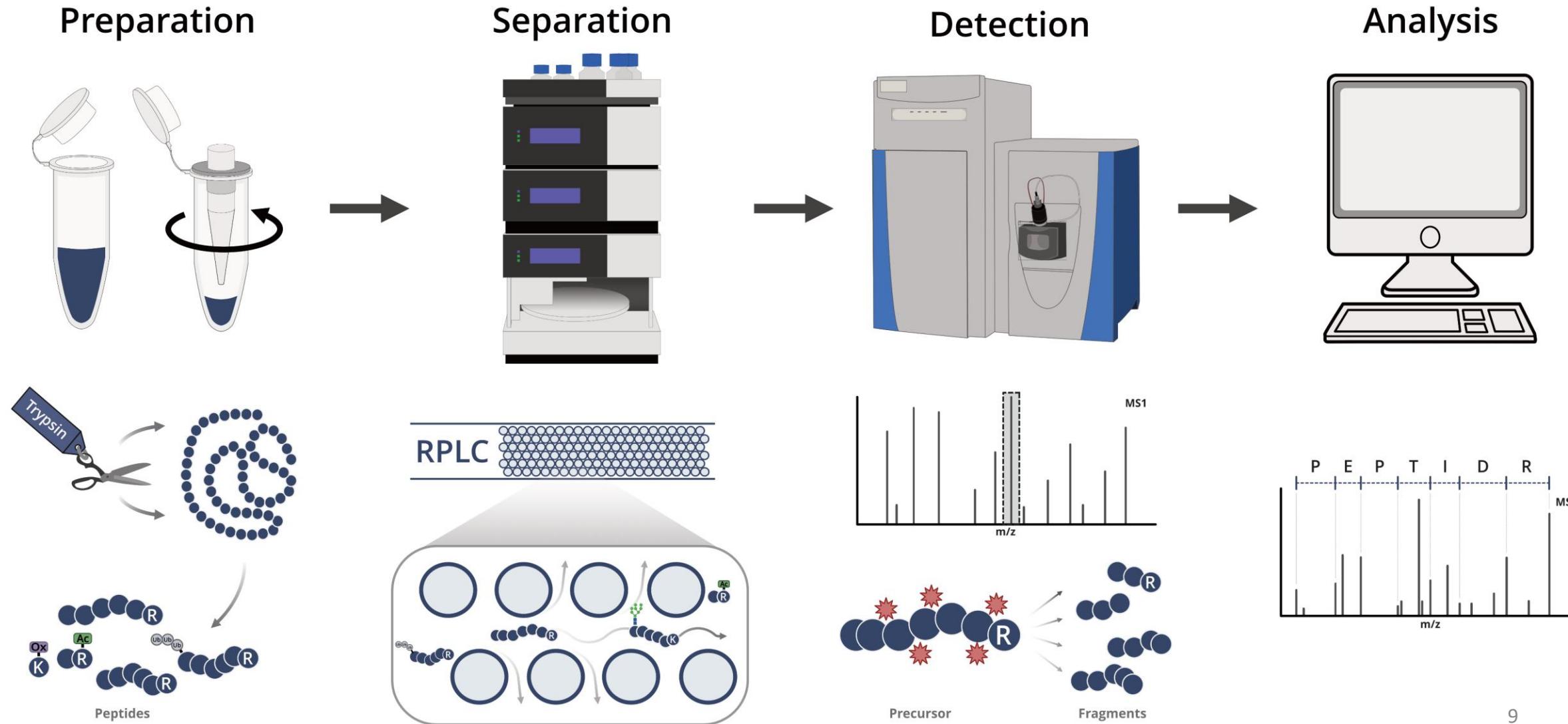
Mass Spectrometry-Based Proteomics



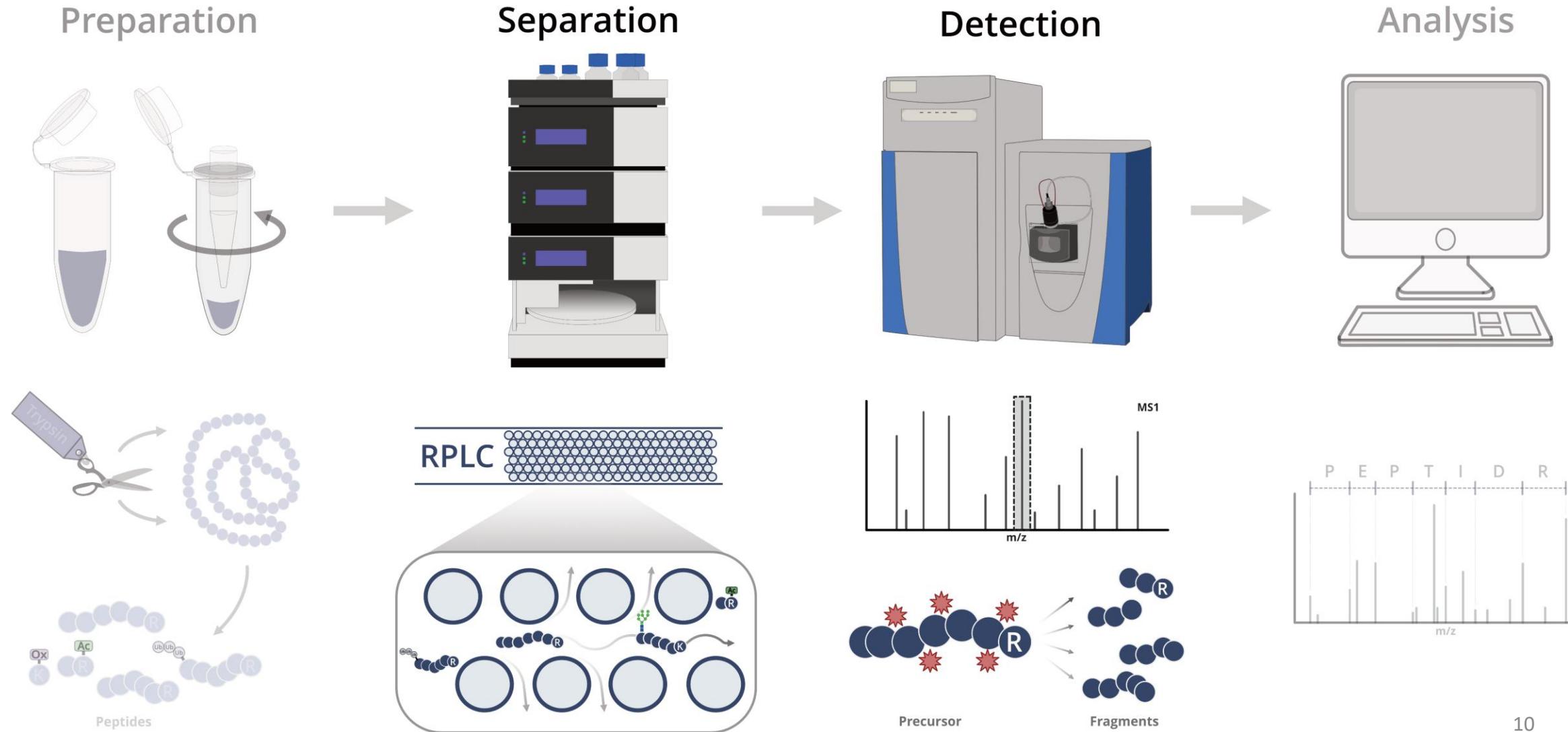
Mass Spectrometry-Based Proteomics



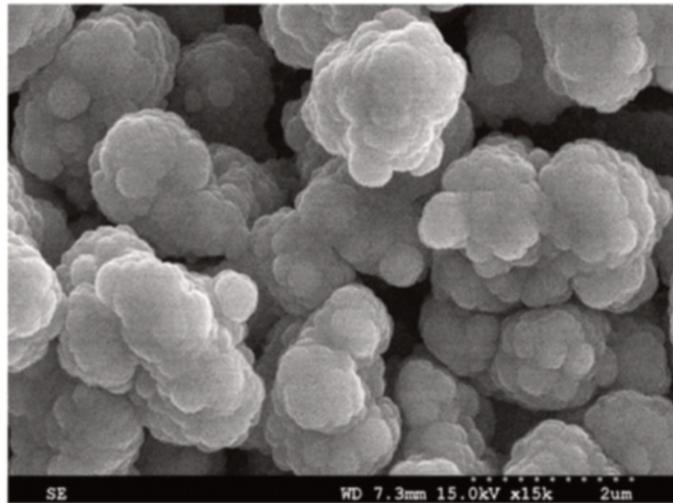
Mass Spectrometry-Based Proteomics



Mass Spectrometry-Based Proteomics



Utilizing Alternative Separations

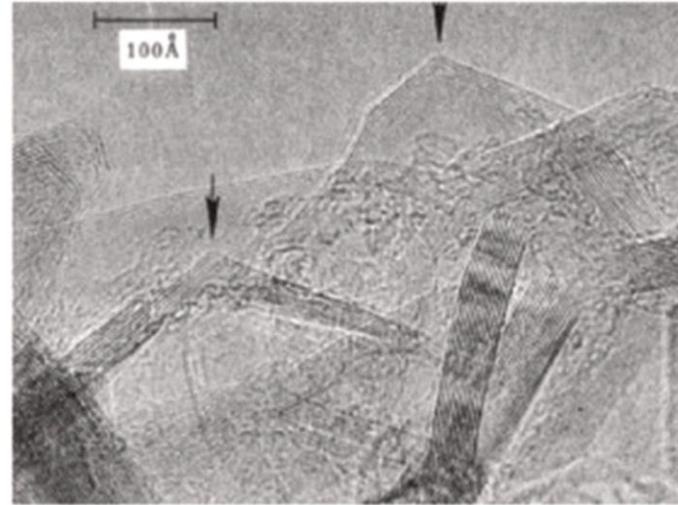


RPLC C18

Utilizing Alternative Separations



RPLC C18



PGC

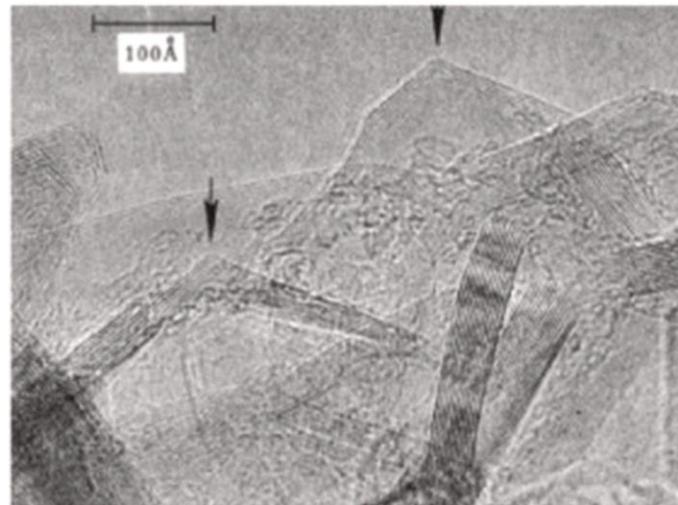
Porous Graphitic Carbon

- Retention based on polar and electrostatic interactions
- Solvent flexibility, amenable to traditional water-organic systems
- Retention orthogonal to RPLC without the need for phase change
- Reproducible, available

Utilizing Alternative Separations



RPLC C18



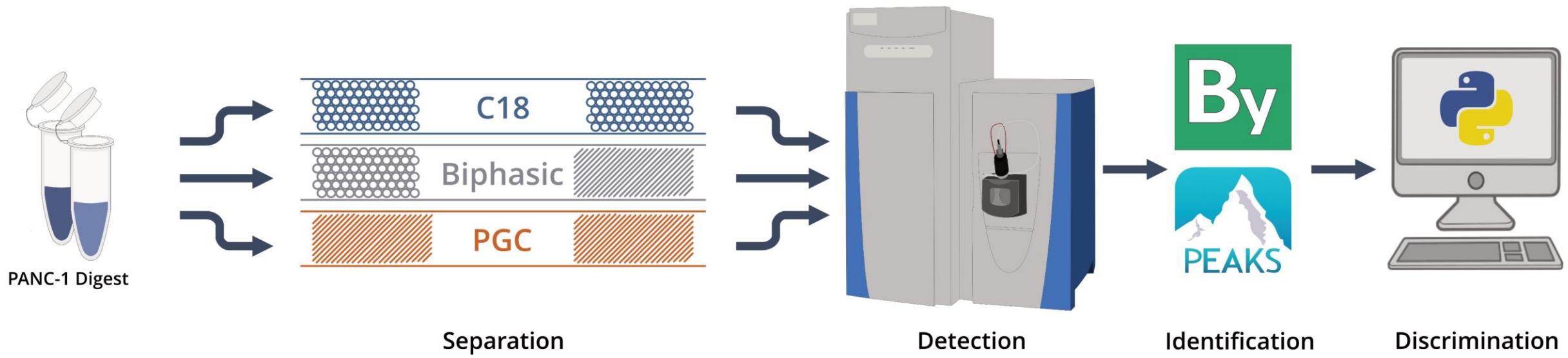
PGC

Porous Graphitic Carbon

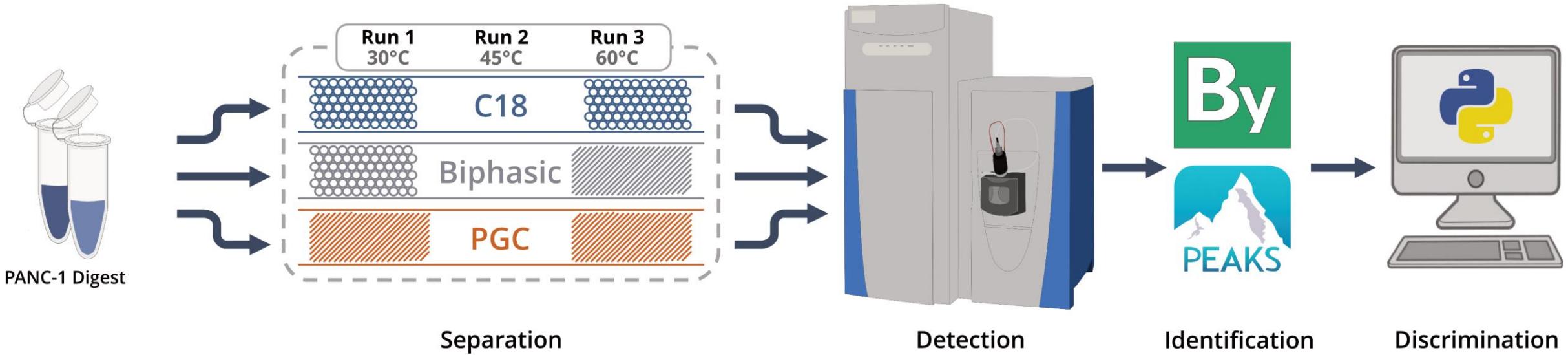
- Retention based on polar and electrostatic interactions
- Solvent flexibility, amenable to traditional water-organic systems
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- Reproducible, available

Does PGC offer an amenable way to enhance proteome access?

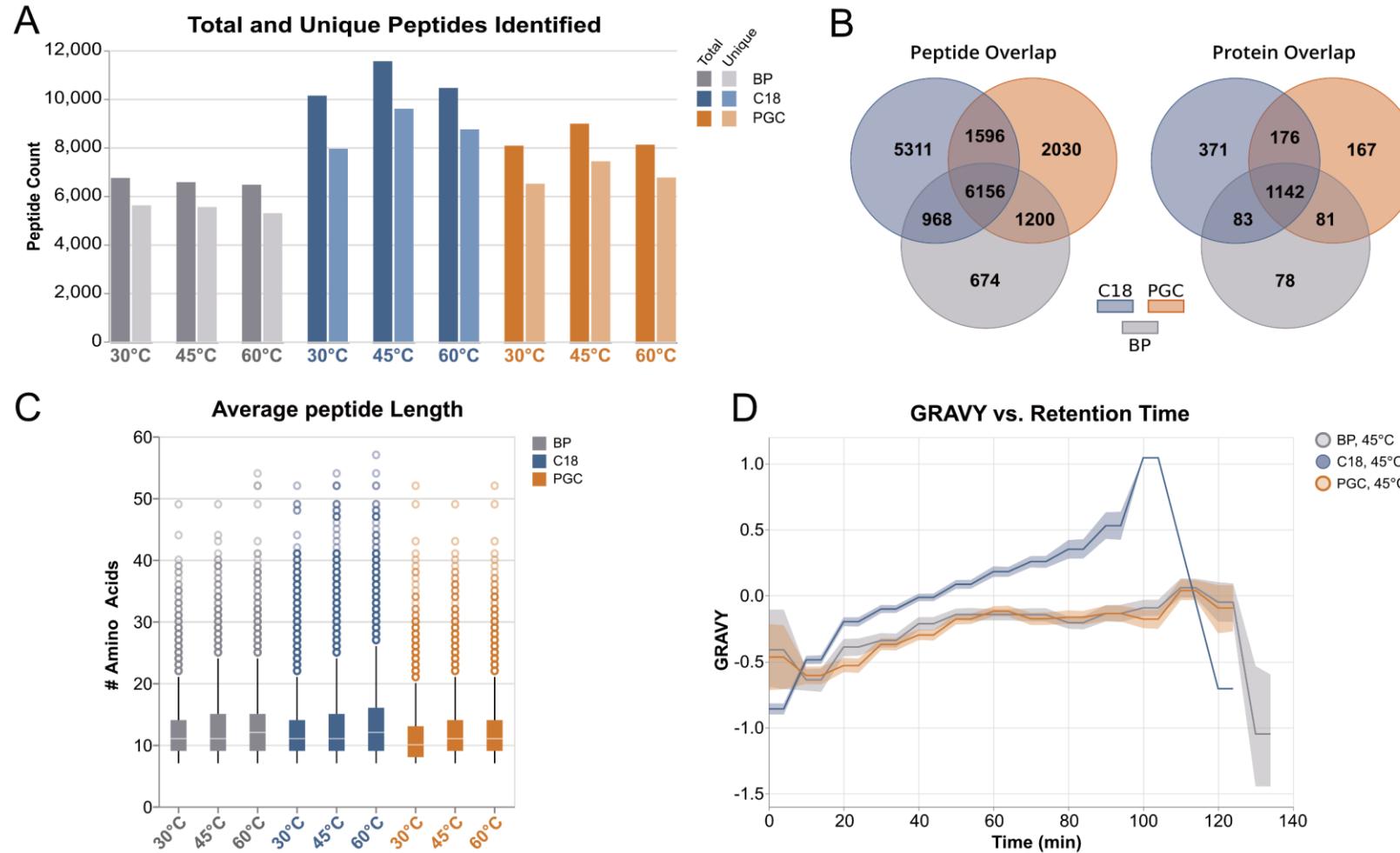
PGC-Based Shotgun Proteomics



PGC-Based Shotgun Proteomics



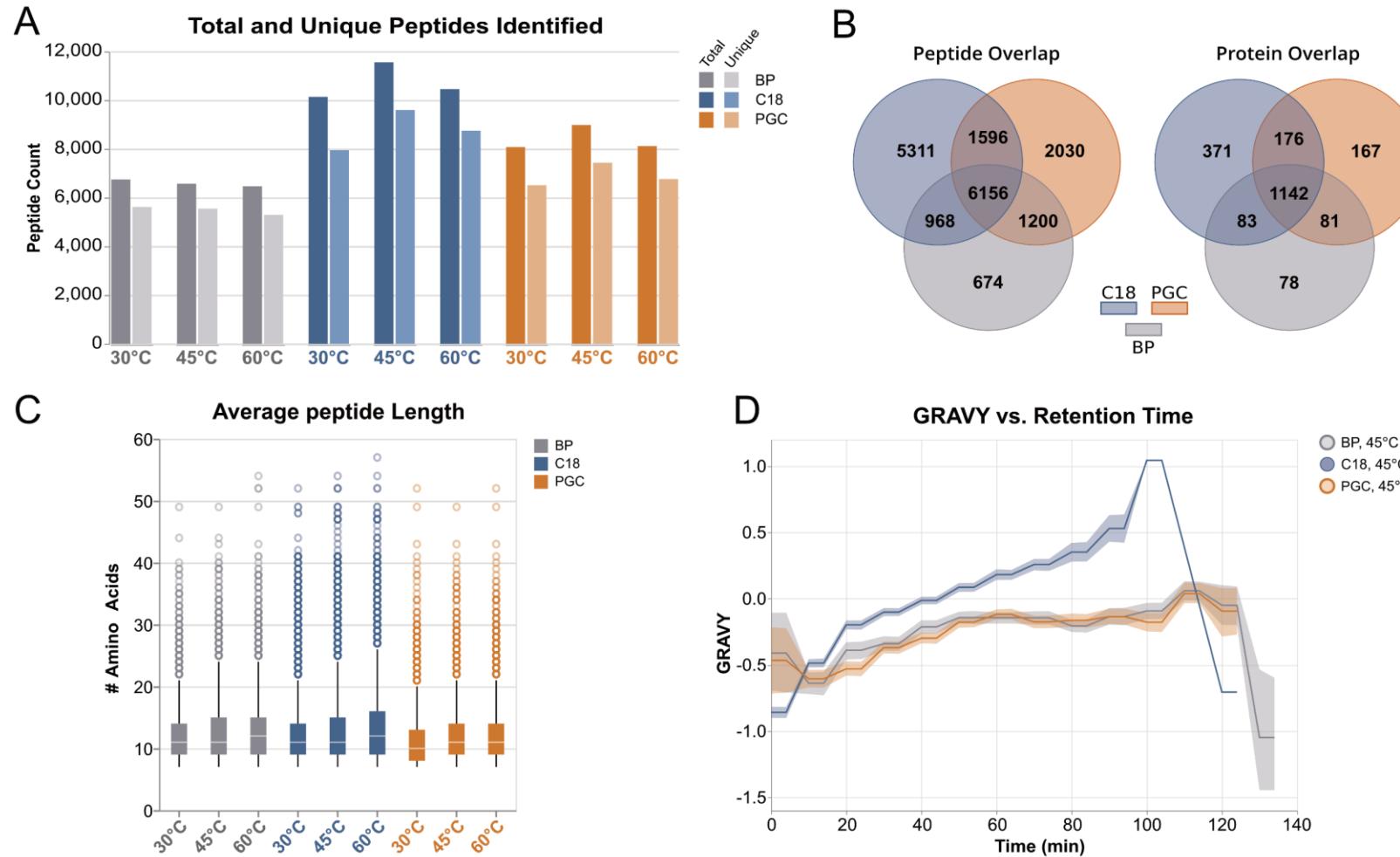
PGC Complements RPLC Analyses



Key Results

- PGC separations provide additional 15-20% peptide and protein identifications
- Preferential retention of smaller, hydrophilic peptides
- Complementary sample coverage though not orthogonal

PGC Complements RPLC Analyses

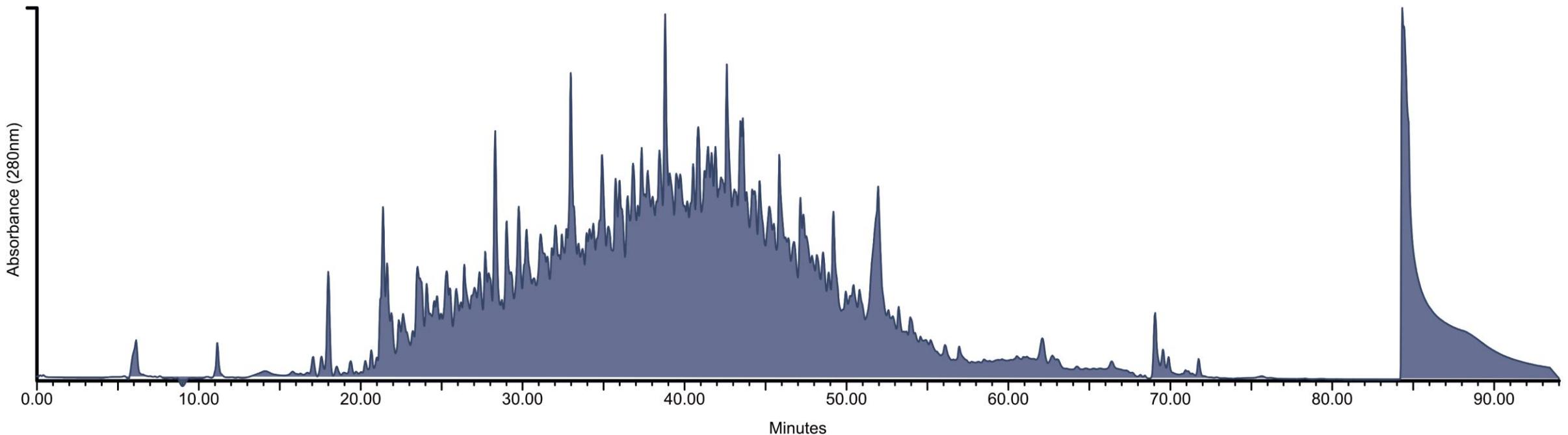


Key Results

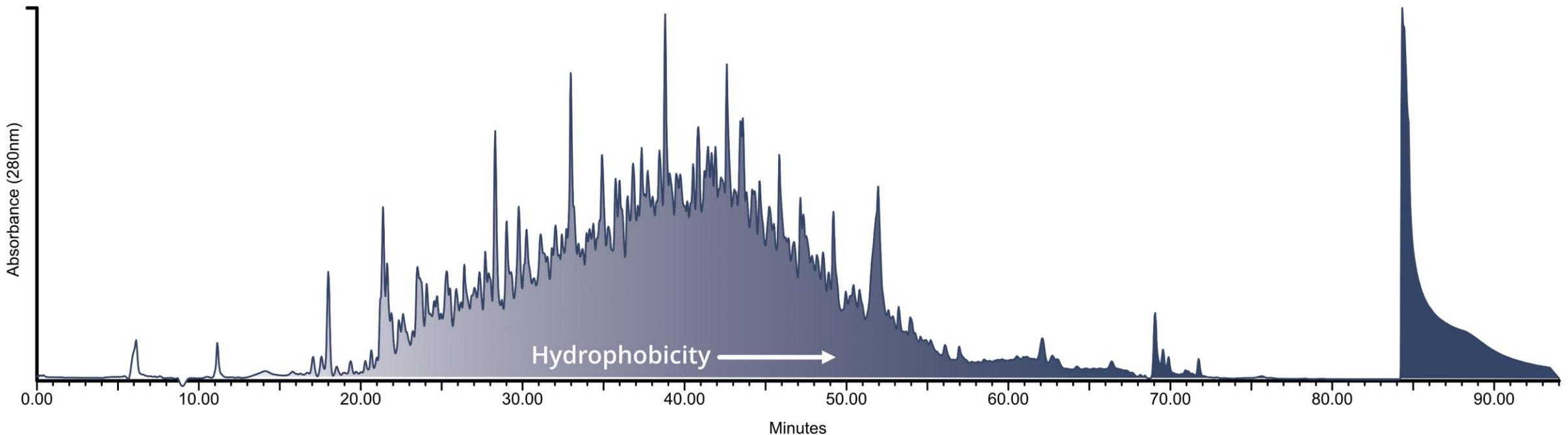
- PGC separations provide additional 15-20% peptide and protein identifications
- Preferential retention of smaller, hydrophilic peptides
- Complementary sample coverage though not orthogonal

Knowing each separation provides access to different sample components, stepping away from shotgun proteomics will increase efficiency and decrease redundancy.

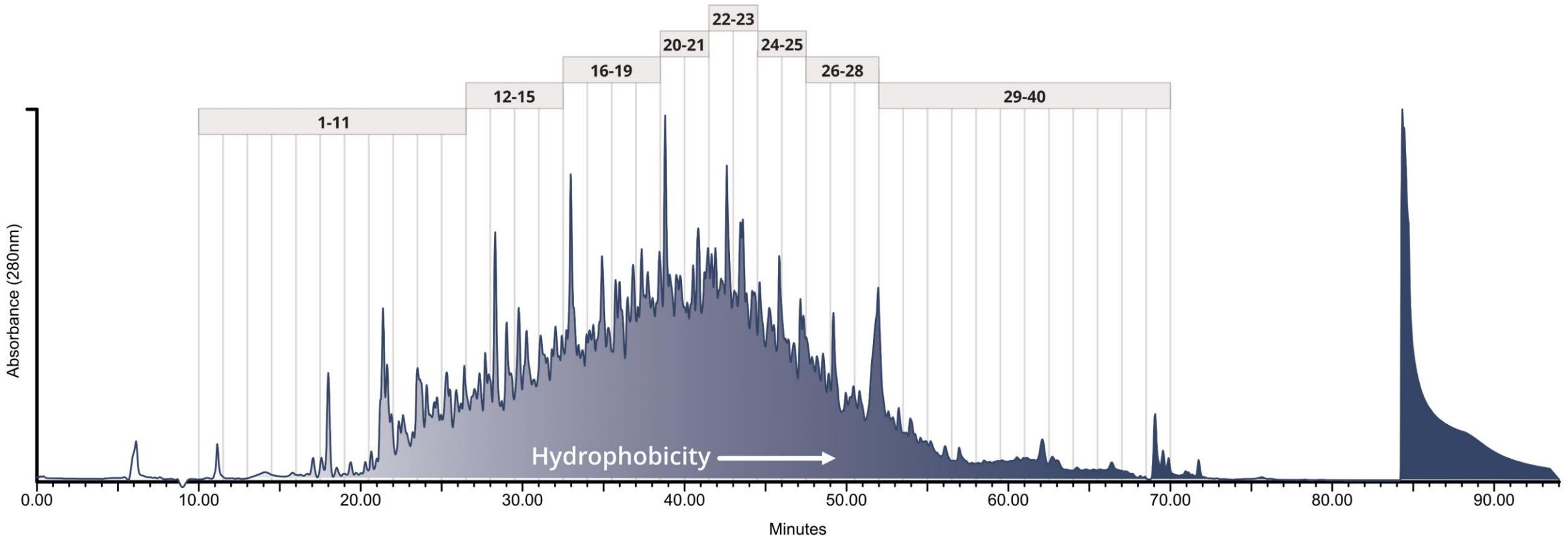
More Efficient Use of PGC-RPLC



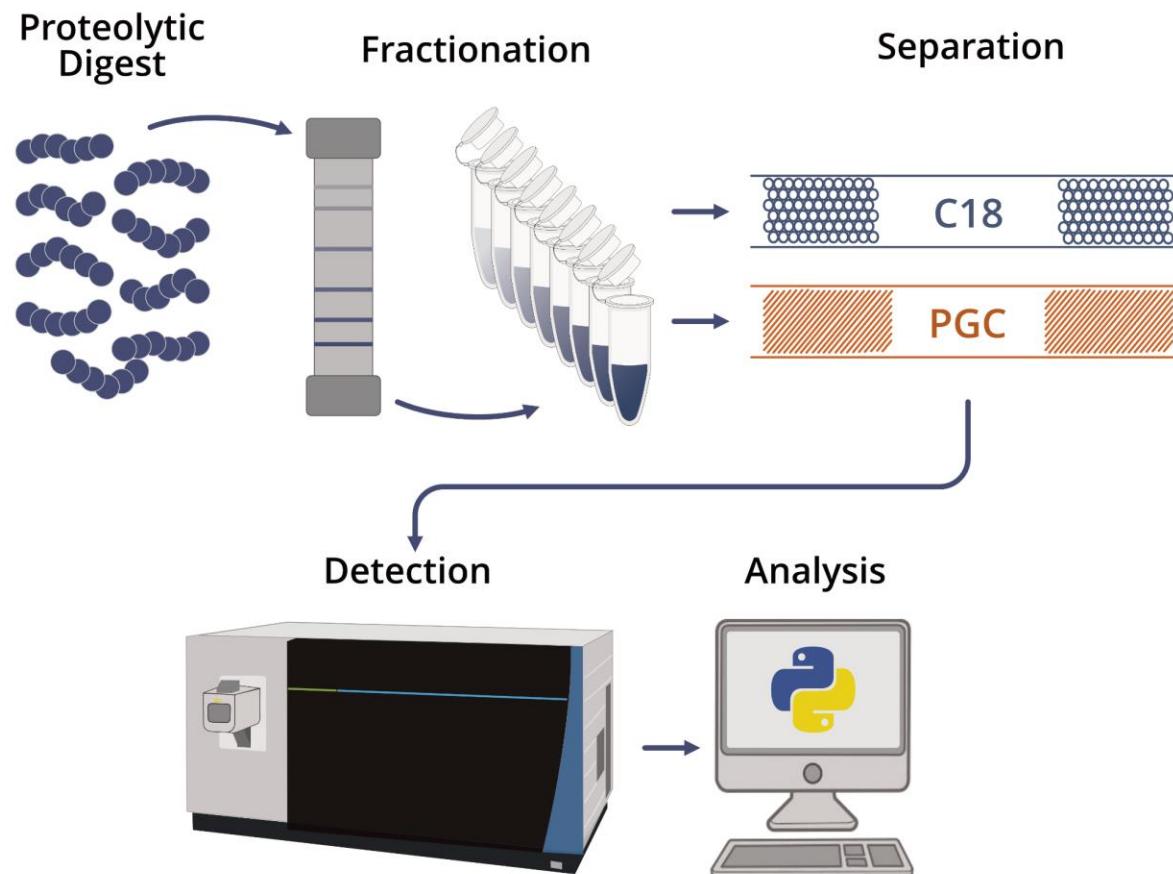
More Efficient Use of PGC-RPLC



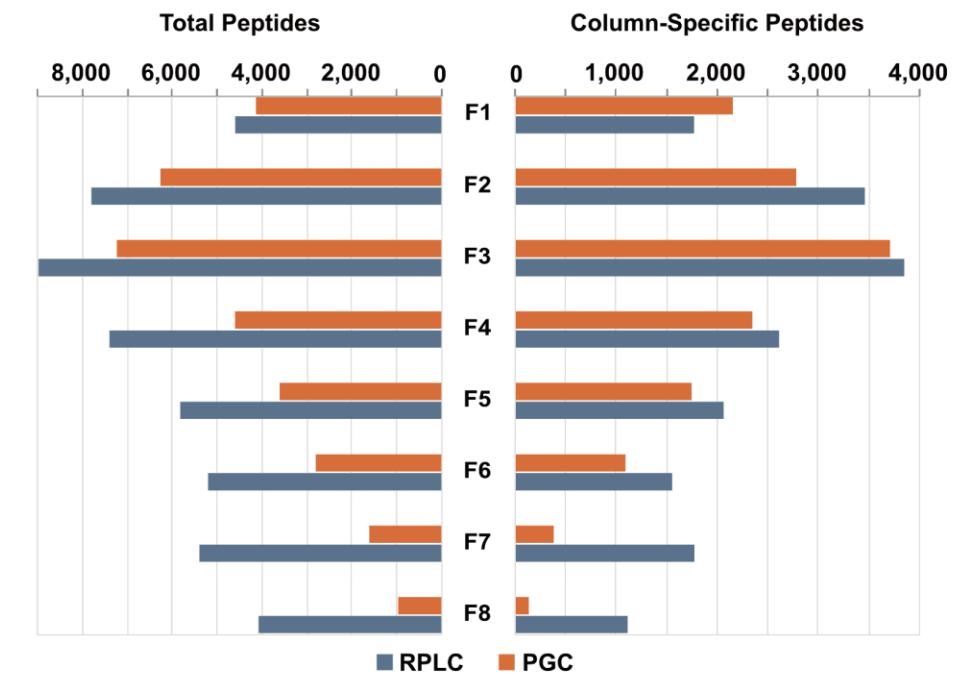
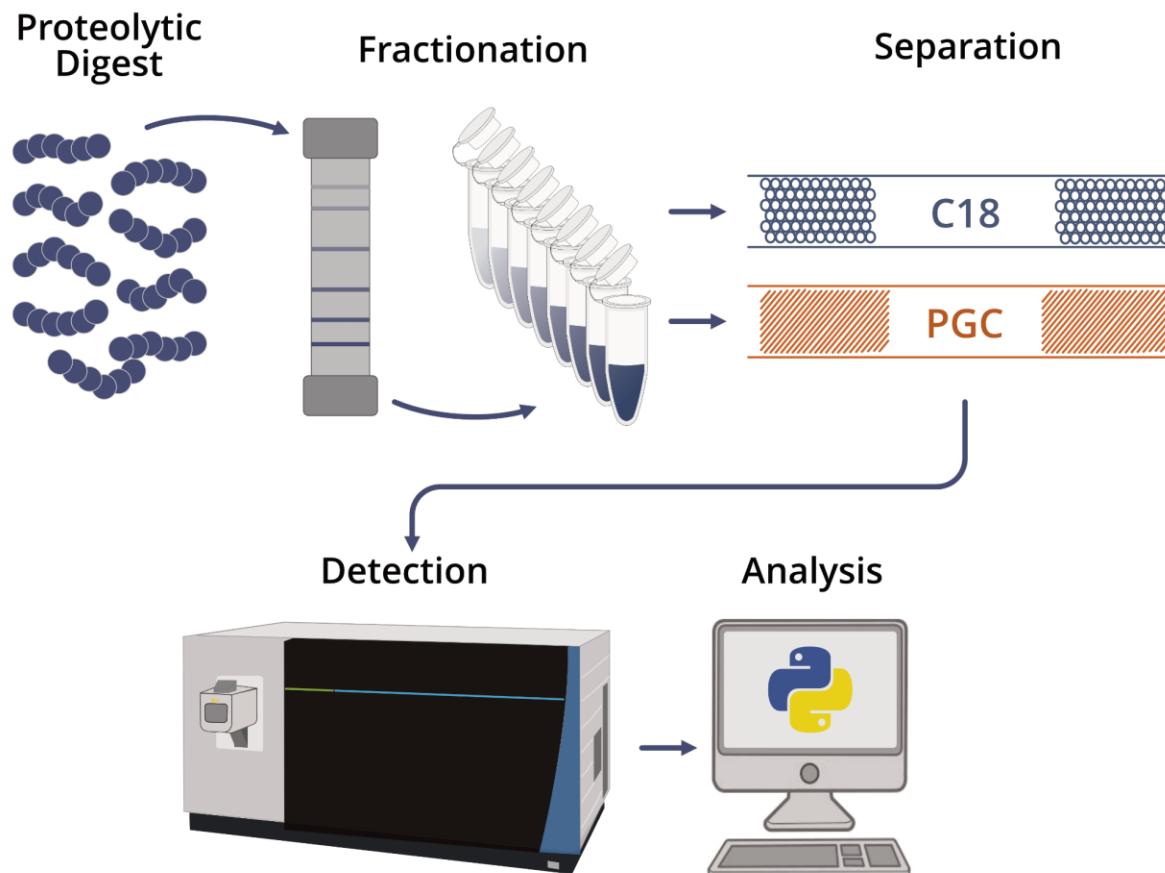
More Efficient Use of PGC-RPLC



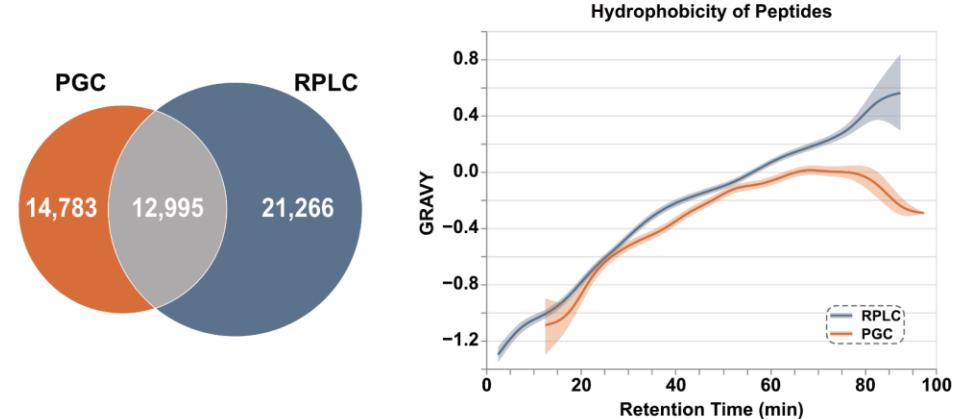
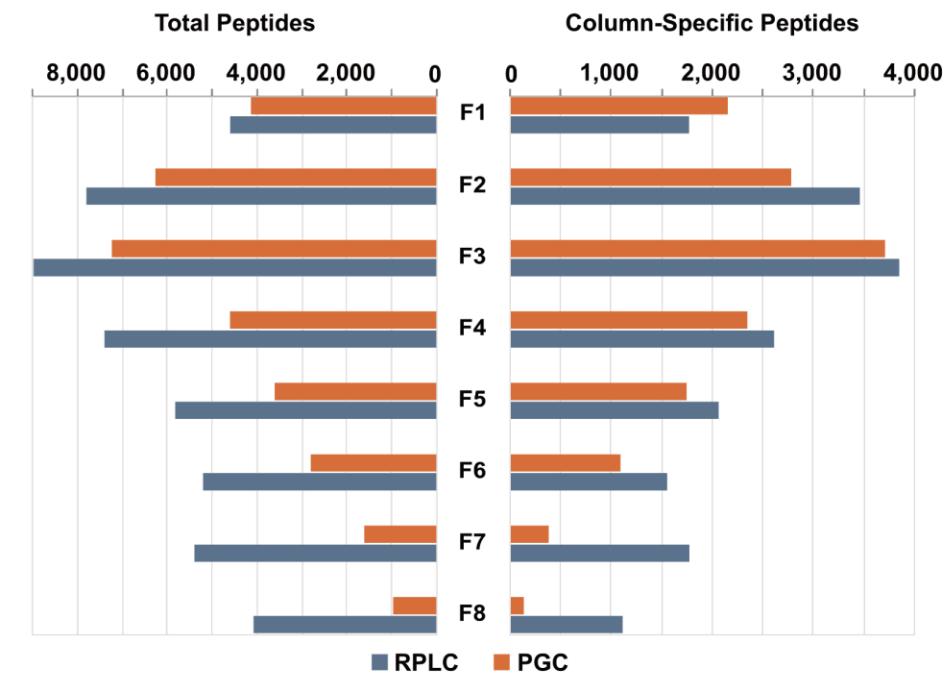
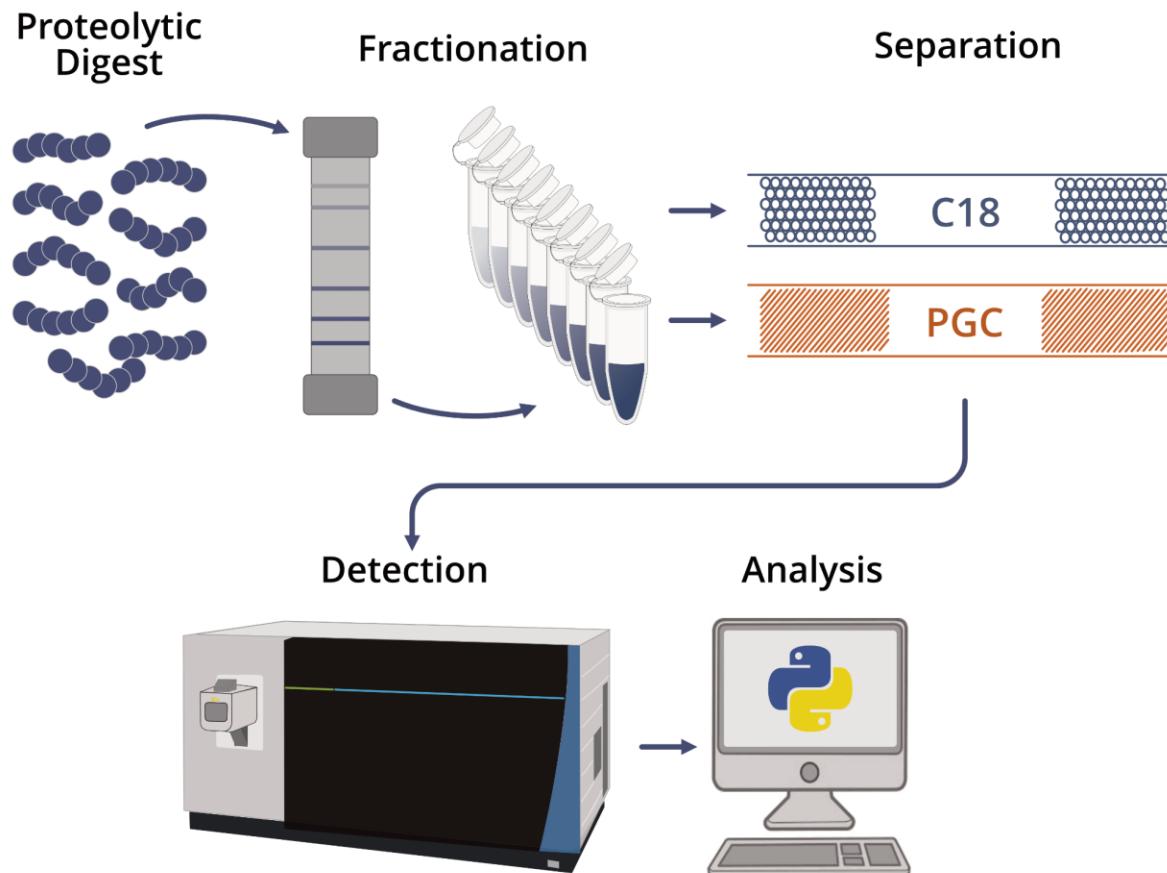
PGC is Competitive at Early Timepoints



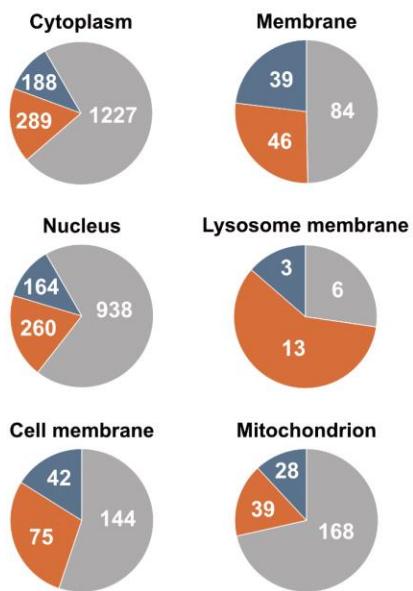
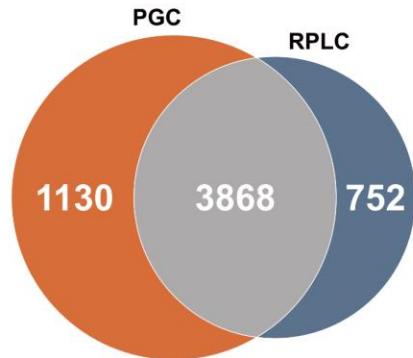
PGC is Competitive at Early Timepoints



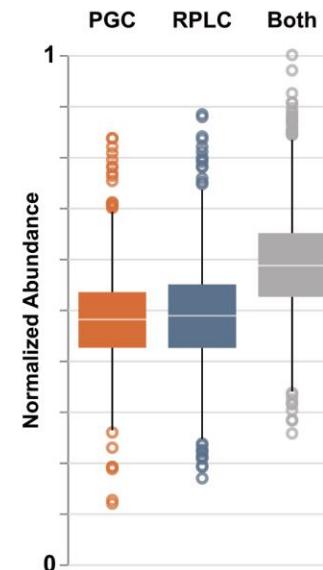
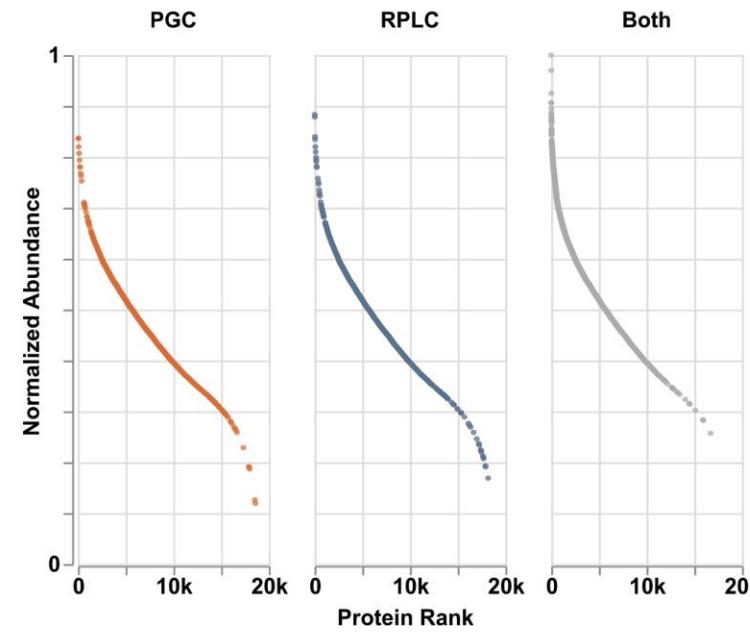
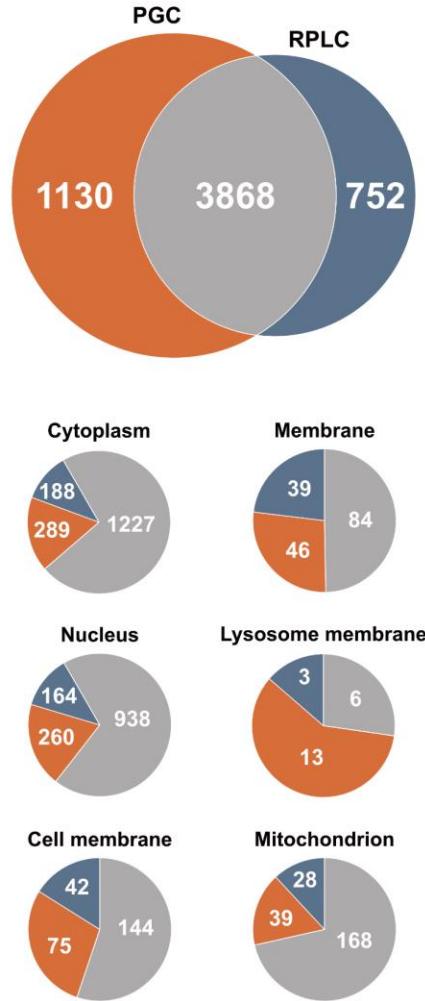
PGC is Competitive at Early Timepoints



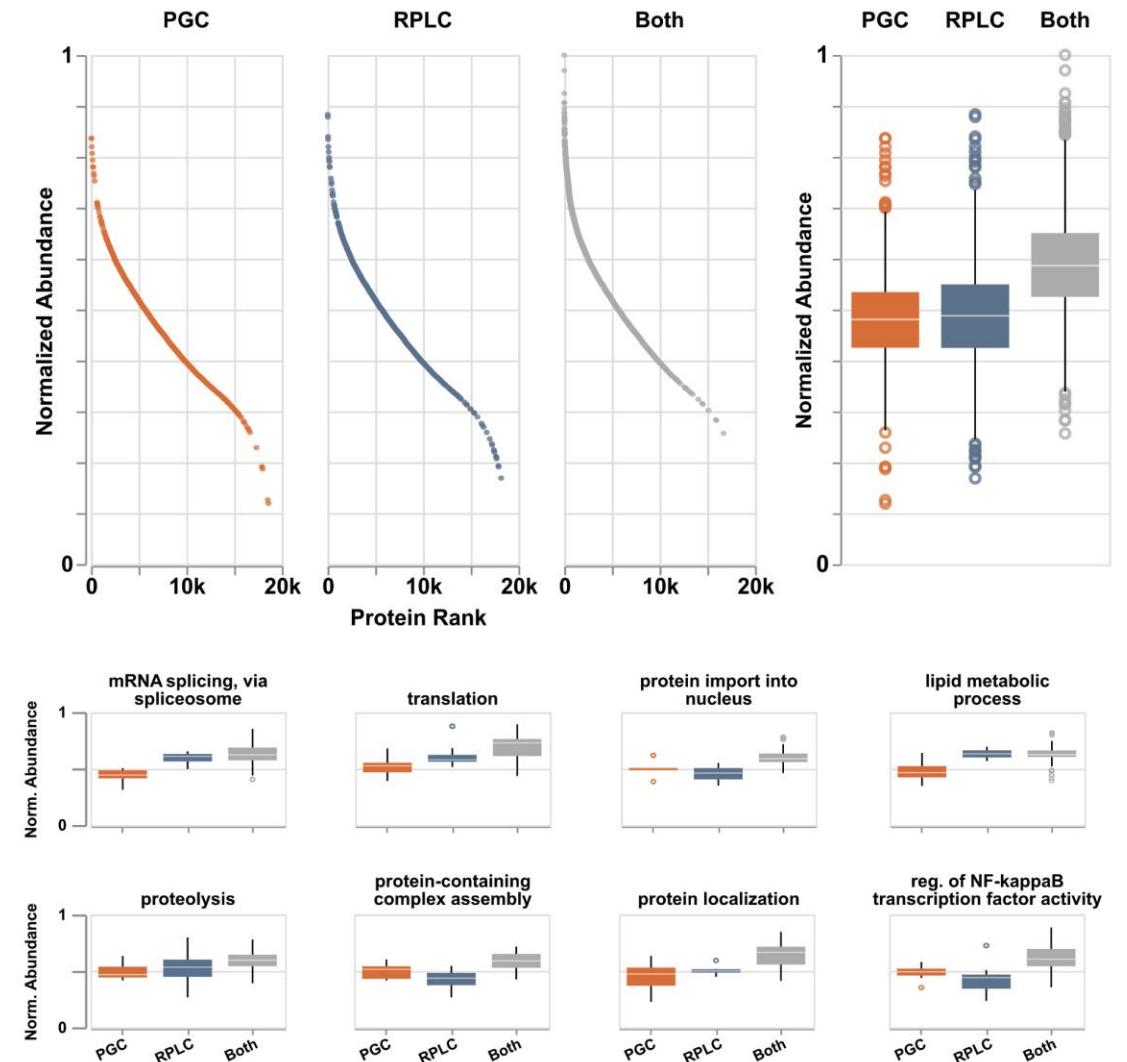
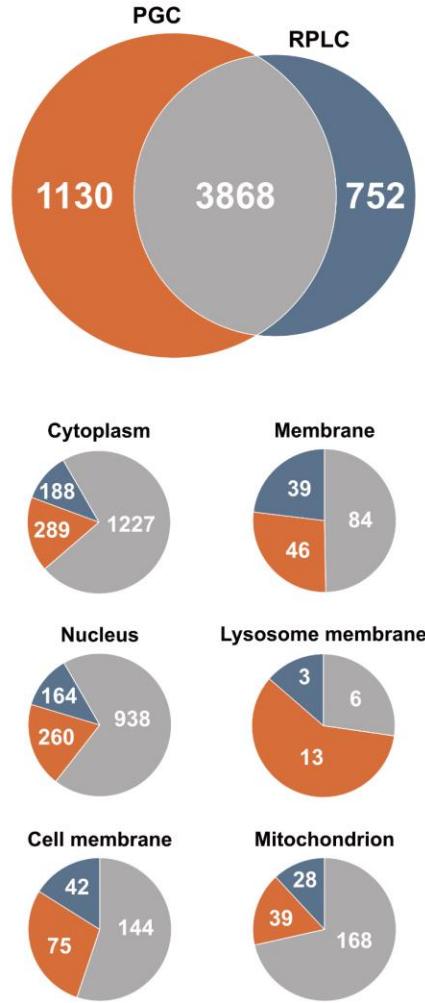
Greater Protein Identification, Regardless of Depth



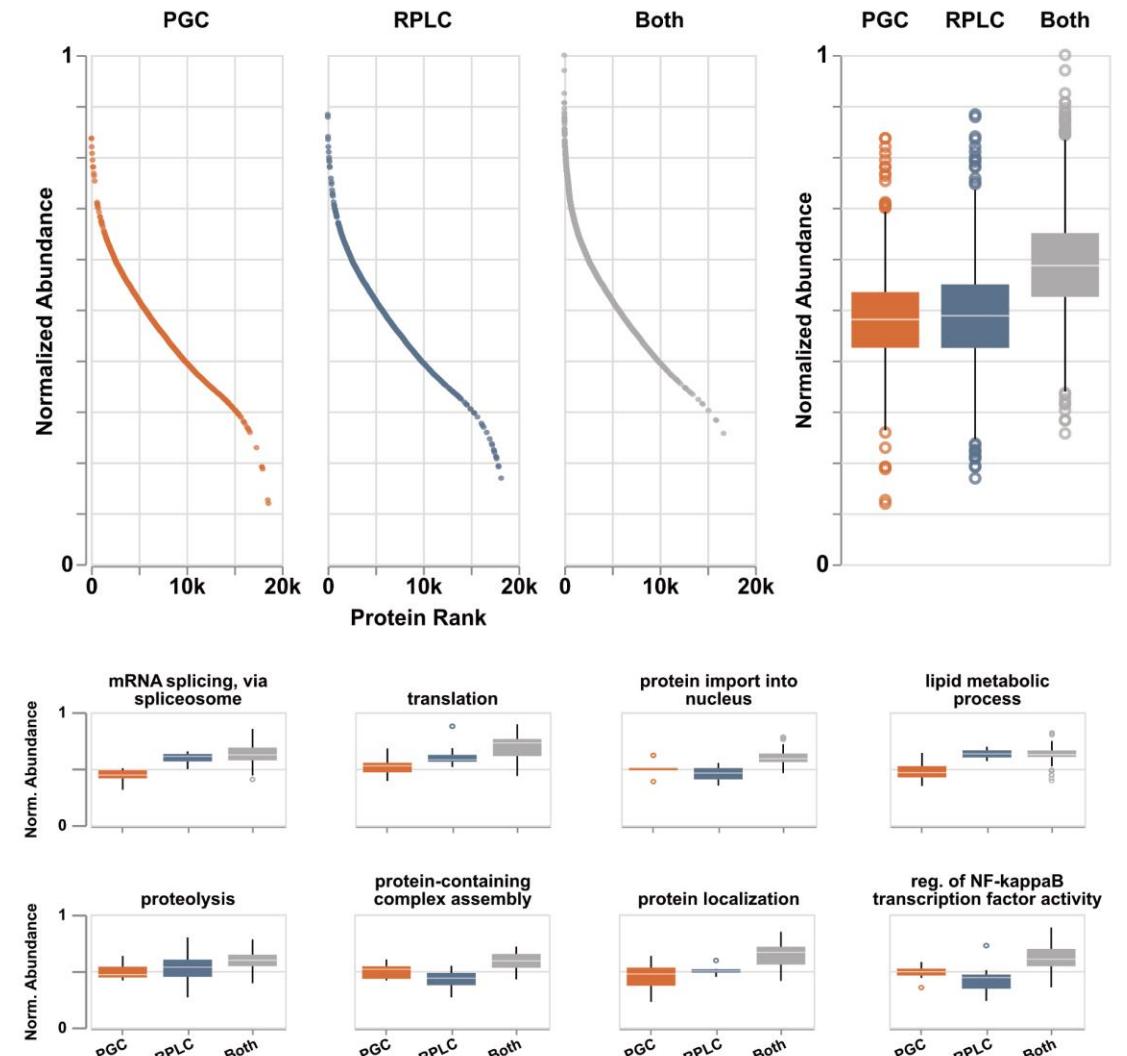
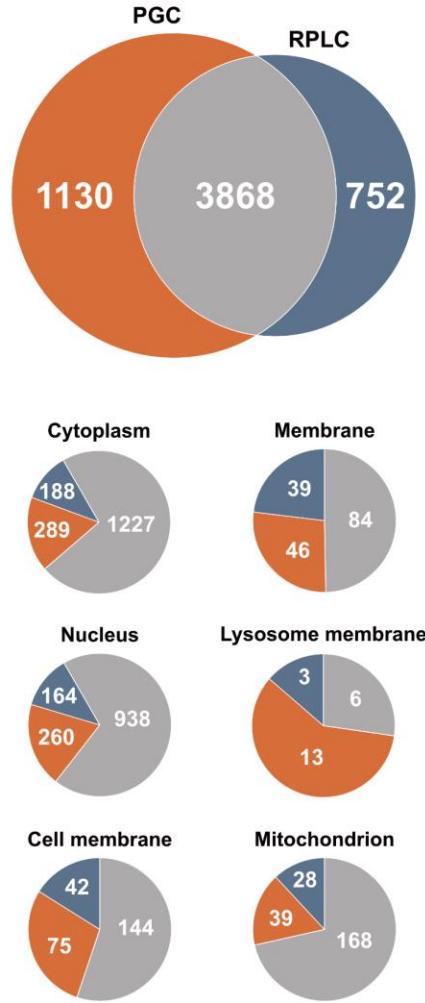
Greater Protein Identification, Regardless of Depth



Greater Protein Identification, Regardless of Depth



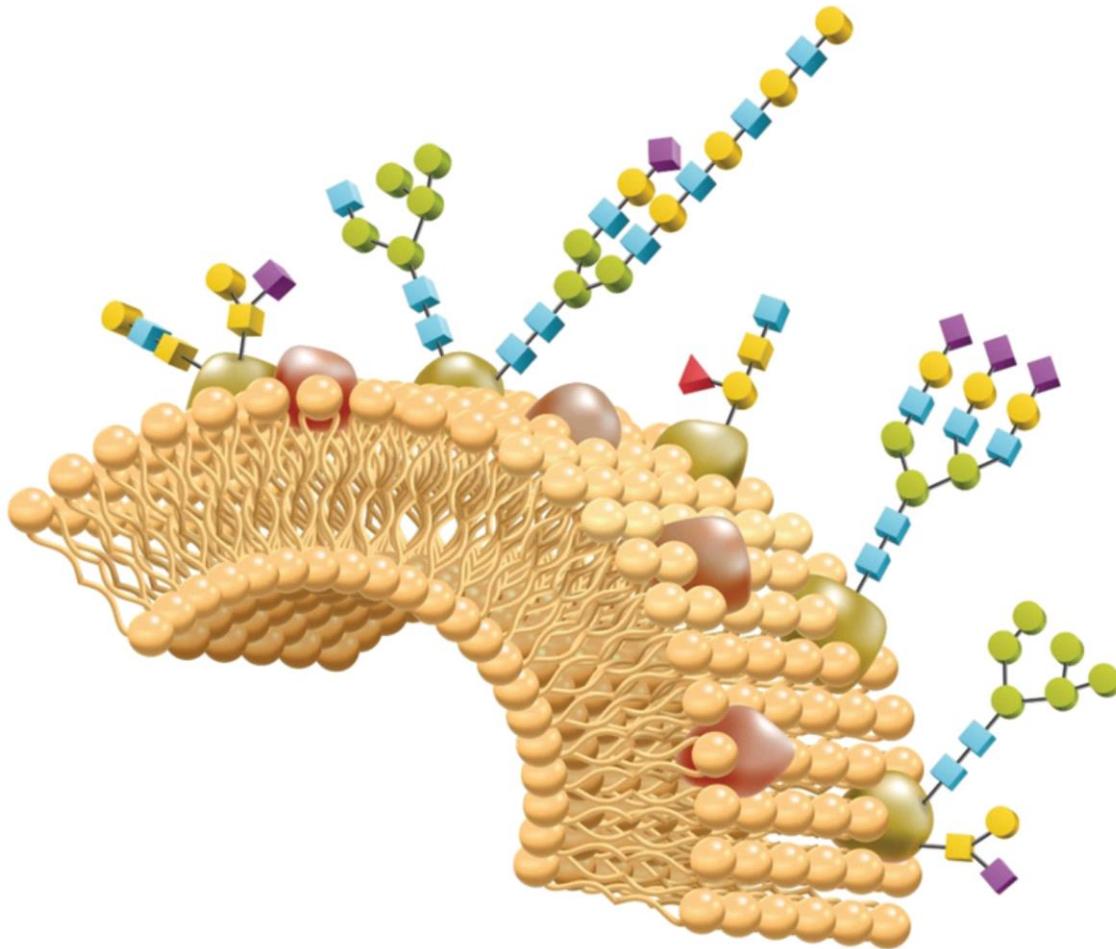
Greater Protein Identification, Regardless of Depth



PGC for Proteomics

- With optimization, PGC improves peptide and protein identification by up to 40%
- Complementary retention regardless of sample complexity
- Profiling breadth readily achieved, depth is on the horizon

Glycoproteomics



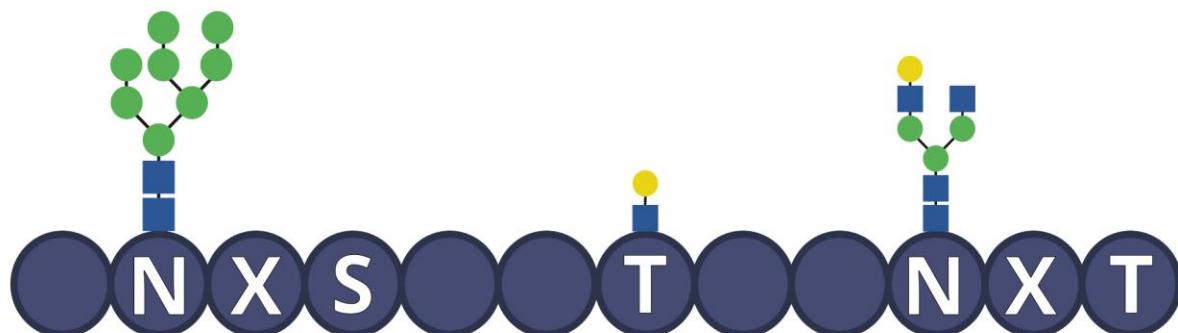
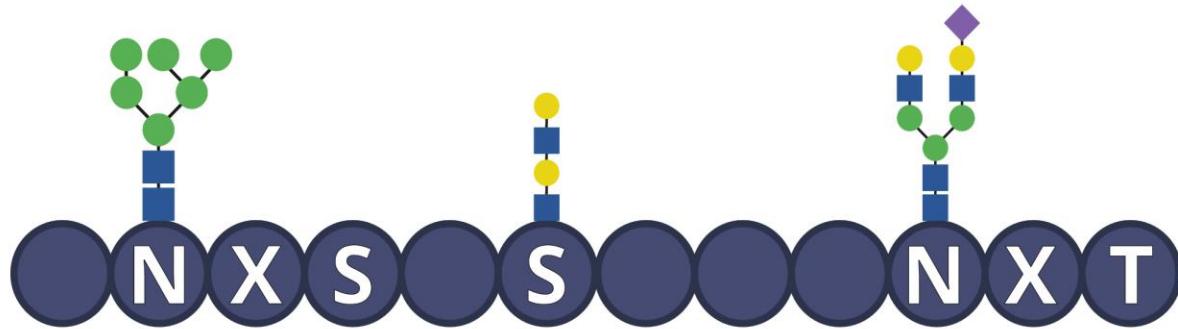
Function

Cellular communication and immune response
Intrinsic/extrinsic signaling pathways
Protein folding and viability

Disease

Target for pathogen invasion
Altered expression during disease propagation
Aberrant profiles across numerous disease

Glycoproteomics

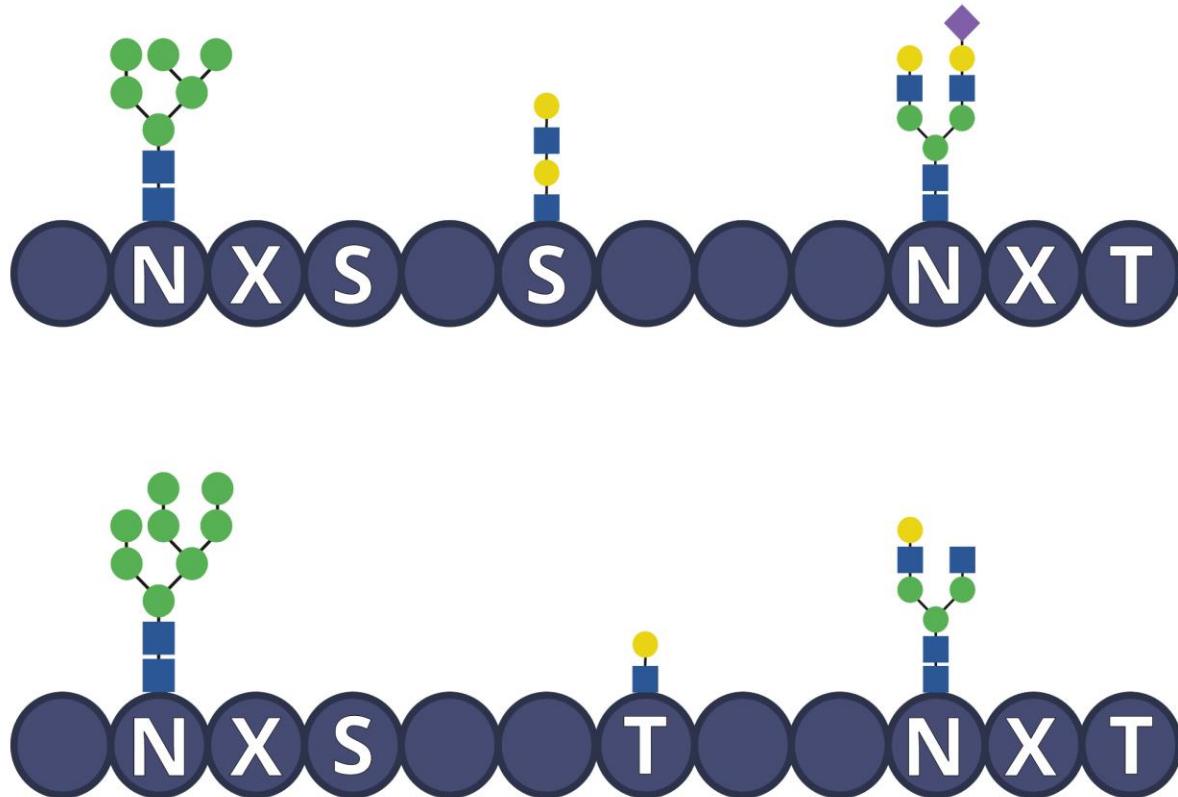


Biological

- **Class**
 - N-linked or O-linked
- **Composition**
 - 12 commonly occurring monosaccharides
- **Structure**
 - α/β orientation
 - Connectivity

Challenges

Glycoproteomics



Challenges

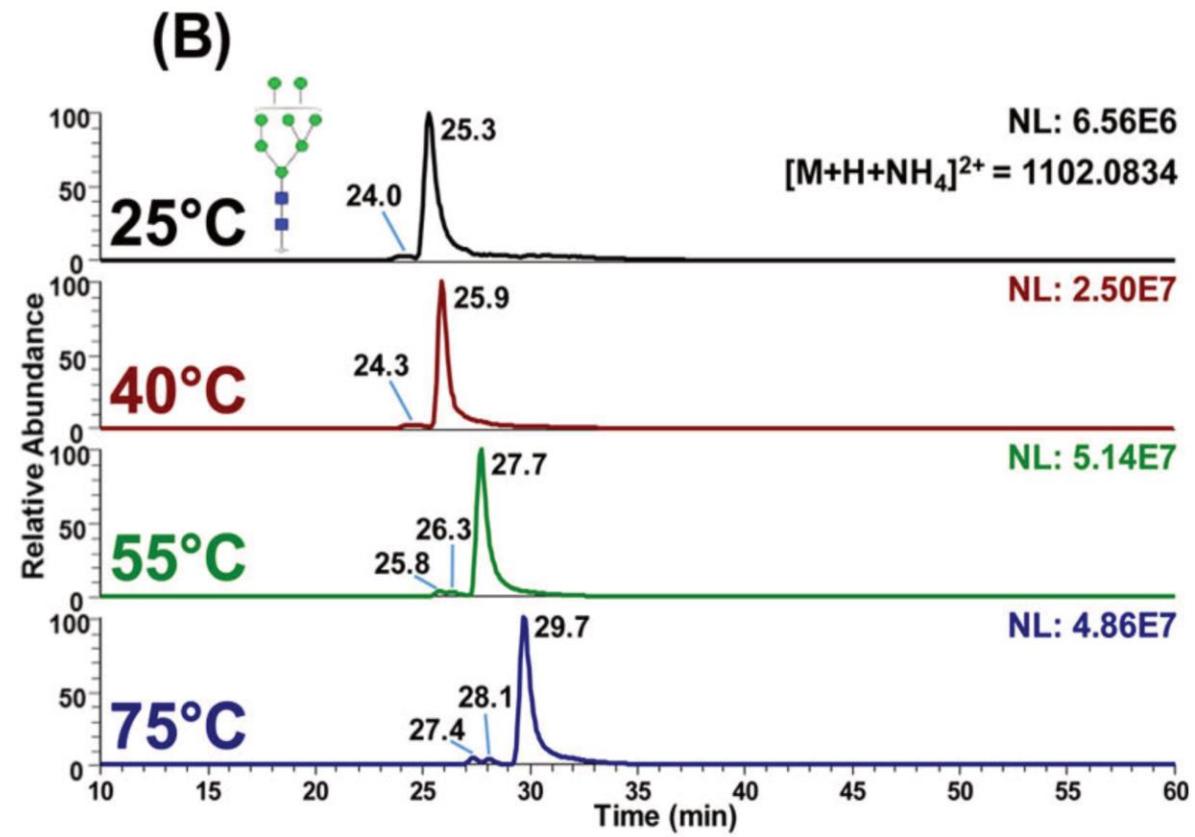
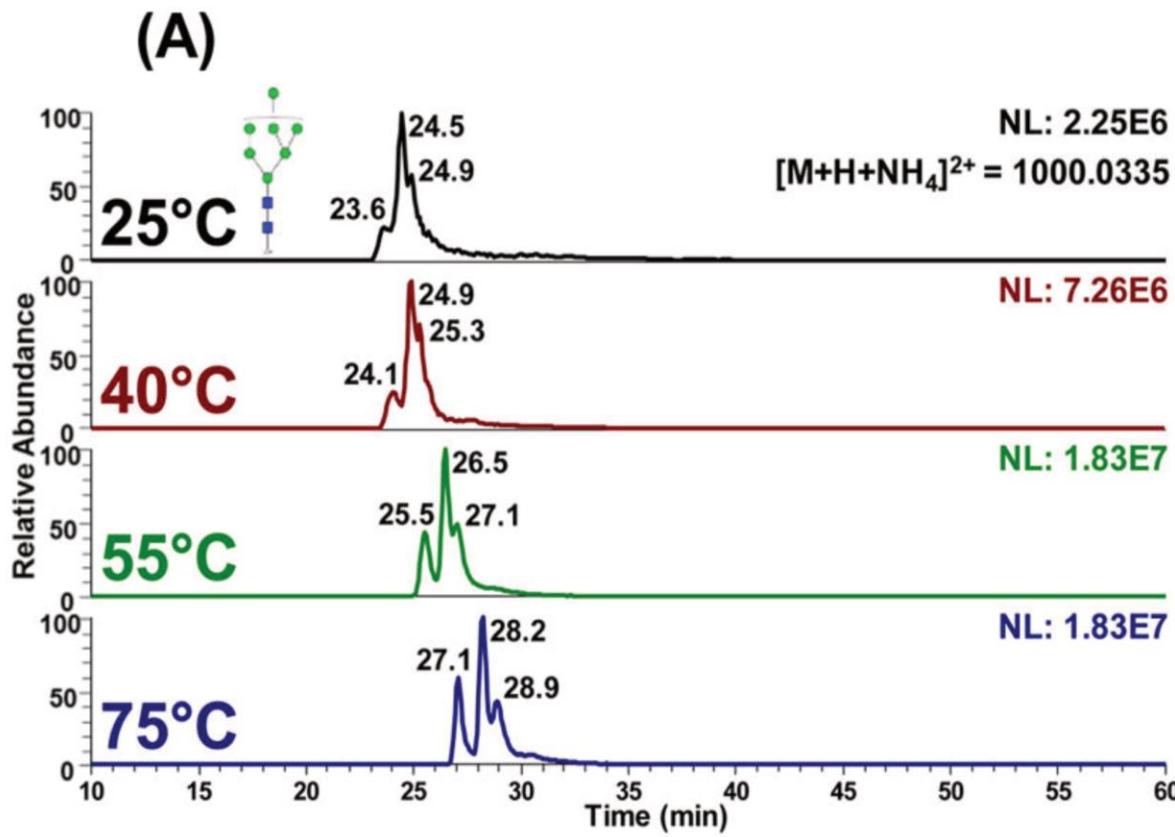
Biological

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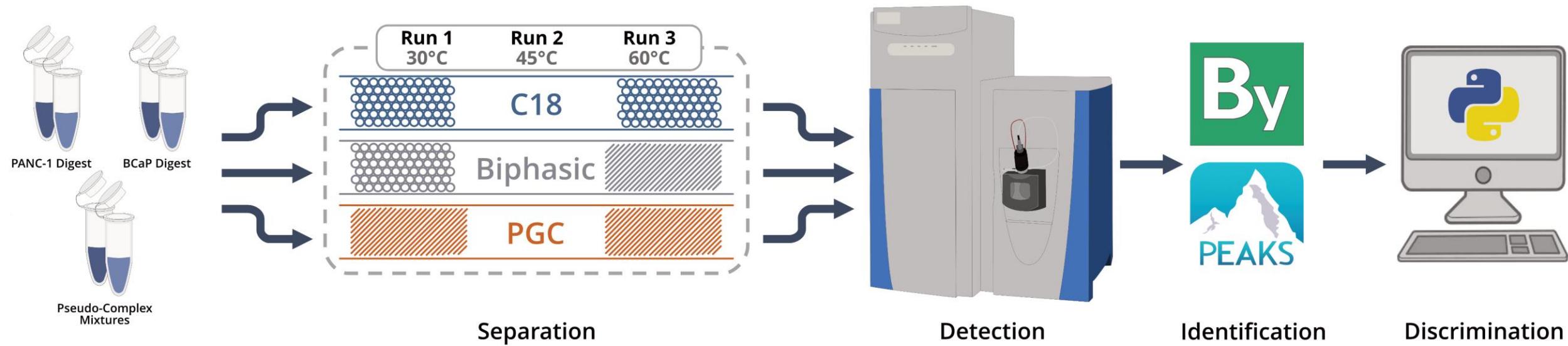
Analytical

- **Retention**
 - Hydrophilic moiety
- **Charge suppression**
 - Outcompeted by hydrophobic, basic peptides
- **Heterogeneity**
 - Site occupation
 - Structure

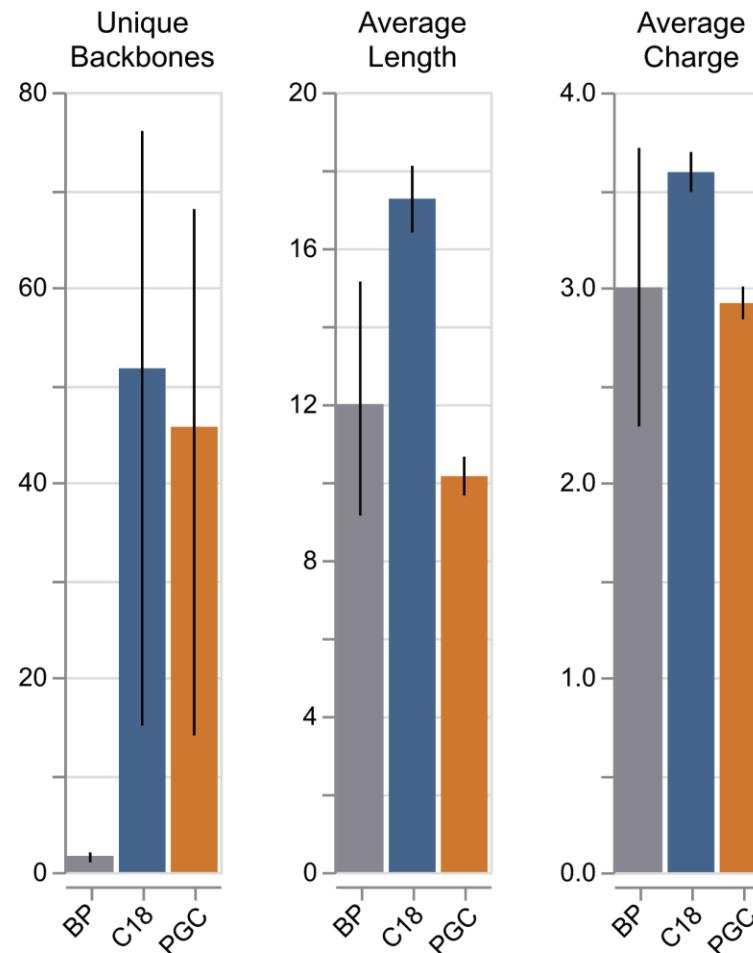
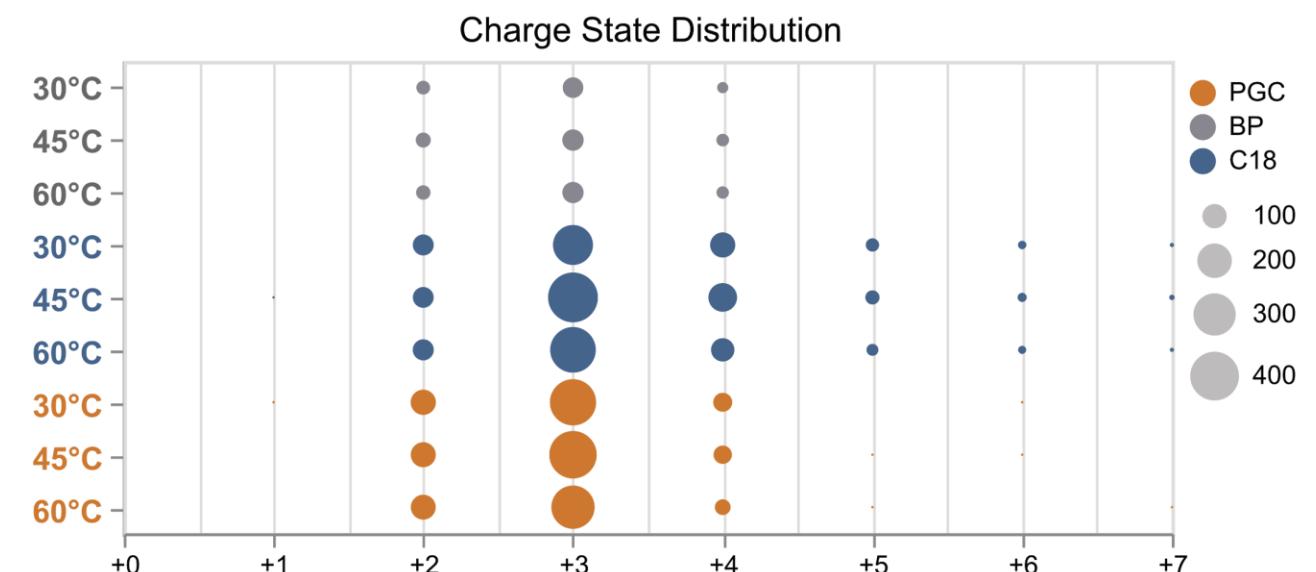
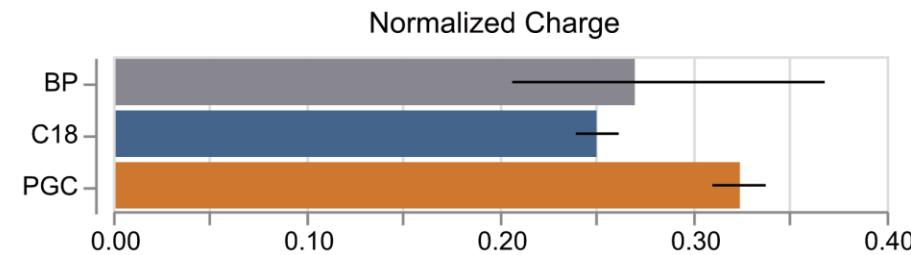
PGC for Glycoproteomics



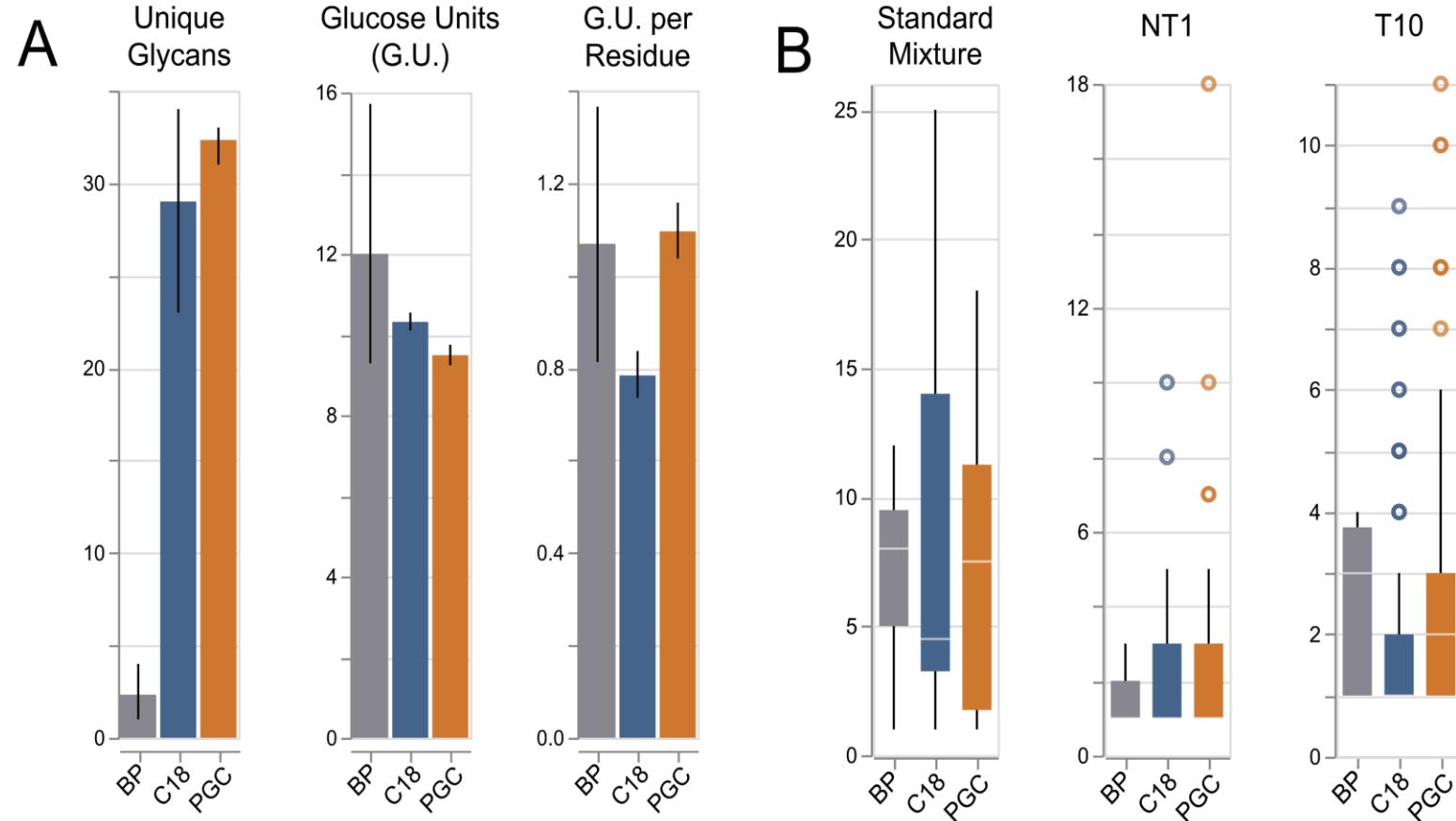
PGC-Based Shotgun Proteomics



Glycopeptide Character

A**B****C**

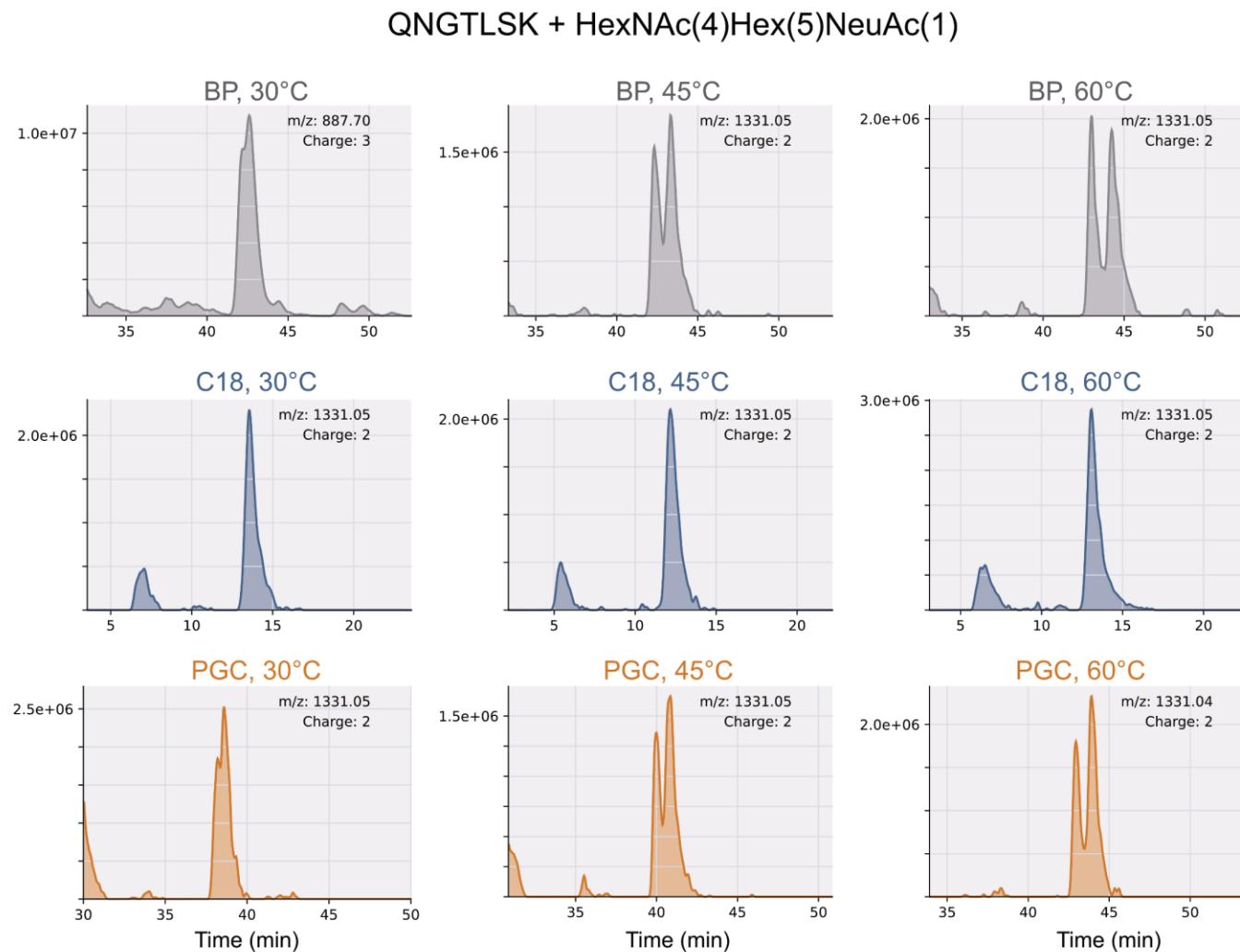
Glycopeptide Character



Outcomes

- PGC complements RPLC separations, providing access to the hydrophilic portion of the glycoproteome
- Selective retention reduces charge state suppression
- PGC improved microheterogeneity recognition

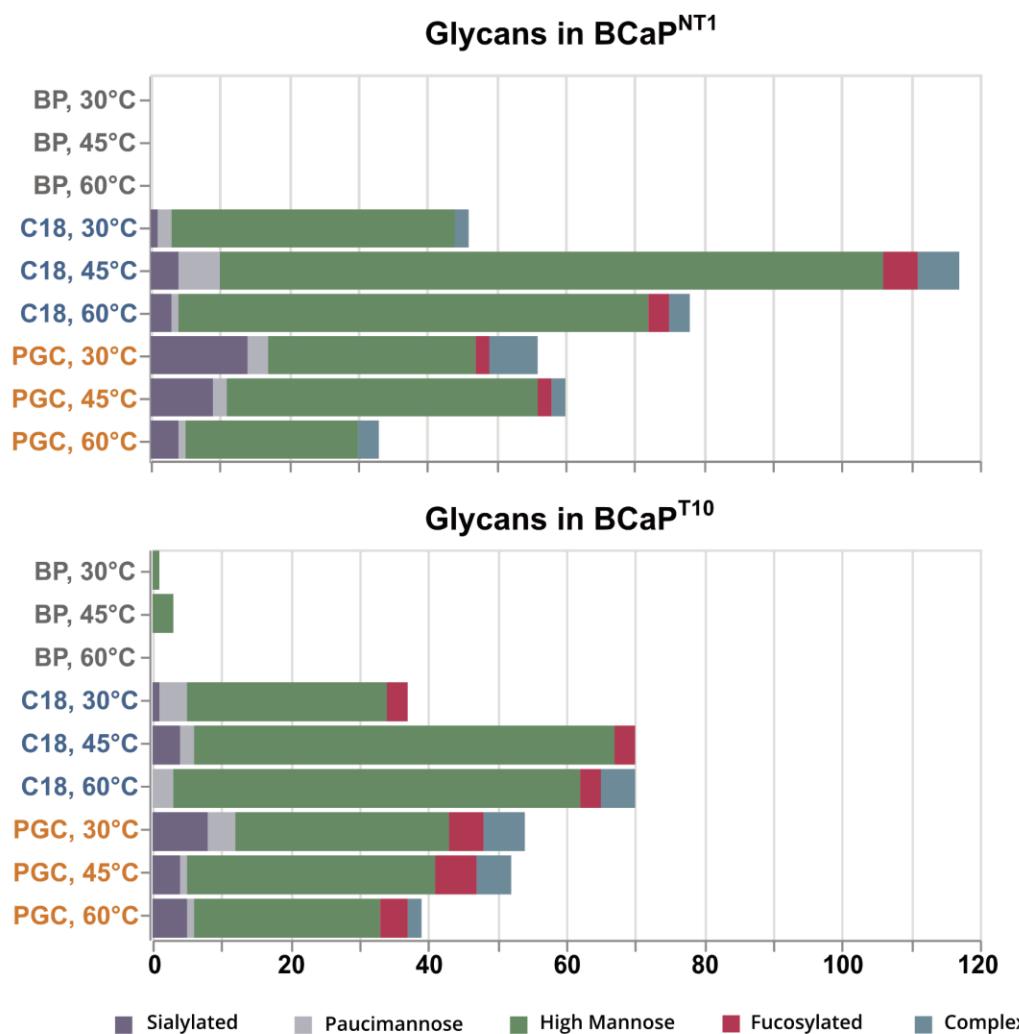
Isomeric Separation



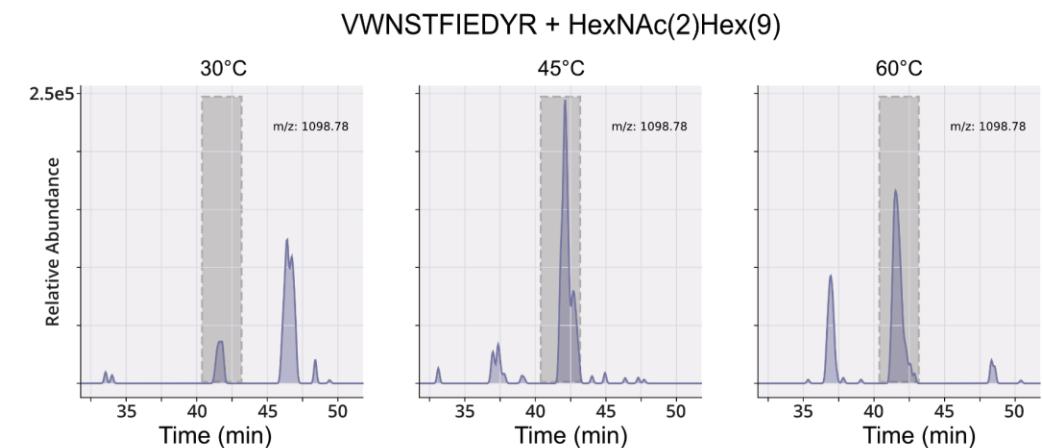
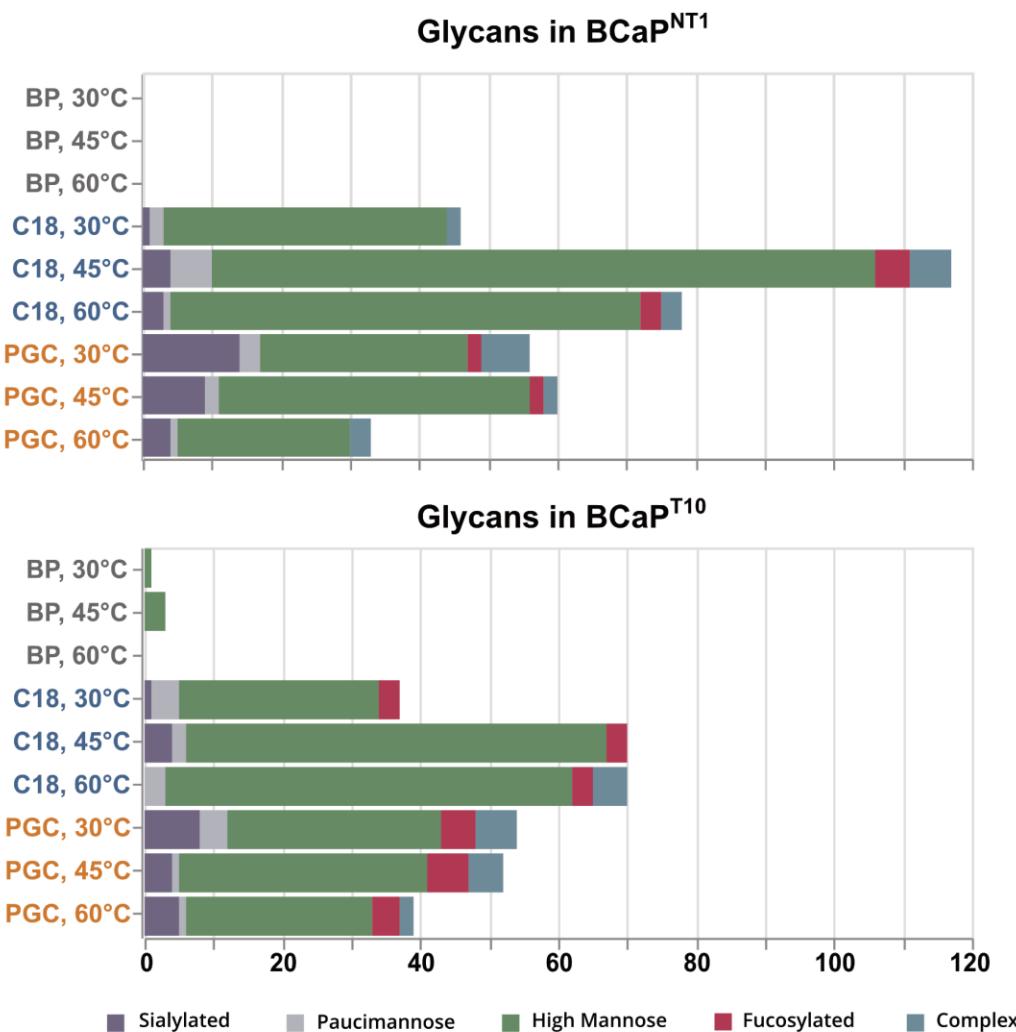
Outcomes

- PGC complements RPLC separations, providing access to the hydrophilic portion of the glycoproteome
- Selective retention reduces charge state suppression
- PGC improved microheterogeneity recognition
- PGC demonstrates temperature-dependent liquid-phase resolution - can reveal isomeric species.

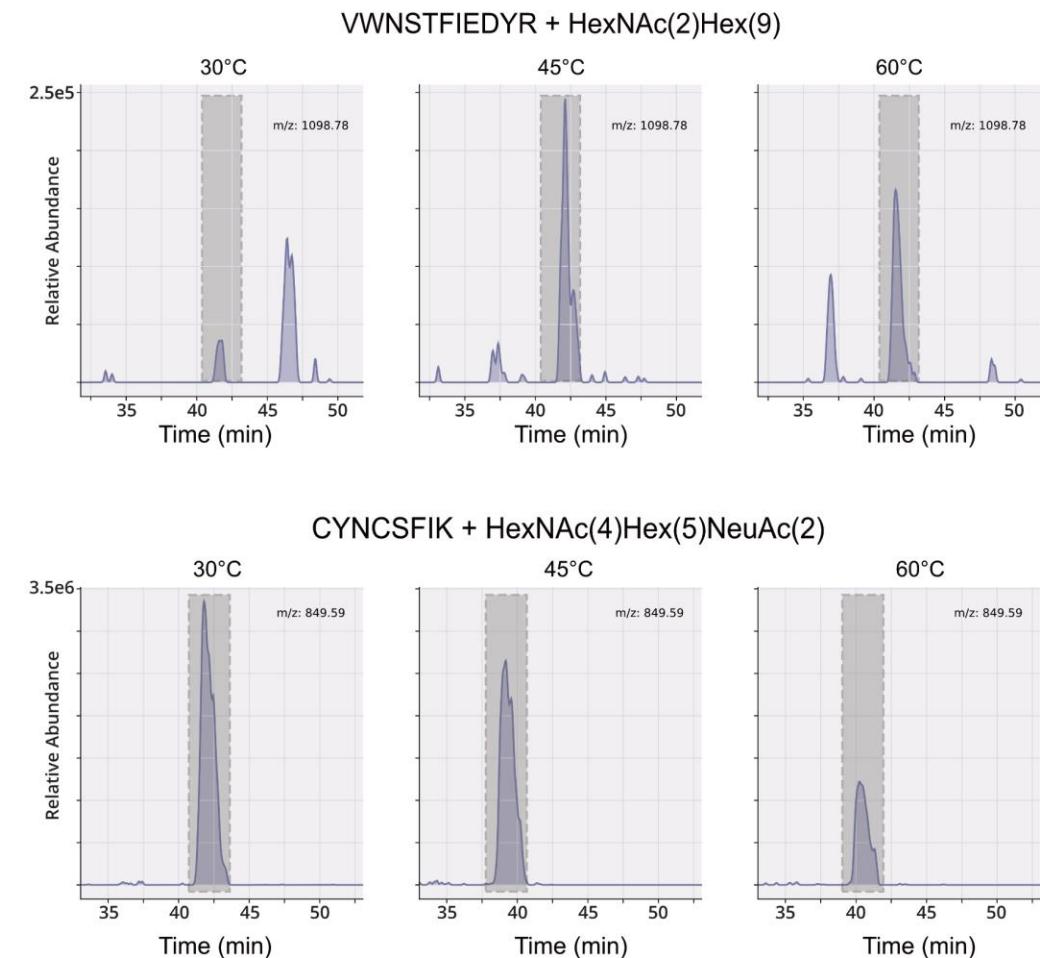
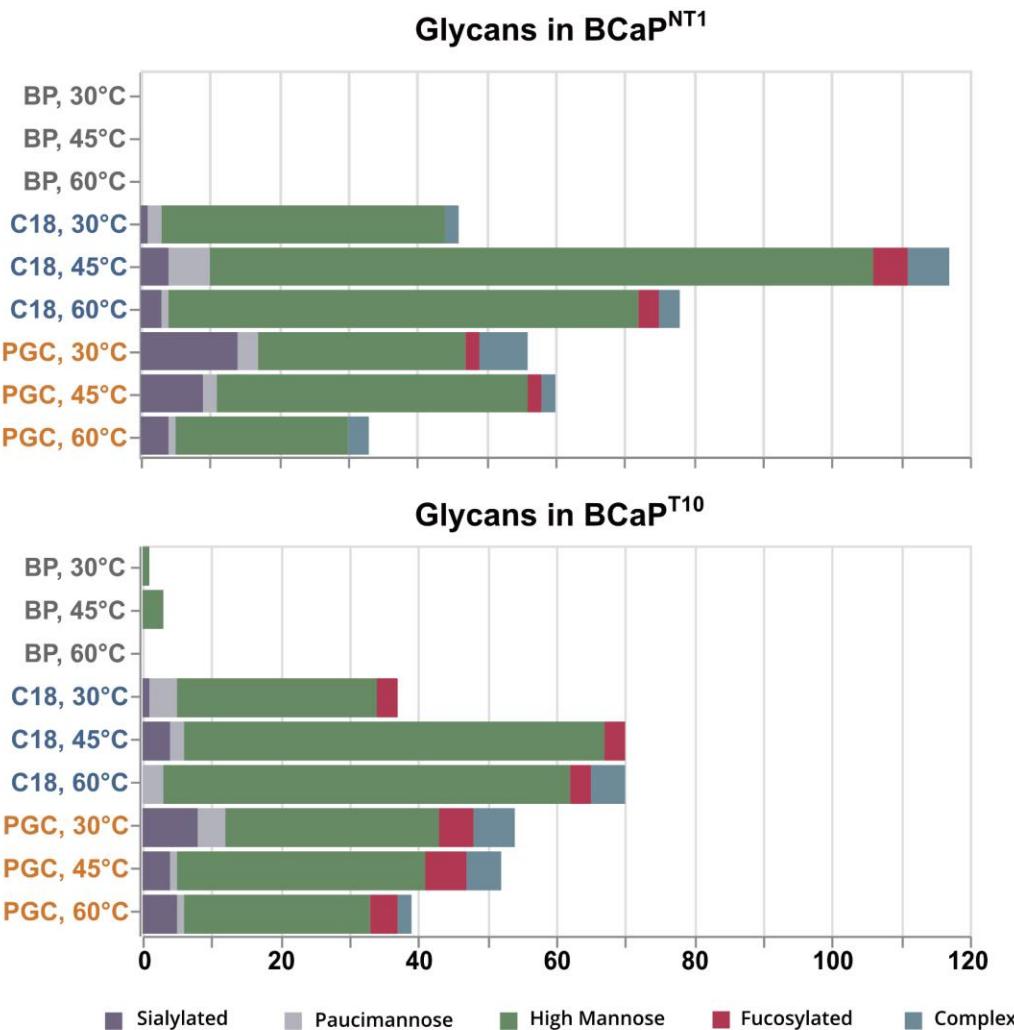
Temperature Affects Glycopeptide Identifications



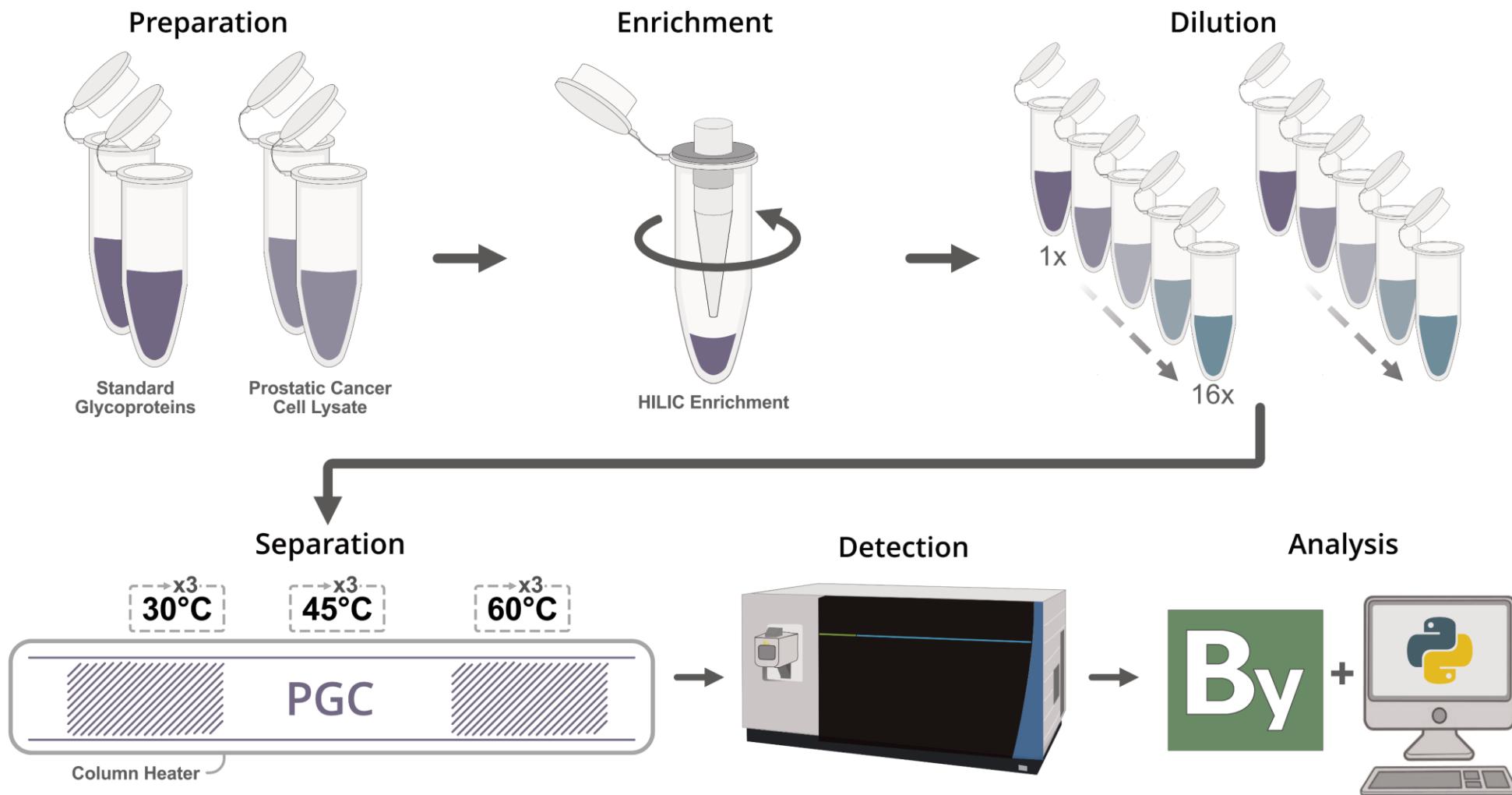
Temperature Affects Glycopeptide Identifications



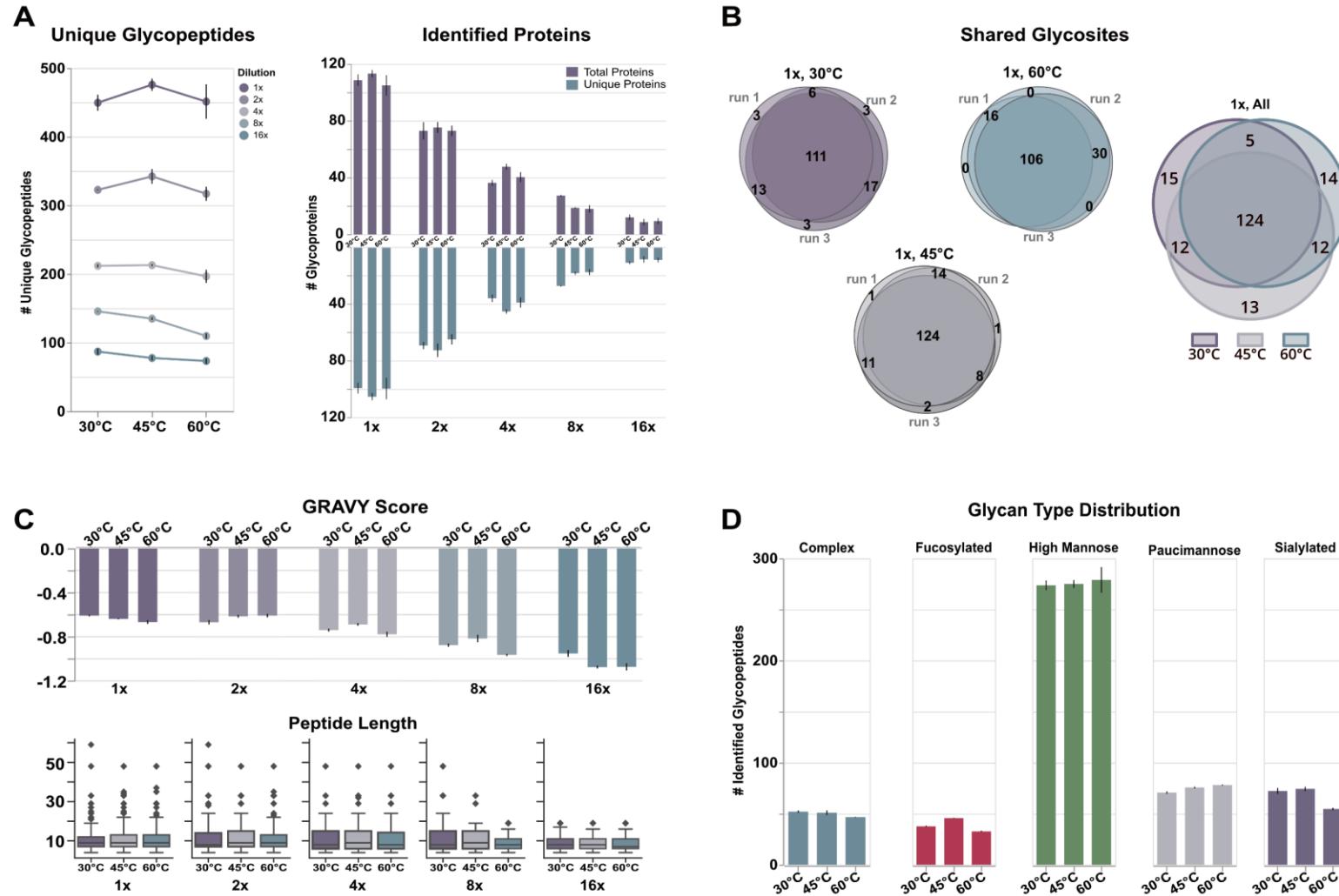
Temperature Affects Glycopeptide Identifications



Investigating Temperature-based Aberrations



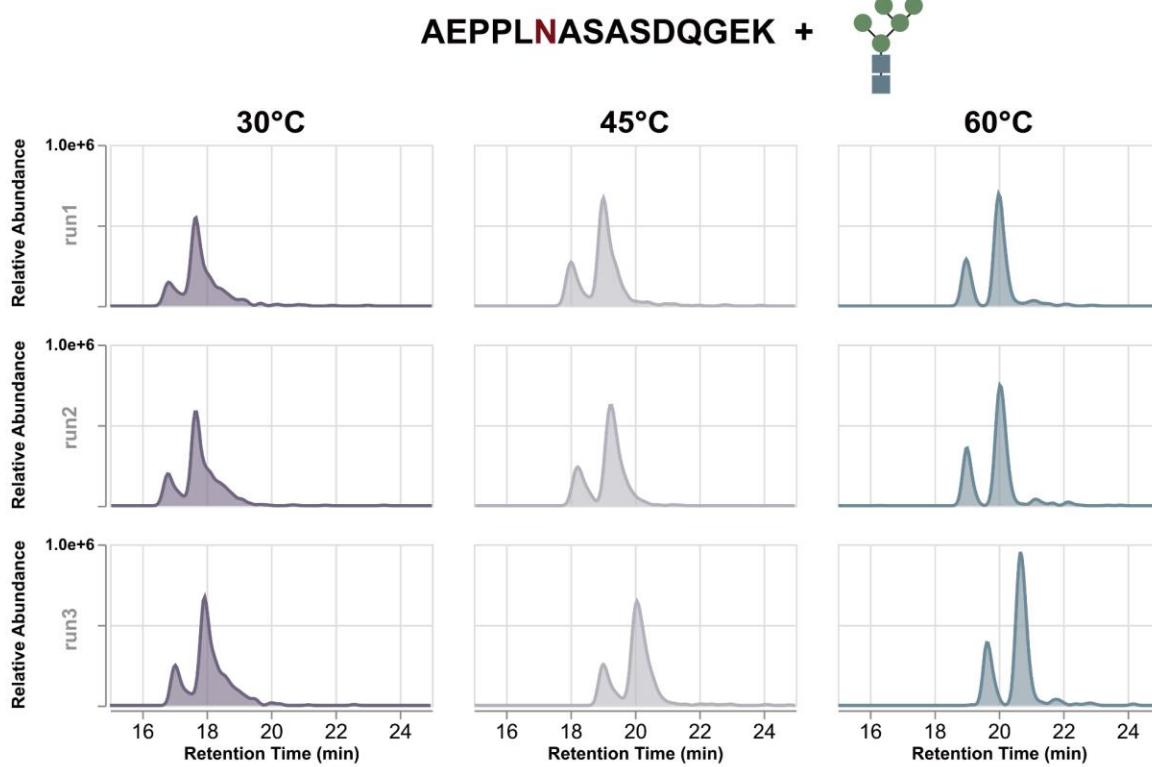
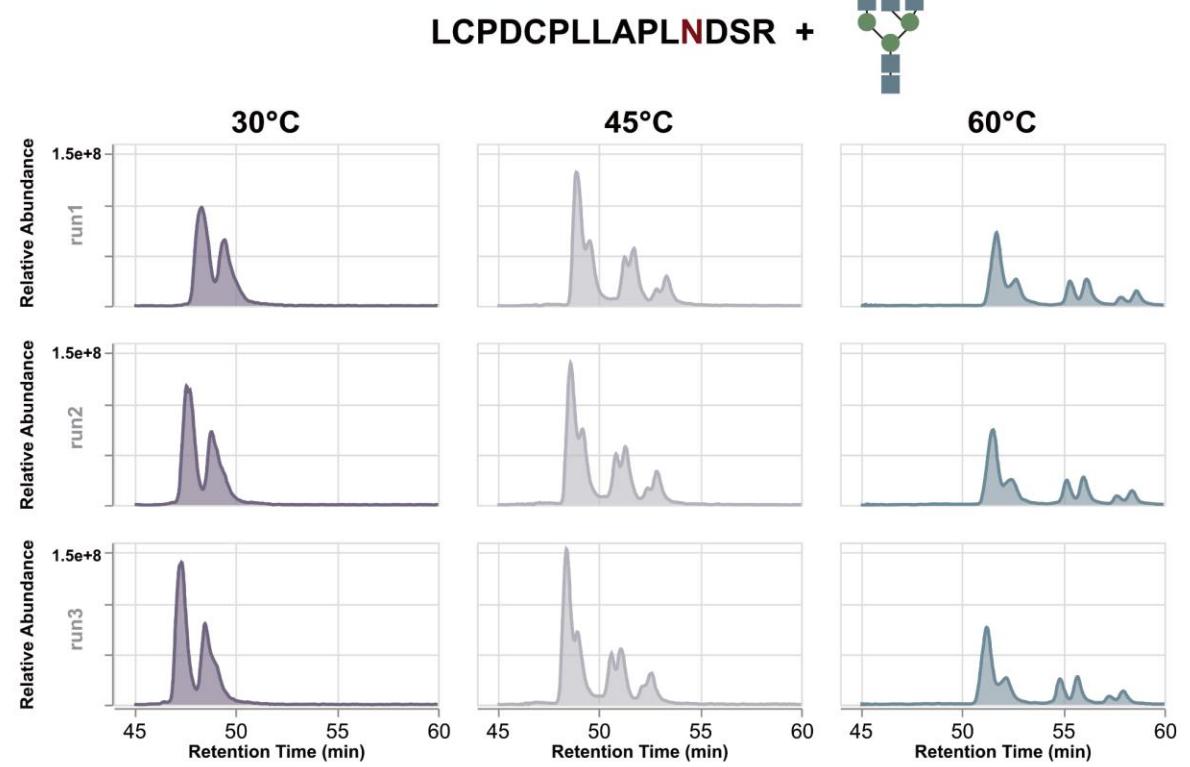
Investigating Temperature-based Aberrations



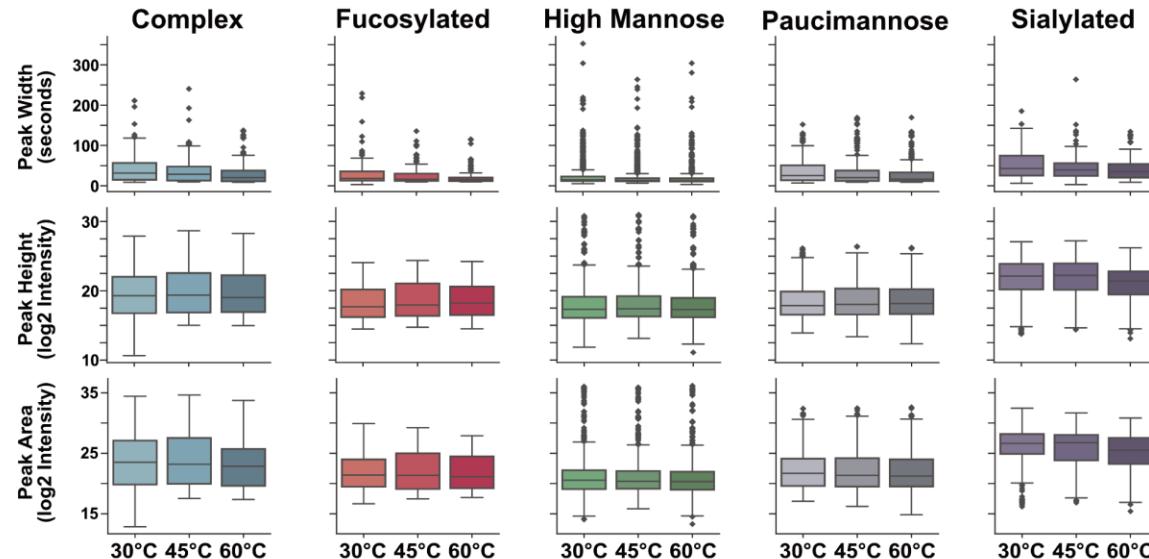
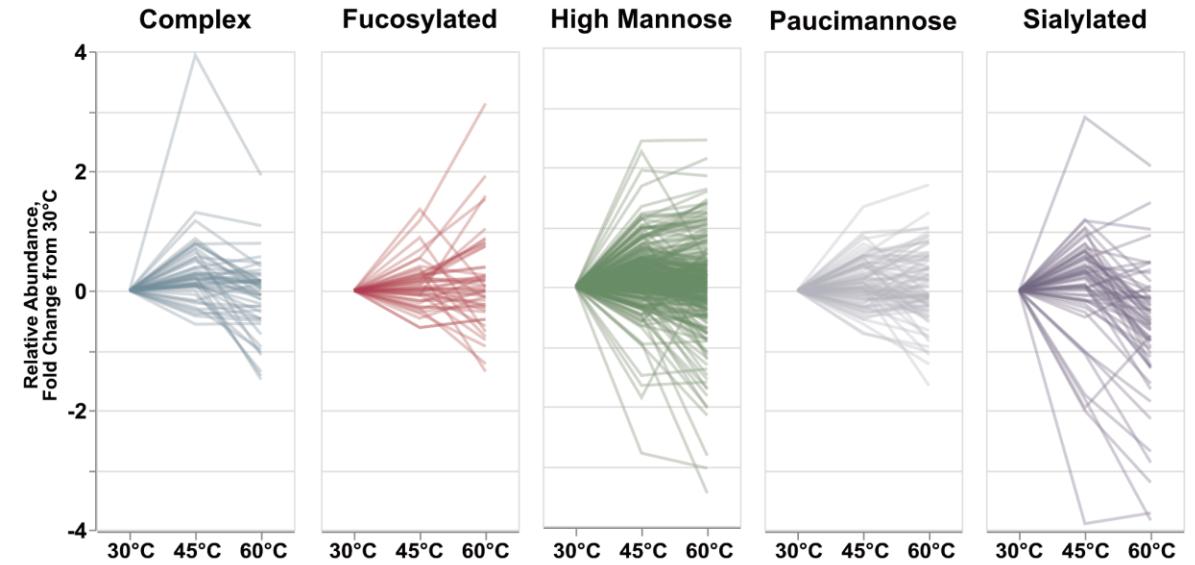
Complications

- Data rearticulate identification improvements at 45°C and drop off at 60°C
- Analytical variation and heterogeneity are not a factor
- Mannose type glycopeptides seem to 'survive' at high temperatures, others do not

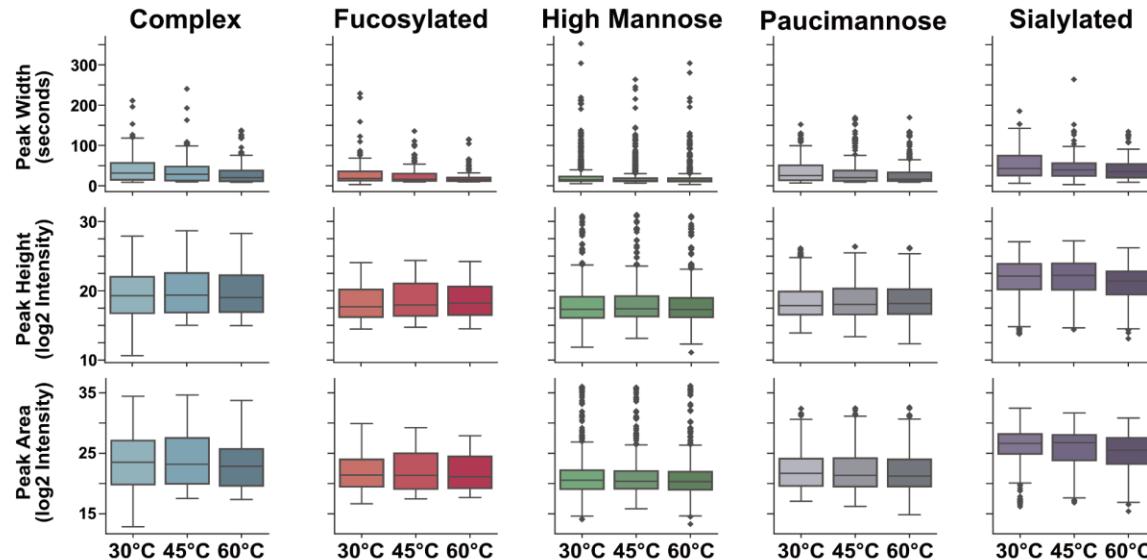
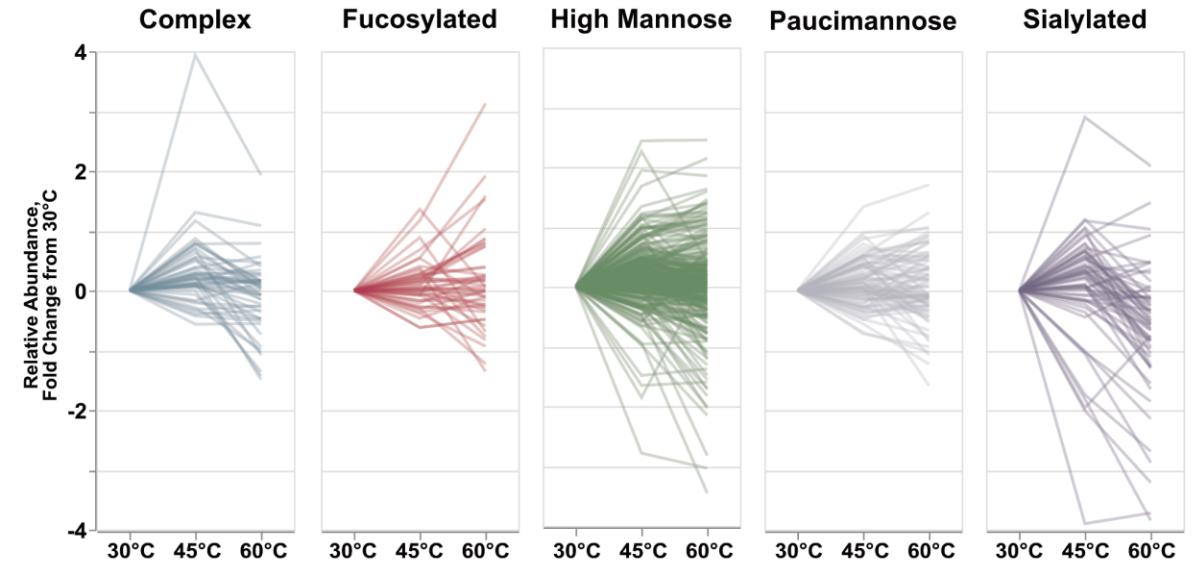
Temperature Inspired Chromatographic Changes

A**B**

Resolution vs. Signal Intensity

A**B**

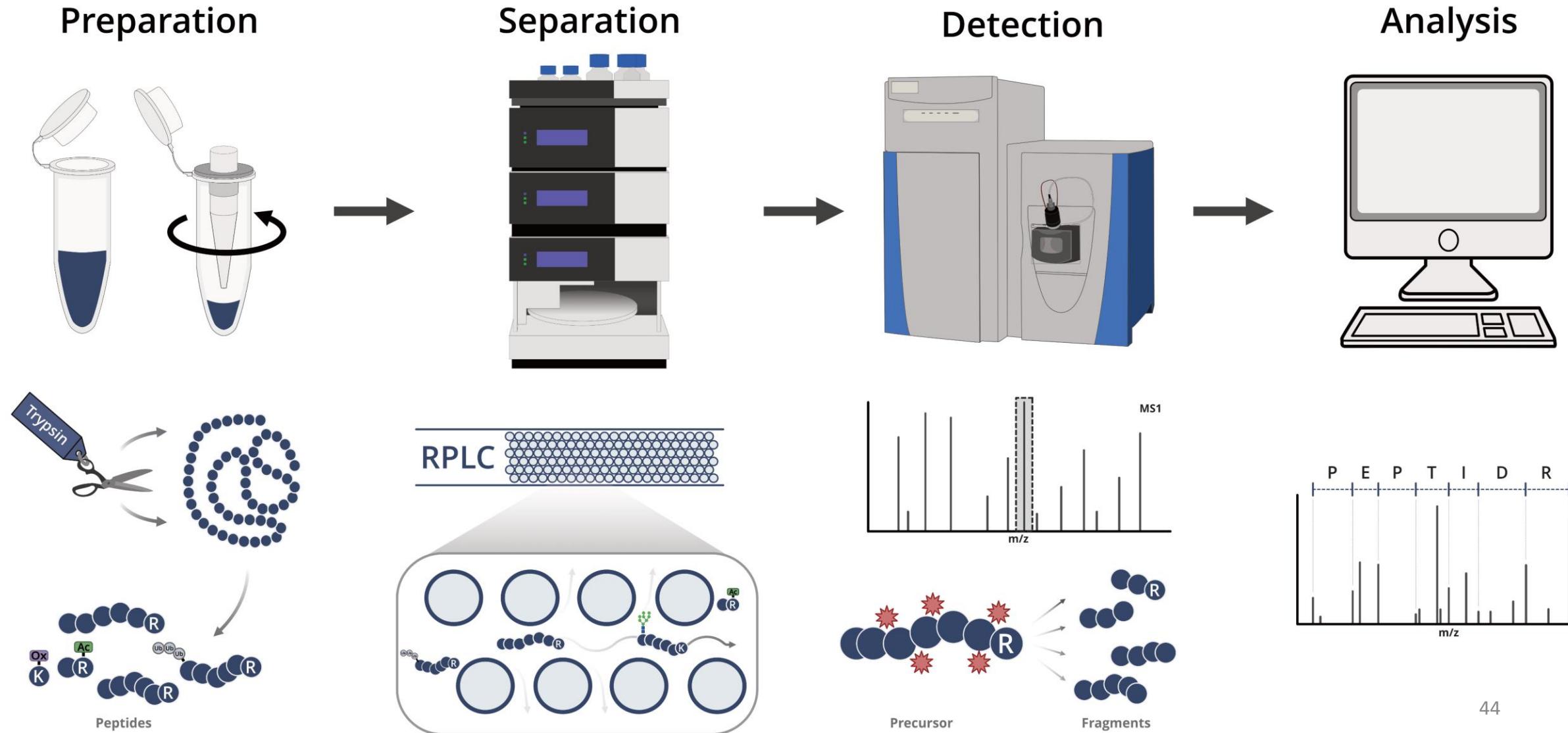
Resolution vs. Signal Intensity

A**B**

Conclusions

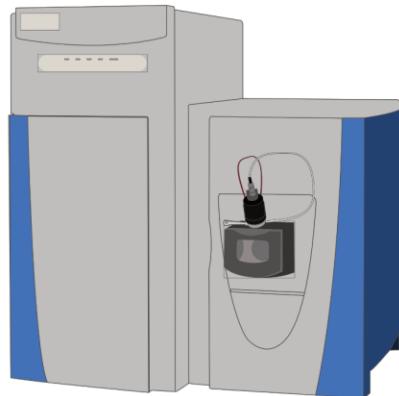
- PGC separations demonstrate great utility in glycopeptide analyses, but must be tailored to experimental goals
- Glycopeptide discovery is most efficient at 45°C where reporting signal is highest
- Isomeric separation is best at $\geq 60^\circ\text{C}$ but adversely affects labile glycan moieties

MS-Based Proteomics

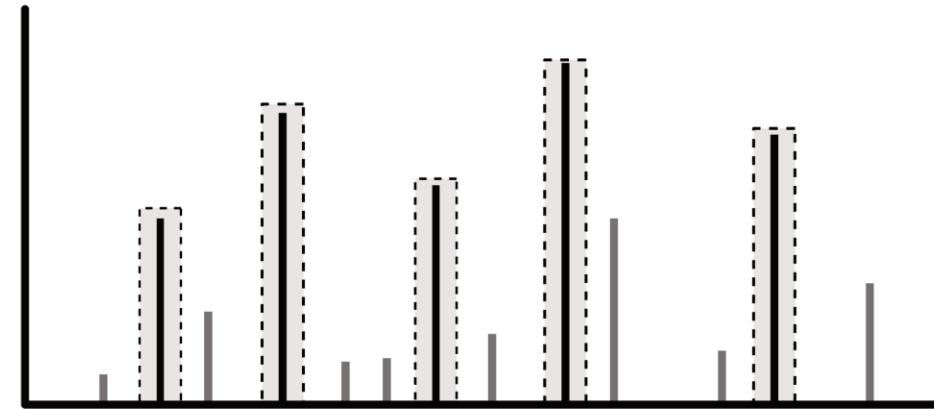


Dependent vs. Independent Acquisition

Detection

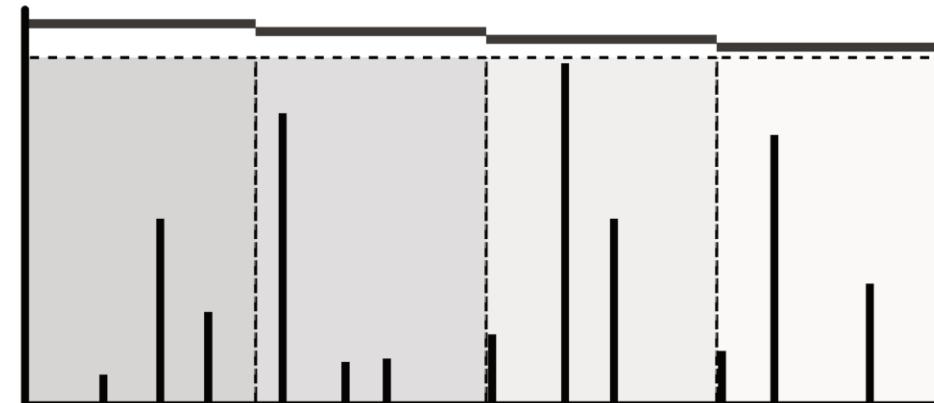


Data-Dependent Acquisition (DDA)

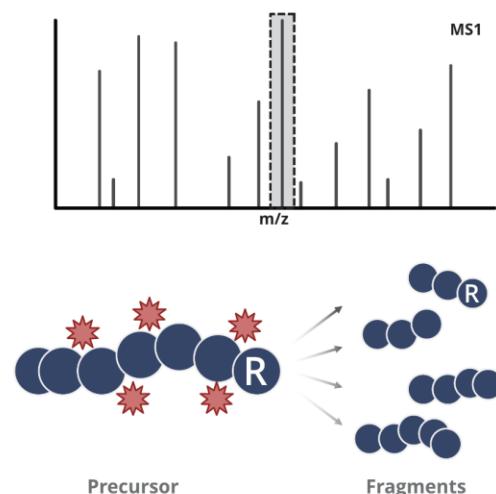


Confident and clean
but biased towards
highly-abundant
analytes

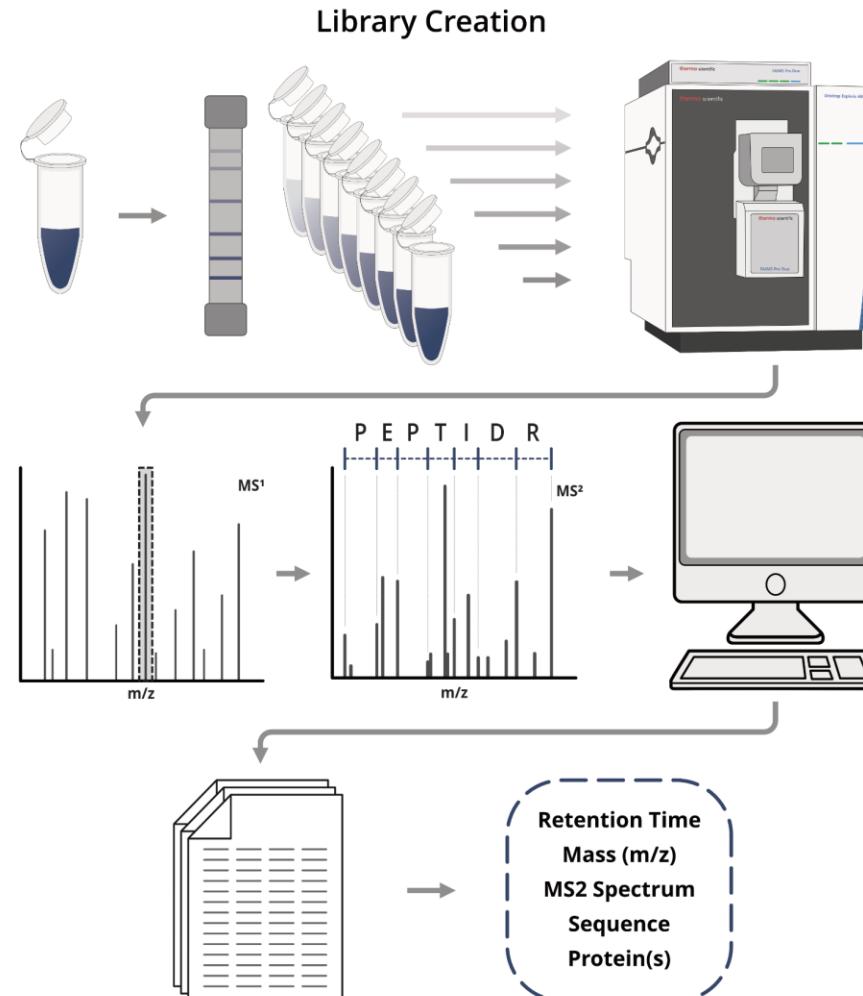
Data-Independent Acquisition (DIA)



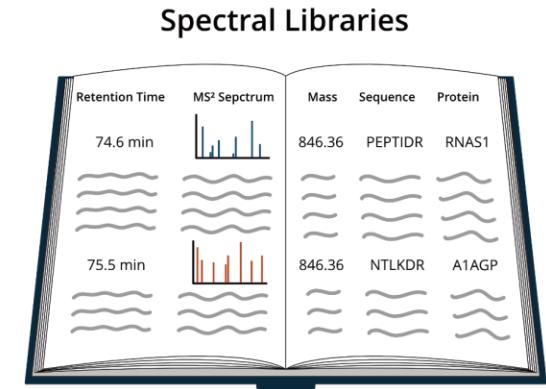
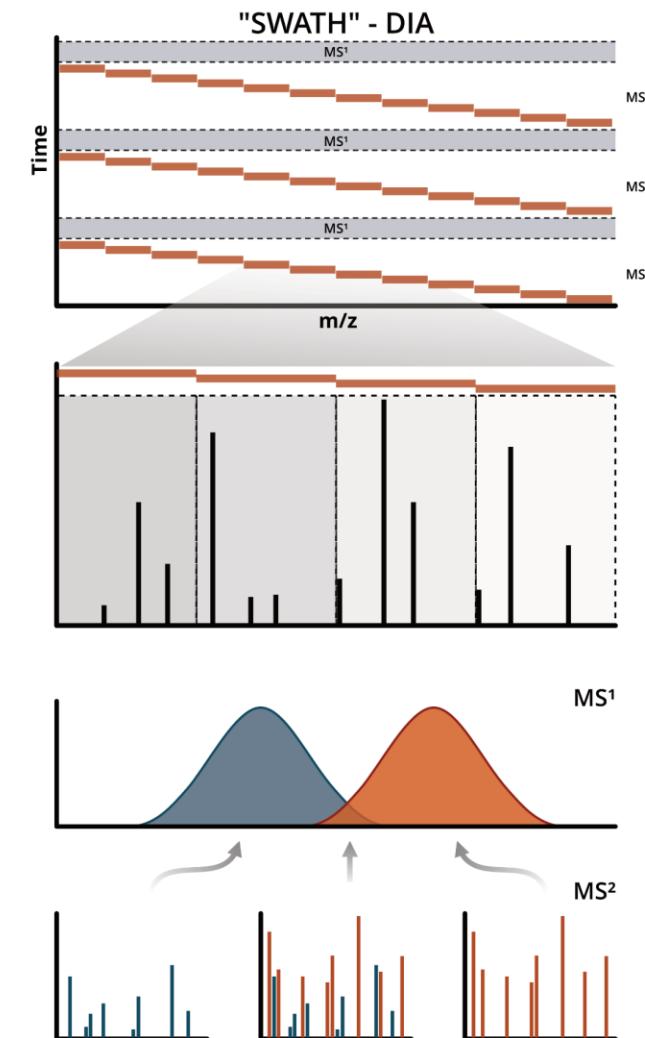
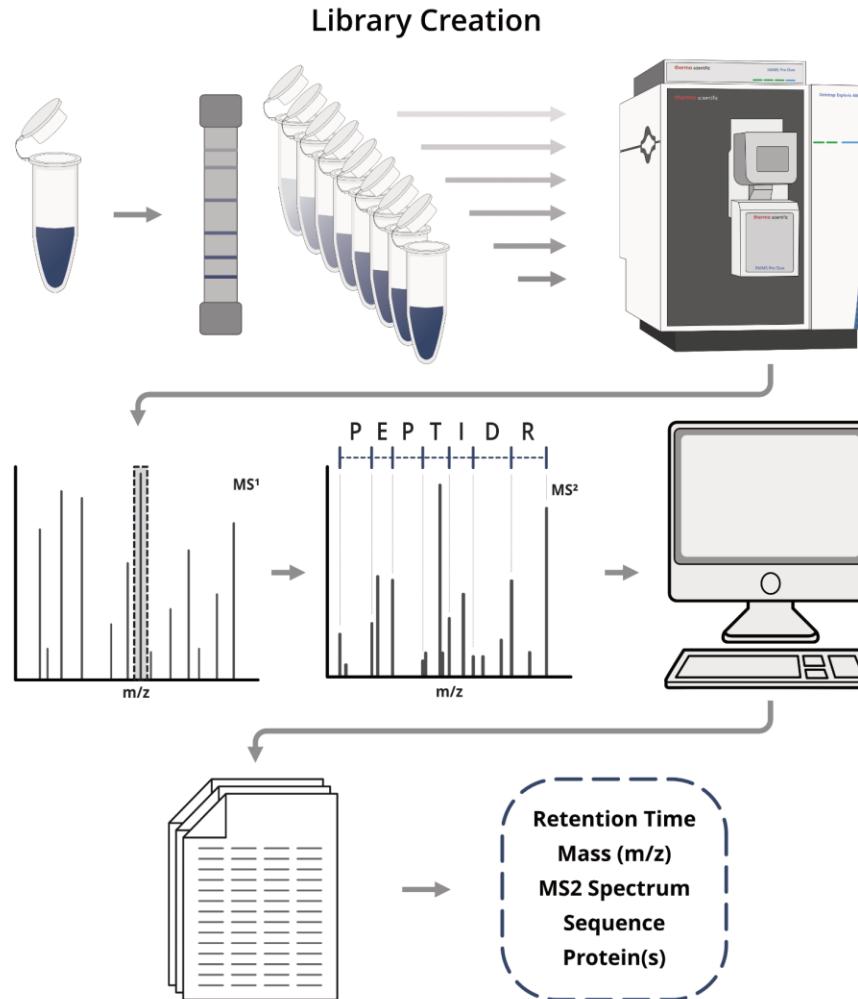
Unbiased and higher
throughput but data
processing is more
complex



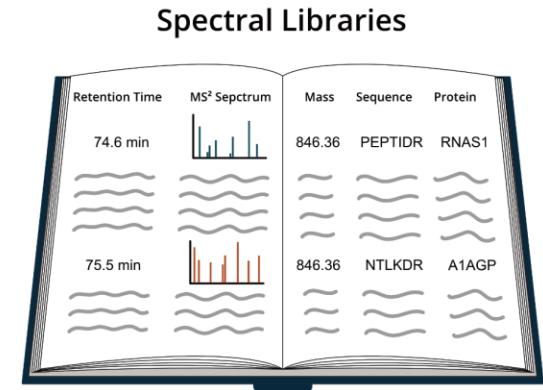
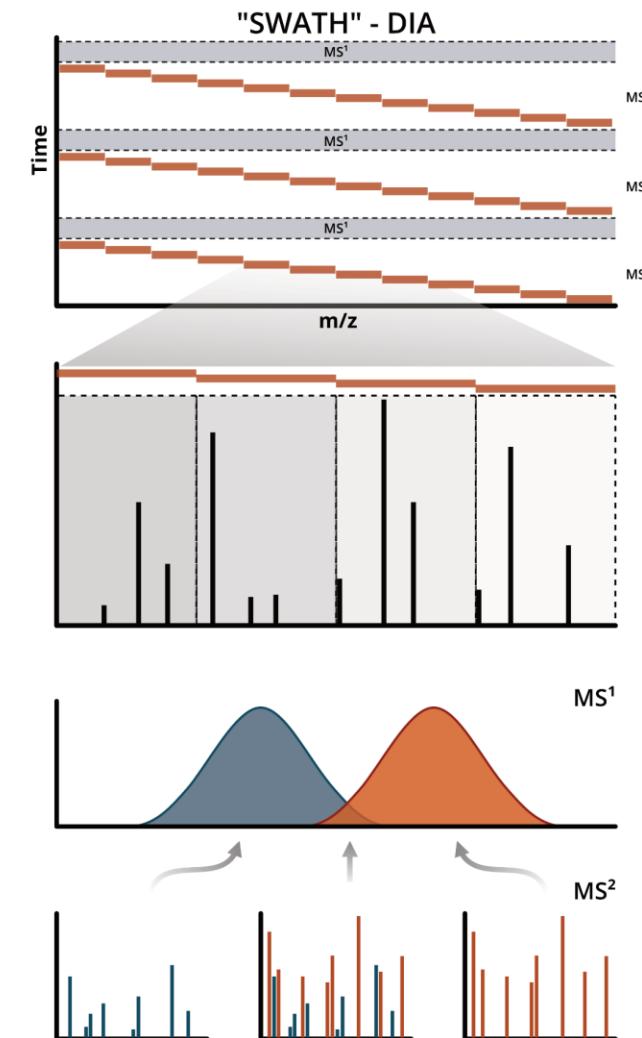
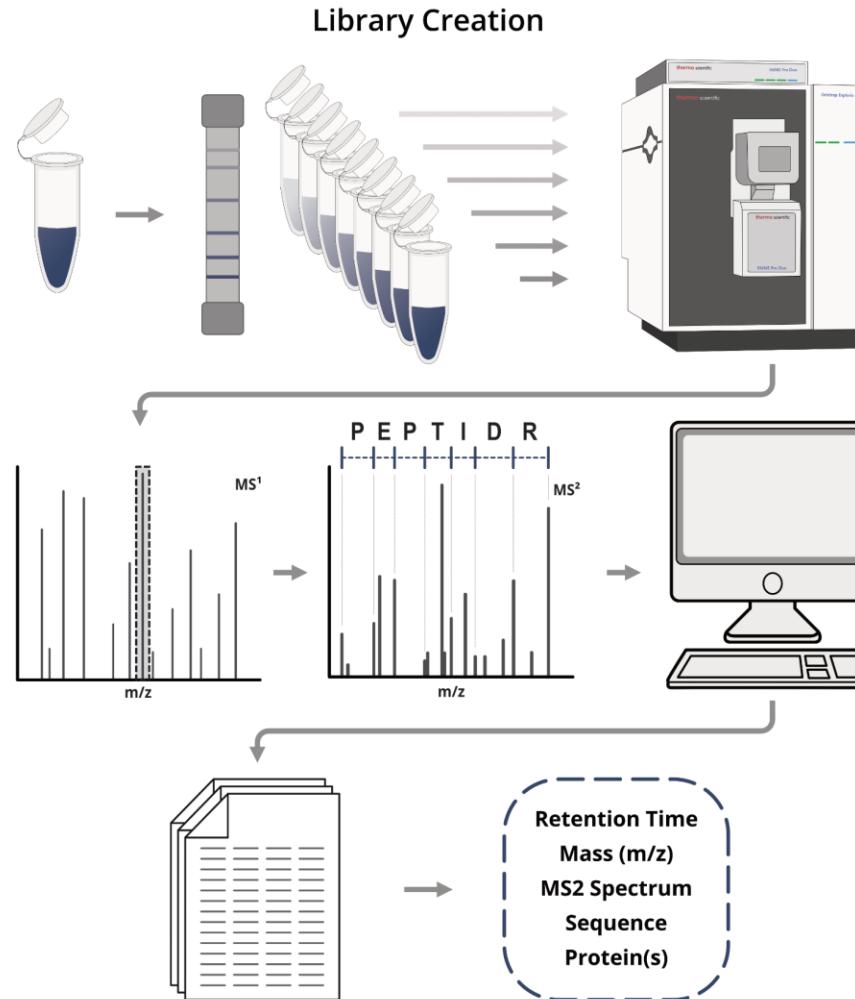
DIA and Spectral Libraries



DIA and Spectral Libraries

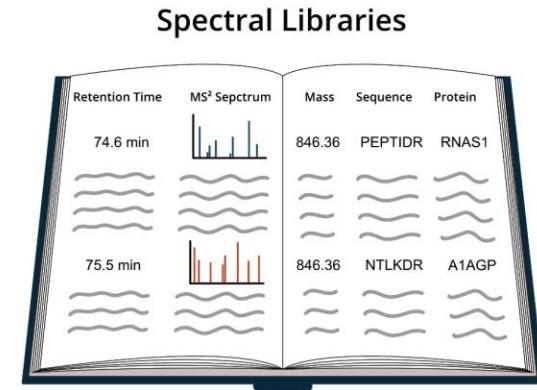
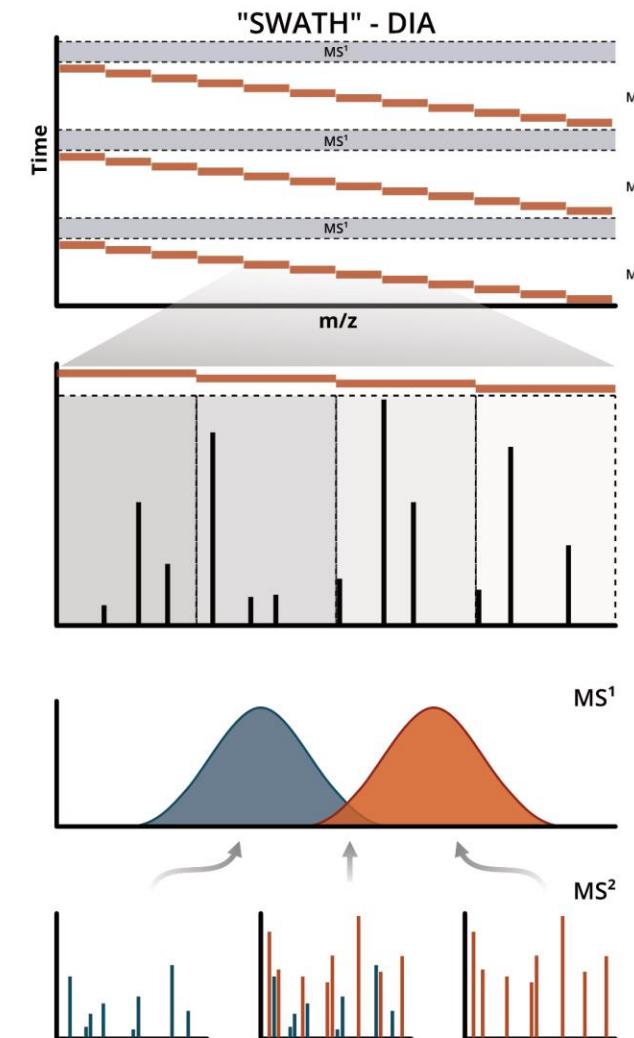
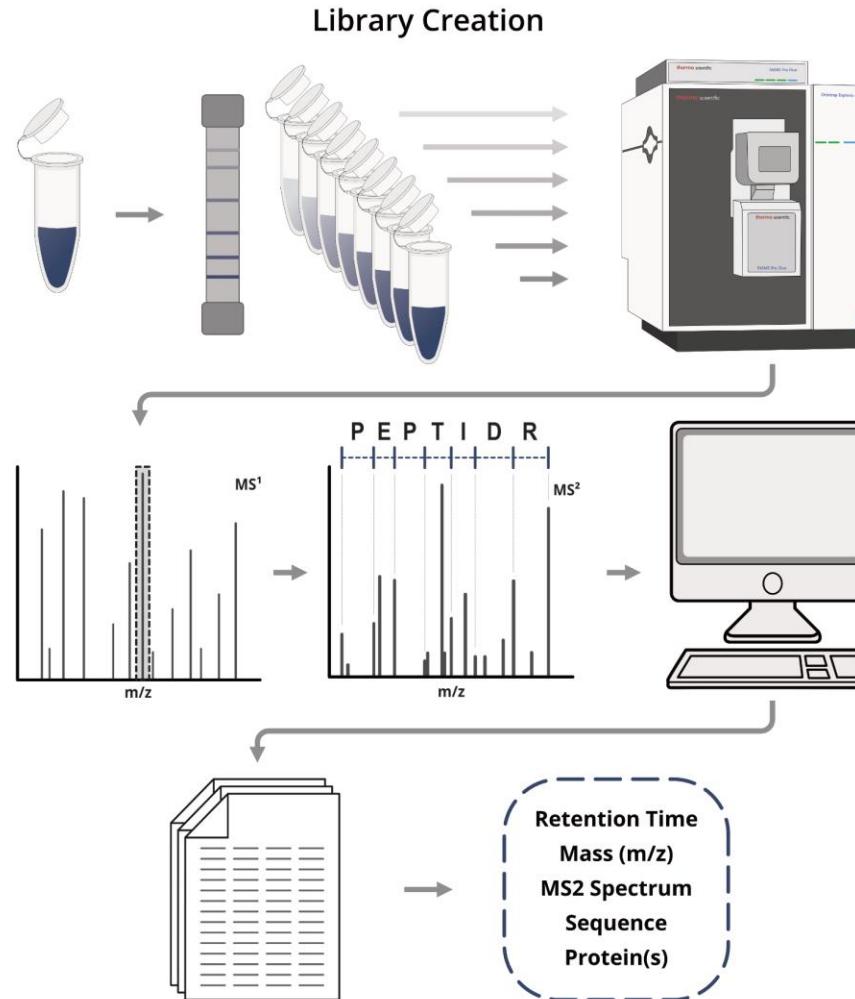


DIA and Spectral Libraries



- + Efficient DIA deconvolution
- + Sample-specific, evidence-based
- Increases sample requirement
- Profiling depth limited by quality of library

DIA and Spectral Libraries



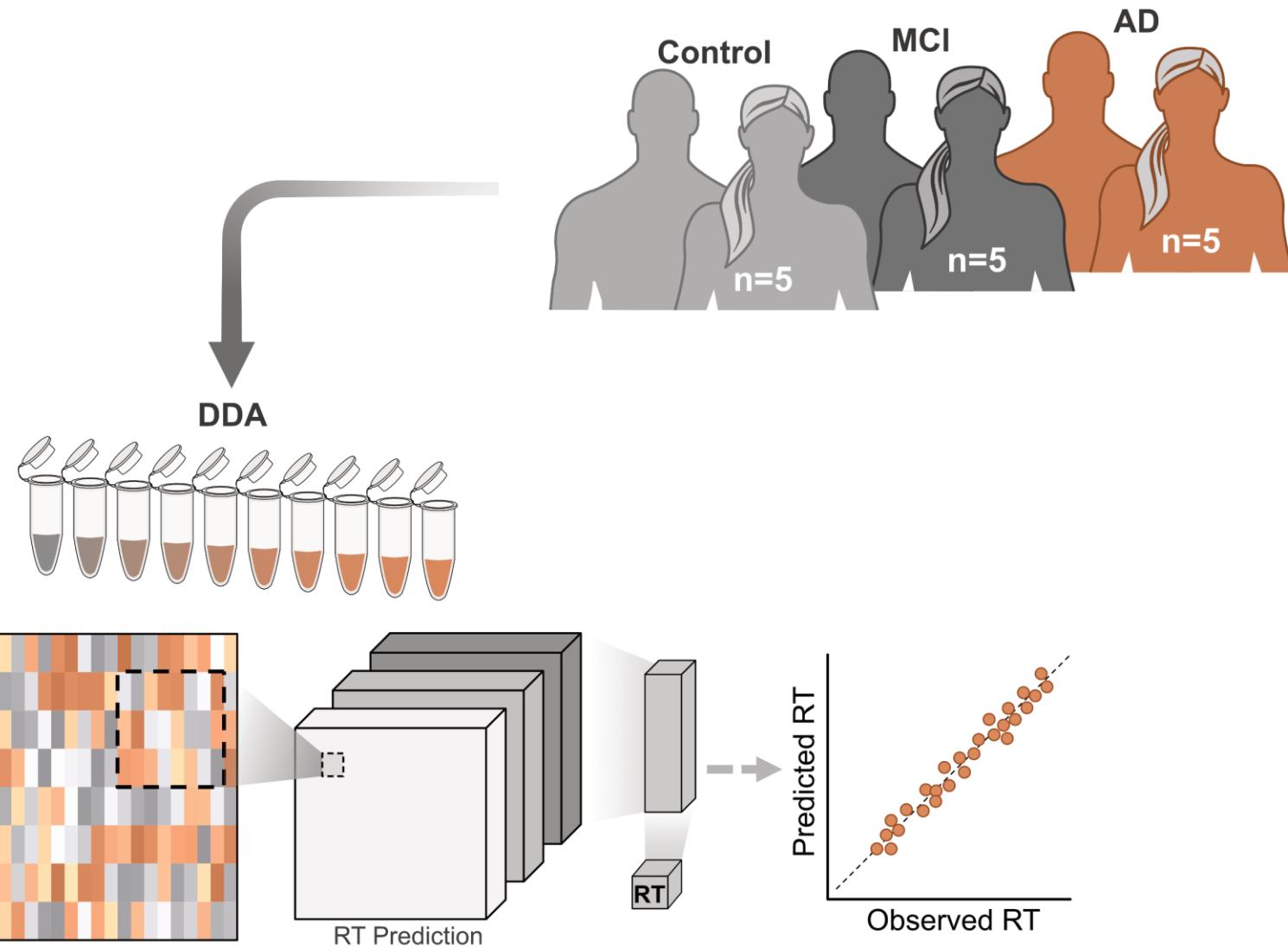
- + Efficient DIA deconvolution
- + Sample-specific, evidence-based
- Increases sample requirement
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Is it possible to adapt spectral libraries from existing analyses to a new experiment?

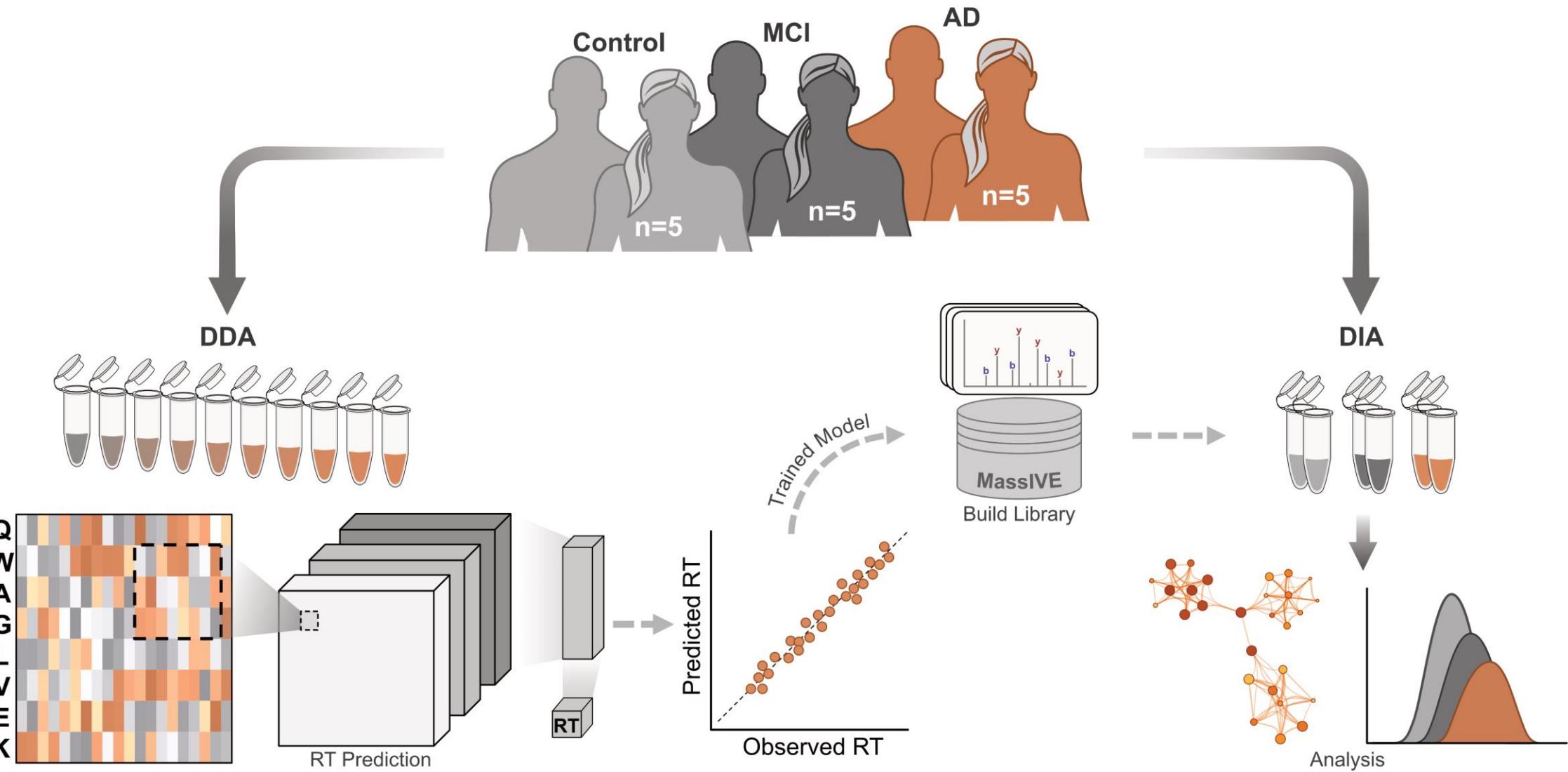
Questions and Experimental Goals

- Can we validate the ability to correlate prior proteomic measurements to new experimental conditions?
- Can we re-purpose prior measurements for biomolecular discovery?
How does it compare to traditional approaches?
- Is quantitation accurate and reliable?
- Can we utilize this information to discover proteomic dysregulation in clinically-relevant applications?

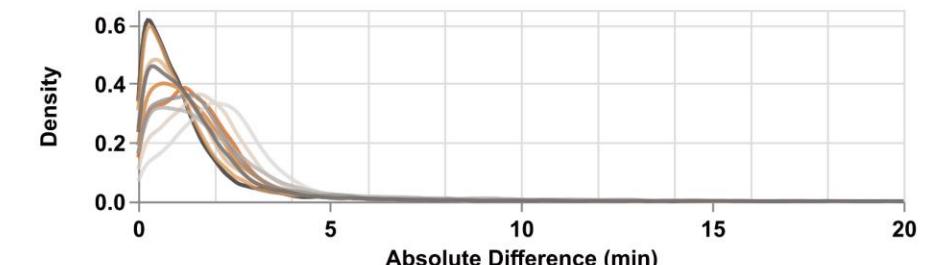
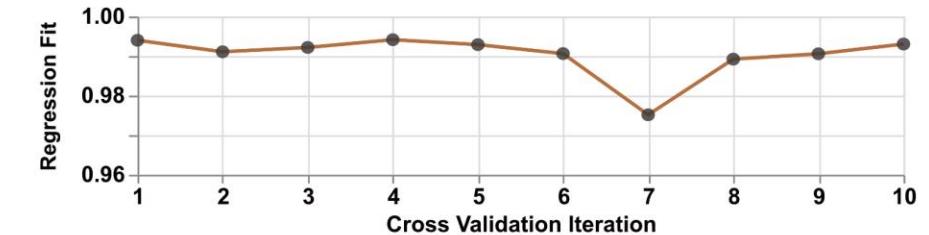
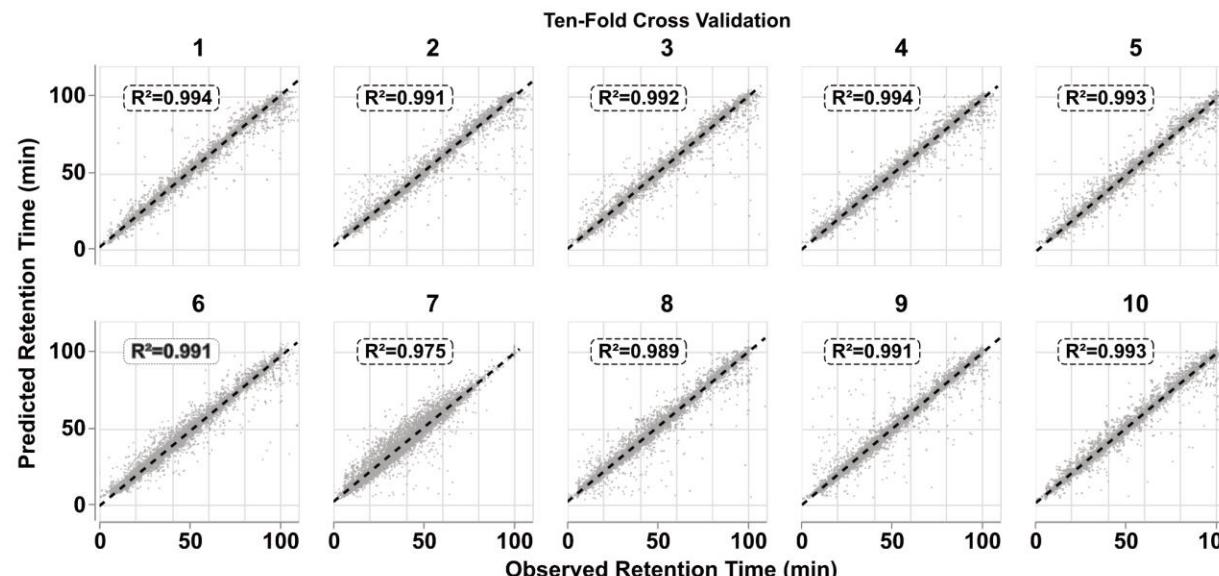
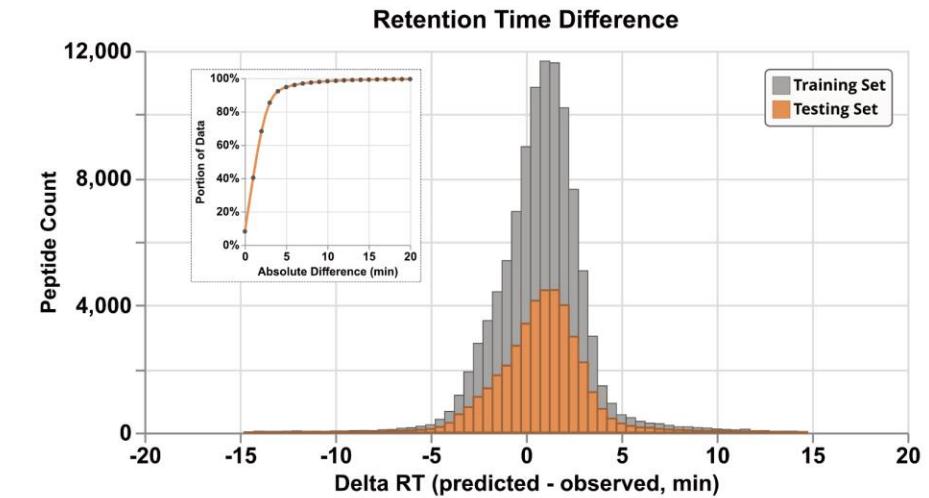
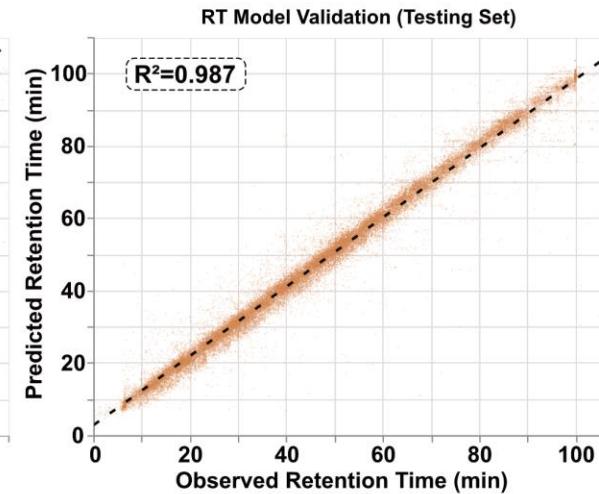
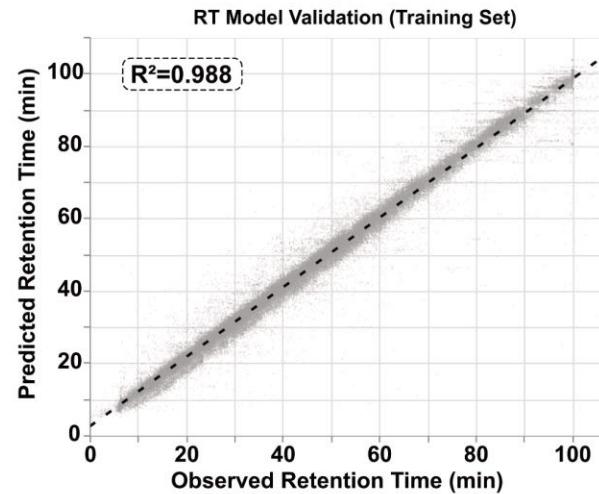
Parallel Workflow



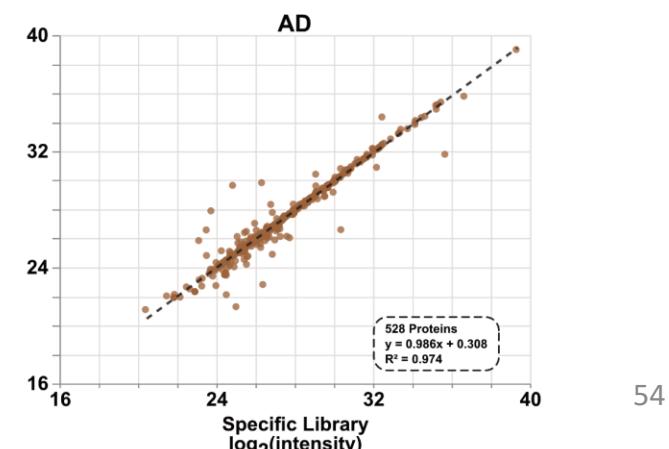
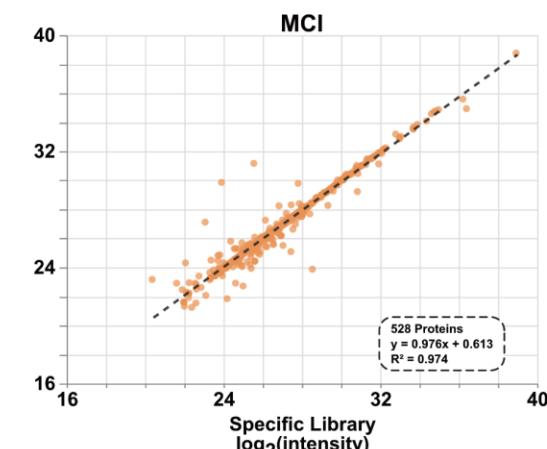
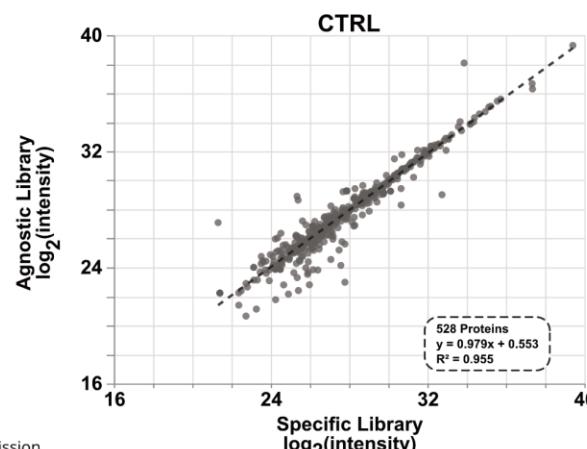
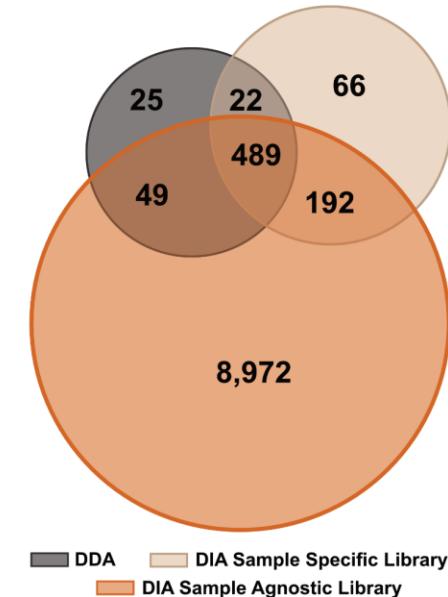
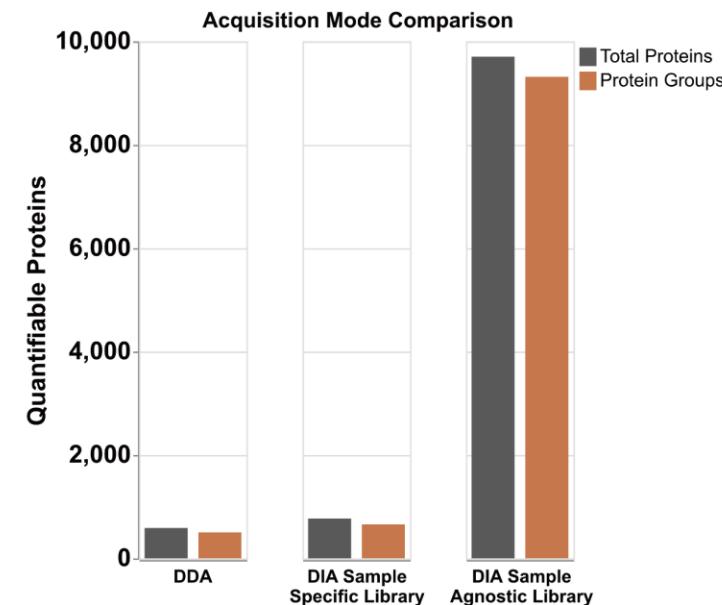
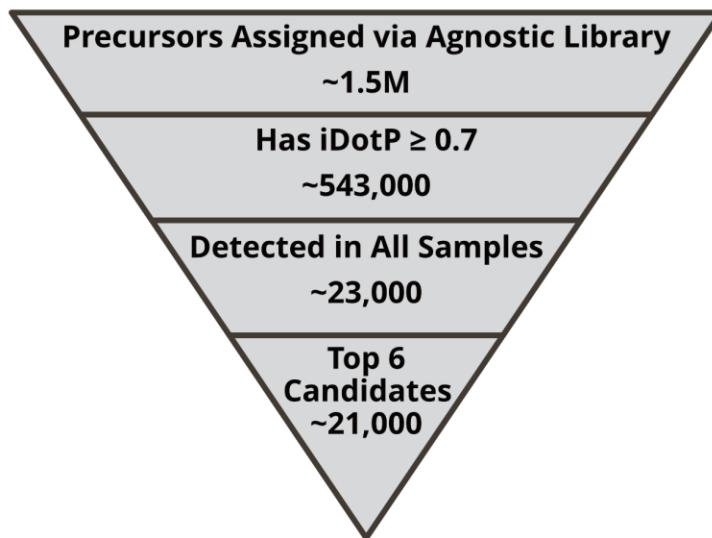
Parallel Workflow



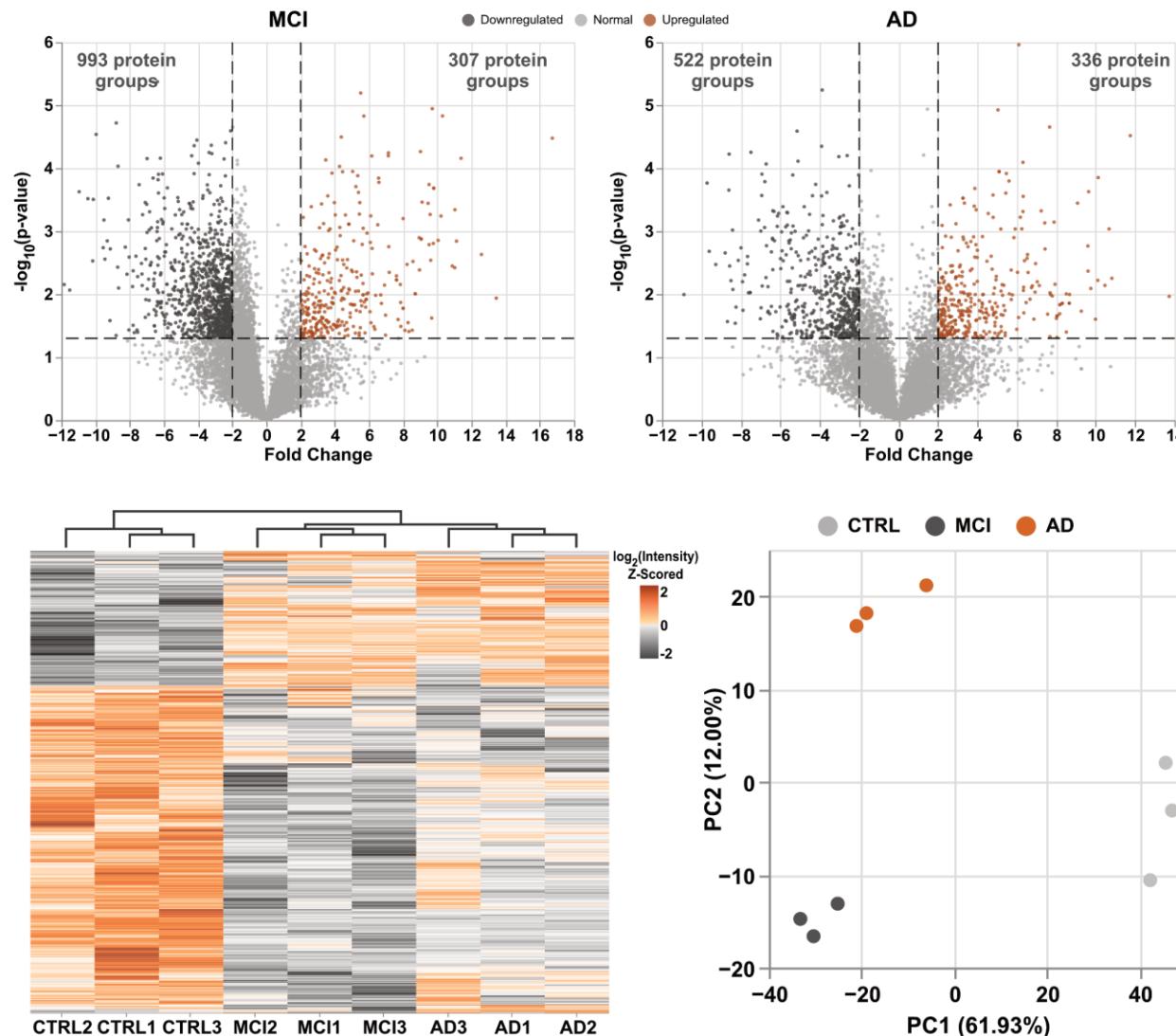
Model Validation



Quantitative Comparison



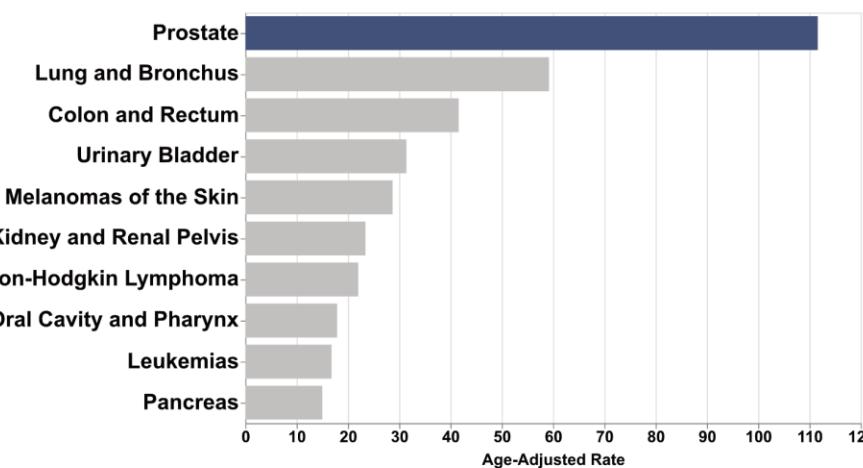
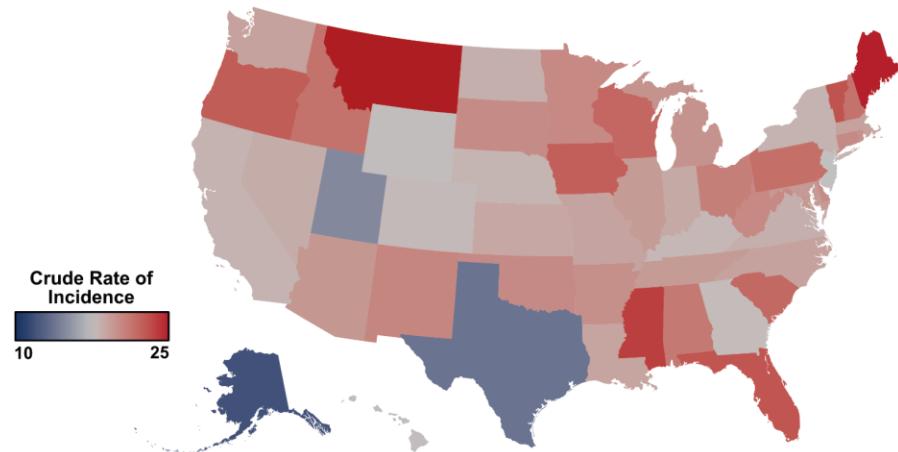
Disease State Discrimination



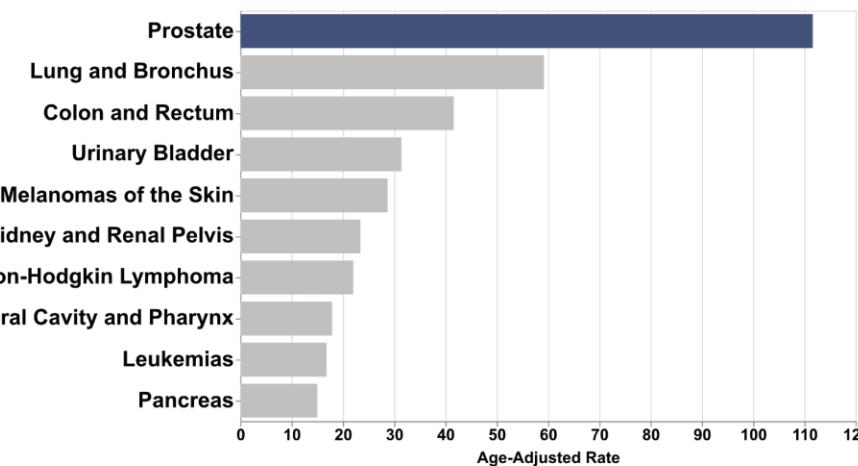
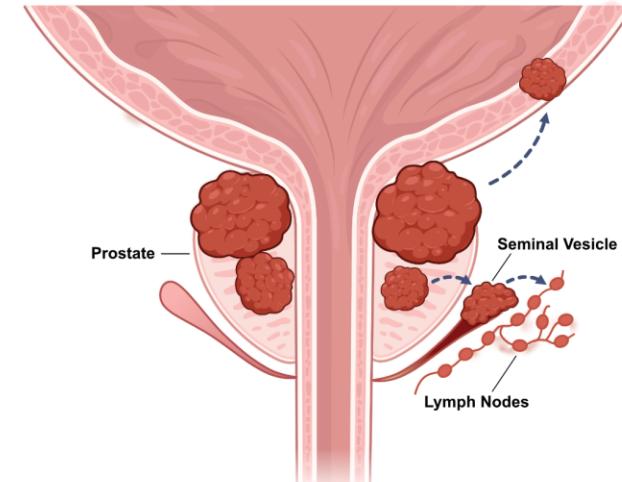
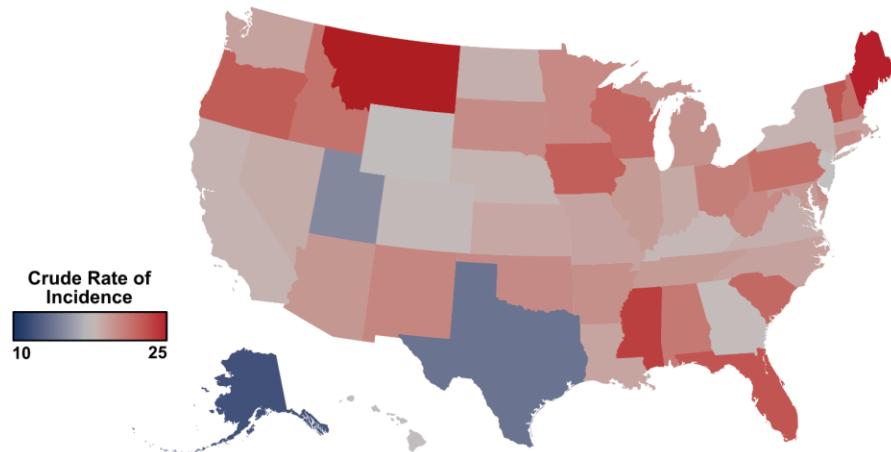
Outcomes

- Using machine learning, spectral libraries can be effectively calibrated to a new experiment.
- Agnostic libraries provide significantly improved profiling depth.
- We revealed 1,642 significantly dysregulated proteins, highlighting remodeling of extracellular matrix and tubulin disruption.
- We advocate for a stronger community-driven approach to translate proteomic analyses to clinical application.
- Presents a path forward when utilizing novel chromatographic separations where peptide retention times are significantly different.

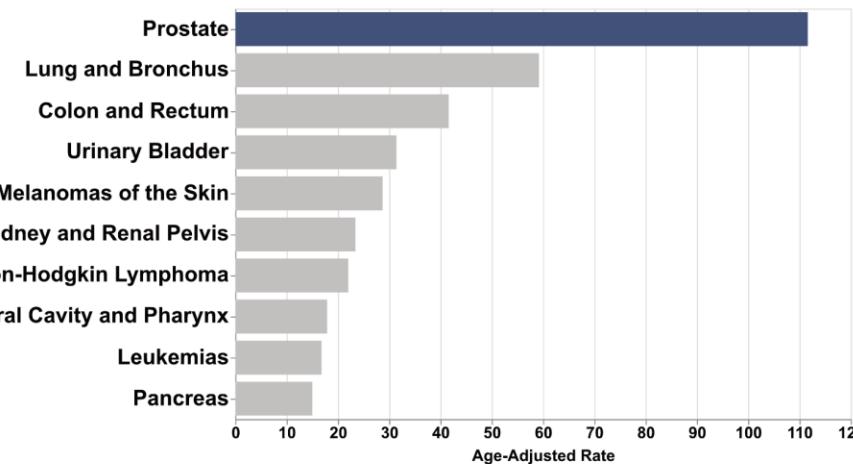
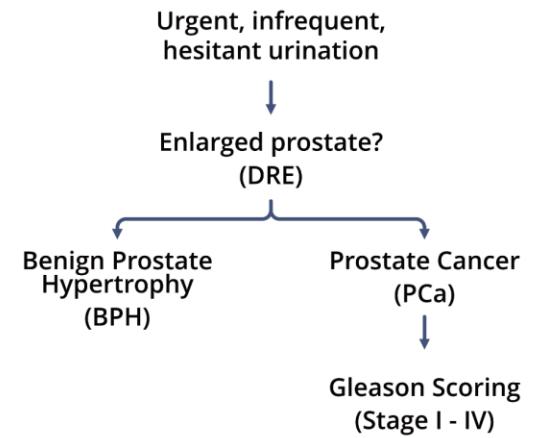
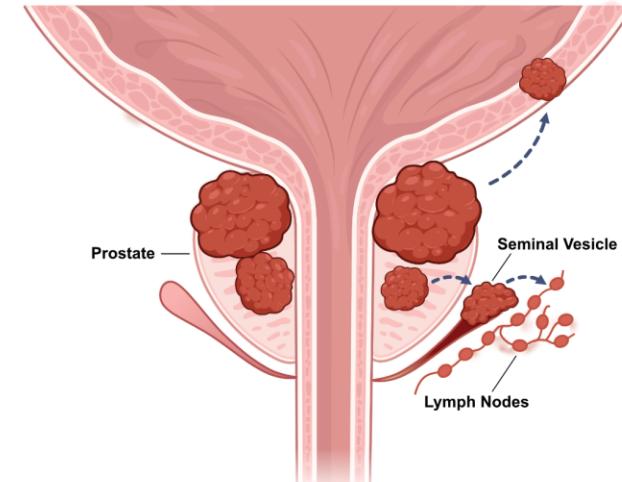
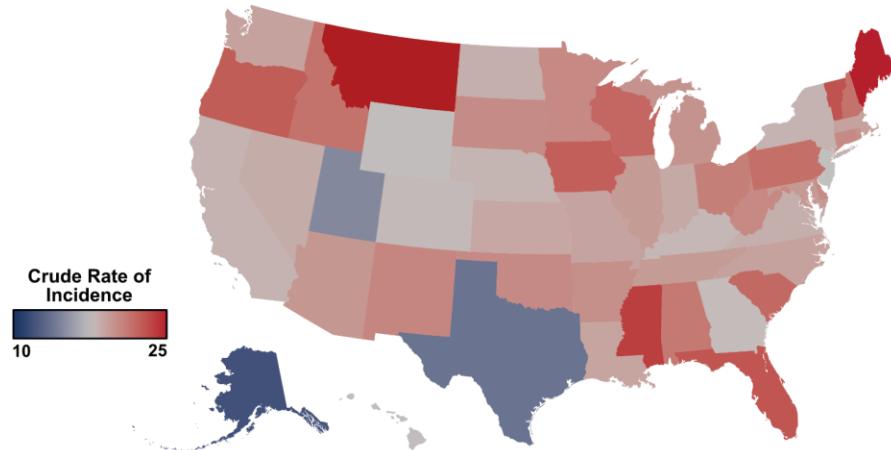
Prostate Cancer Incidence and Outlook



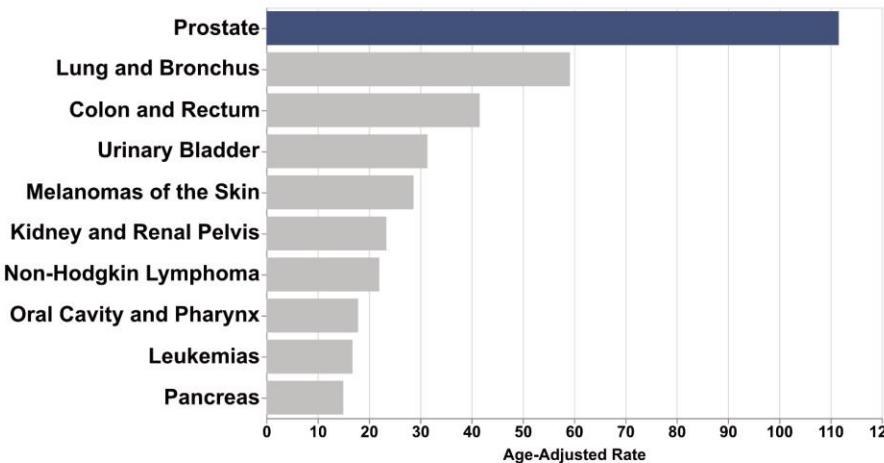
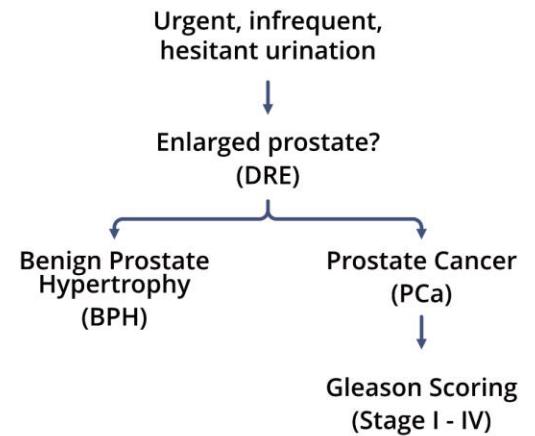
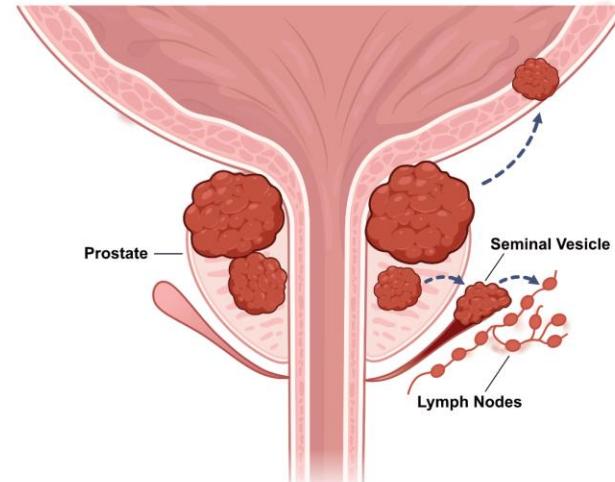
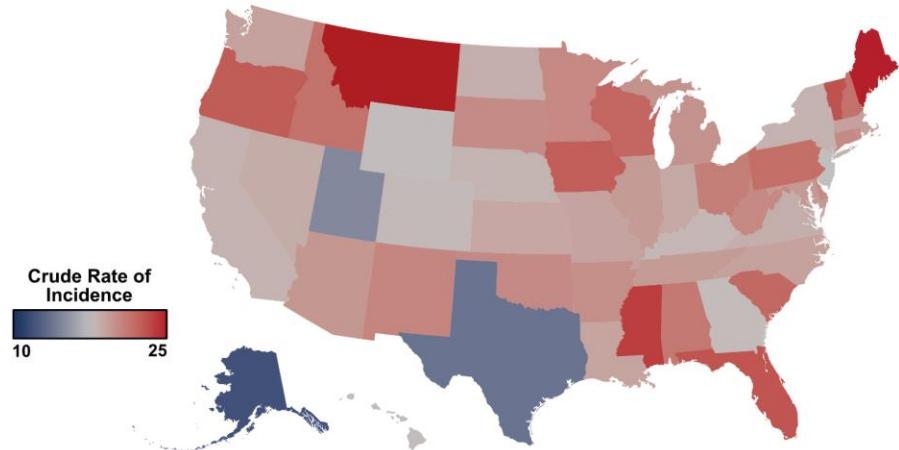
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Prostate Cancer Incidence and Outlook

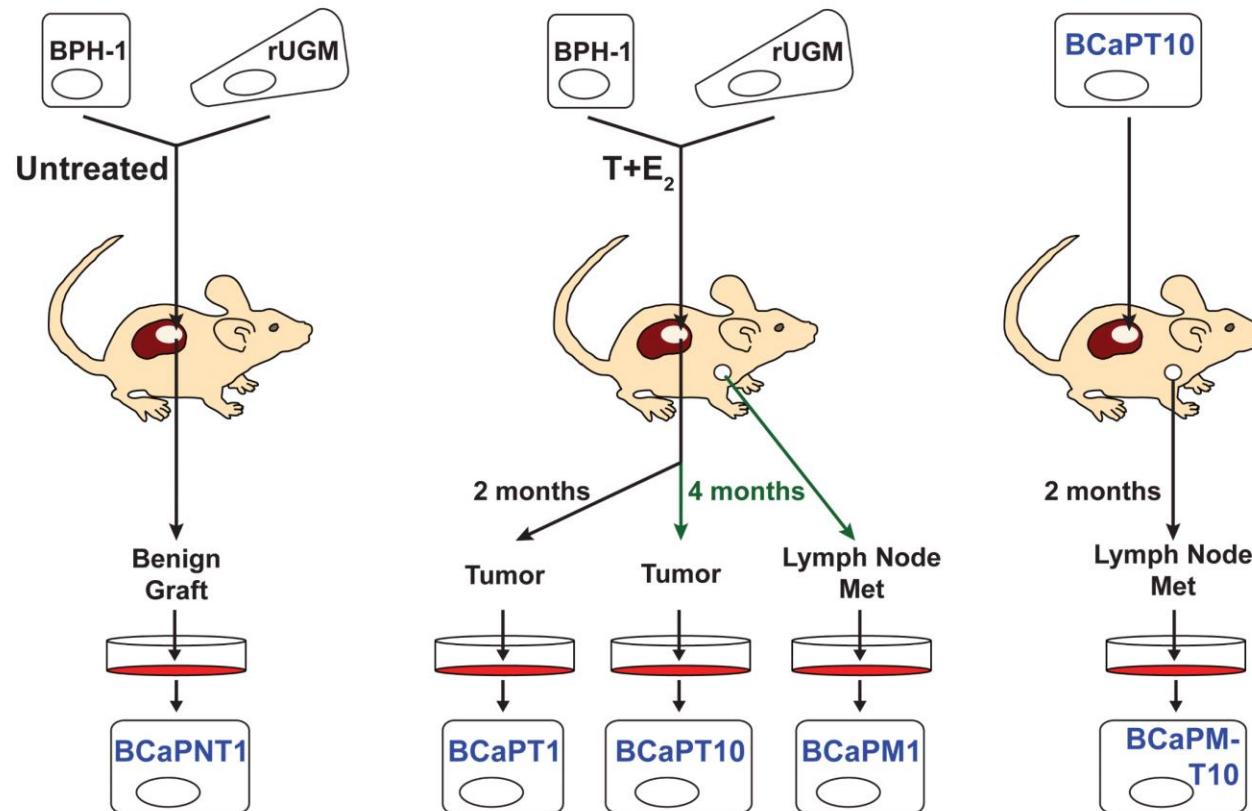


Prostate Cancer Incidence and Outlook



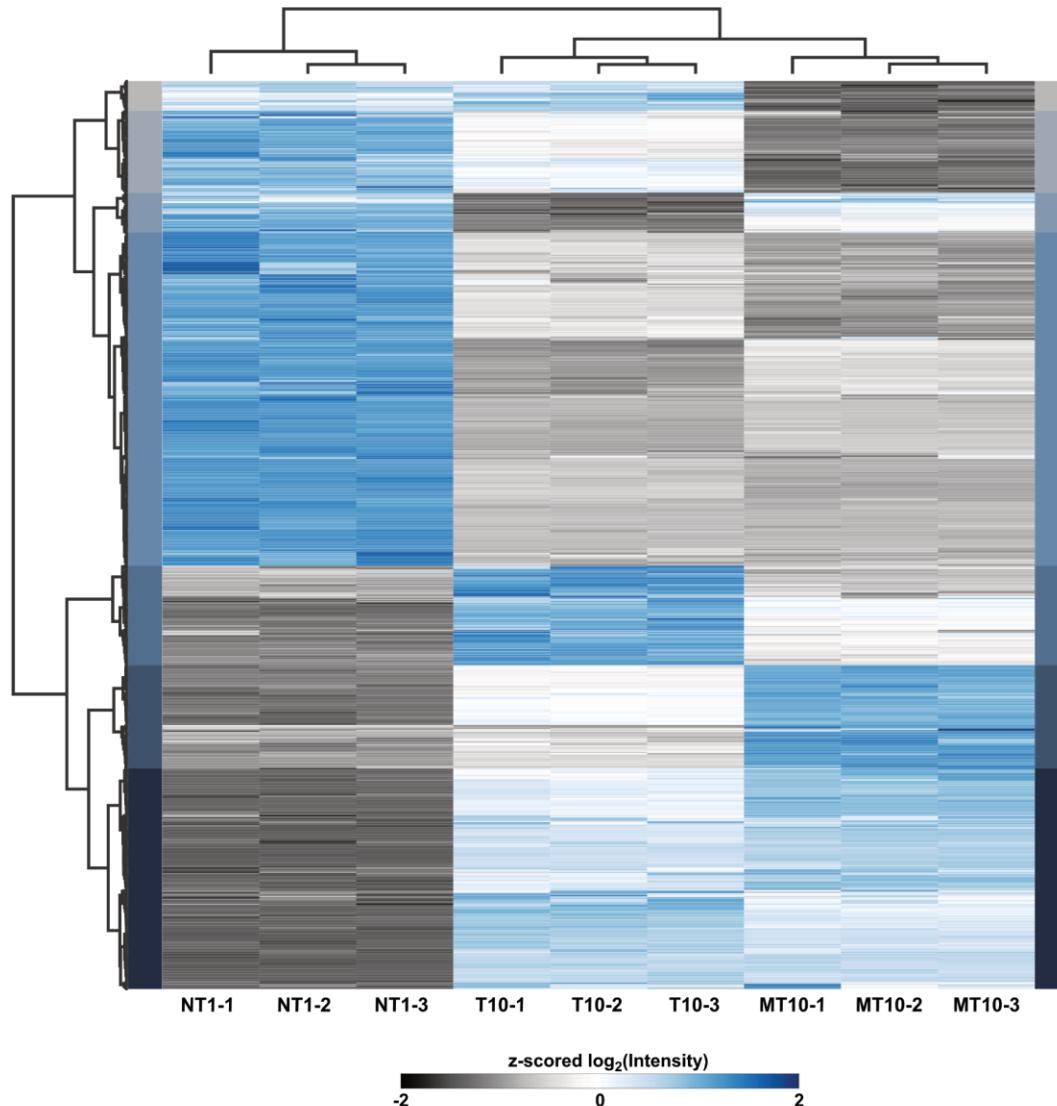
- Leading rates of incidence and mortality necessitate preventative screening
- Urological screening (e.g., DRE) remain the most direct means of PCa detection but still require biopsy and serological confirmation
- Therapeutic monitoring and PCa progression dependent on a single biomarker
- There is substantial need to uncover protein panels that provide unambiguous diagnosis, stratification, and monitoring

BPH-1-Derived Cancer Progression Cell Model

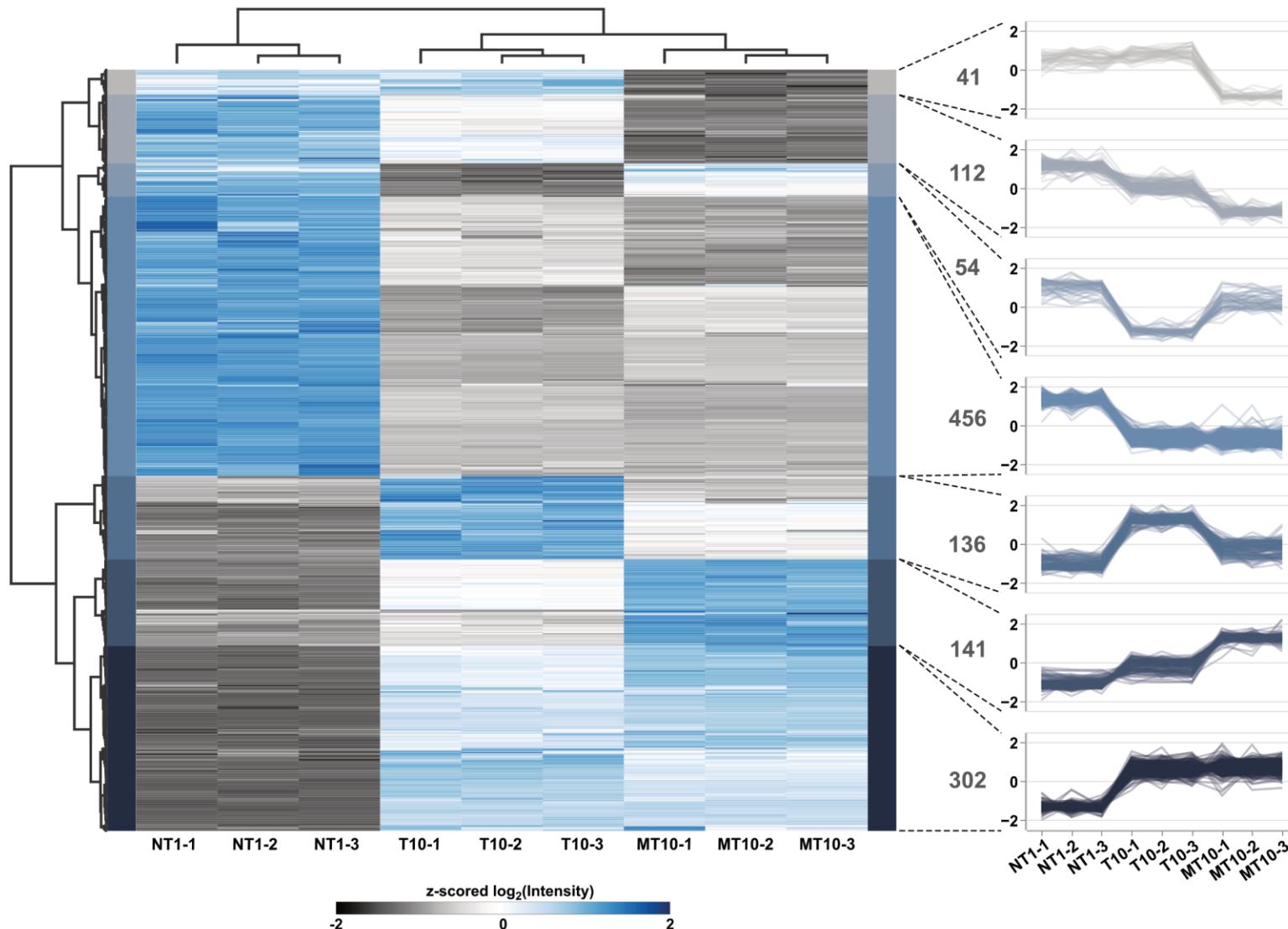


- Early investigations demonstrated Benign Prostatic Hypertrophy (BPH) can progress to cancer in presence of hormones
- Ricke and colleagues developed cell line model of cancer progression that mimics the genetic, phenotypic, and molecular characteristics of PCa
- Proteomic analyses of discrete phenotypes will help illuminate the proteomic organization specific to PCa progression

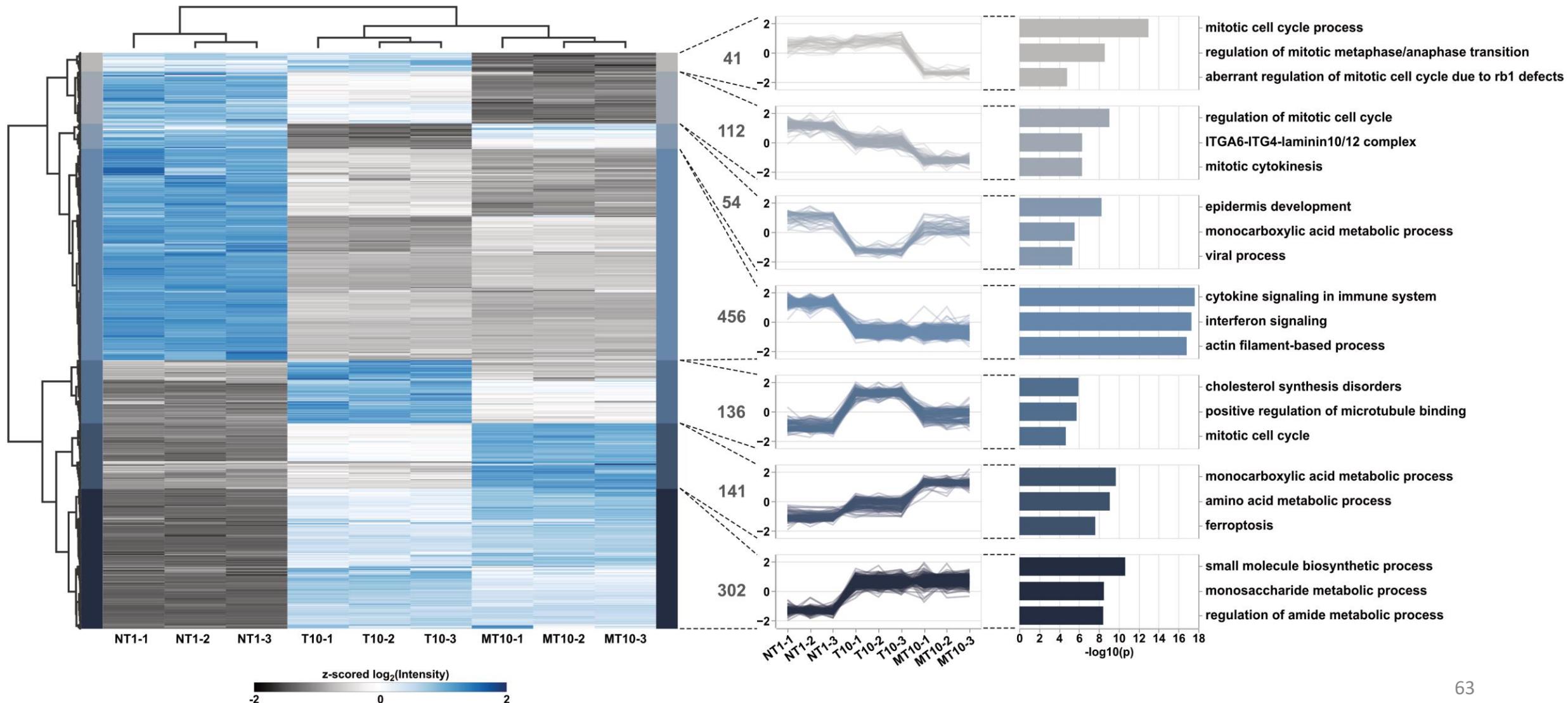
PCa Proteomic Fingerprinting



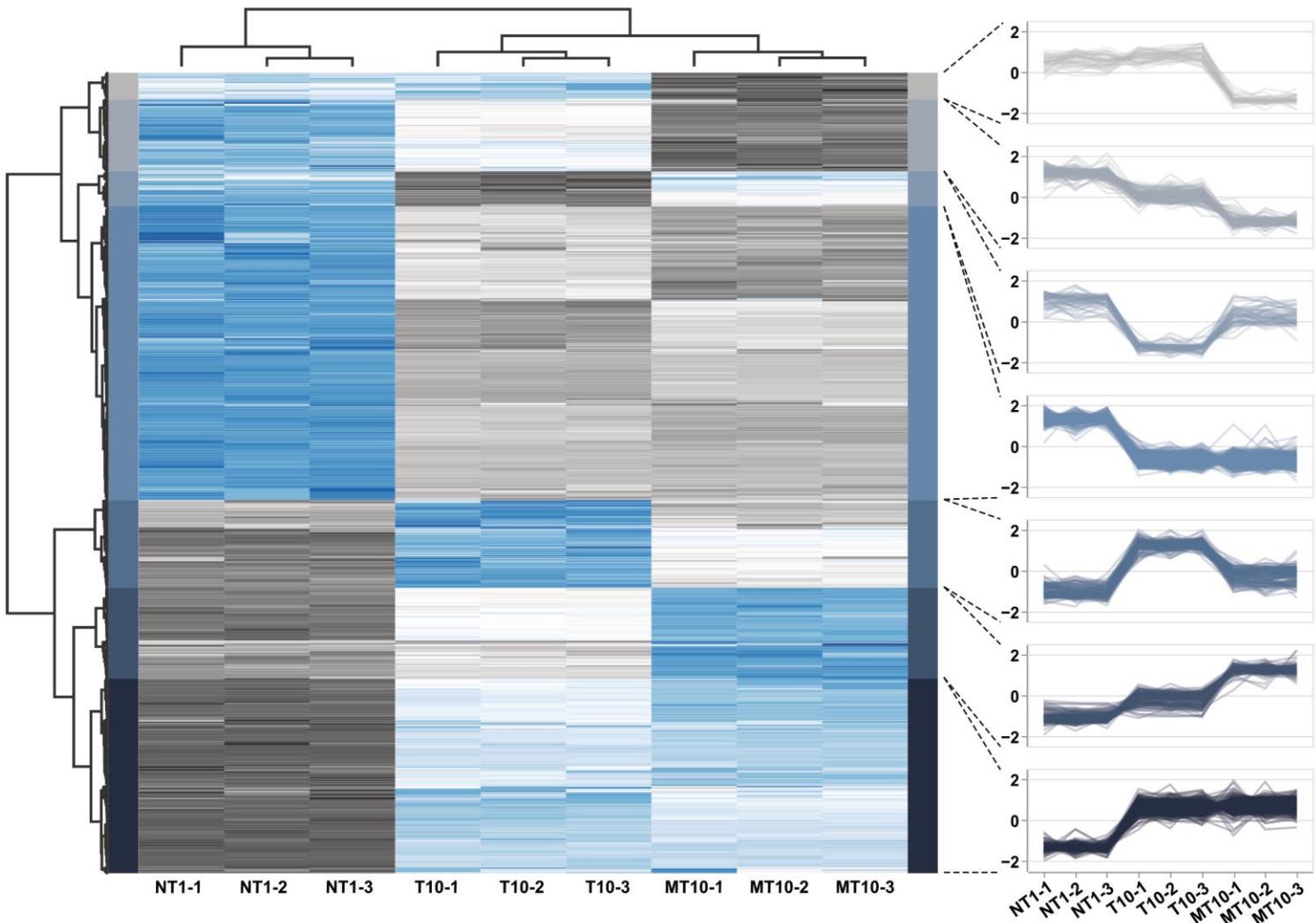
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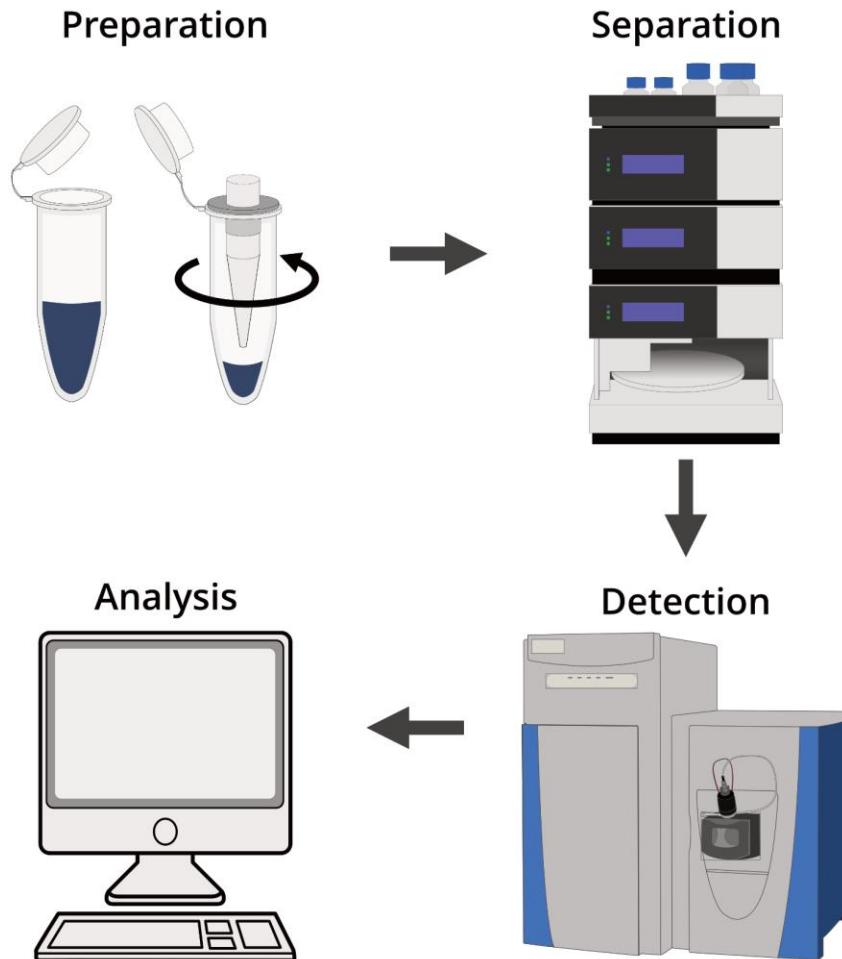
Future Directions



Takeaways

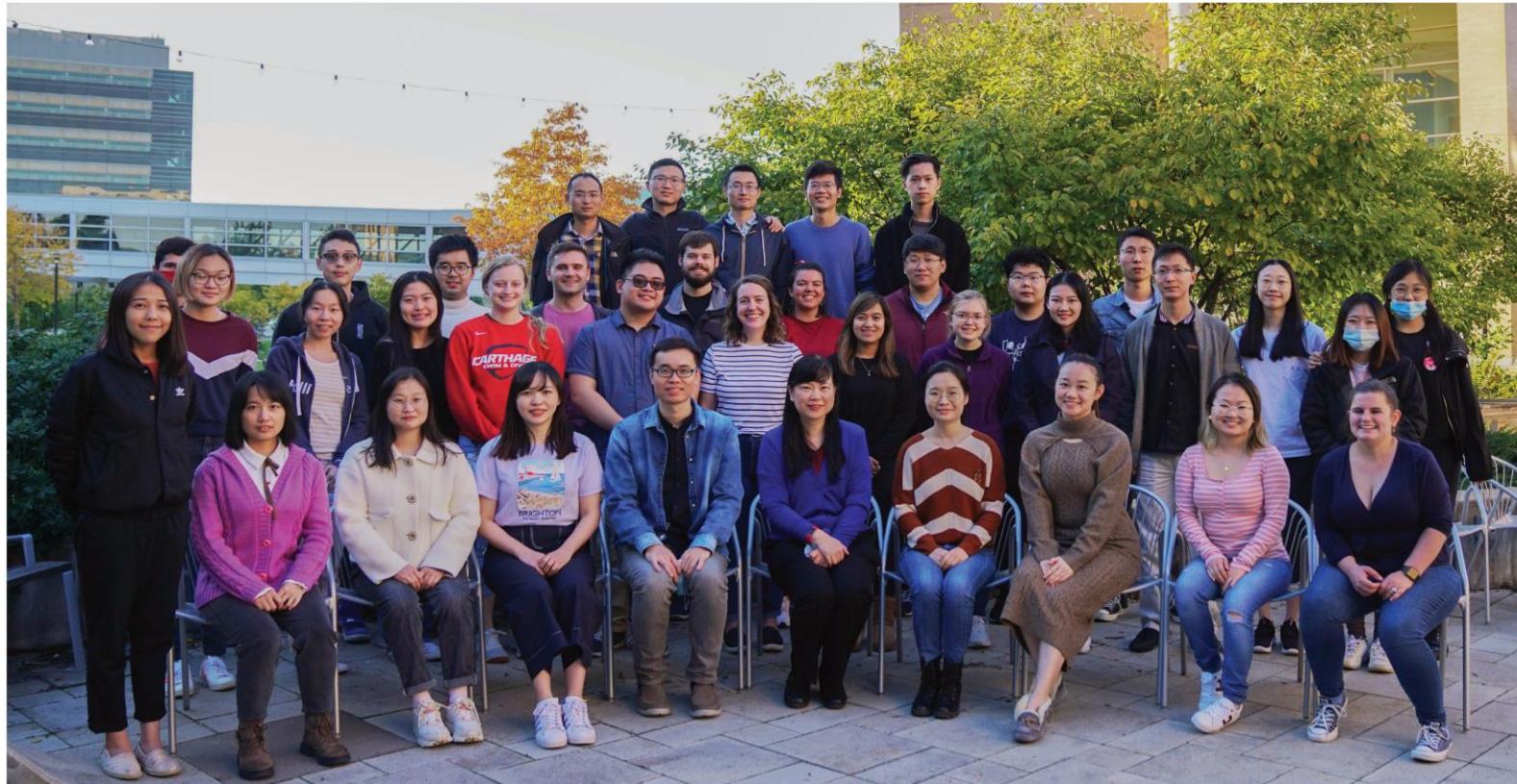
- DIA analyses present unparalleled access to the human proteome and enable reproducible protein quantitation.
- We provide early understanding of the biomolecular fingerprints of BCaP phenotypes.
- Conserved dysregulation of protein communities provide suitable targets for biomarker validation, markers of malignancy and therapeutic monitoring.

Conclusions



- Mass-spectrometry based proteomics serves as a premier method for biomolecular investigations.
- Proteomics will forever be searching for a 'more complete' picture.
- Our interpretation of results and underlying biology are biased by the strategies employed.
- Alternative separations and acquisition strategies compliment our current understanding and traditional strategies.

Acknowledgments



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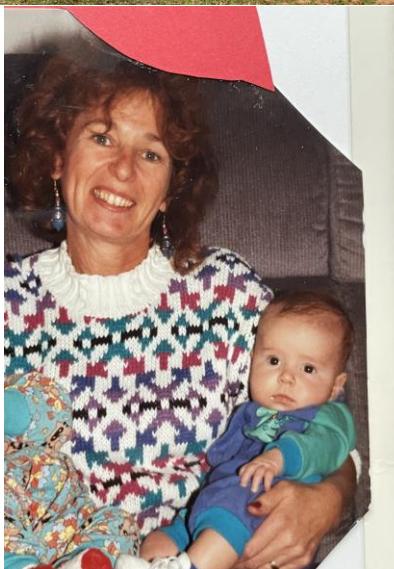
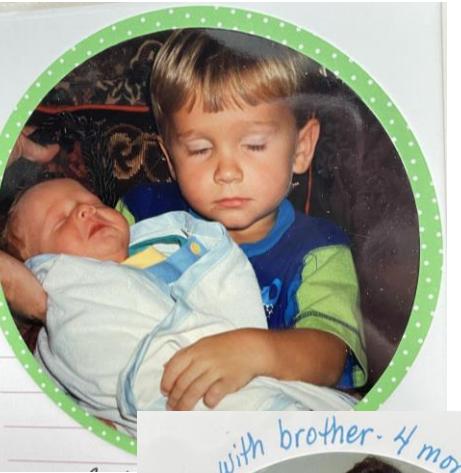


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Disease Research Center
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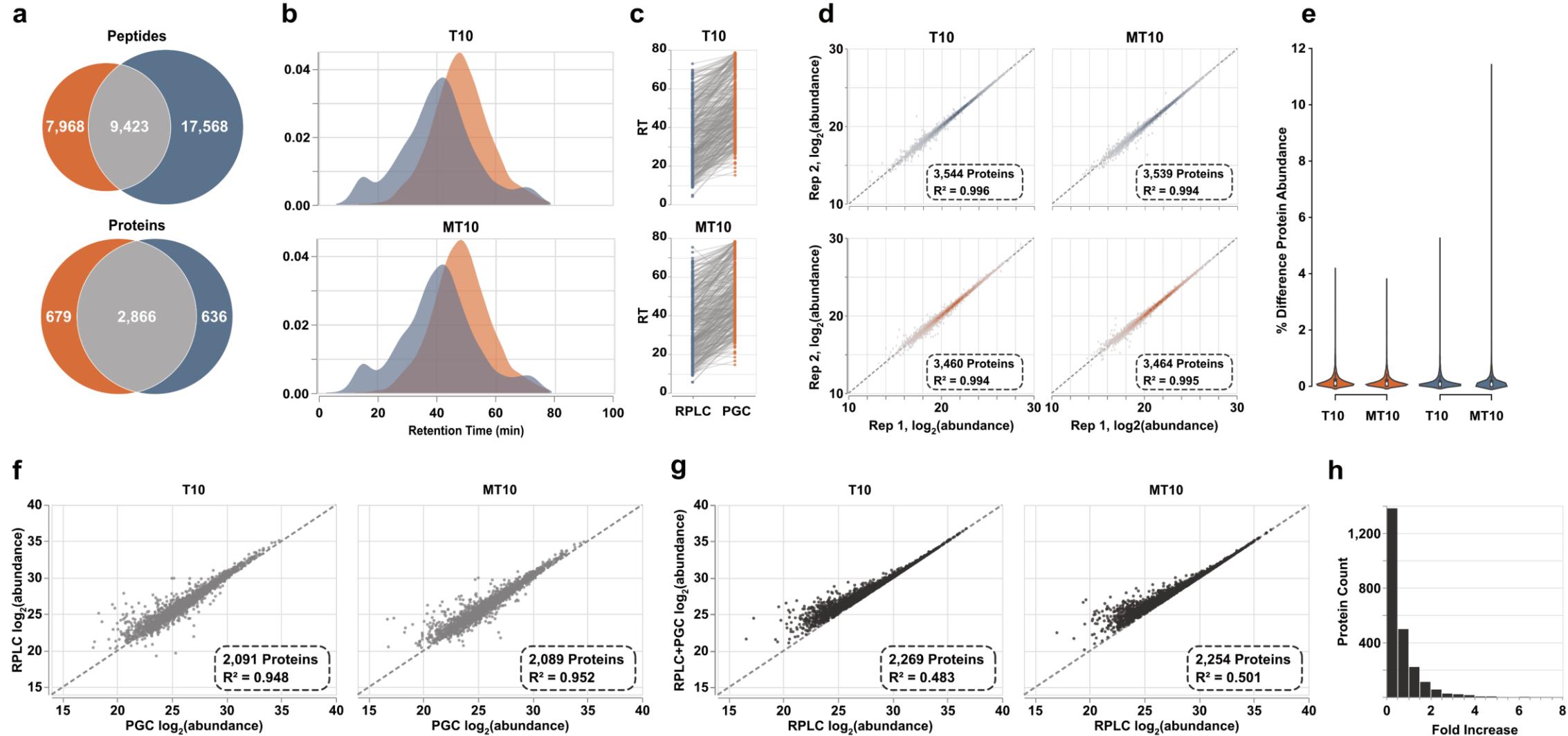
WARF
Wisconsin Alumni Research Foundation



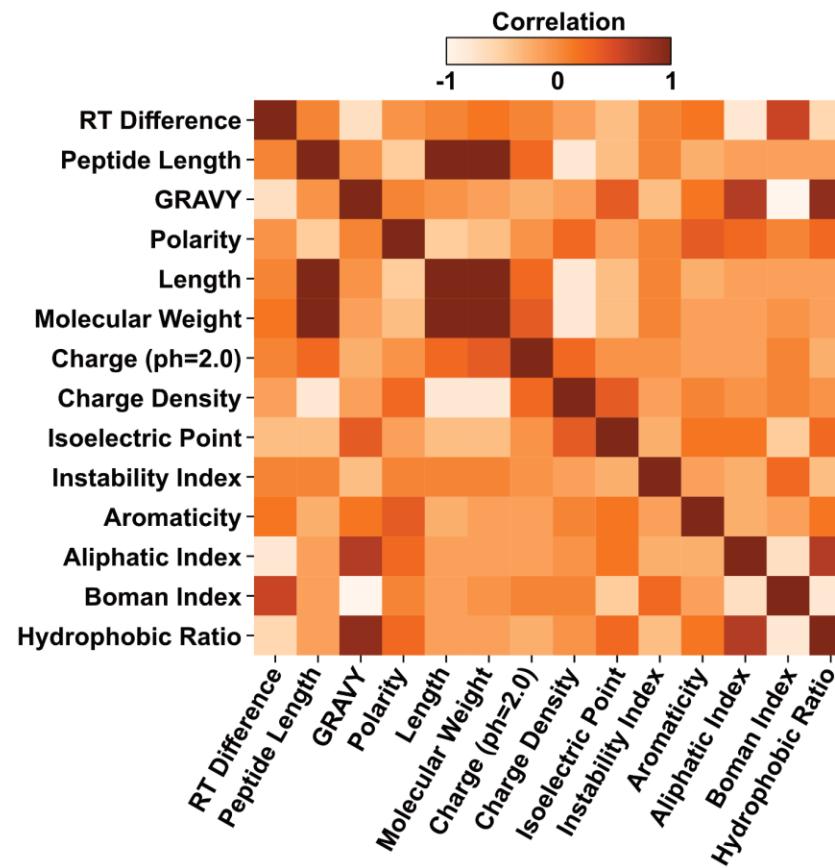
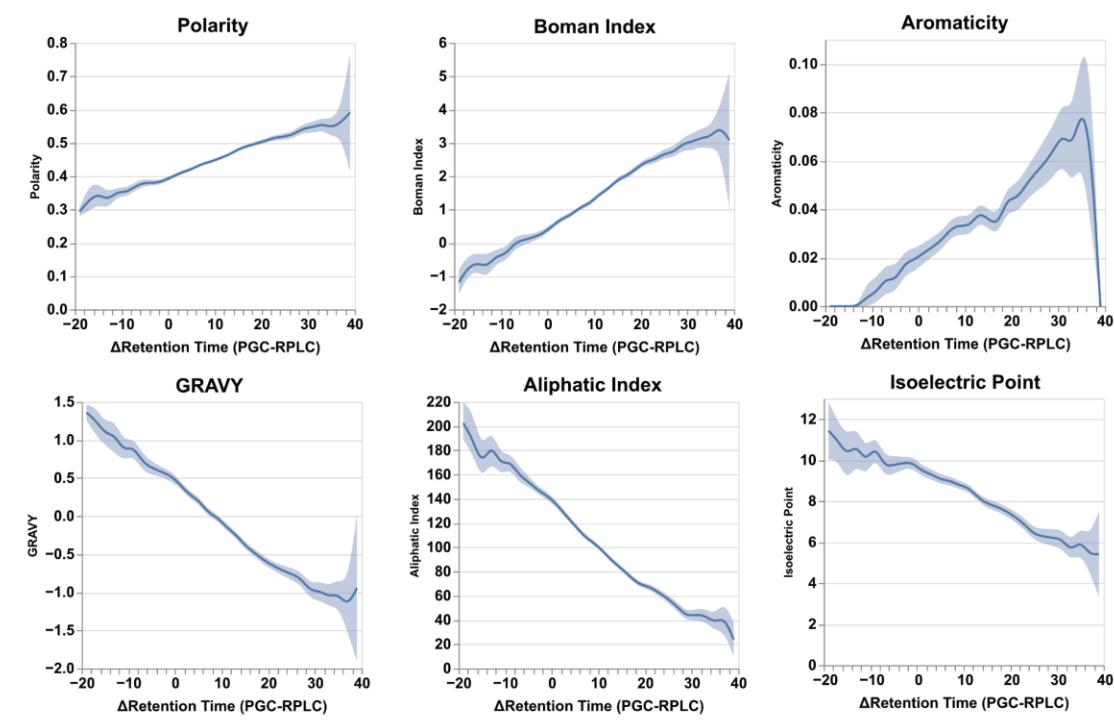


Supplemental

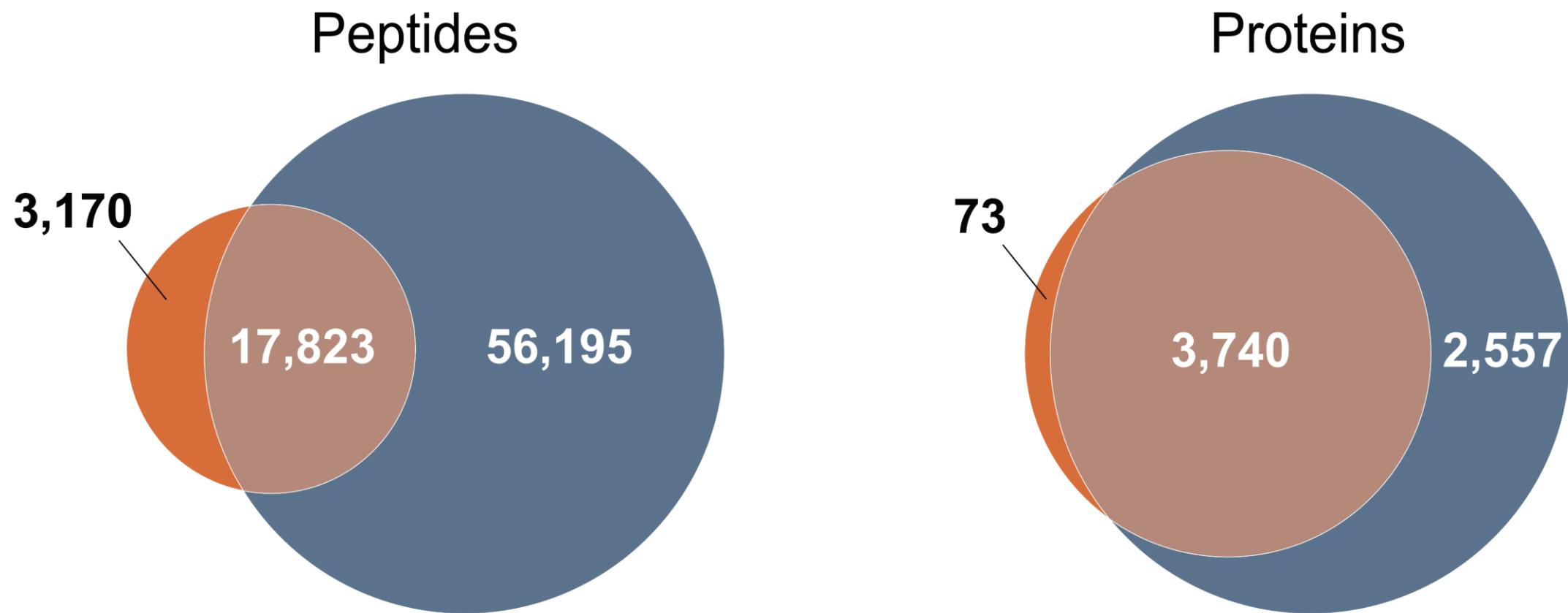
How PGC Separations Affect Proteomic Measurement



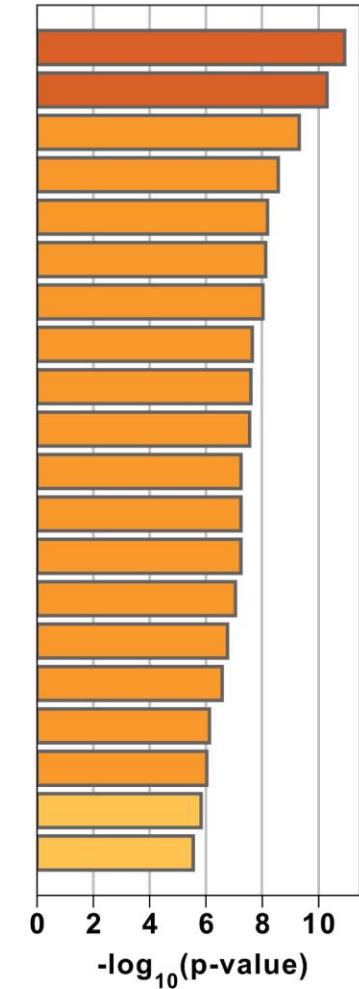
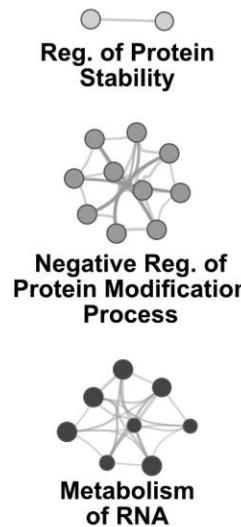
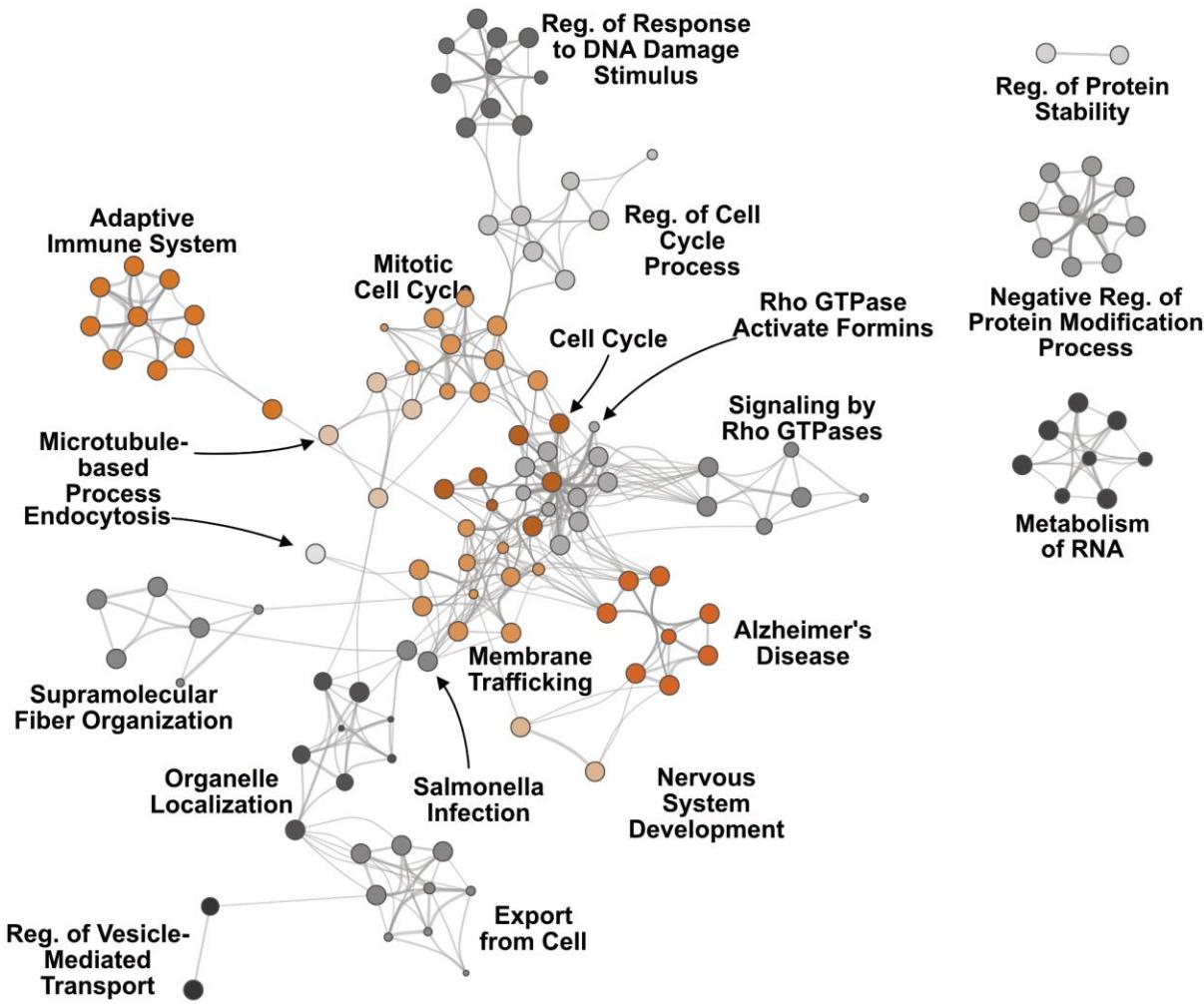
Peptide Character Affecting PGC Retention

a**b**

Library-Free Comparison, RPLC vs. PGC

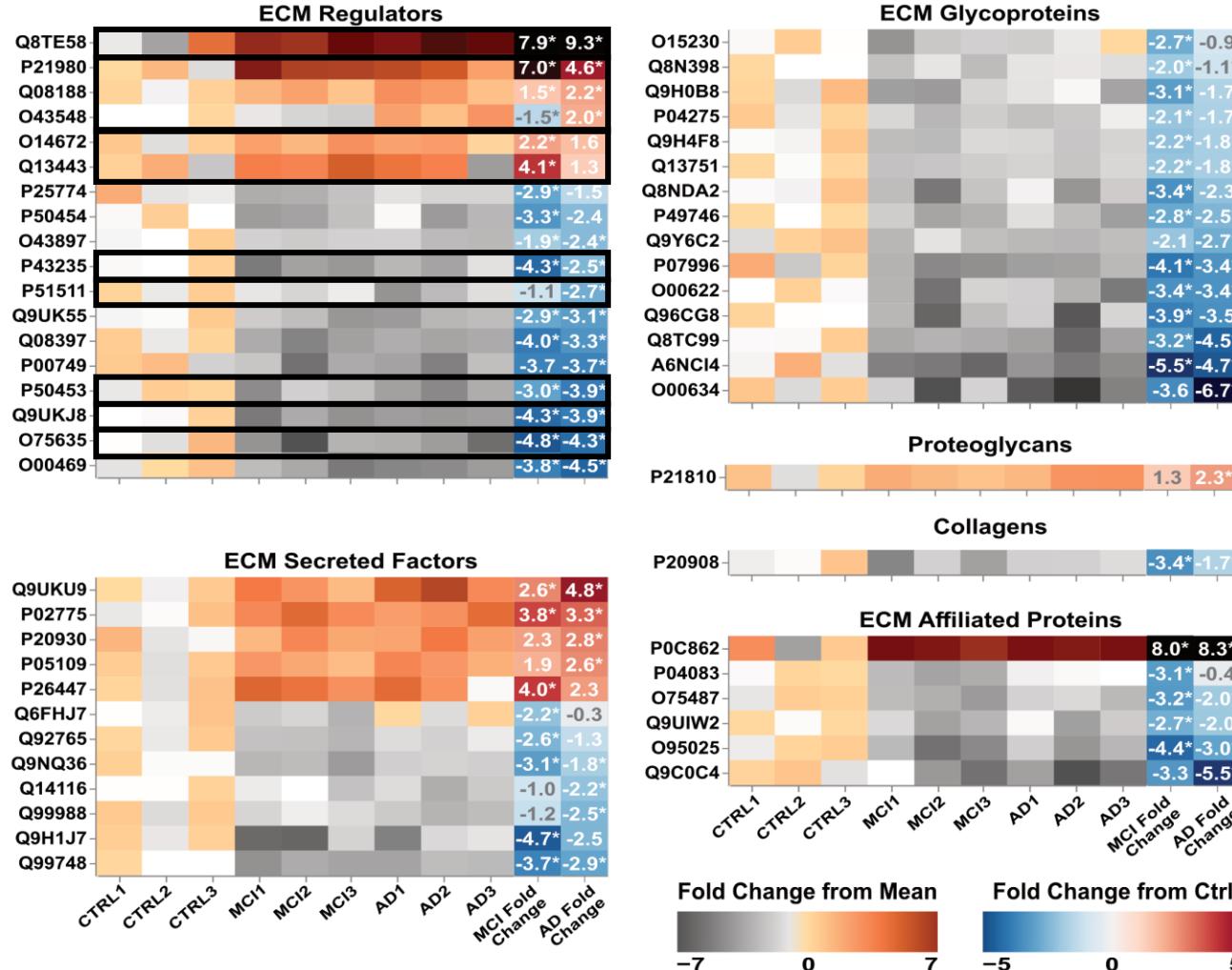


Dysregulated Pathways



Supramolecular fiber organization
Adaptive Immune System
Mitotic cell cycle
Membrane Trafficking
 Export from cell
 Signaling by Rho GTPases
 Cell Cycle
 Regulation of vesicle-mediated transport
 Negative regulation of protein modification process
Microtubule-based process
 Metabolism of RNA
 Organelle localization
 Regulation of response to DNA damage stimulus
 Nervous system development
 Regulation of cell cycle process
RHO GTPases Activate Formins
 Salmonella infection
Alzheimer's disease
 Regulation of protein stability
 Endocytosis

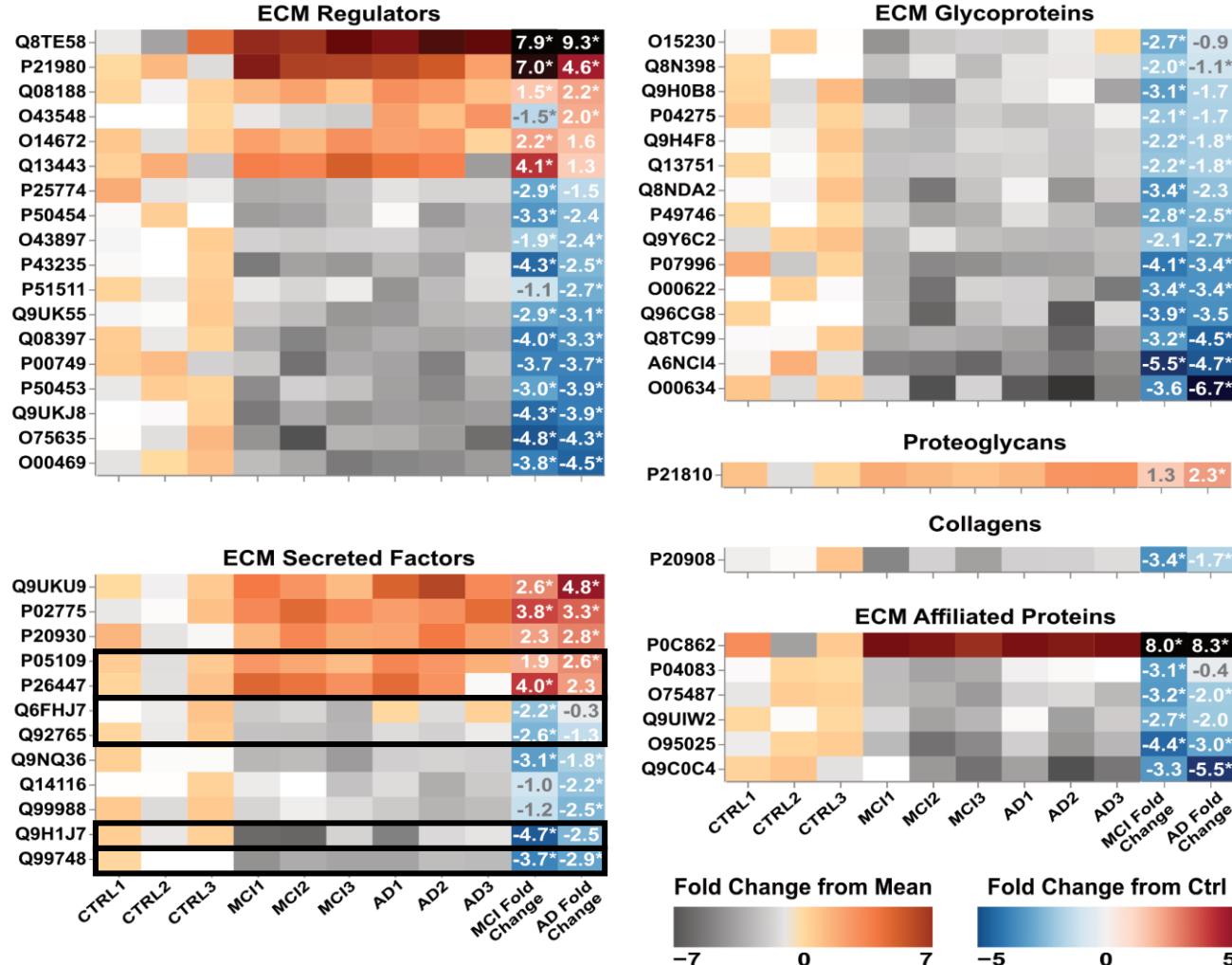
ECM Reorganization



Dysregulated Proteins

- Disentegrin and metalloproteinases
- Gamma glutamyltransferases
- Serpins

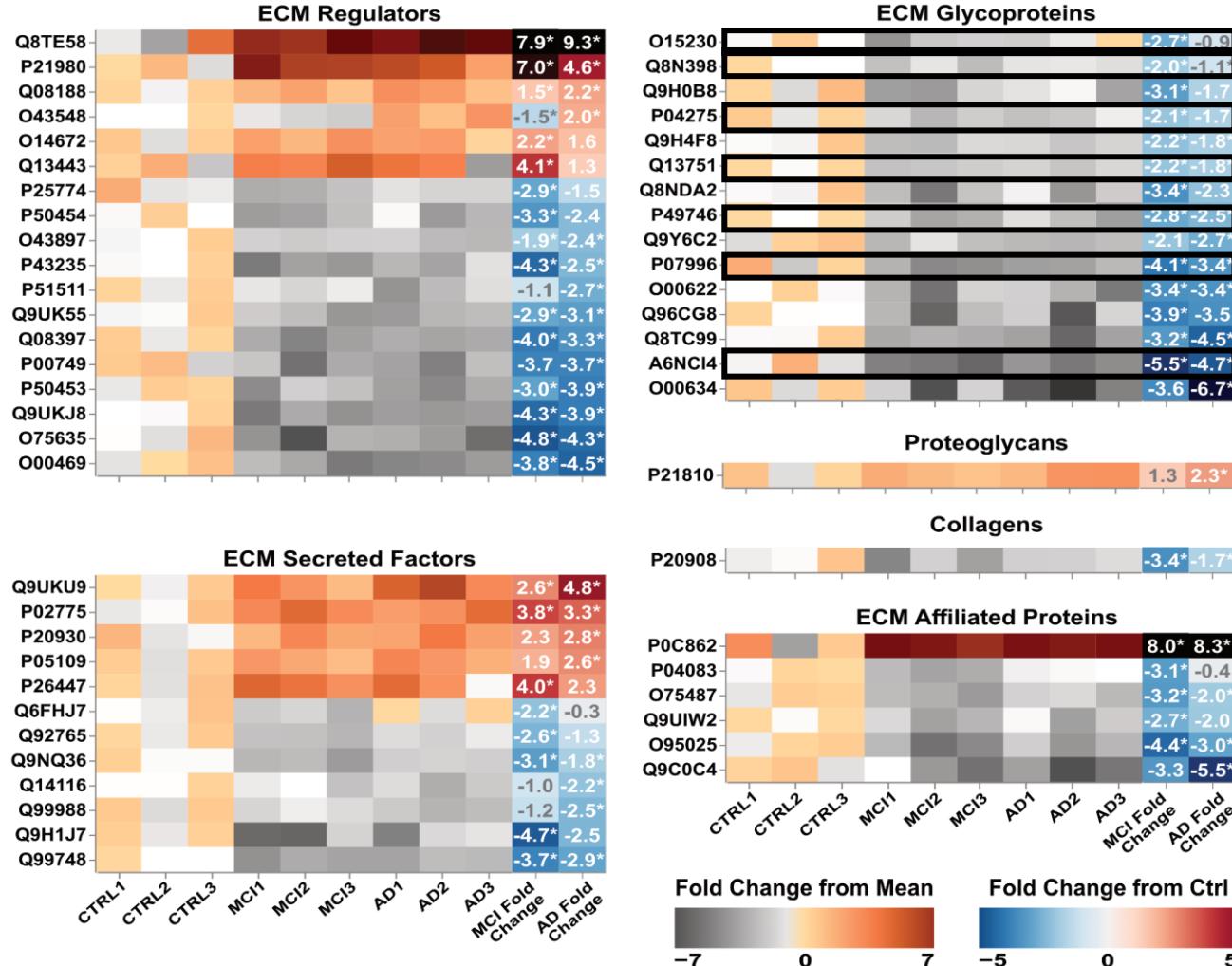
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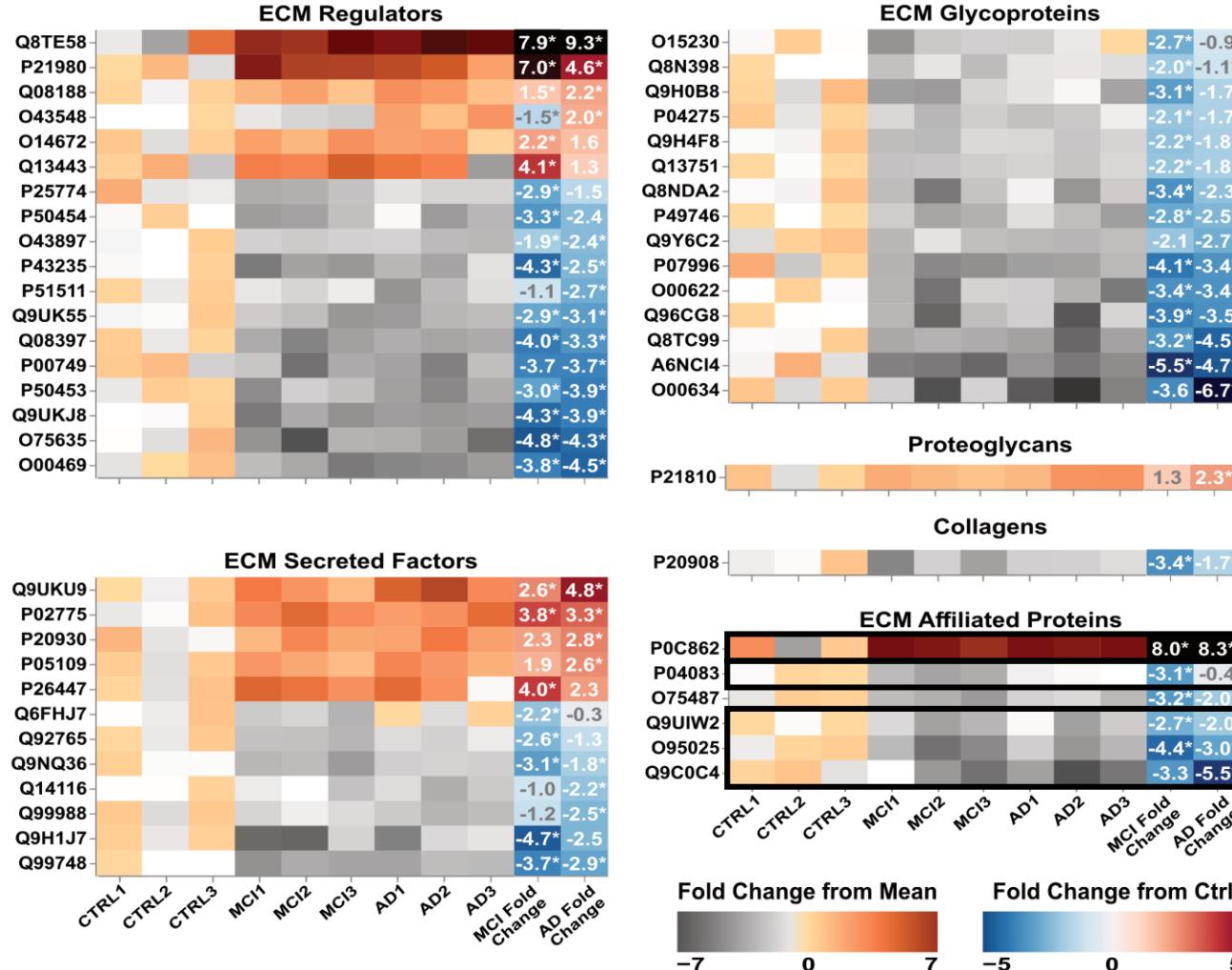
ECM Reorganization



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- Lamanin Subunits
- Thrombospondins

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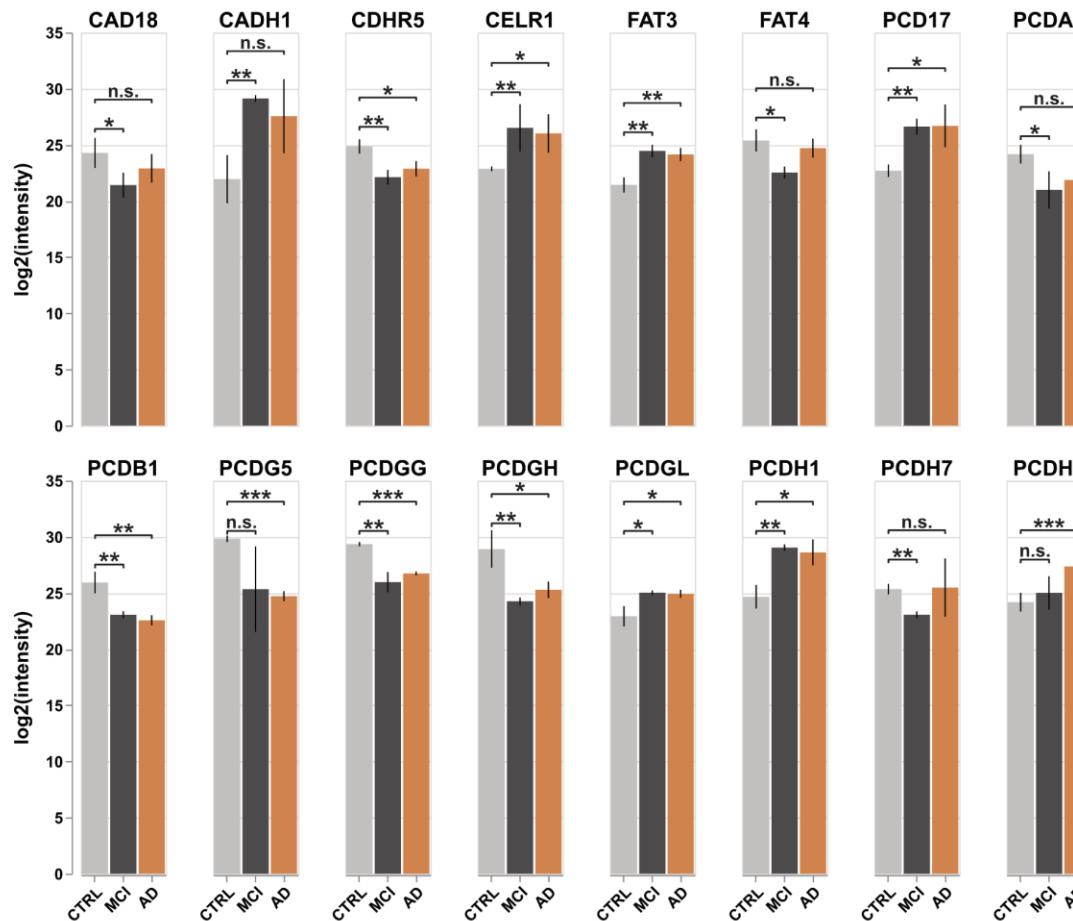


Dysregulated Proteins

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- Gamma glutamyltransferases
- Serpins
- S100 Proteins
- Wnt Pathway Proteins
- Neurturin
- Von Willebrand Factors
- Lamanin Subunits
- Thrombospondins
- Plexin and Semaphorins
- Annexin
- Complement C1q

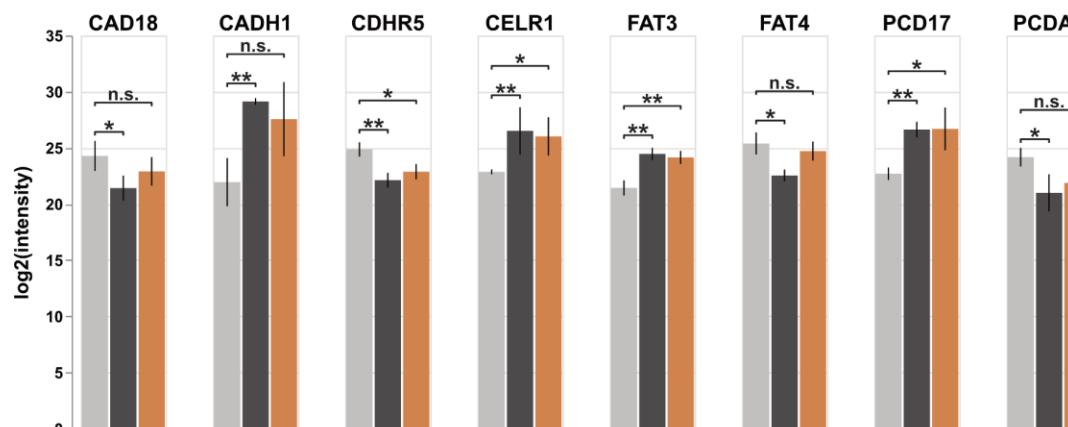
Protein Class Dysregulation

Cadherins & Protocadherins

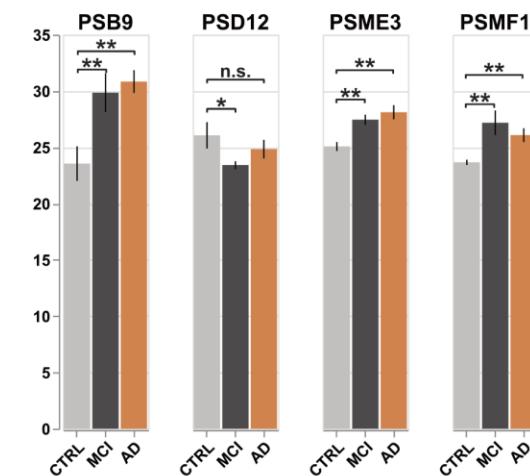
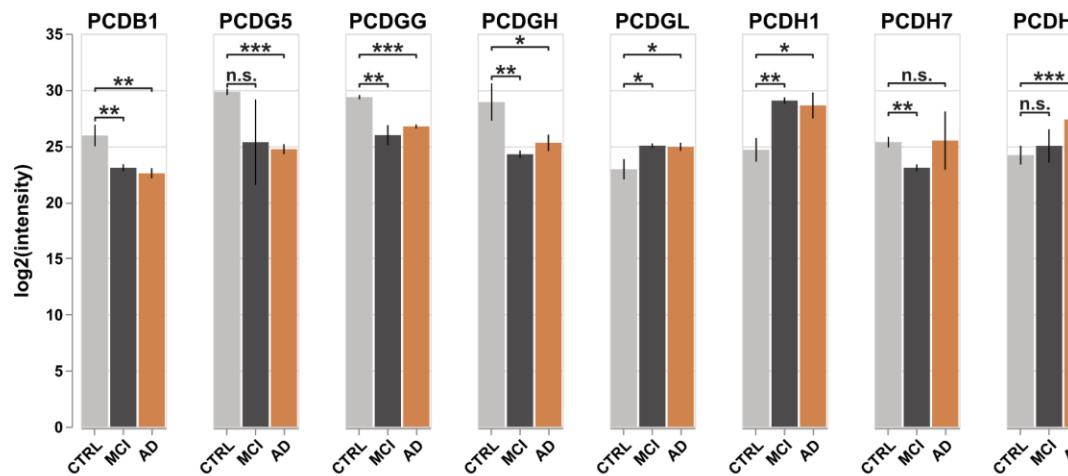
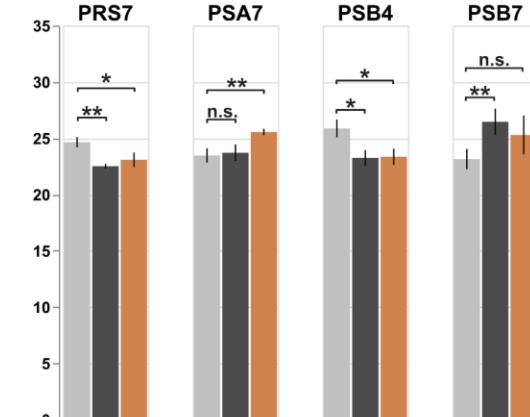


Protein Class Dysregulation

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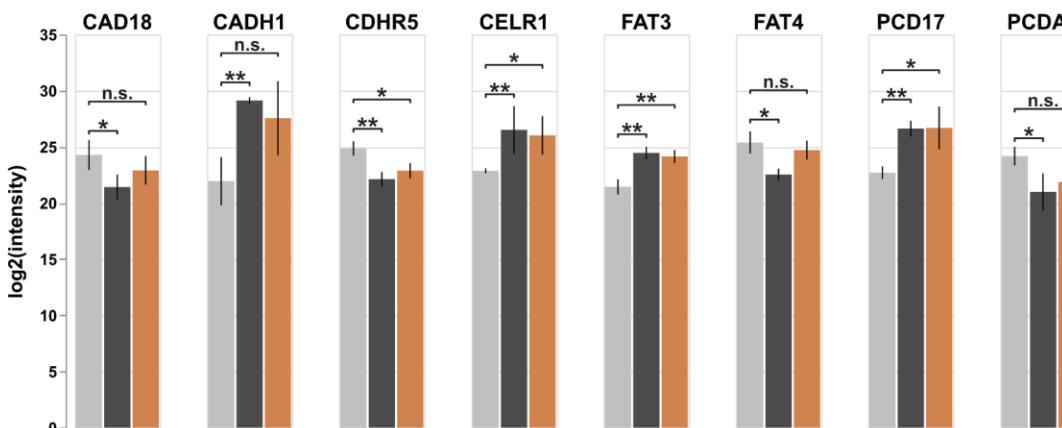


Proteasome

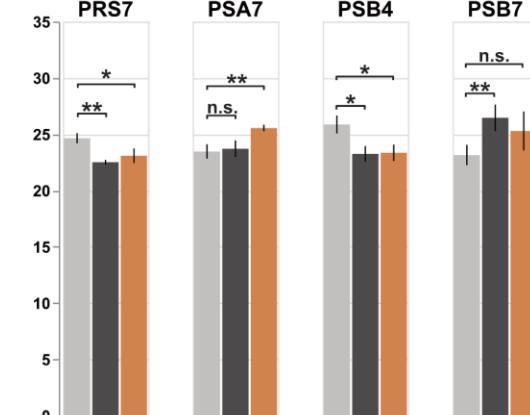


Protein Class Dysregulation

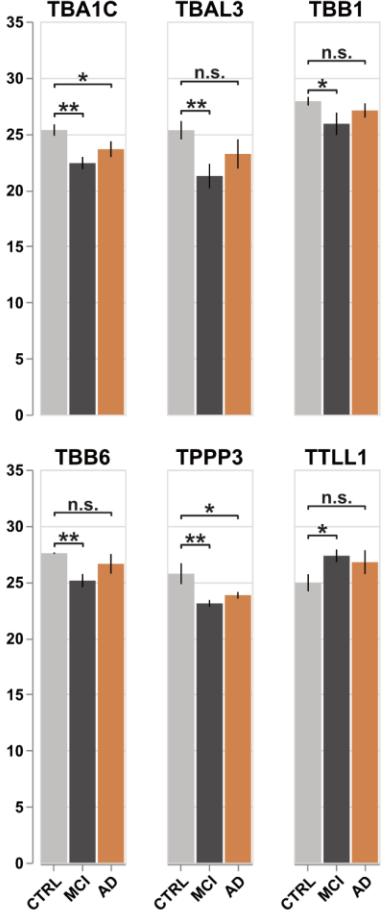
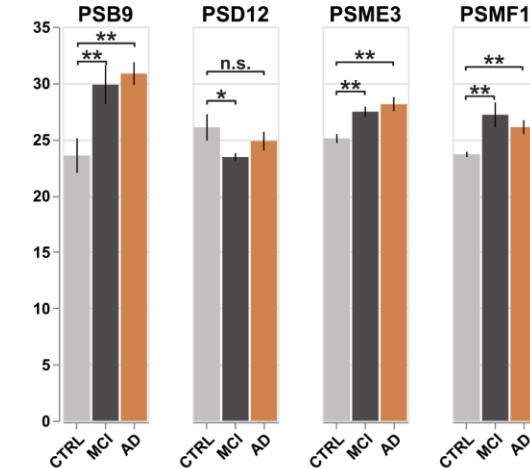
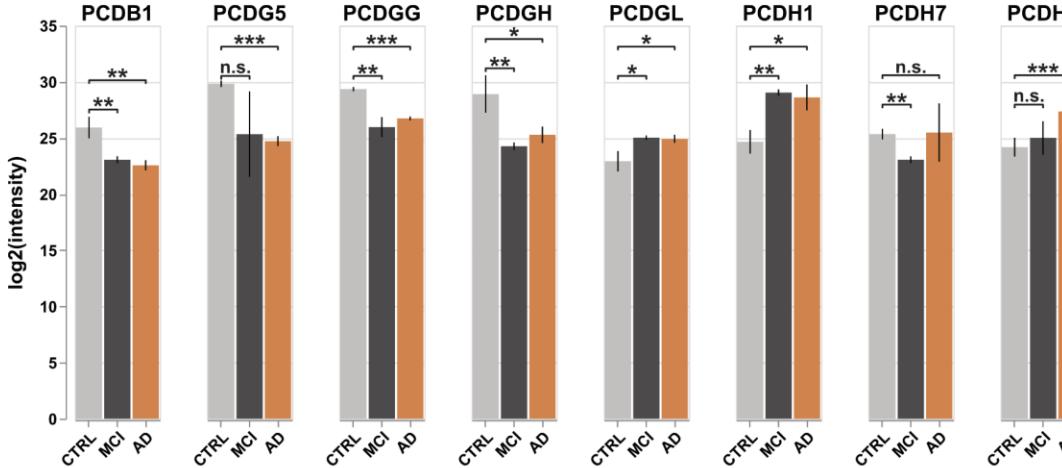
Cadherins & Protocadherins



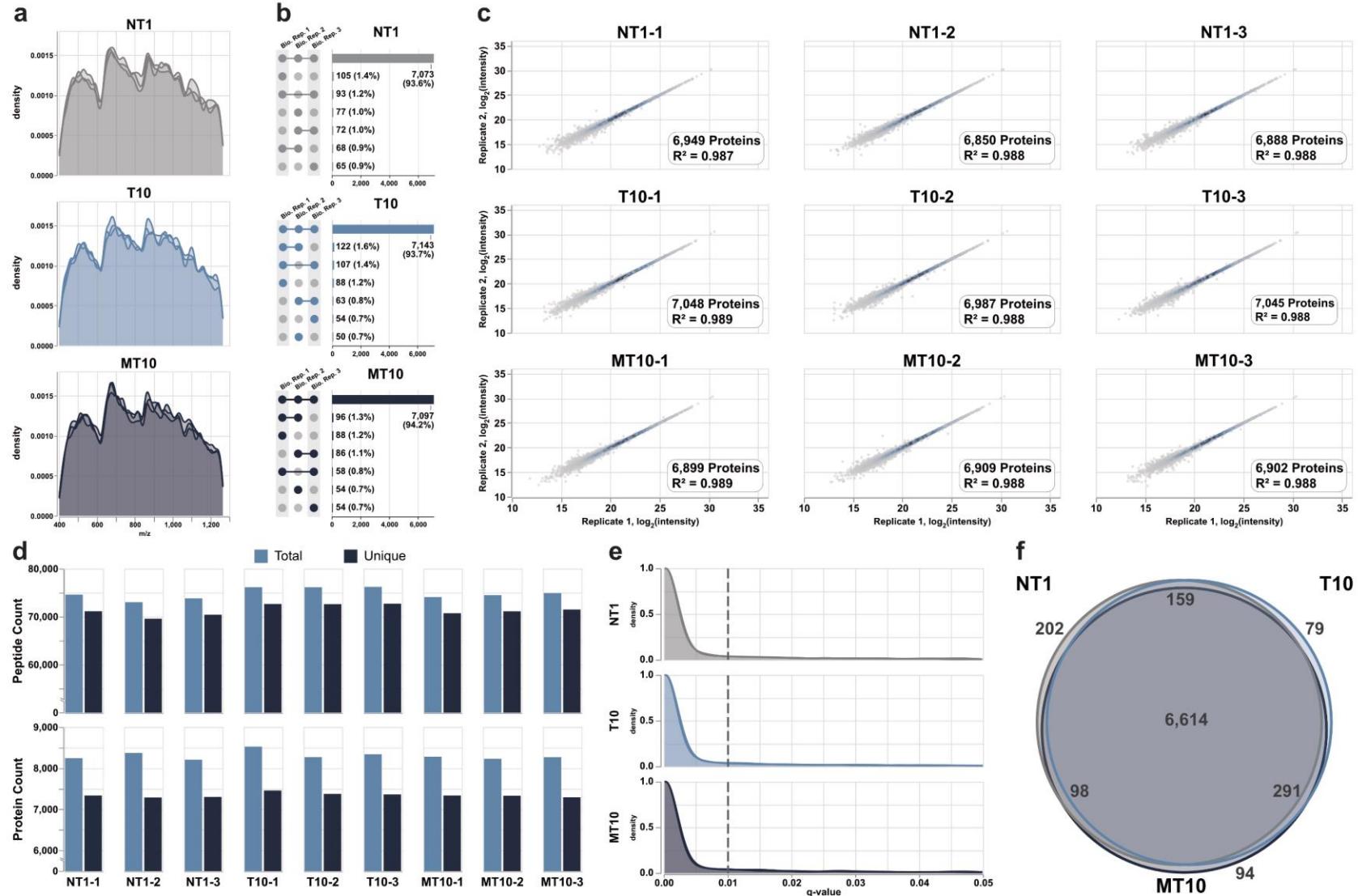
Proteasome



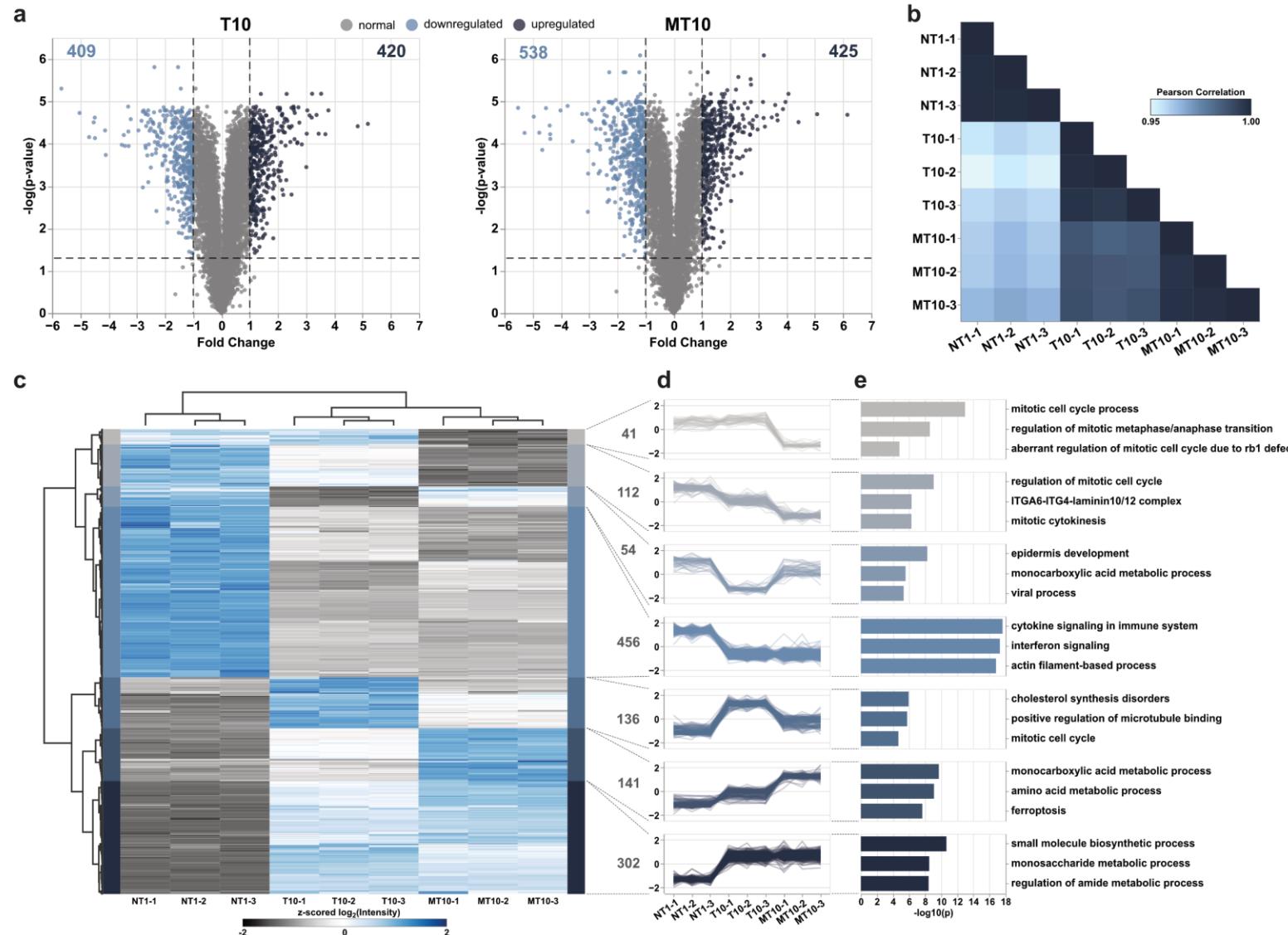
Tubulins



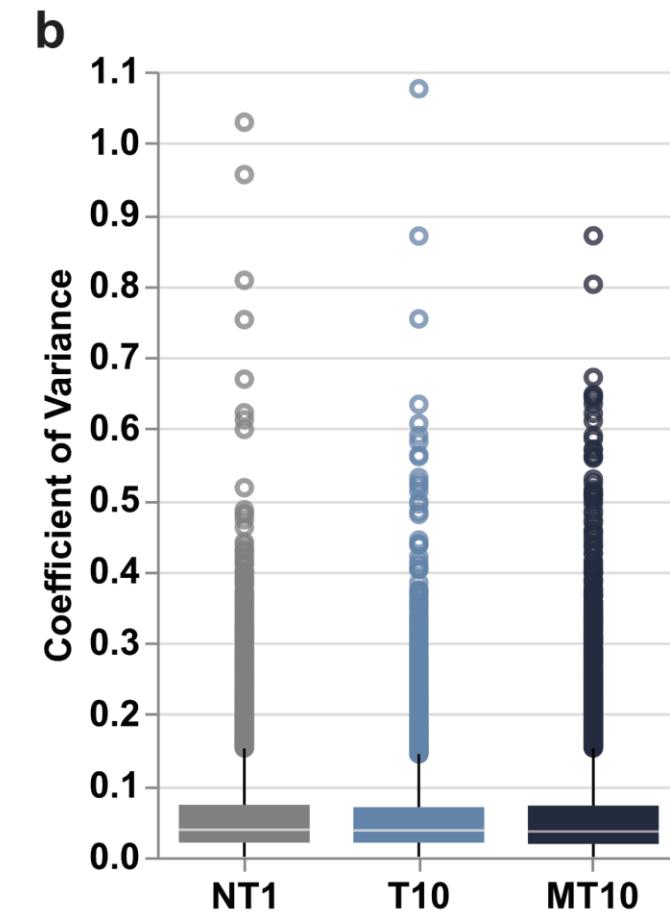
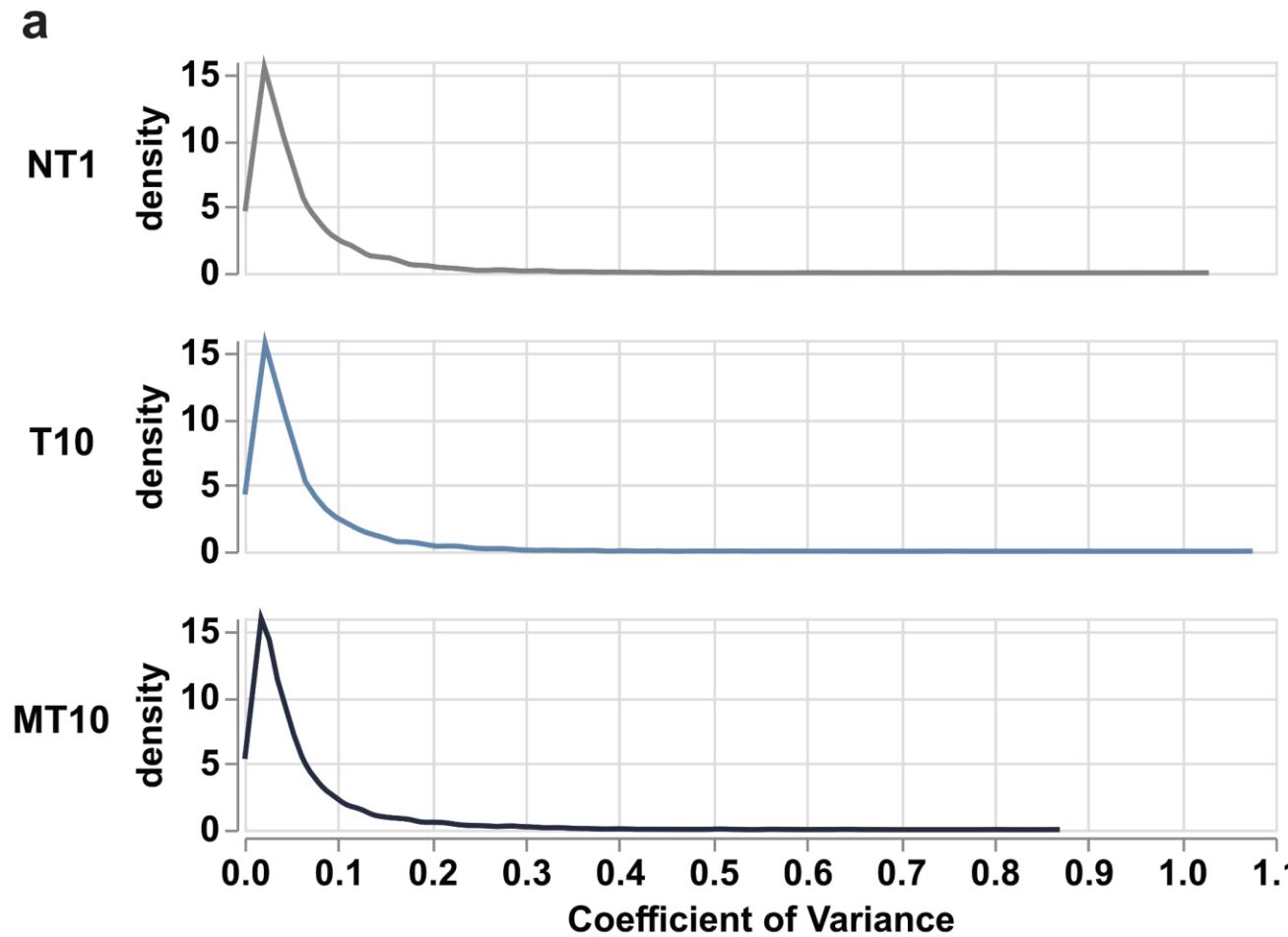
DIA Provides Extensive Profiling Access



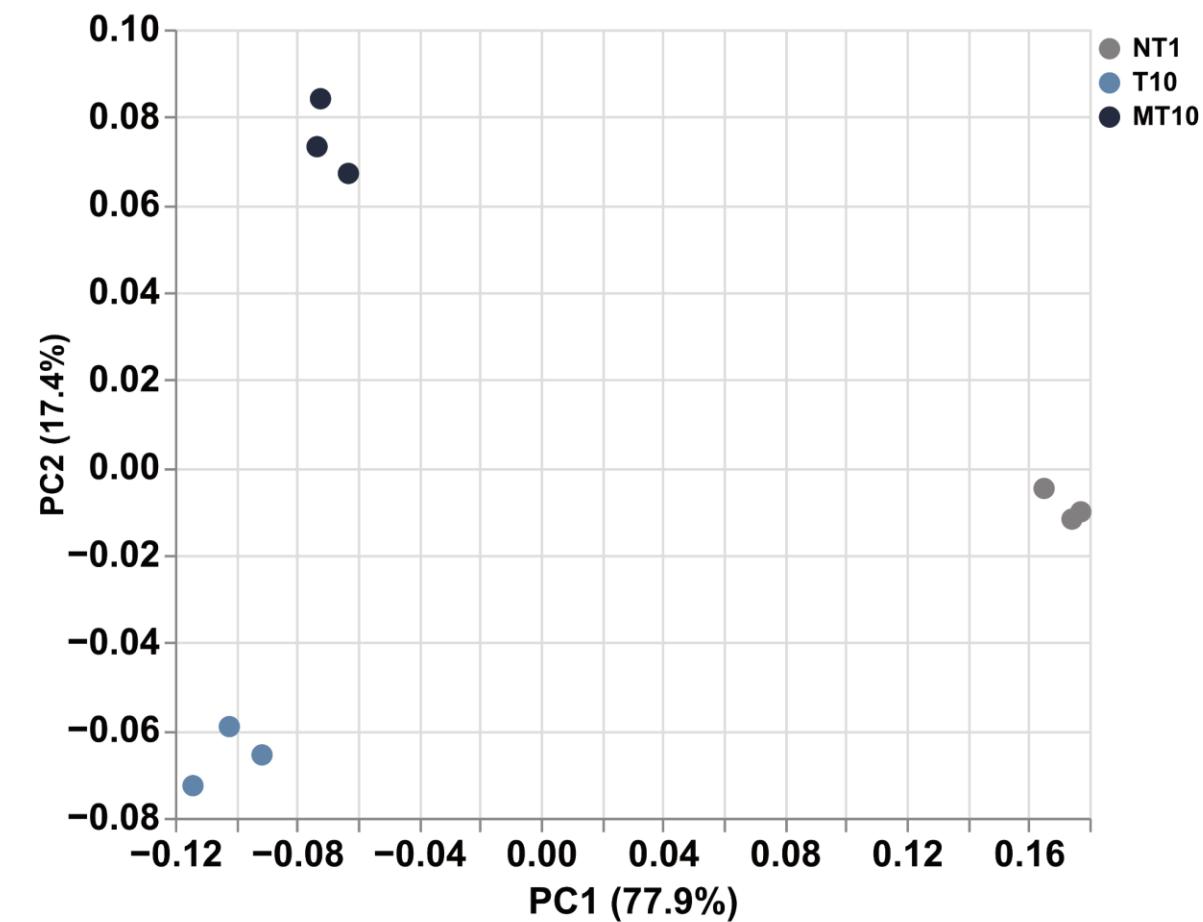
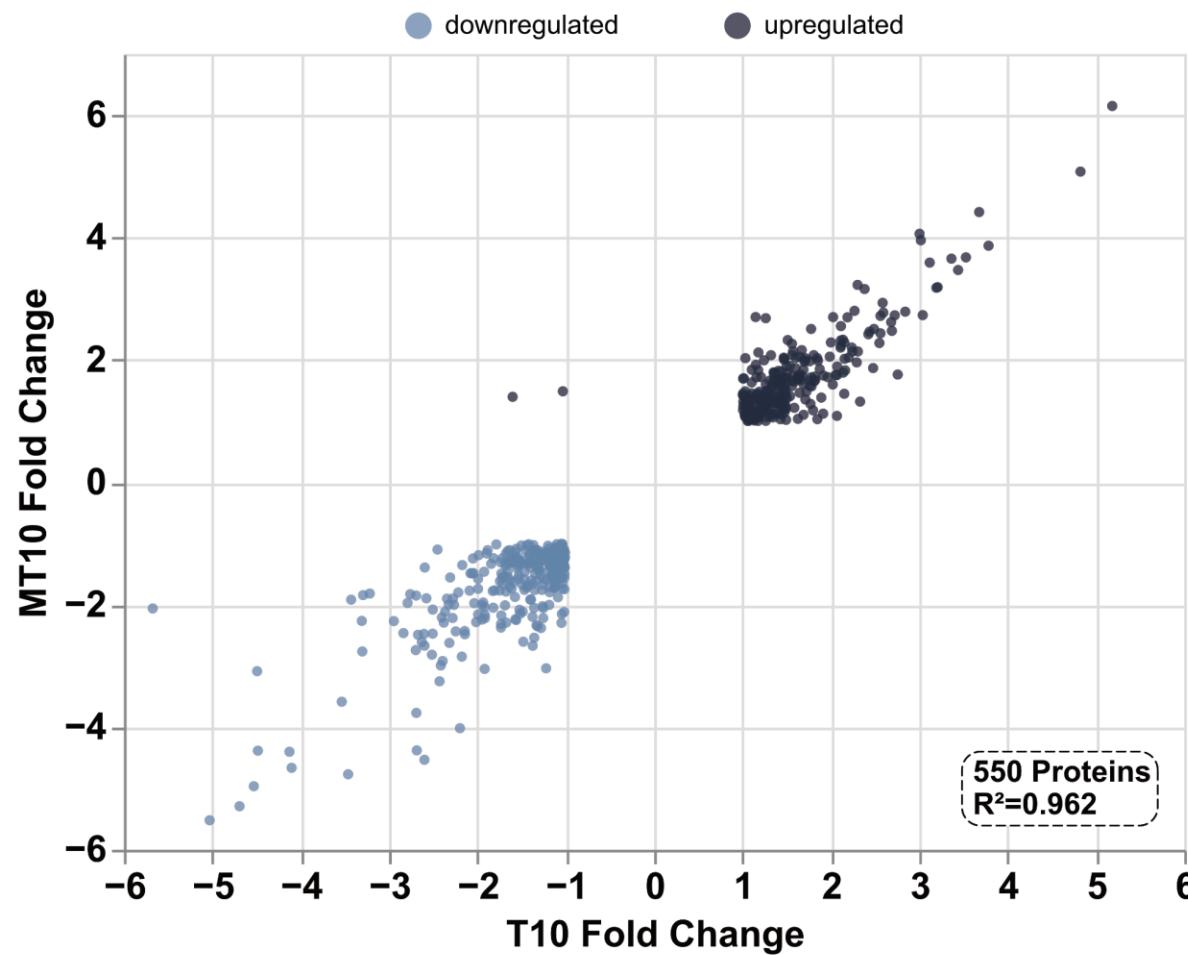
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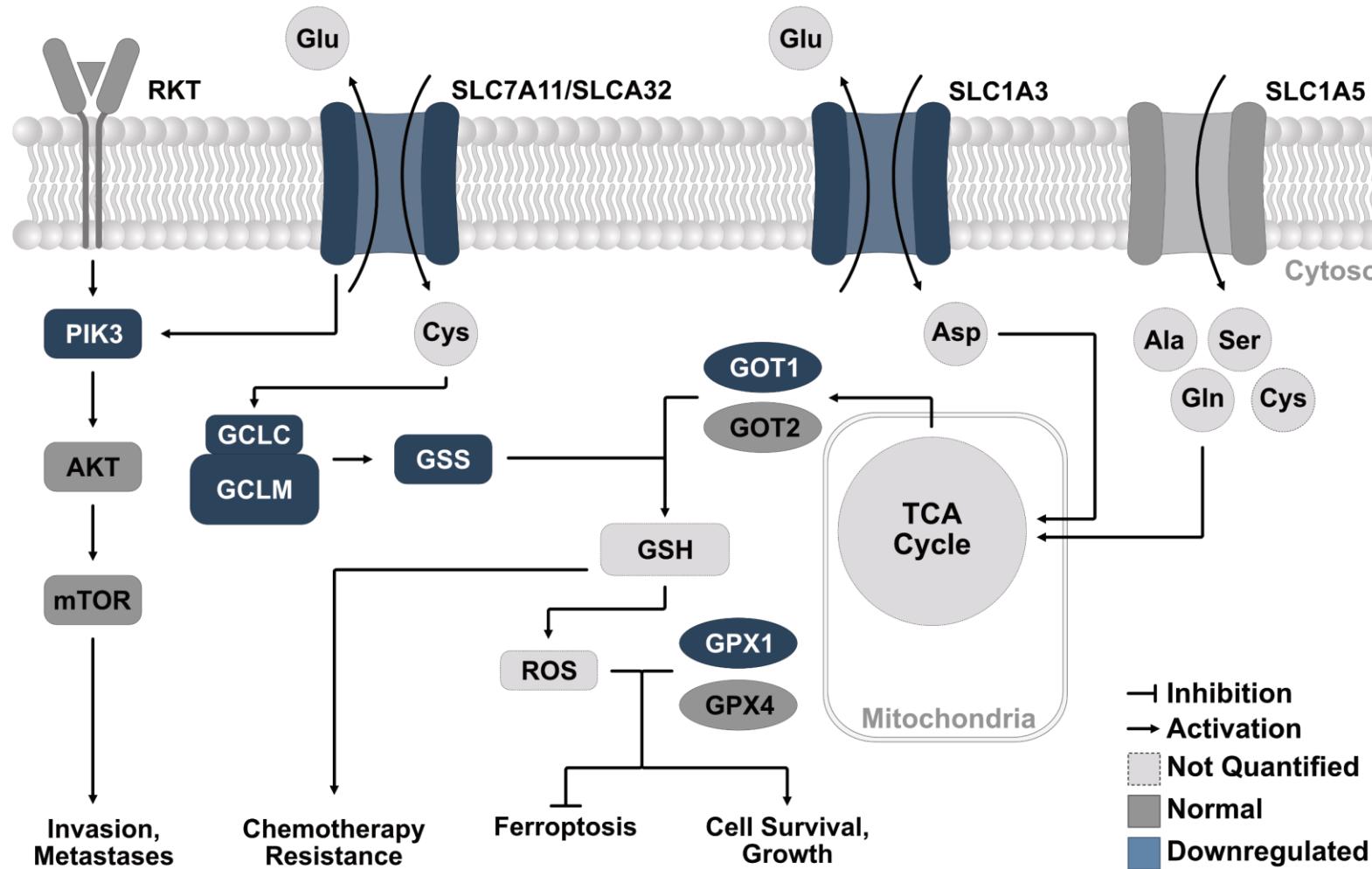
Variance in BCaP DIA Measurements



Correlation and Dimensional Reduction

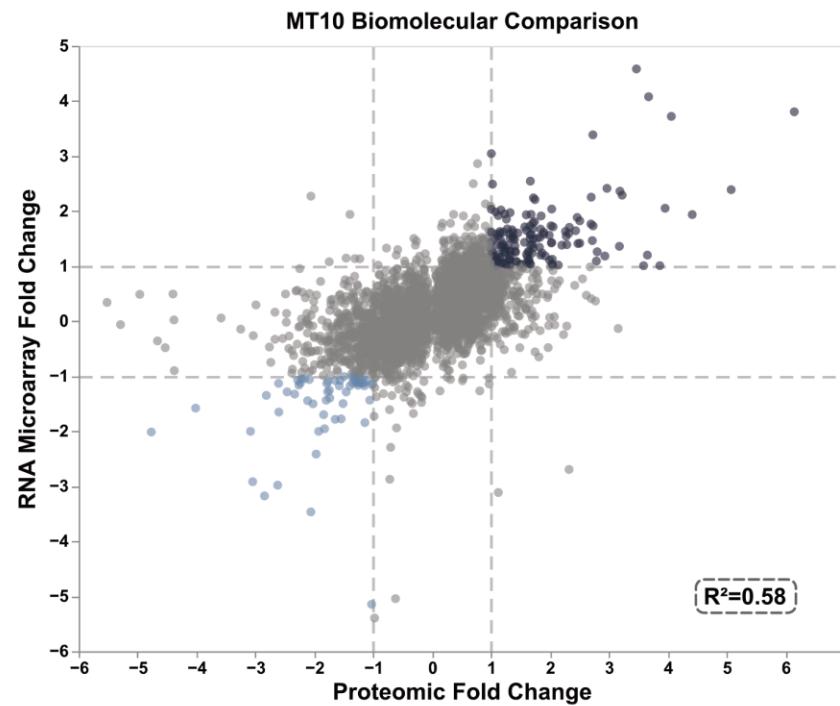


GSH Overproduction in PCa

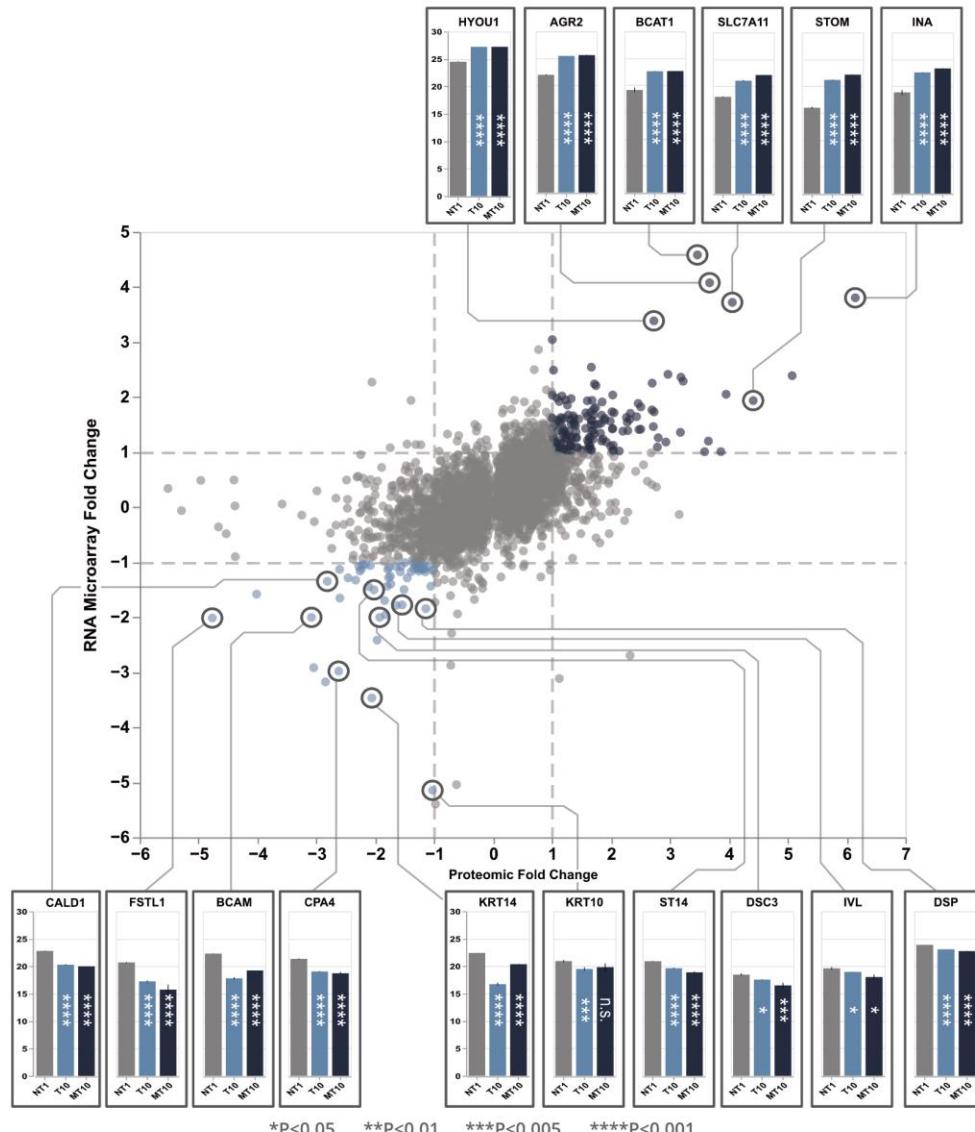


- Proteomic analyses reveal upregulation of proteins involved in GSH production
- Increased GSH is known to confer chemotherapy resistance
- Normal and upregulated expression of glutathione peroxidase suggests inhibition of ferroptosis and increased survival
- Future investigations should seek to directly quantify GSH *in vivo*

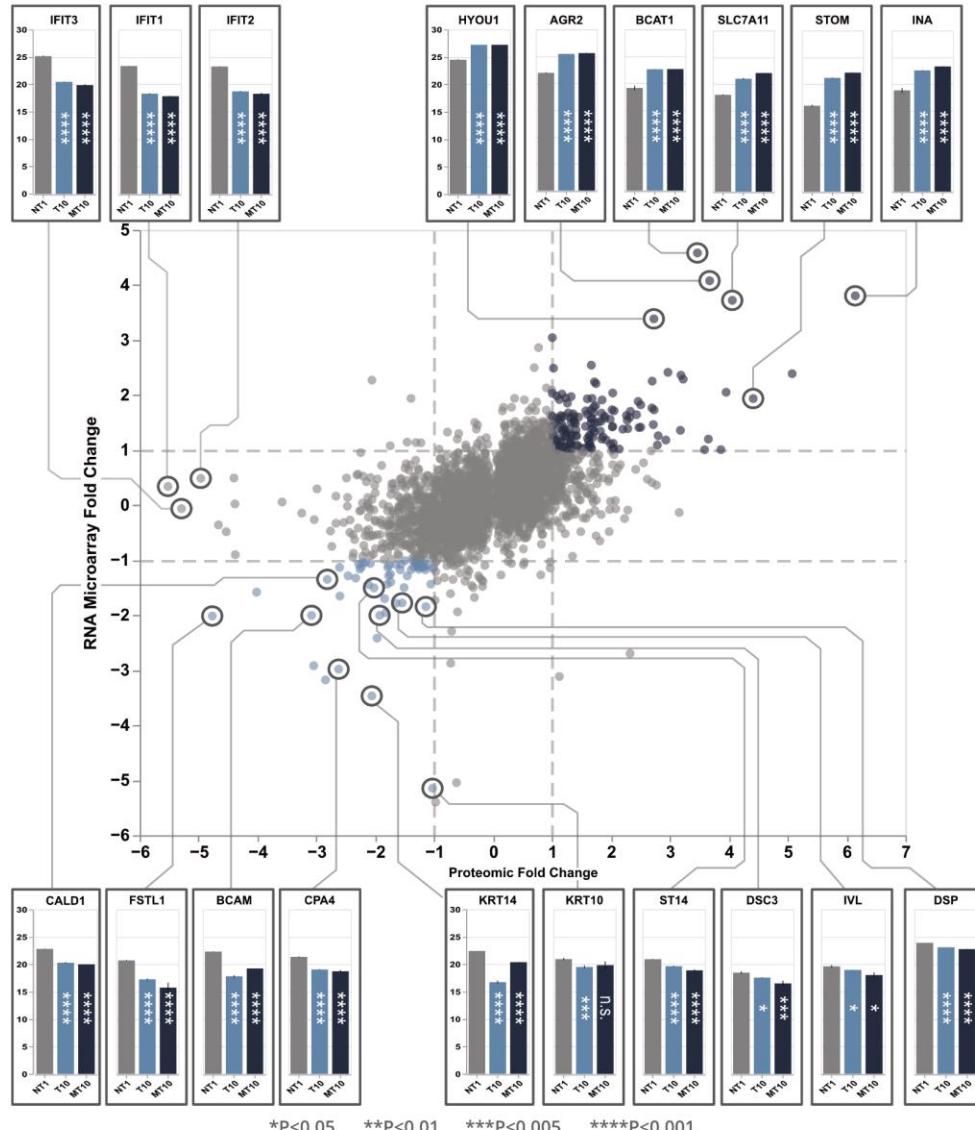
Illuminating Putative Biomarkers



Illuminating Putative Biomarkers



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