



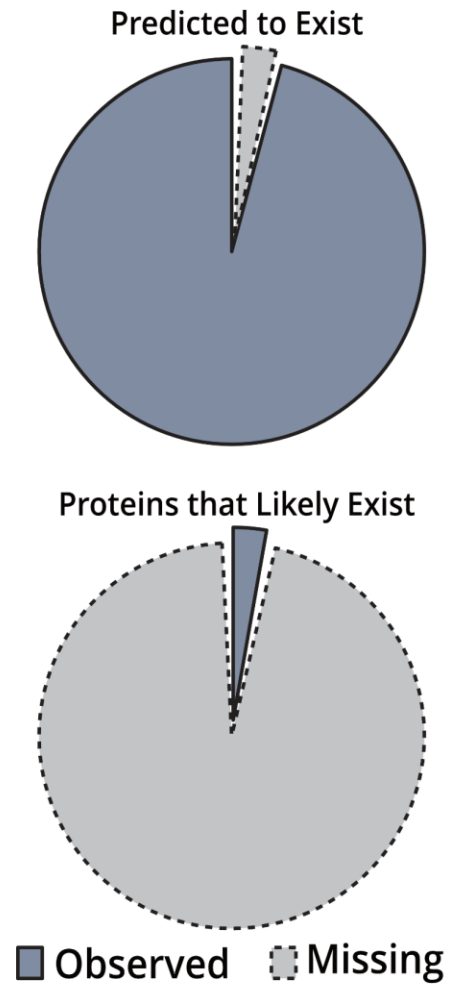
Standing in the Way of Proteome Profiling Depth

*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

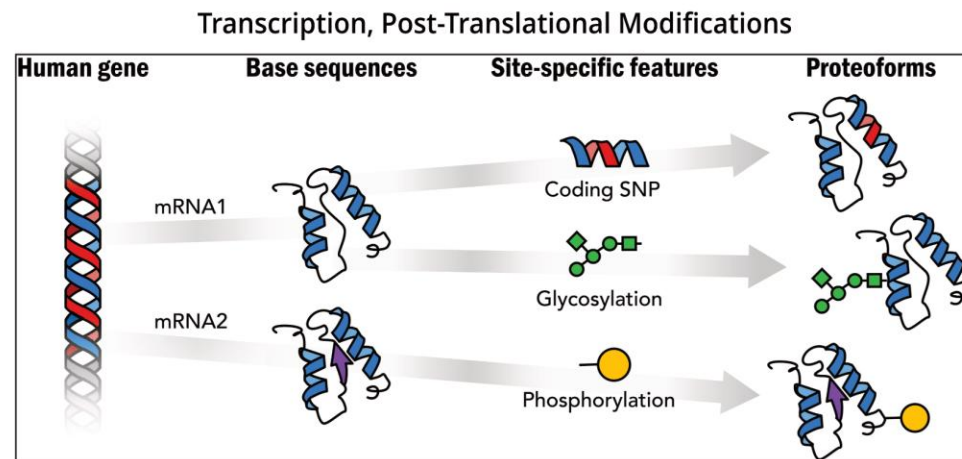
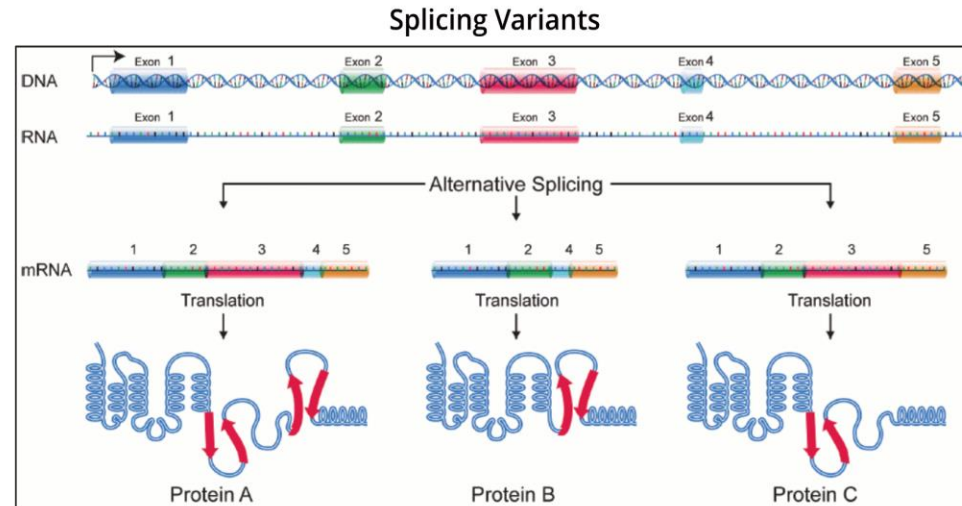
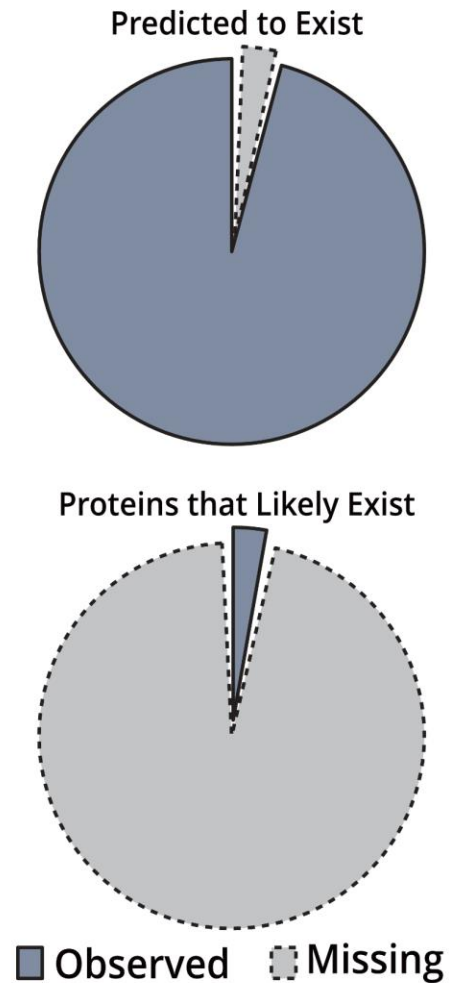


How Proteins Hide

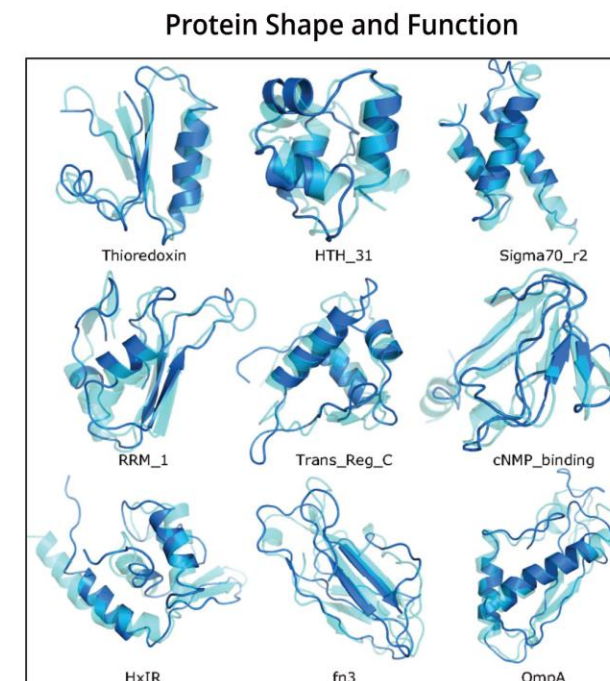
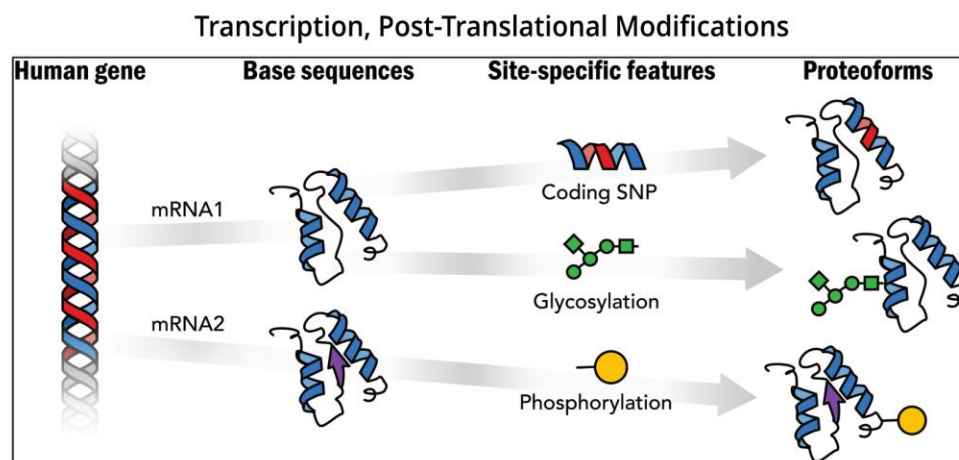
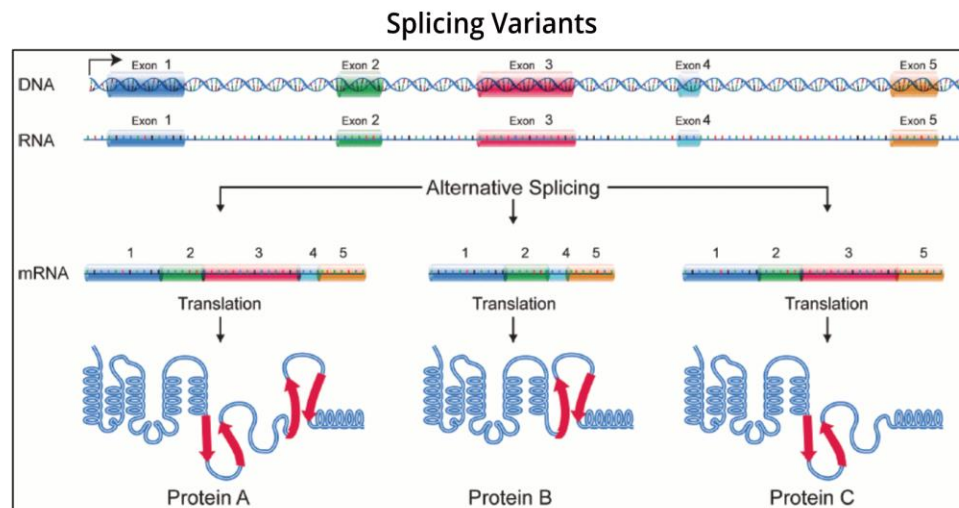
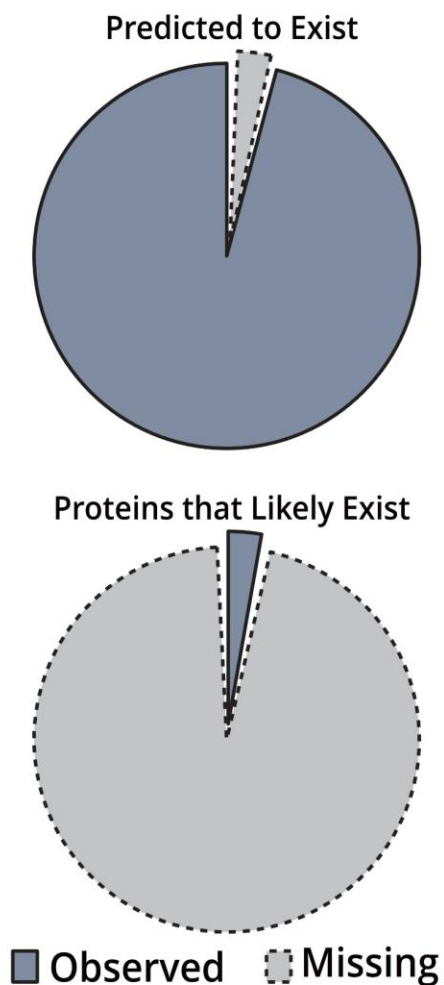




How Proteins Hide

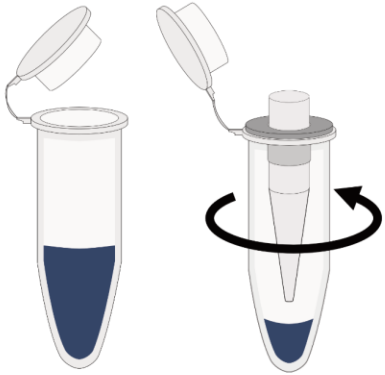


How Proteins Hide



Mass Spectrometry-Based Proteomics

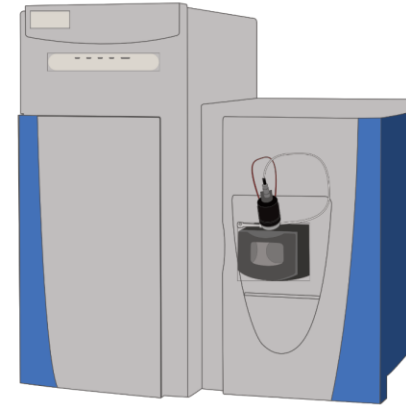
Preparation



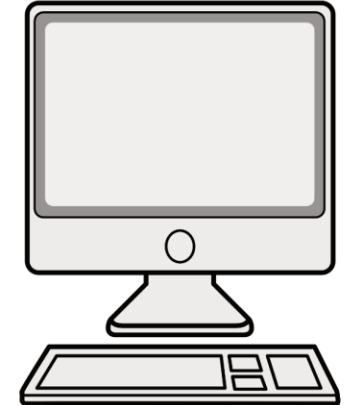
Separation



Detection



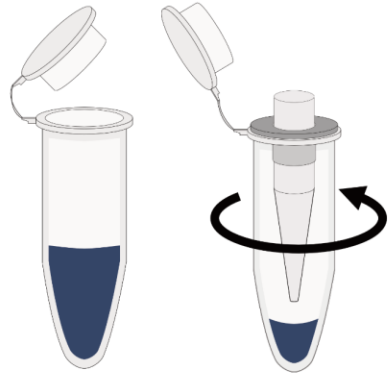
Analysis





Mass Spectrometry-Based Proteomics

Preparation



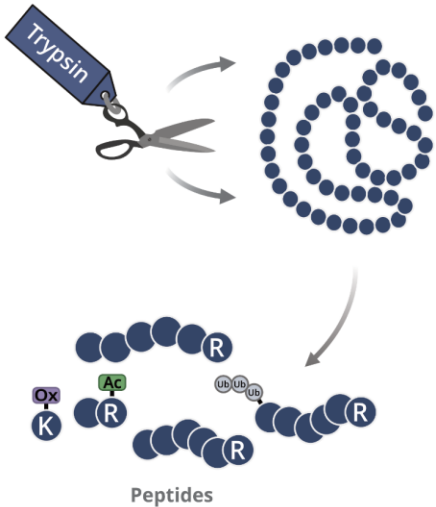
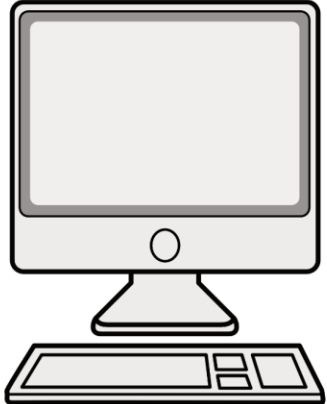
Separation



Detection

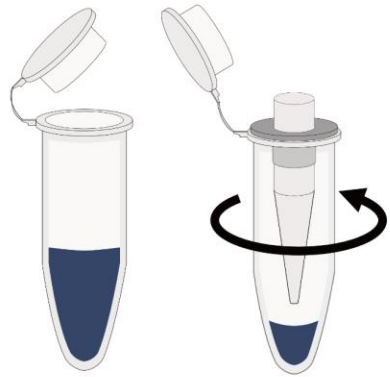


Analysis



Mass Spectrometry-Based Proteomics

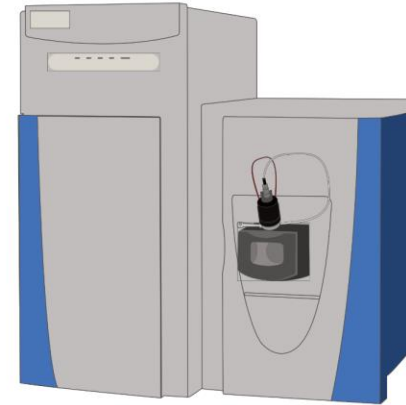
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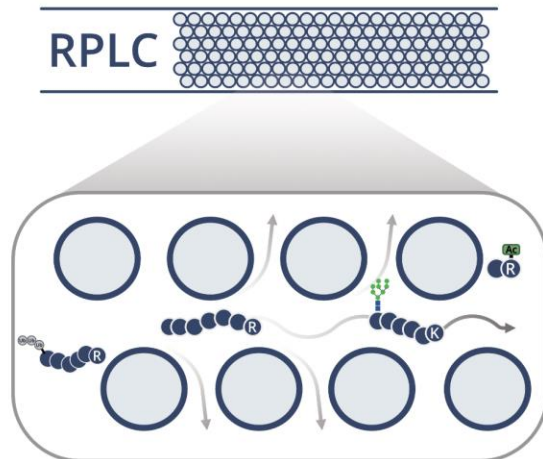
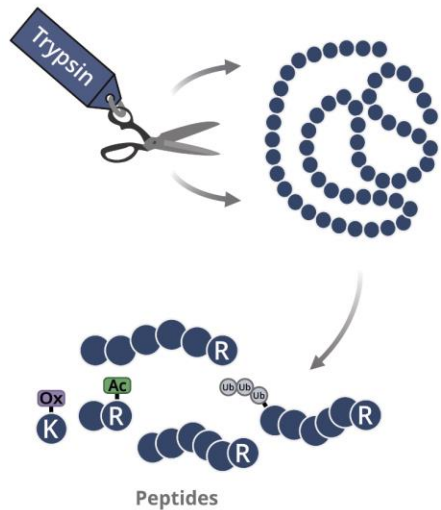
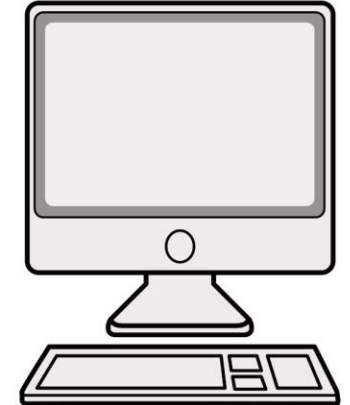
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Detection



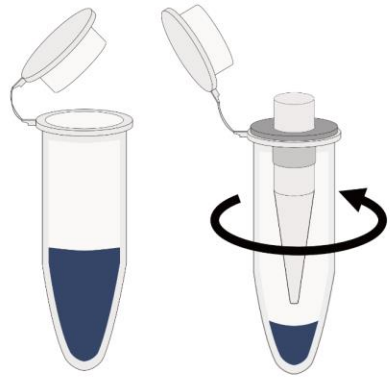
Analysis





Mass Spectrometry-Based Proteomics

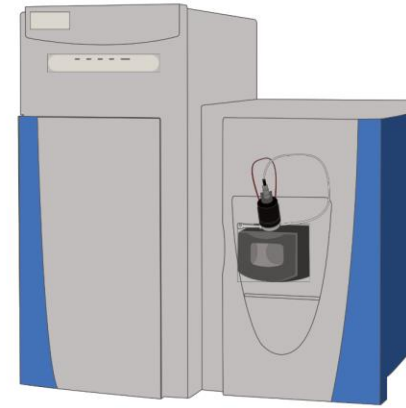
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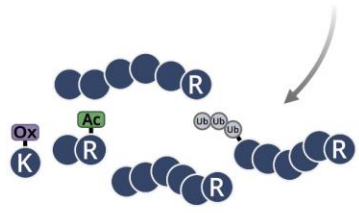
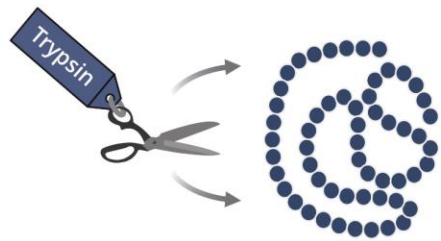
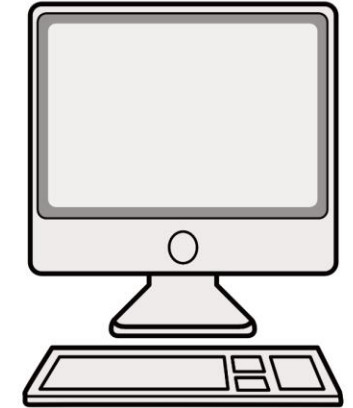
Separation



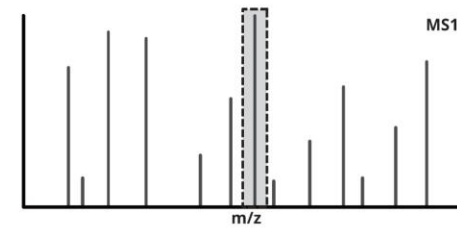
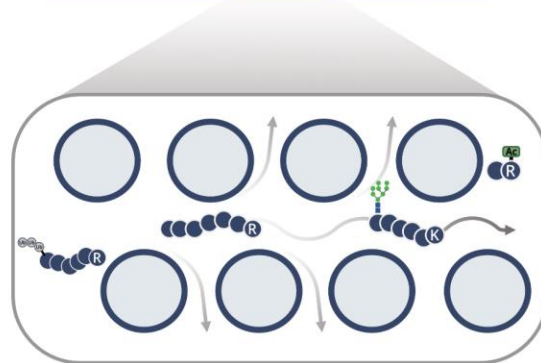
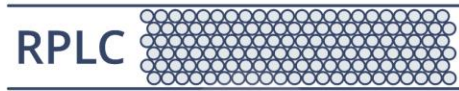
Detection



Analysis



Peptides



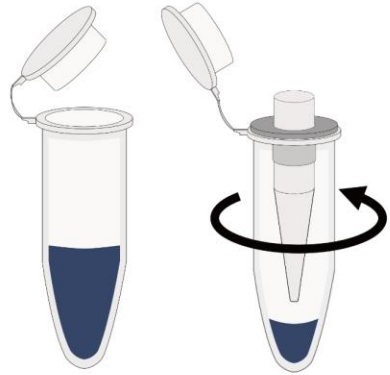
Precursor

Fragments



Mass Spectrometry-Based Proteomics

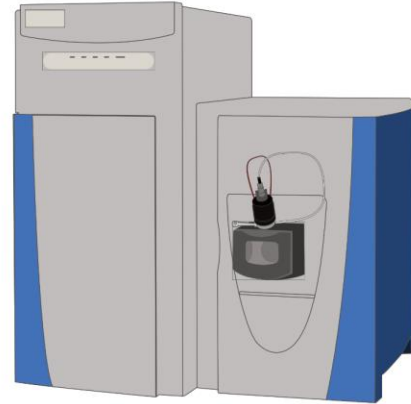
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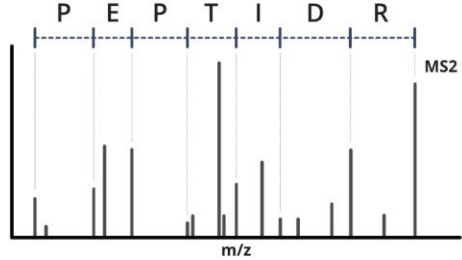
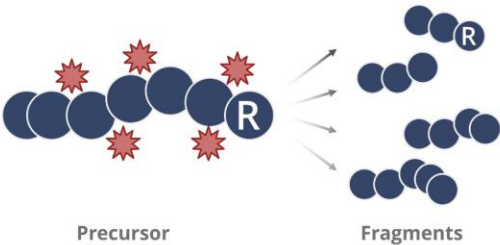
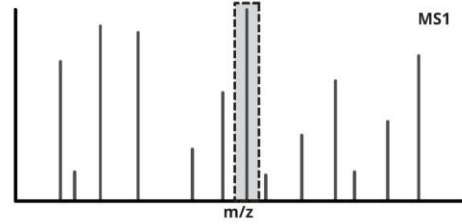
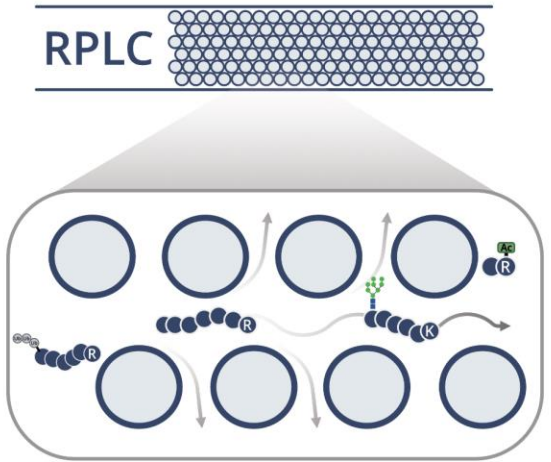
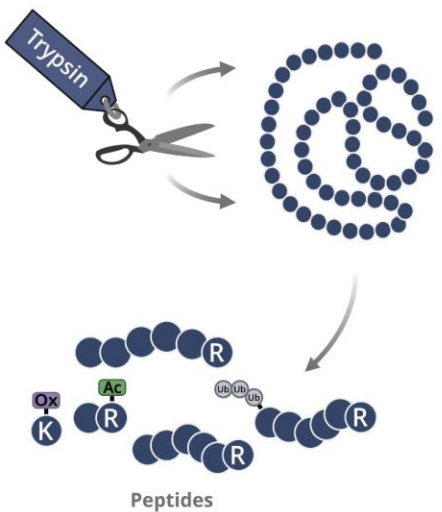
Separation



Detection



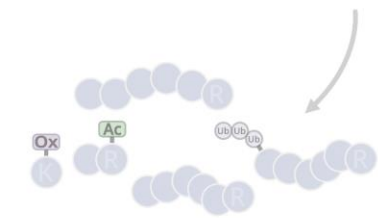
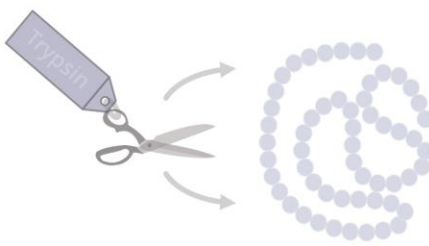
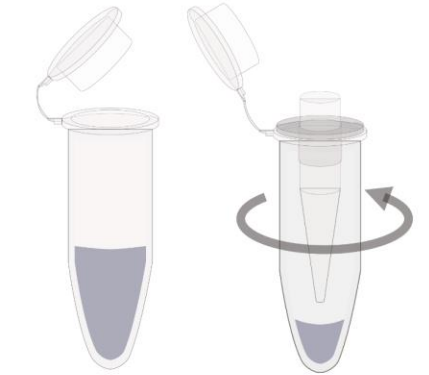
Analysis





Mass Spectrometry-Based Proteomics

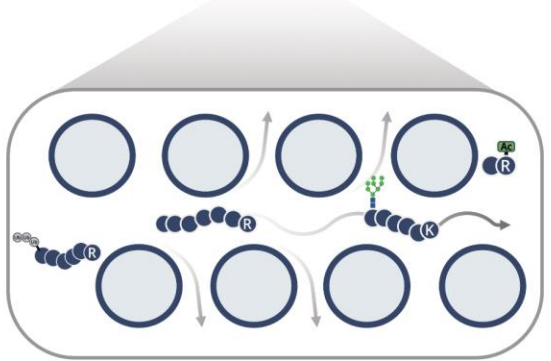
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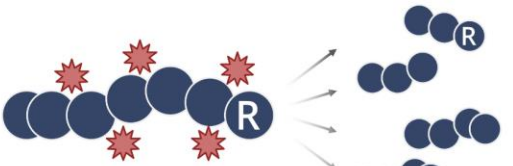
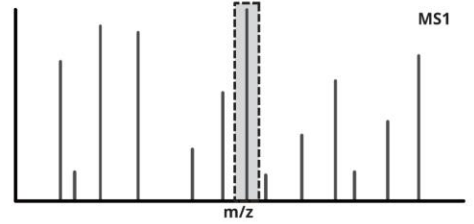
Separation



RPLC



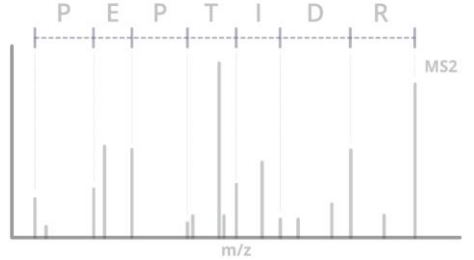
Detection



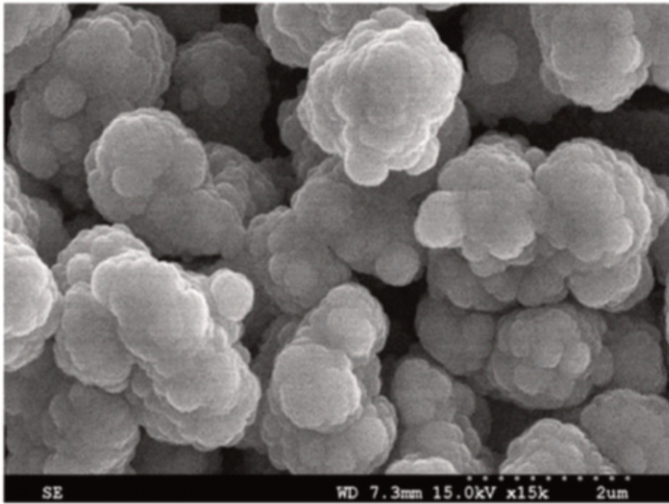
Precursor

Fragments

Analysis



Utilizing Alternative Separations

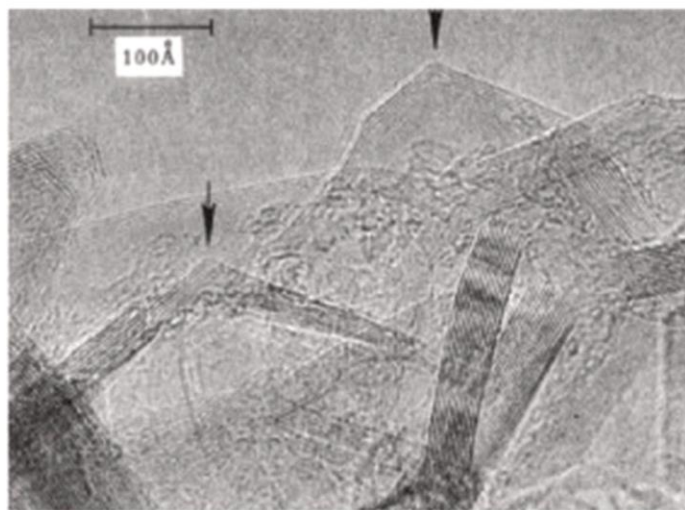


RPLC C18

Utilizing Alternative Separations



RPLC C18



PGC

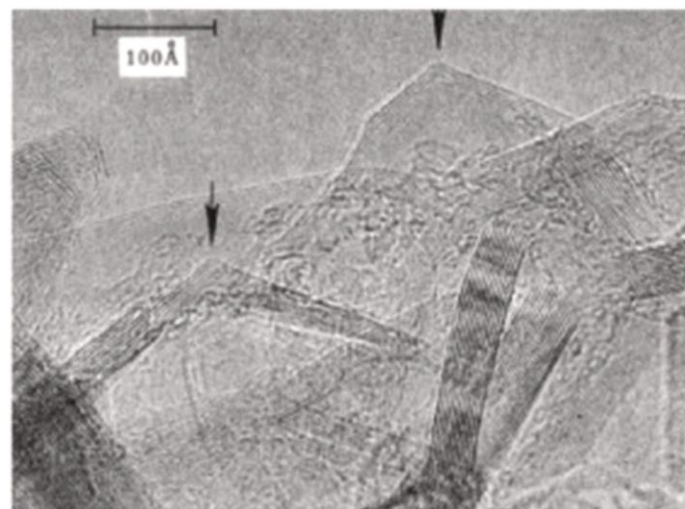
Porous Graphitic Carbon

- 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



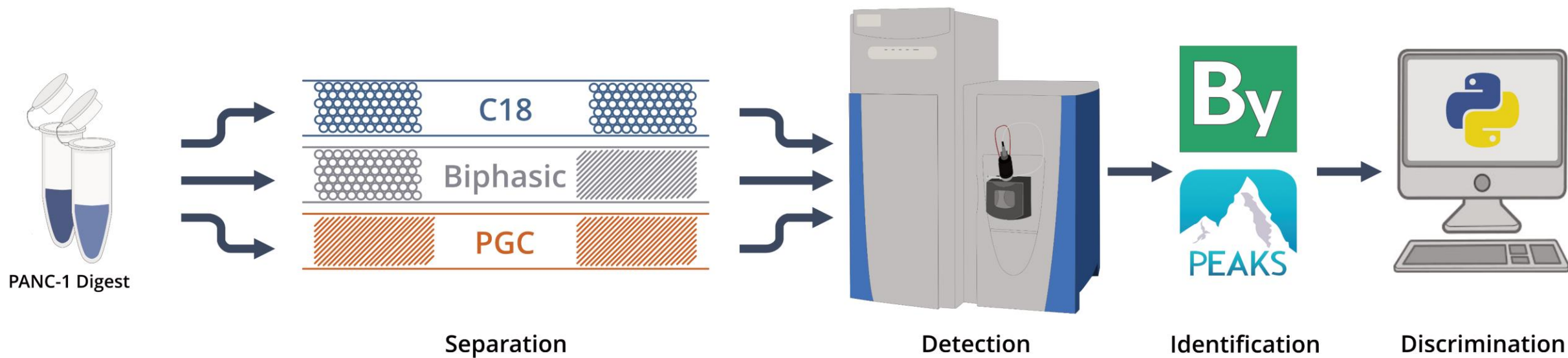
PGC

Porous Graphitic Carbon

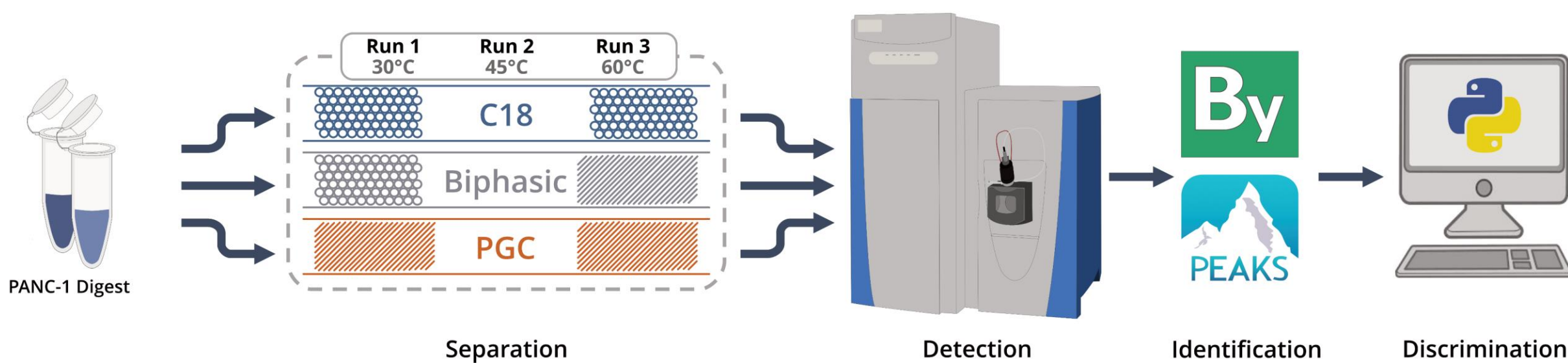
- Retention based on polar and electrostatic interactions
- Solvent flexibility, amenable to traditional water-organic systems
- Retention orthogonal to RPLC without the need for phase change
- Reproducible, available

Does PGC offer an amenable way to enhance proteome access?

PGC-Based Shotgun Proteomics

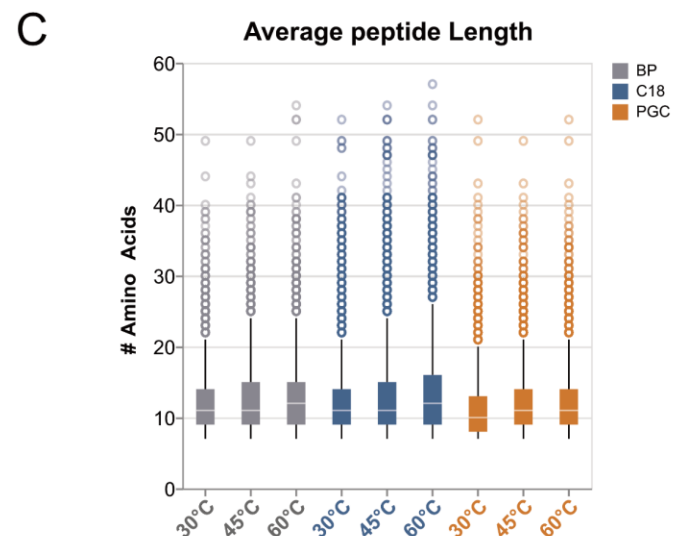
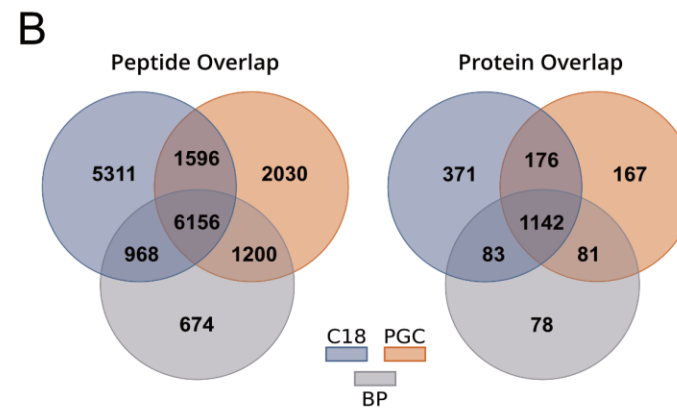
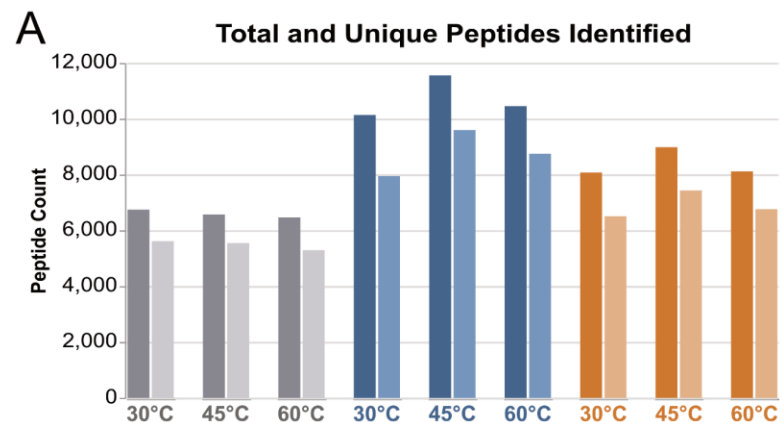


PGC-Based Shotgun Proteomics





PGC Complements RPLC Analyses

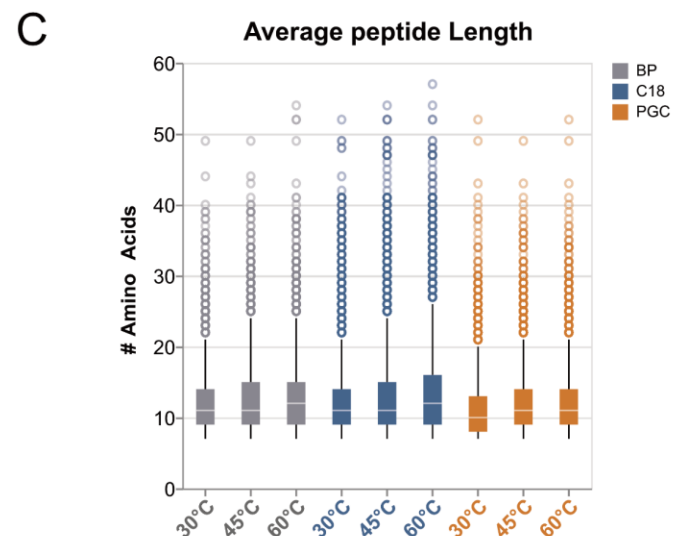
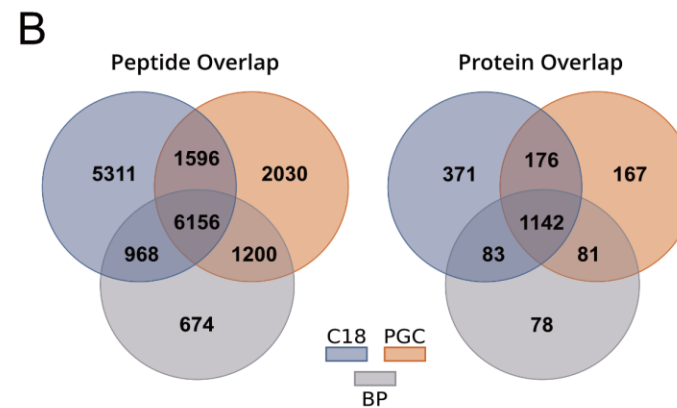
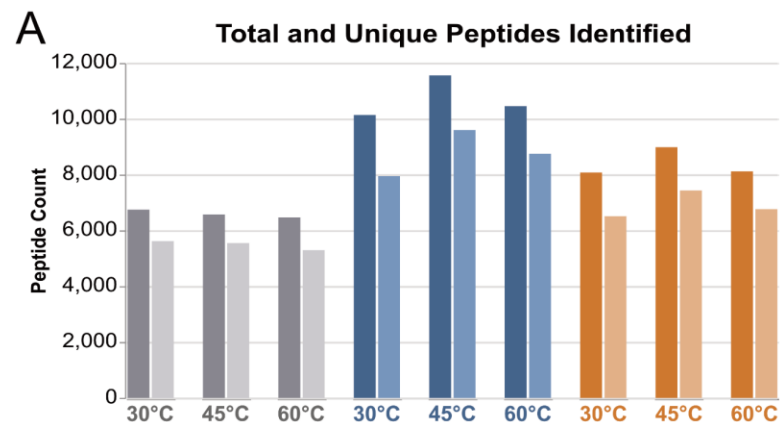


Key Results

- 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



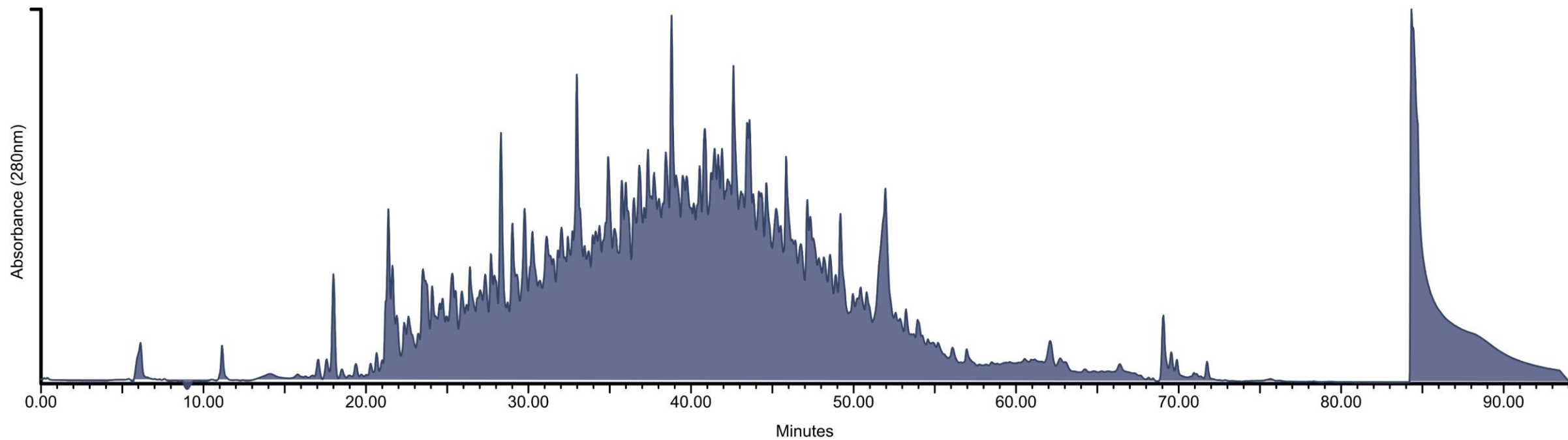
Key Results

- 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.

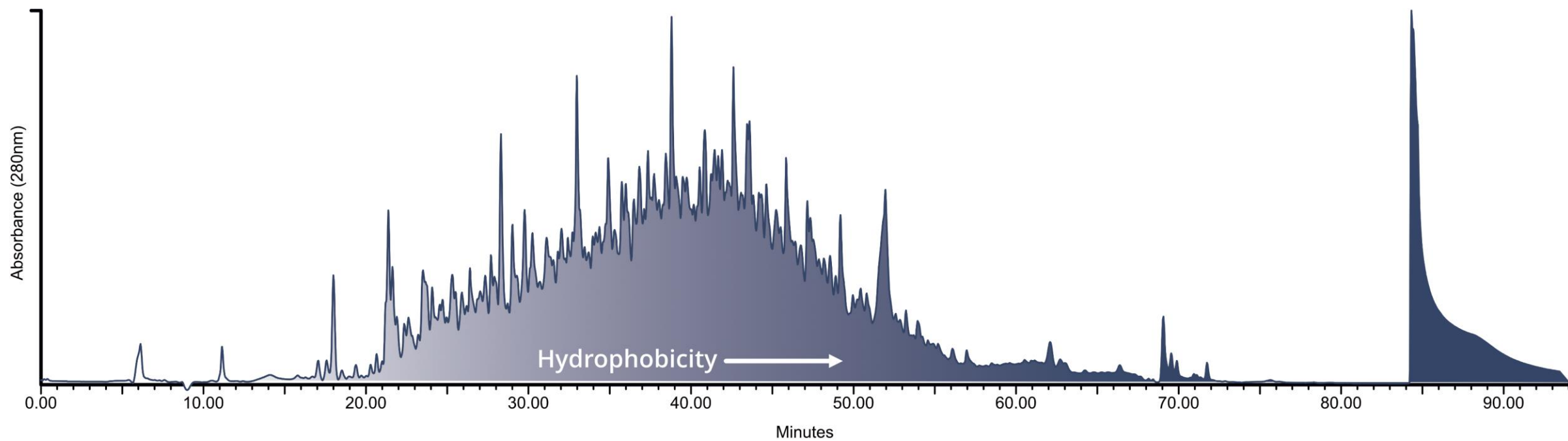


More Efficient Use of PGC-RPLC



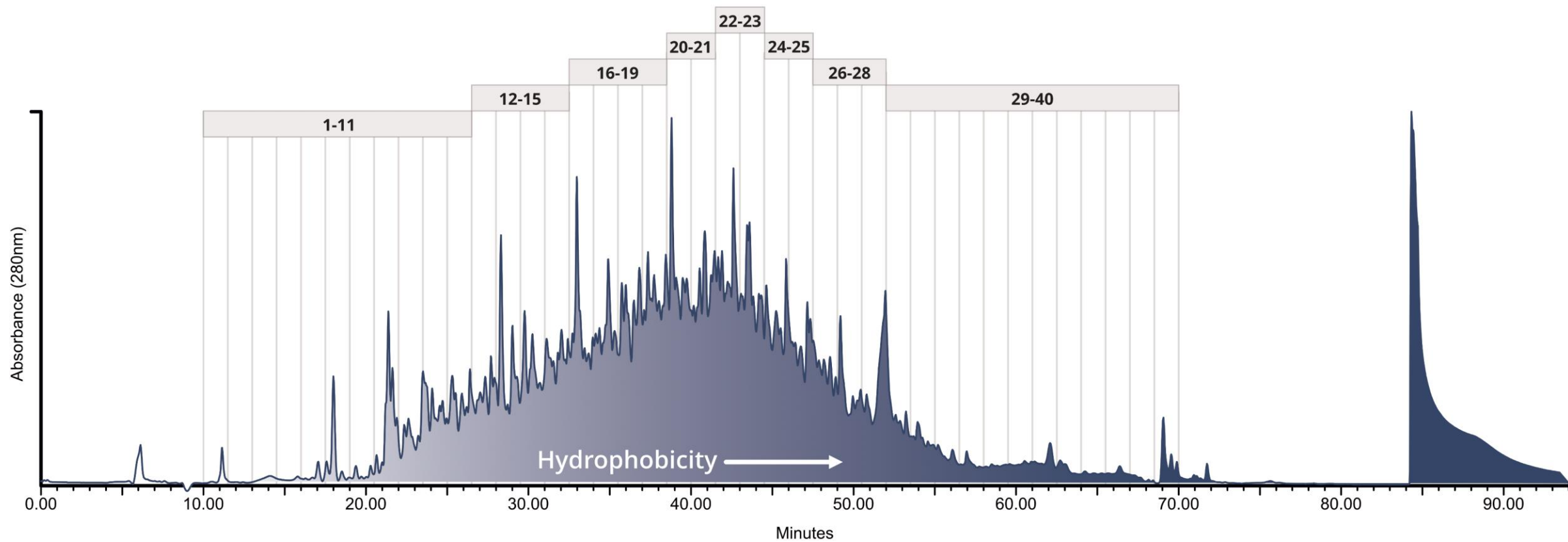


More Efficient Use of PGC-RPLC

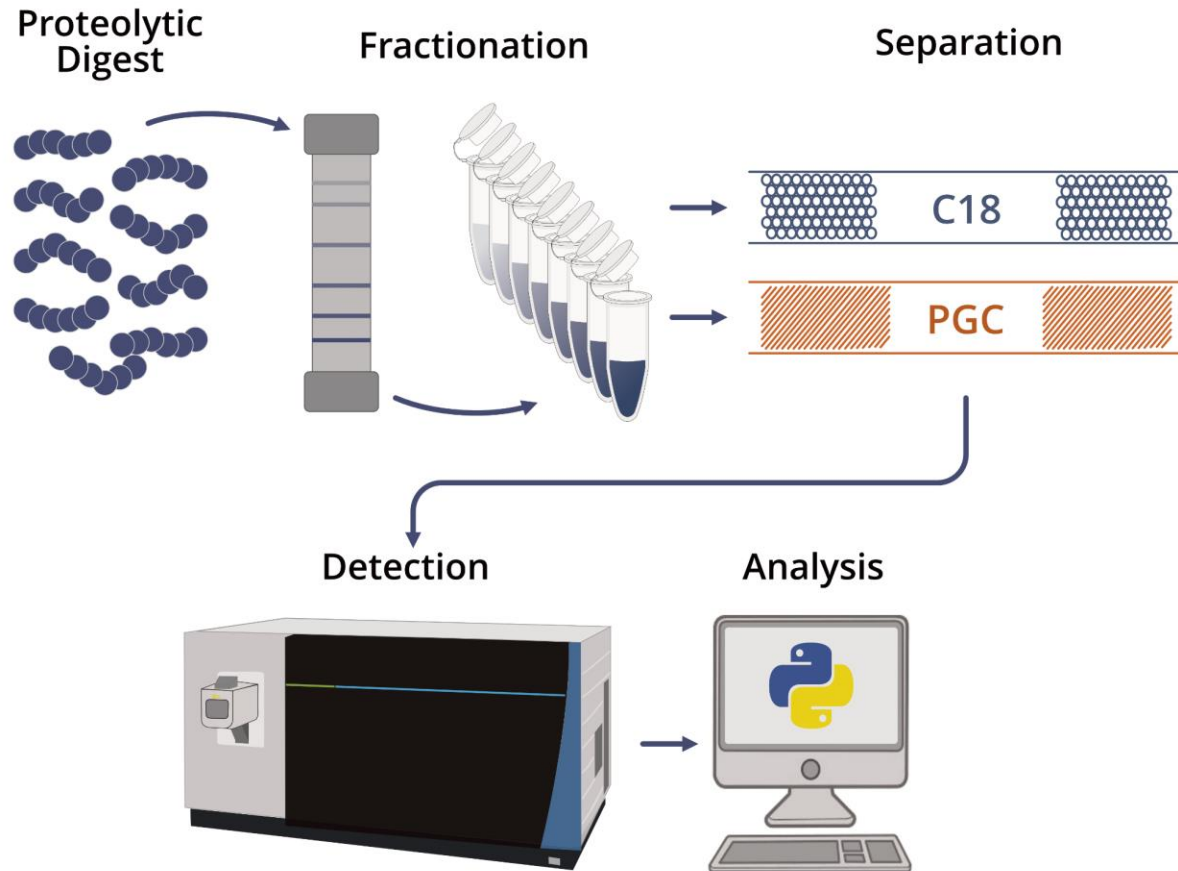




More Efficient Use of PGC-RPLC

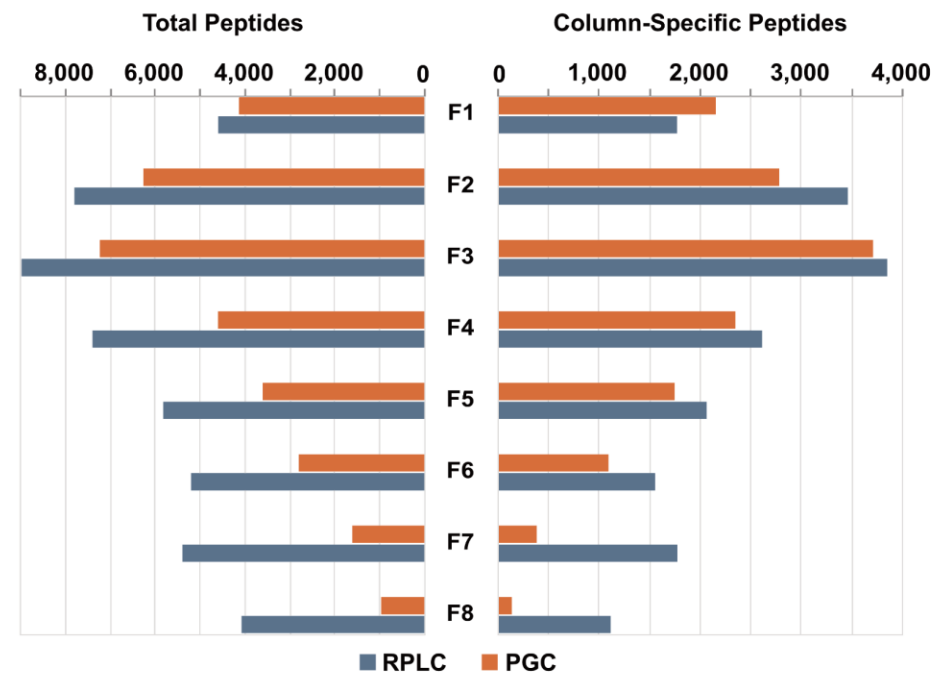
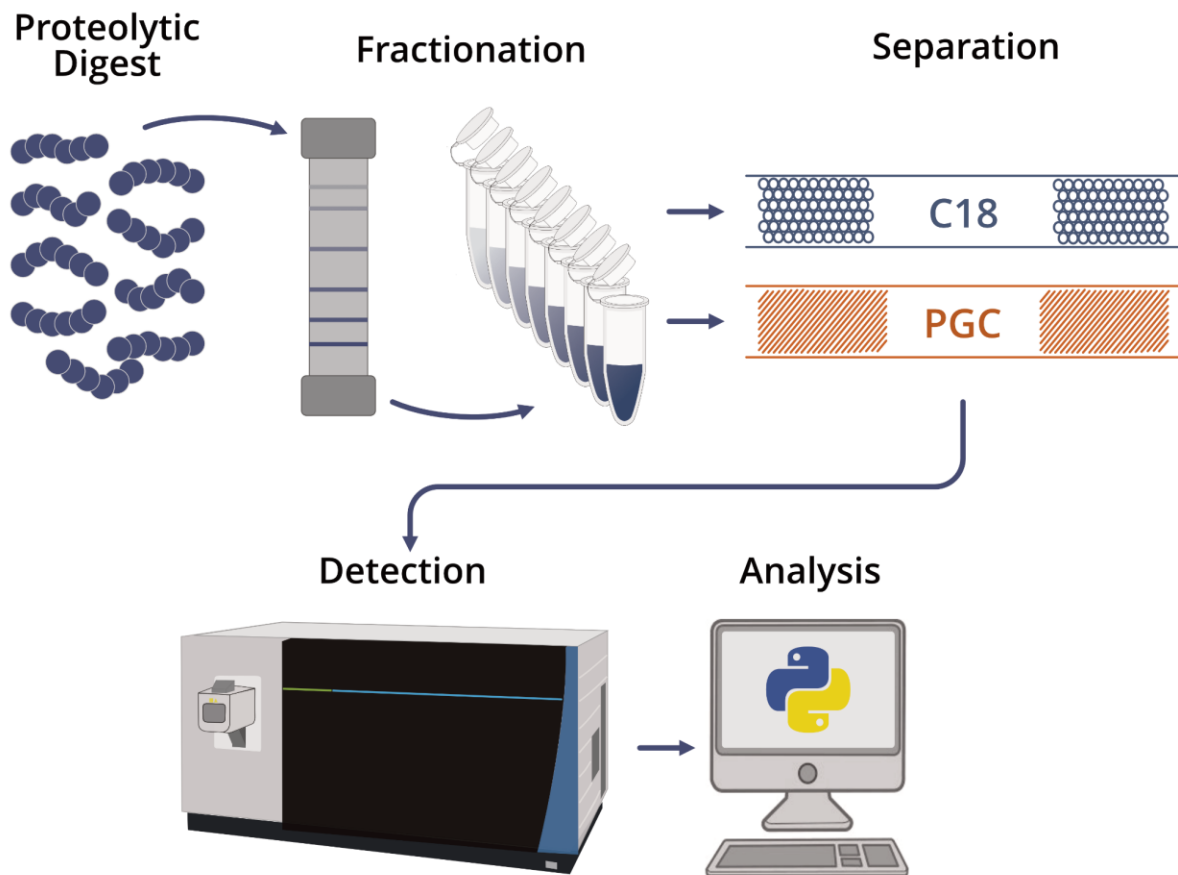


PGC is Competitive at Early Timepoints



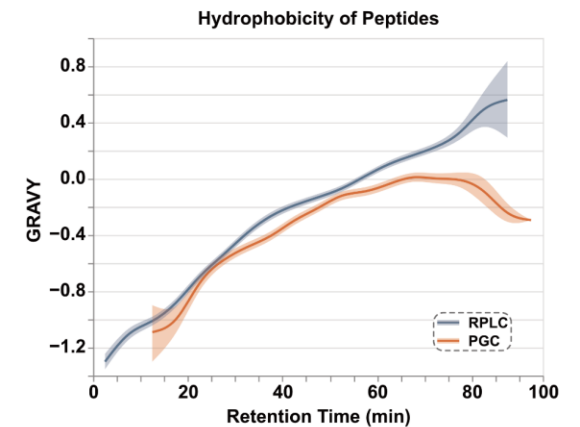
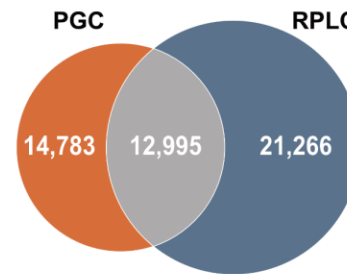
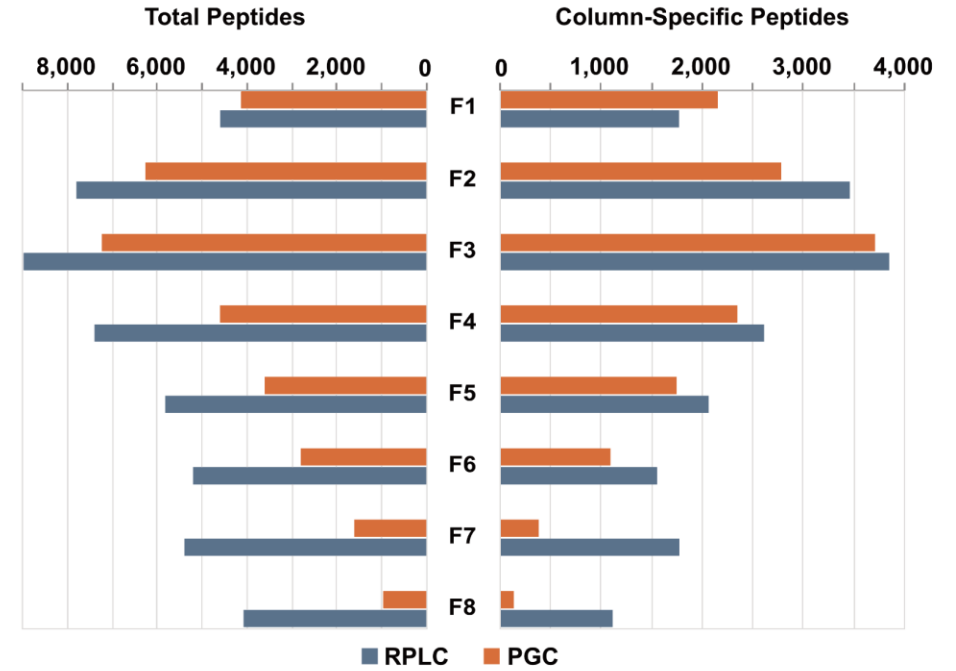
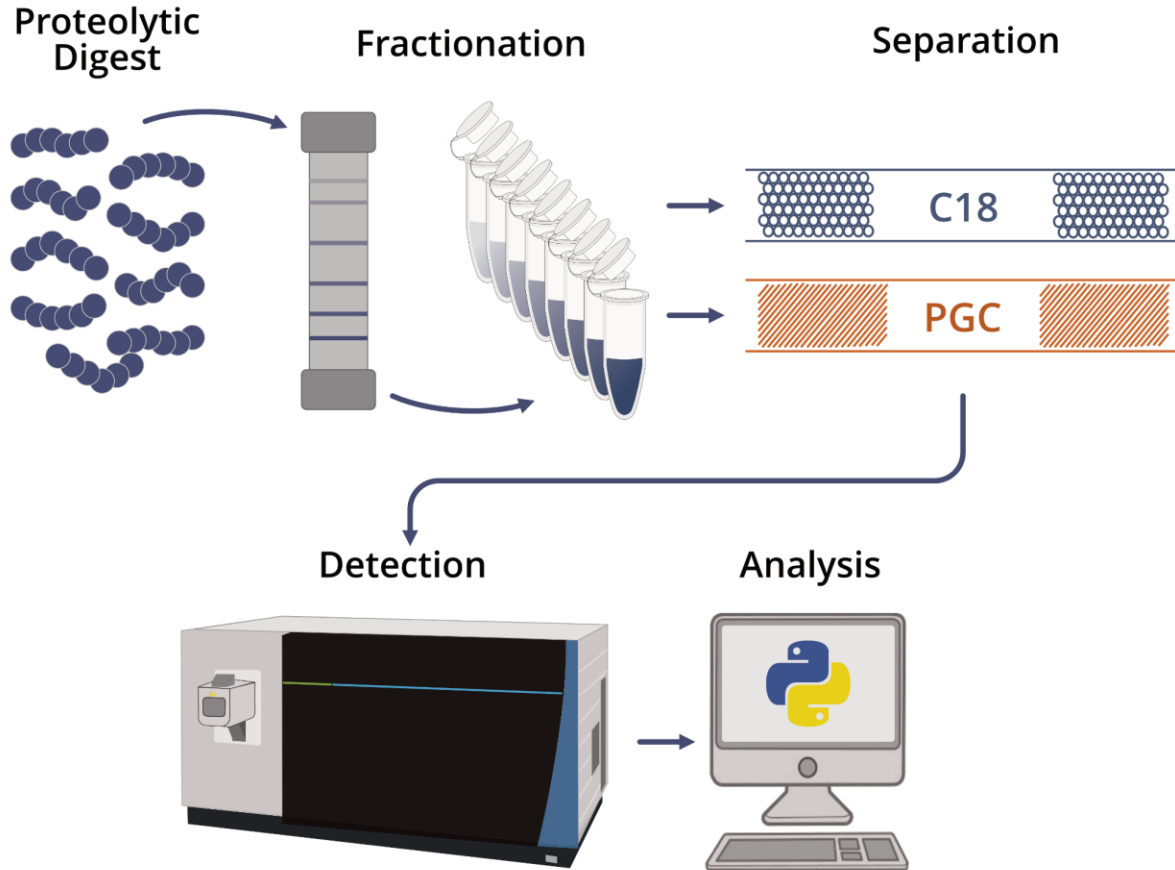


PGC is Competitive at Early Timepoints



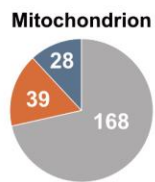
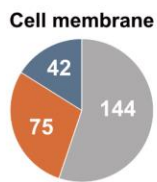
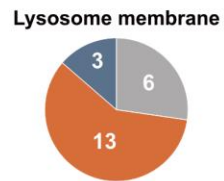
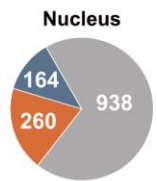
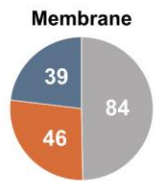
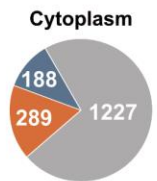
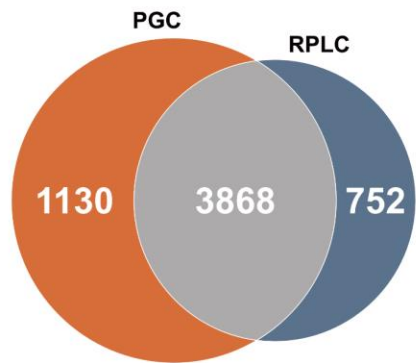


PGC is Competitive at Early Timepoints



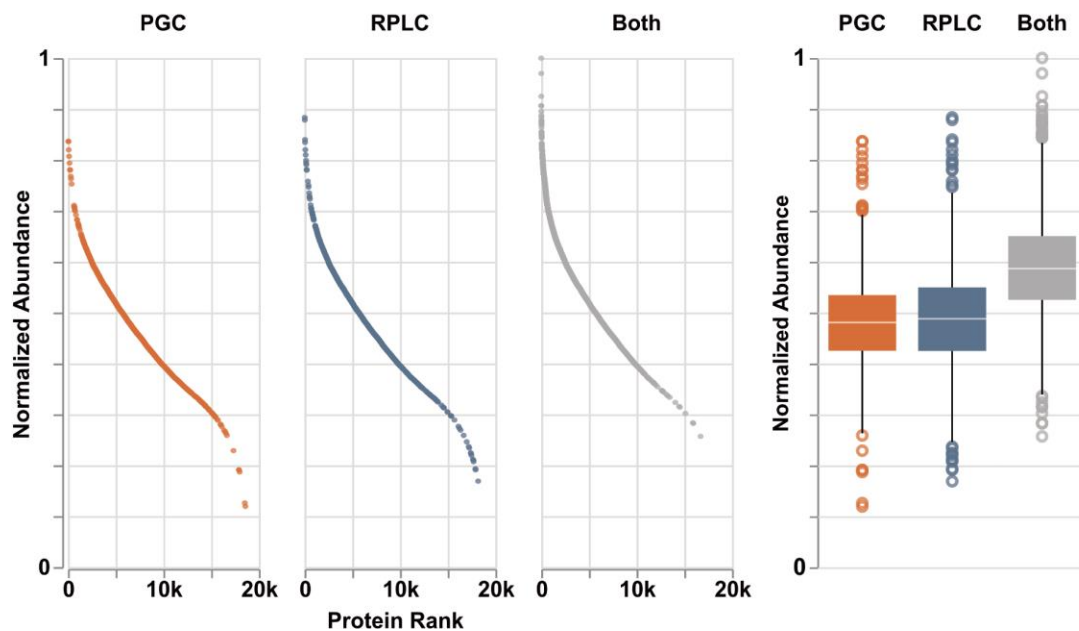
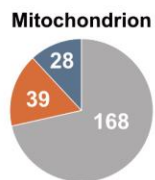
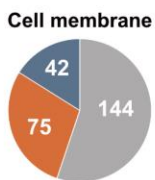
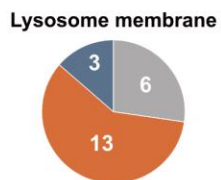
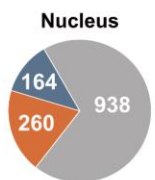
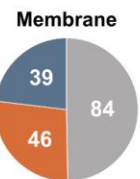
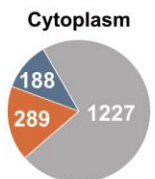
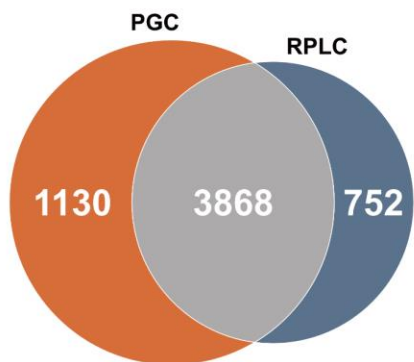


Greater Protein Identification, Regardless of Depth



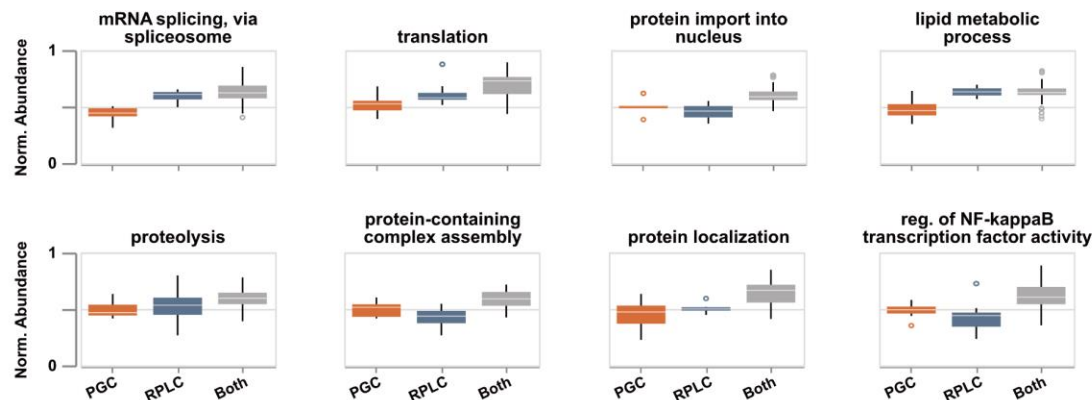
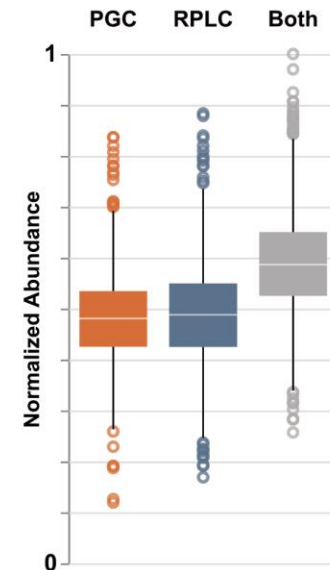
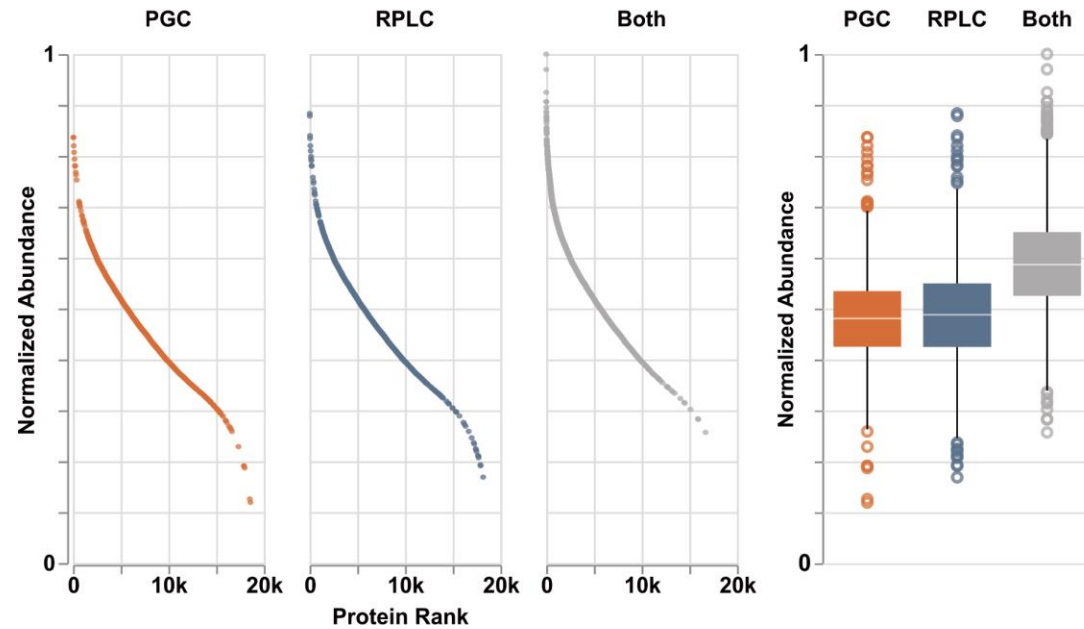
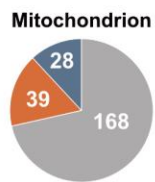
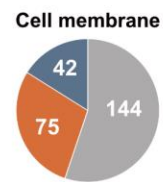
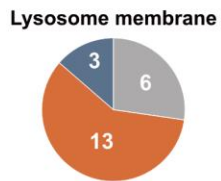
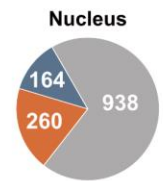
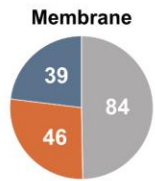
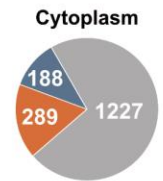
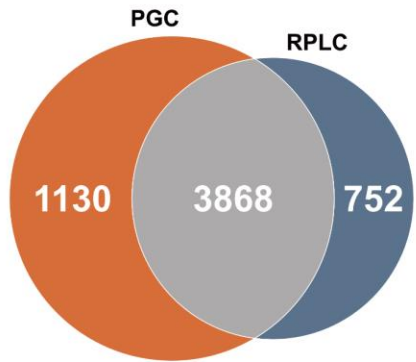


Greater Protein Identification, Regardless of Depth



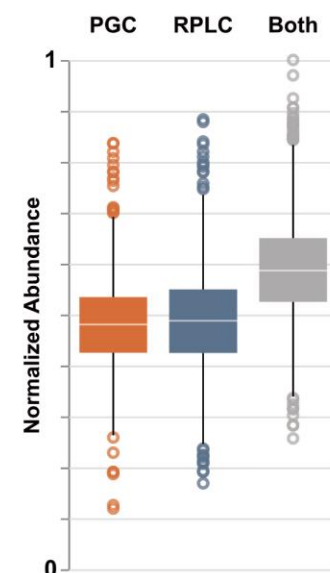
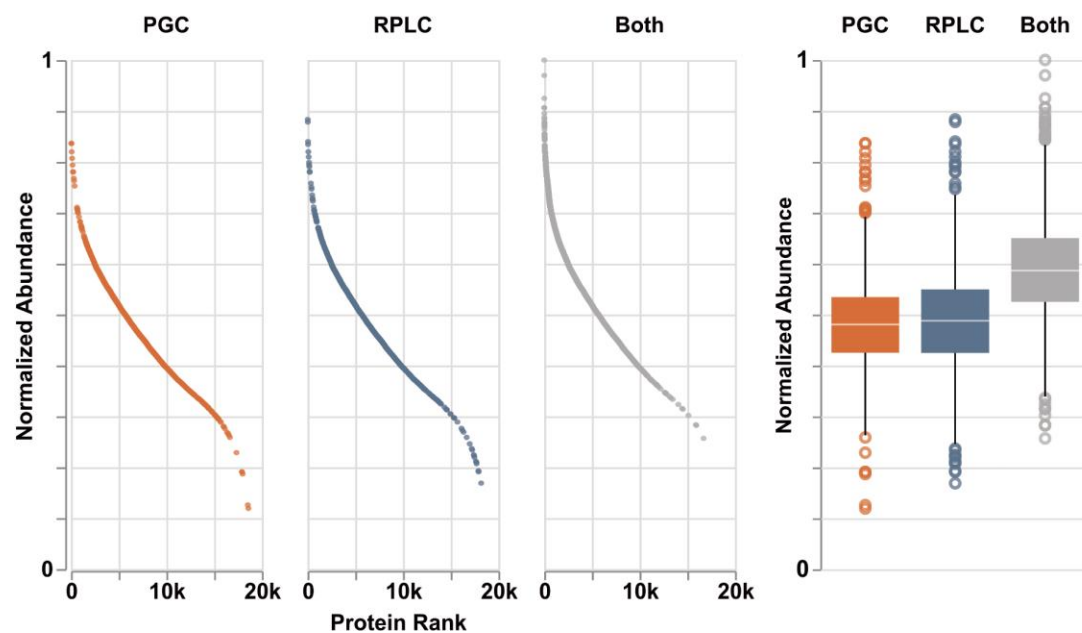
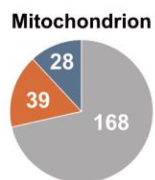
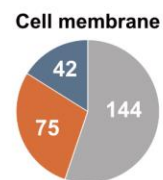
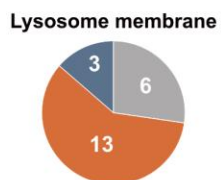
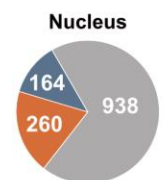
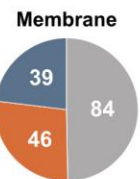
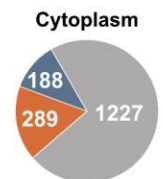
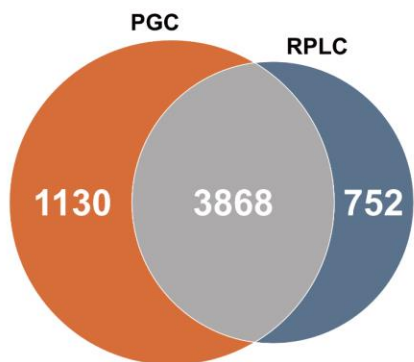


Greater Protein Identification, Regardless of Depth



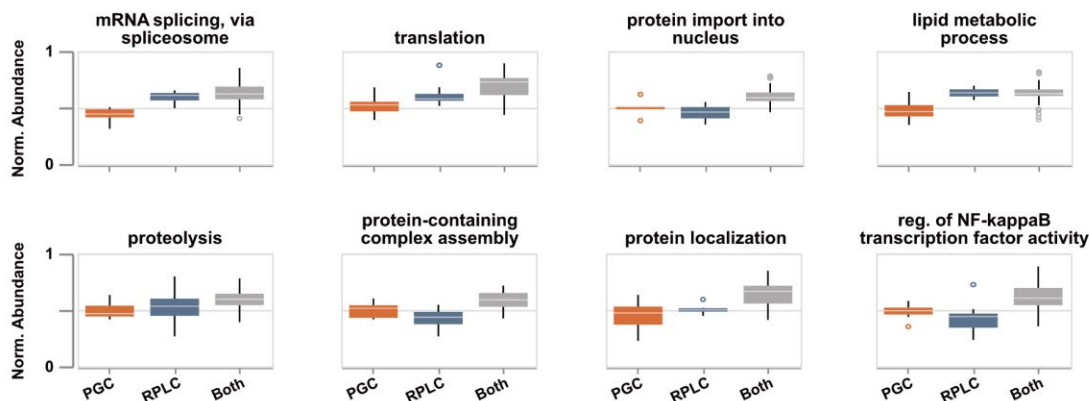


Greater Protein Identification, Regardless of Depth

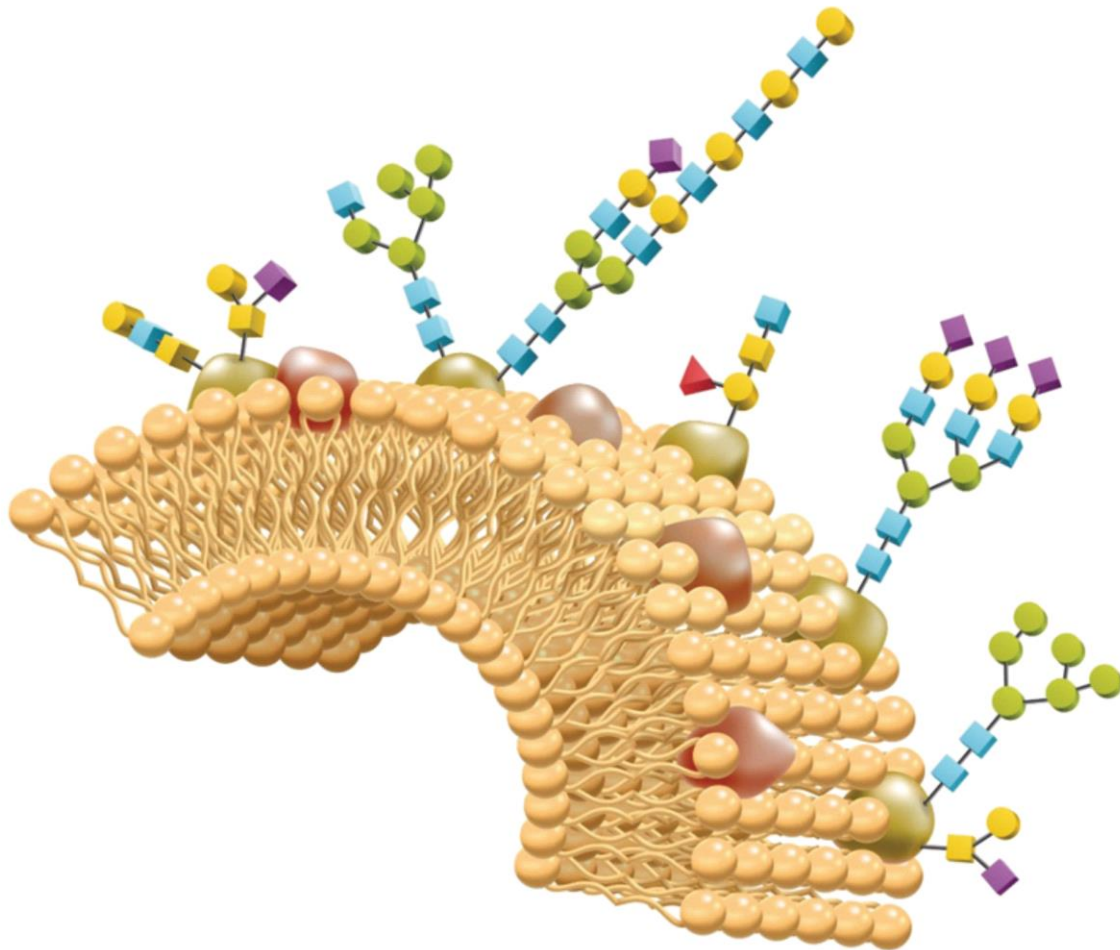


PGC for Proteomics

- 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

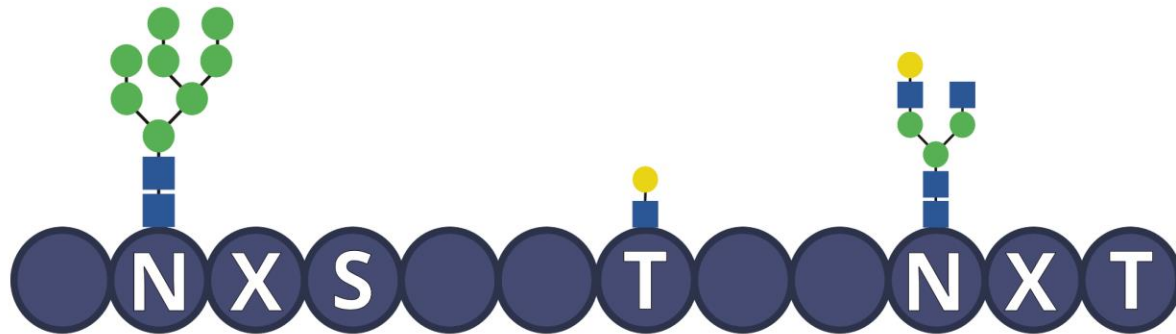
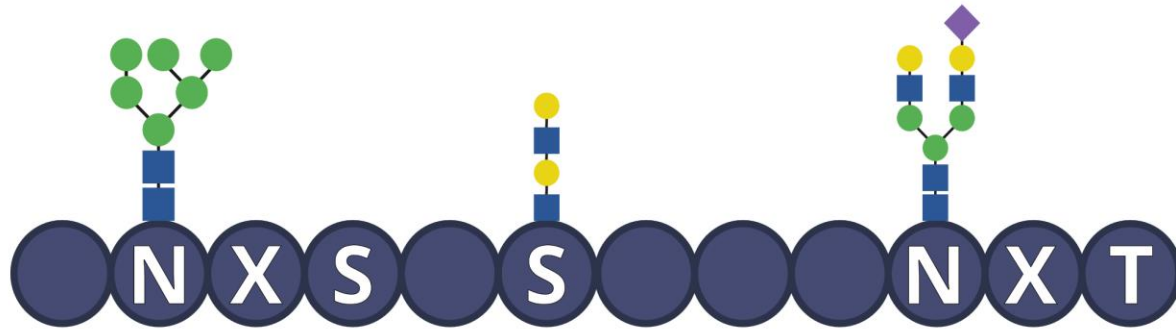
Disease

Target for pathogen invasion

Altered expression during disease propagation

Aberrant profiles across numerous disease

Glycoproteomics

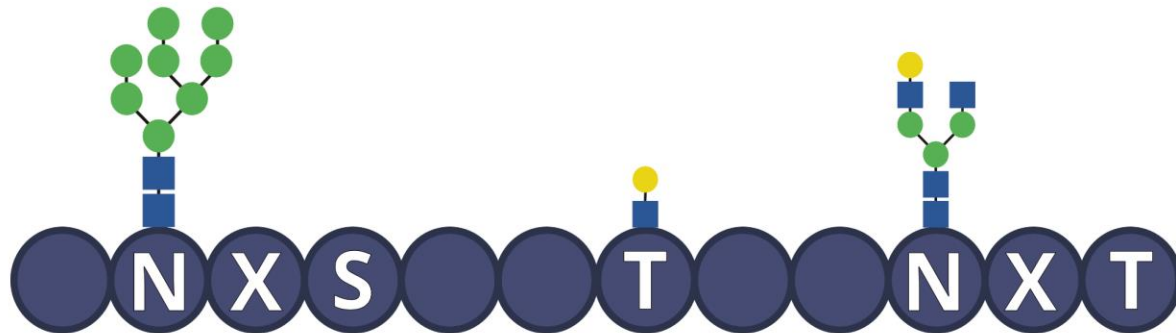
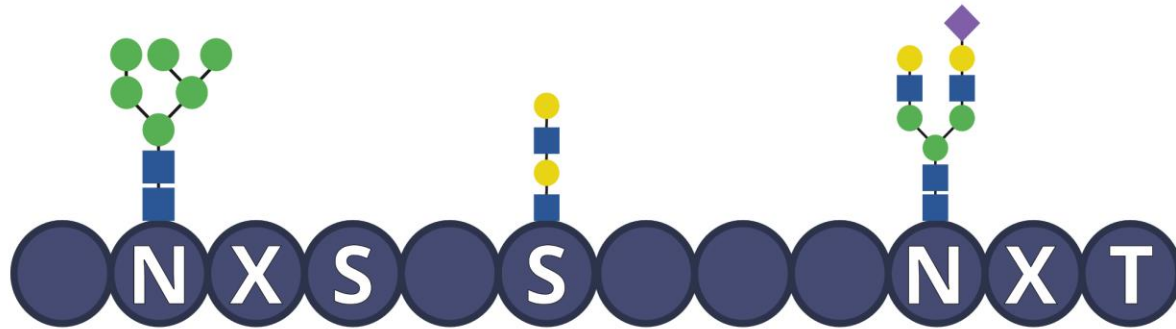


Challenges

Biological

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

Glycoproteomics



Challenges

Biological

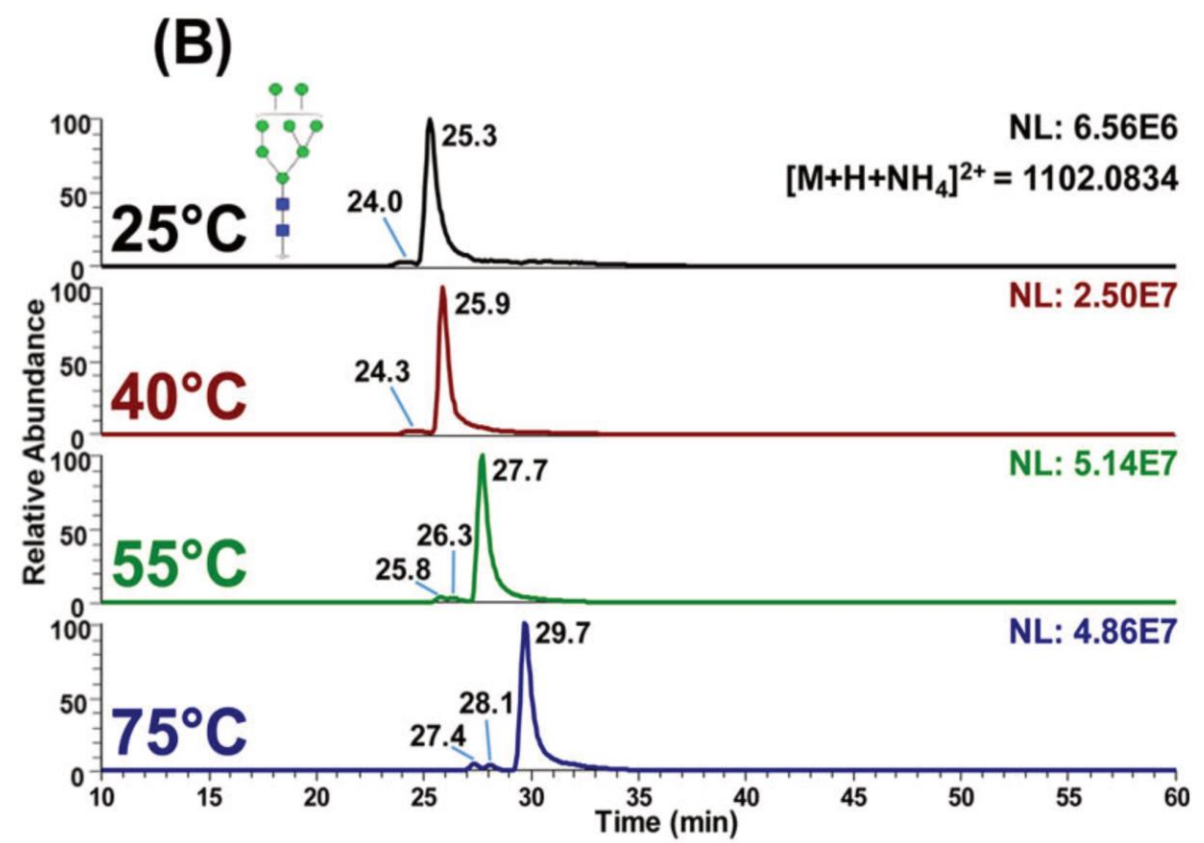
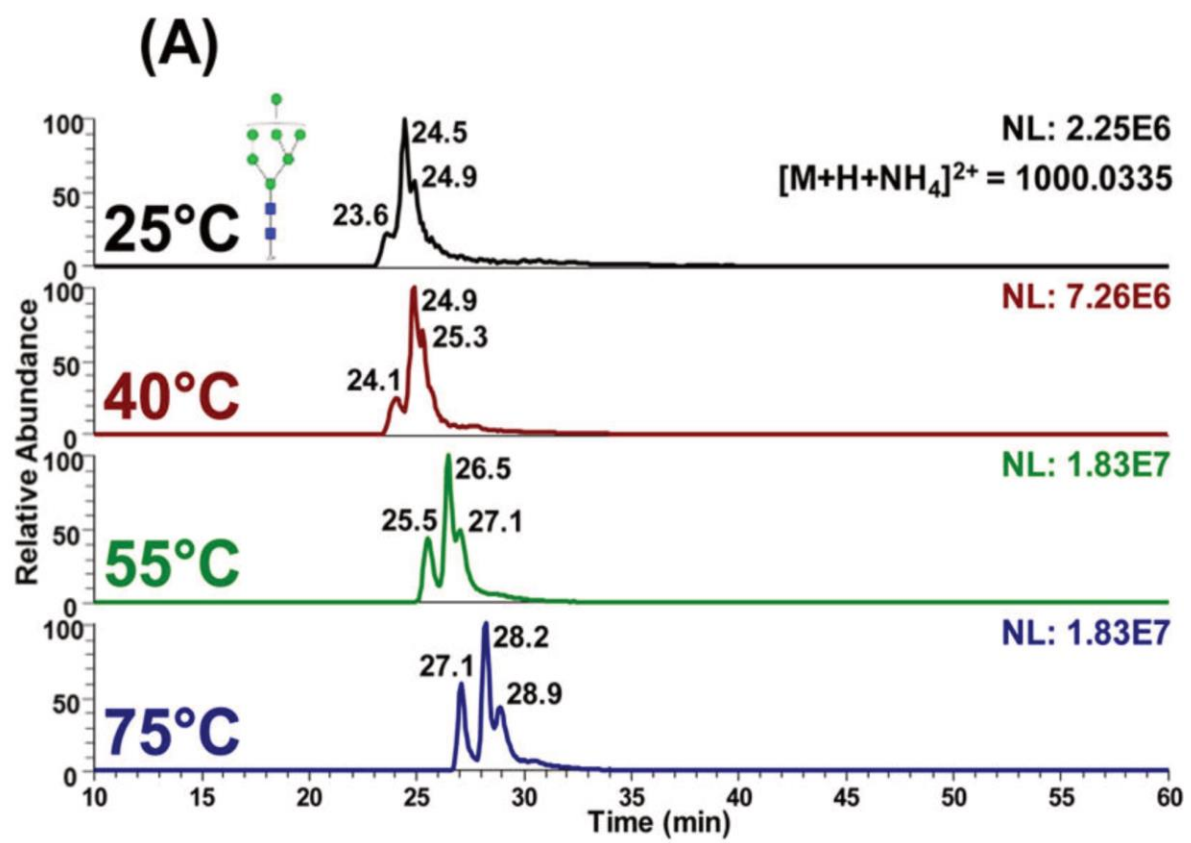
- **Class**
 - N-linked or O-linked
- **Composition**
 - 12 commonly occurring monosaccharides
- **Structure**
 - α/β orientation
 - Connectivity

Analytical

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

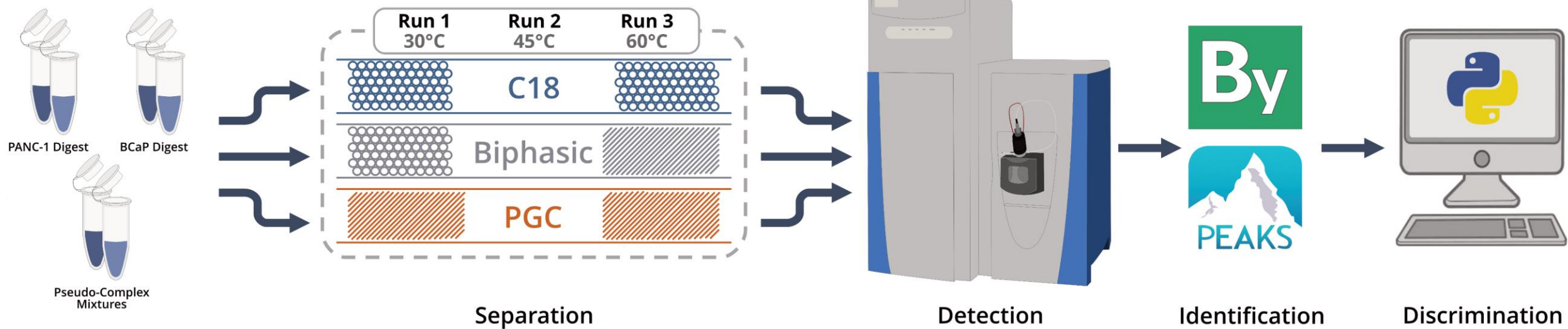


PGC for Glycoproteomics





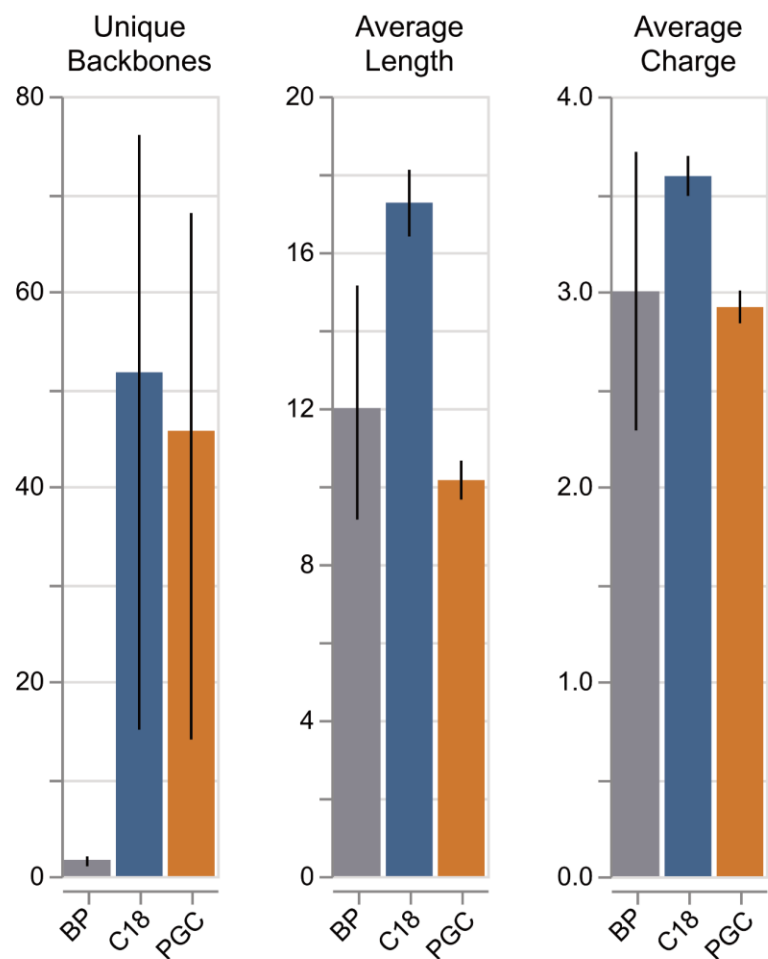
PGC-Based Shotgun Proteomics



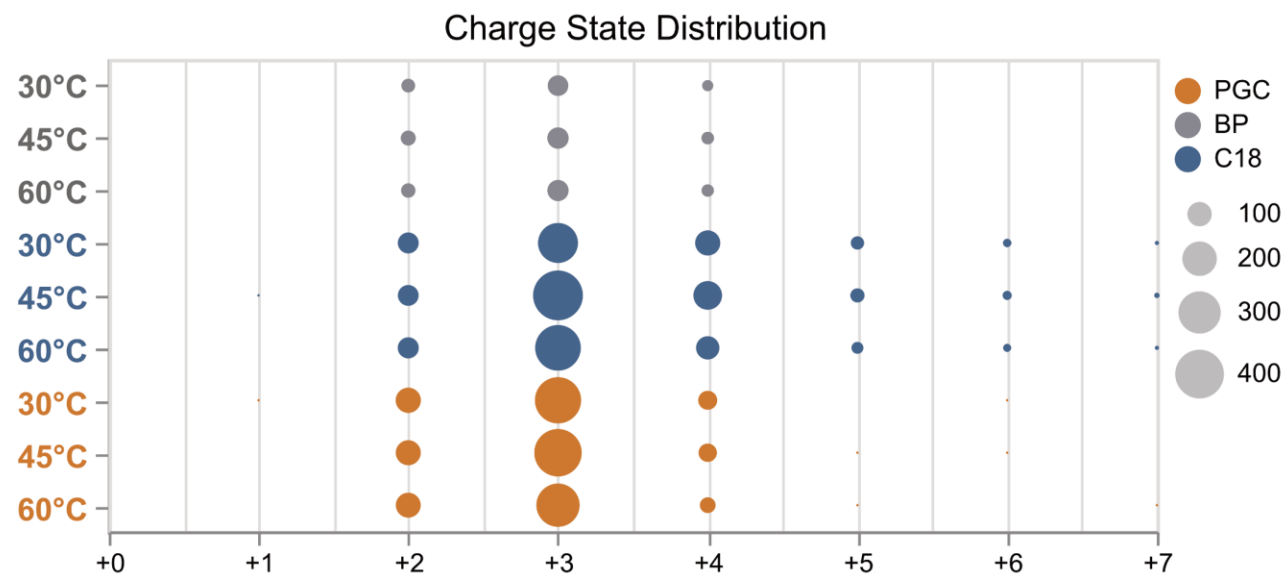


Glycopeptide Character

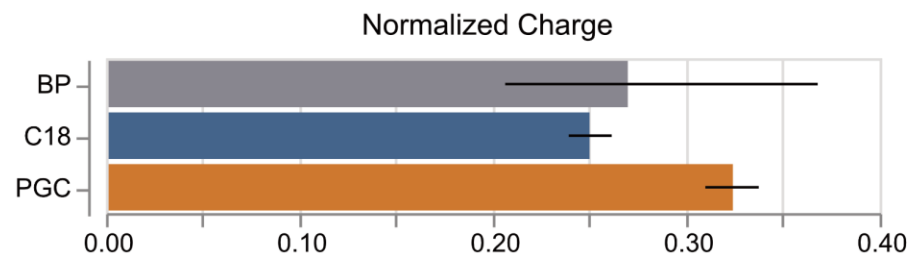
A



B

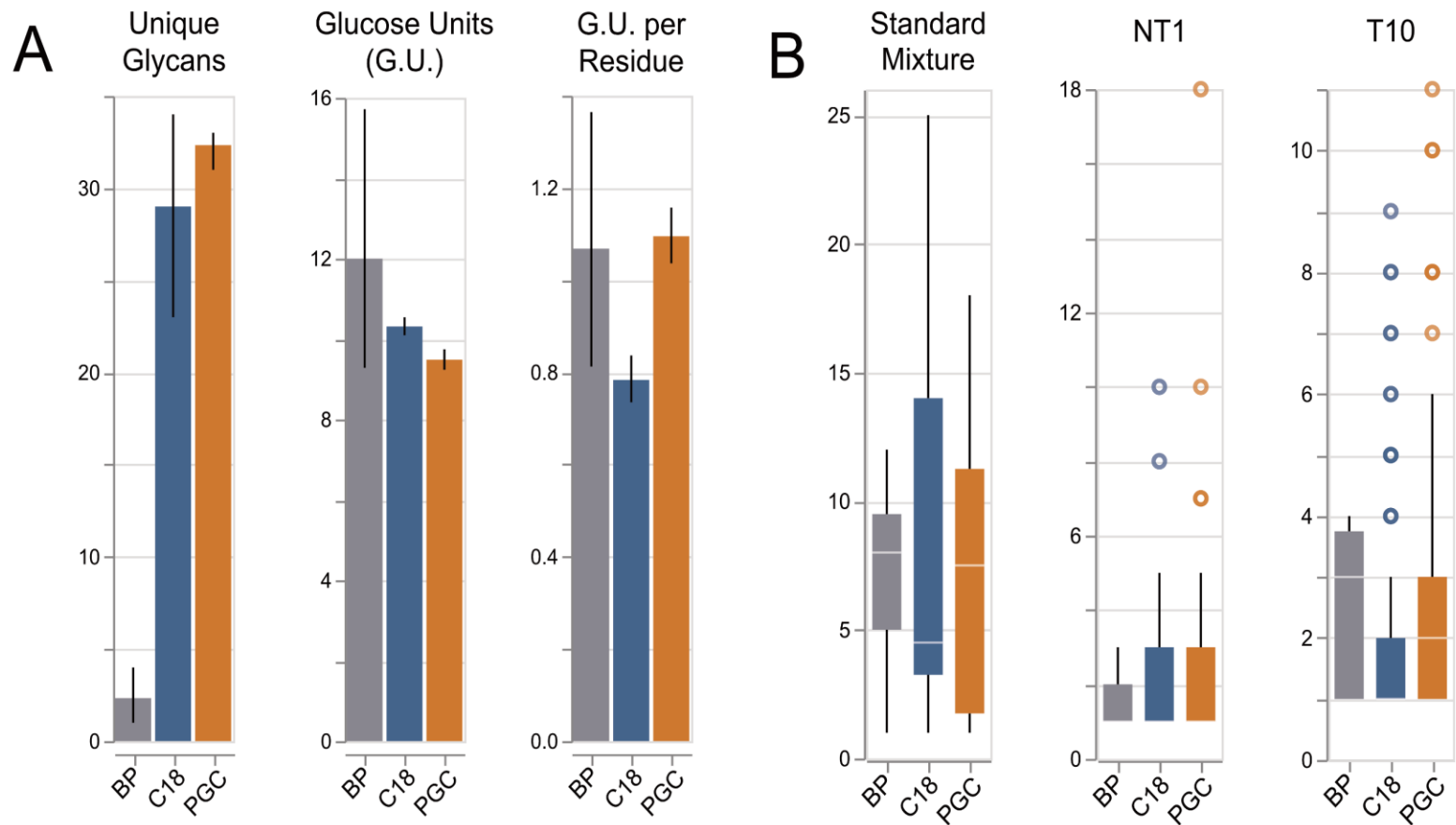


C





Glycopeptide Character



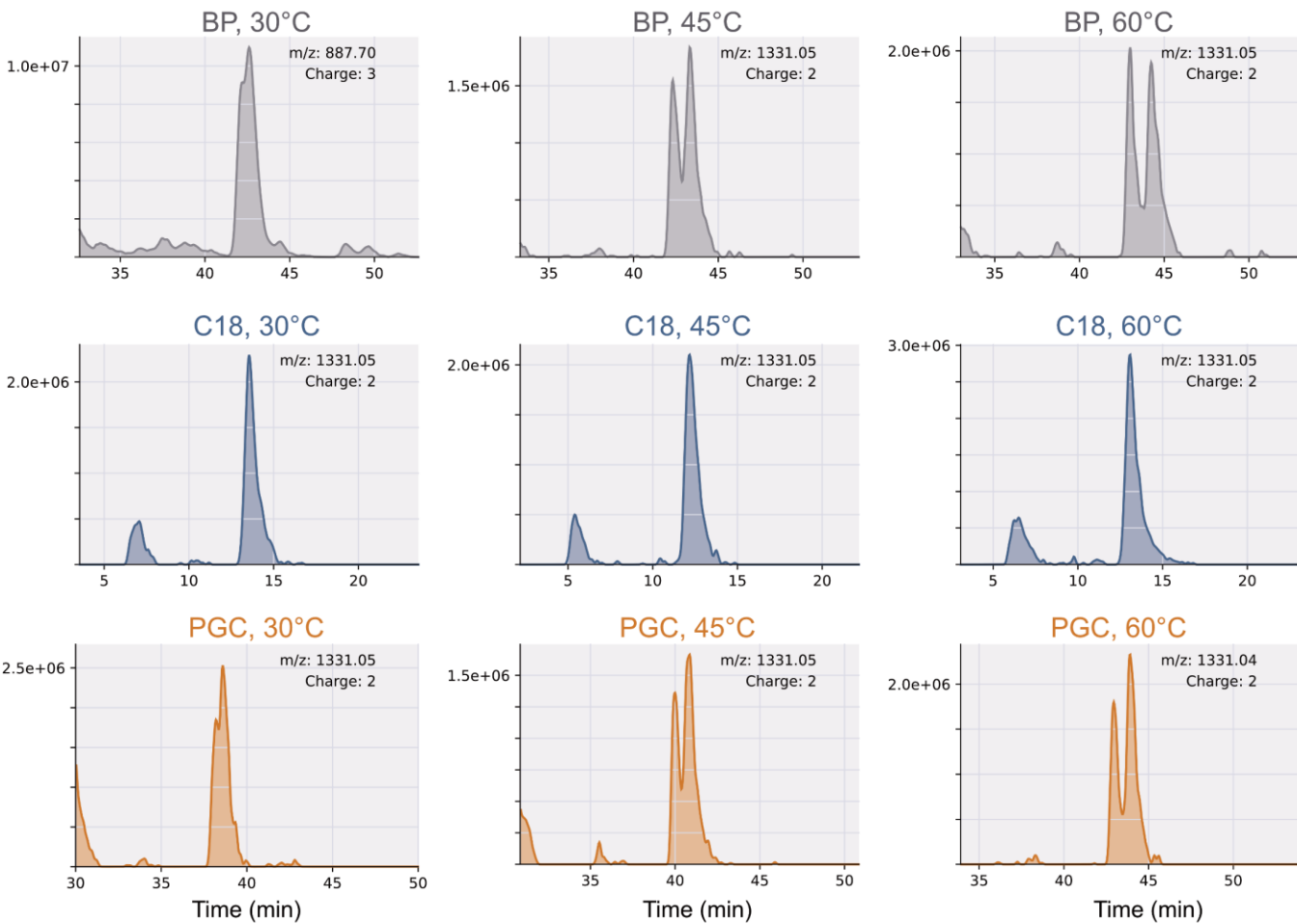
Outcomes

- 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

QNGTLSK + HexNAc(4)Hex(5)NeuAc(1)

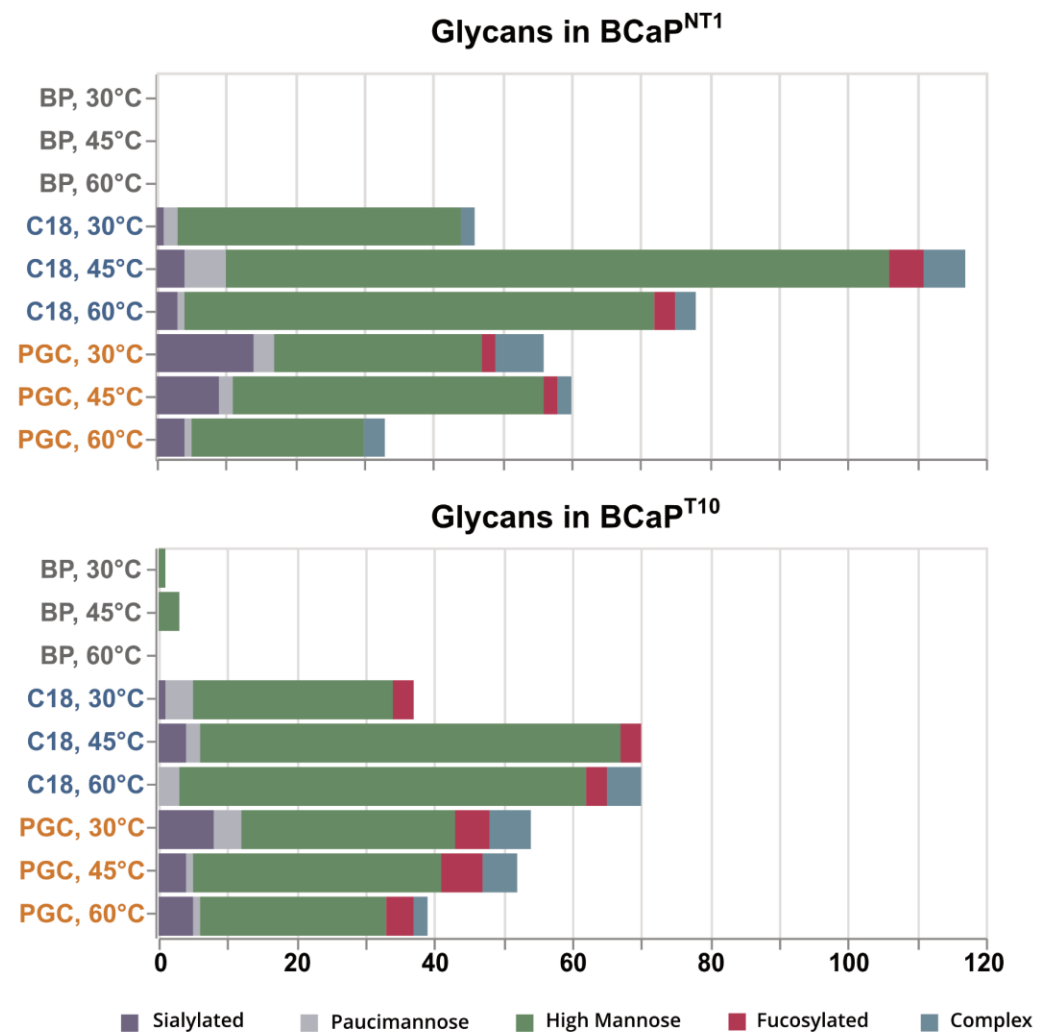


Outcomes

- 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.



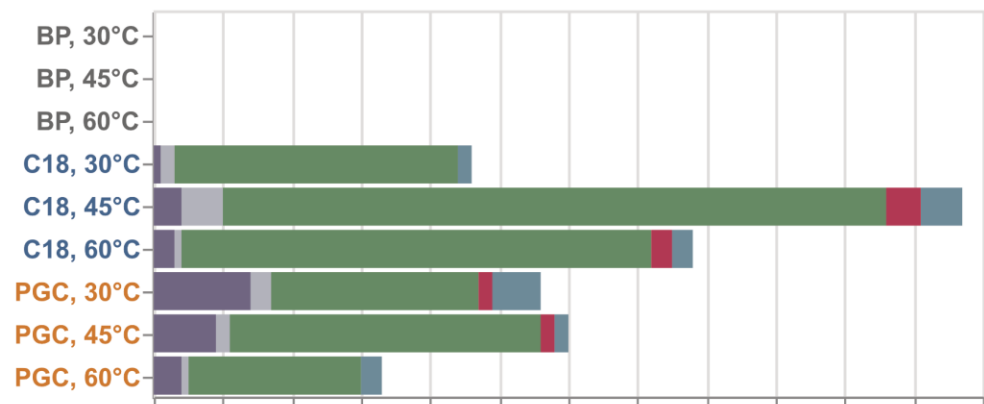
Temperature Affects Glycopeptide Identifications



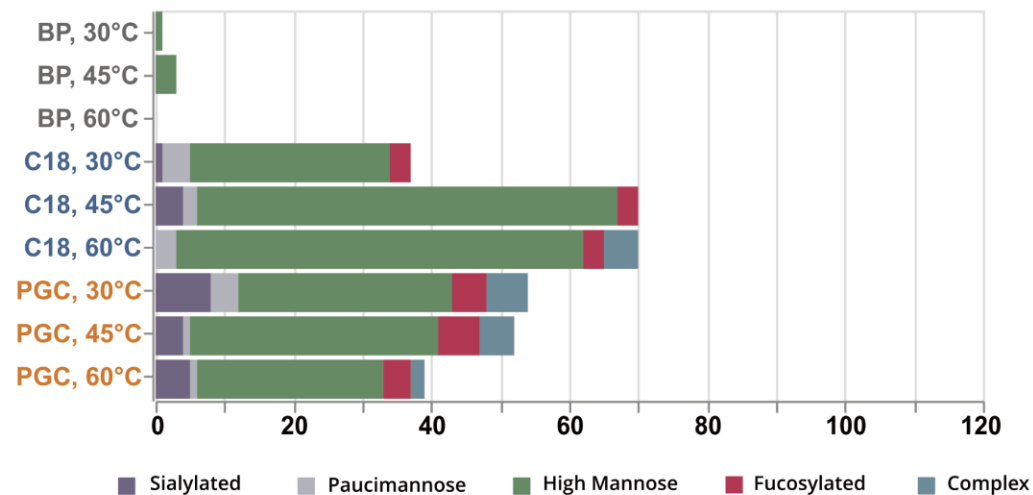


Temperature Affects Glycopeptide Identifications

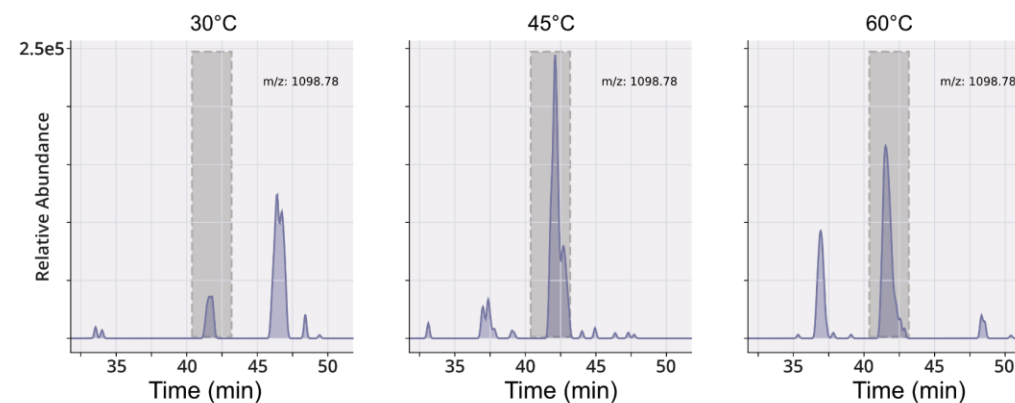
Glycans in BCaP^{NT1}



Glycans in BCaP^{T10}



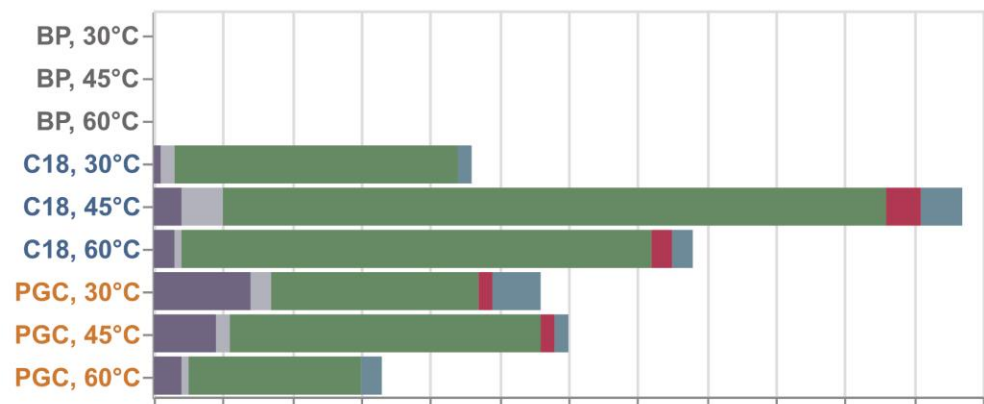
VWNSTFIEDYR + HexNAc(2)Hex(9)



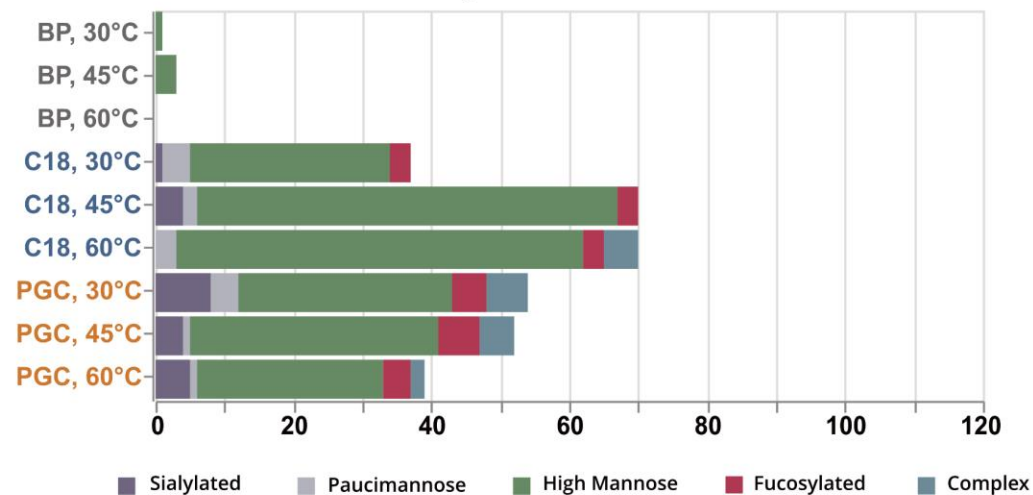


Temperature Affects Glycopeptide Identifications

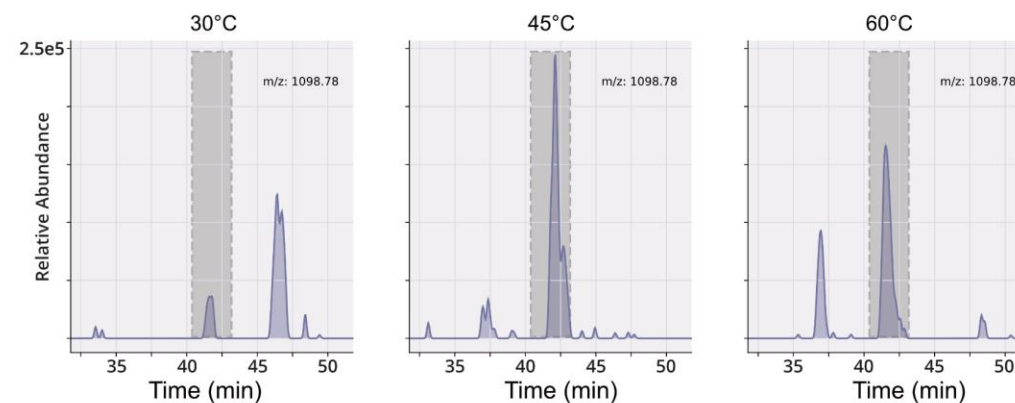
Glycans in BCaP^{NT1}



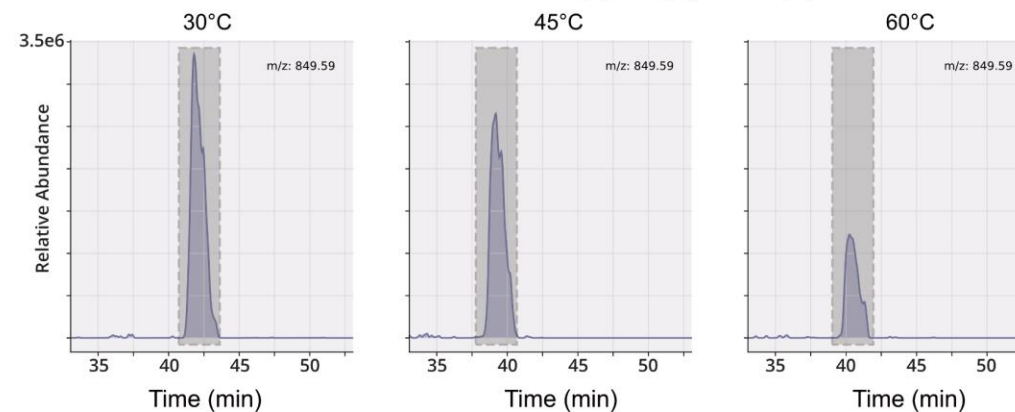
Glycans in BCaP^{T10}



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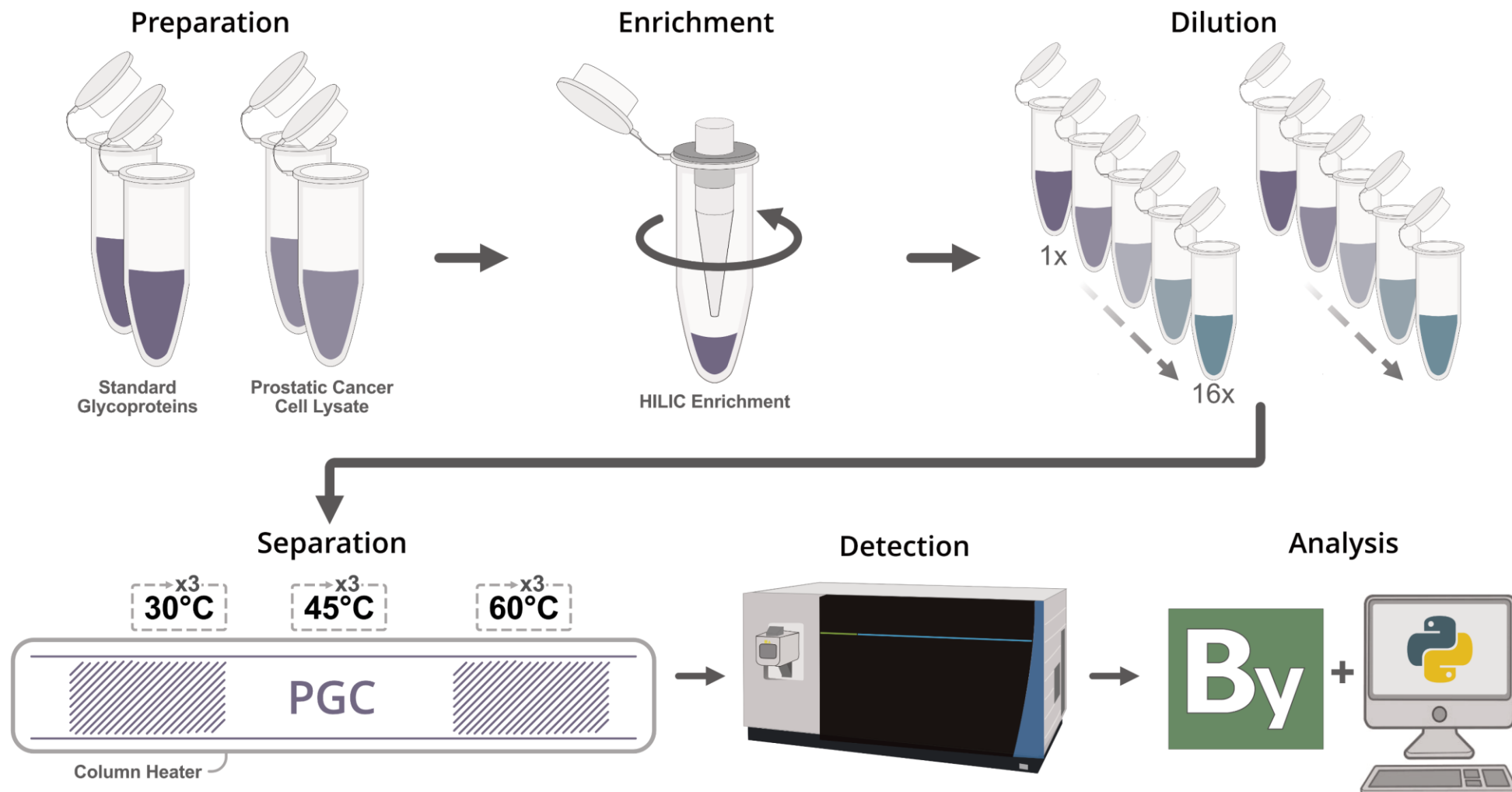


CYNCSFIK + HexNAc(4)Hex(5)NeuAc(2)



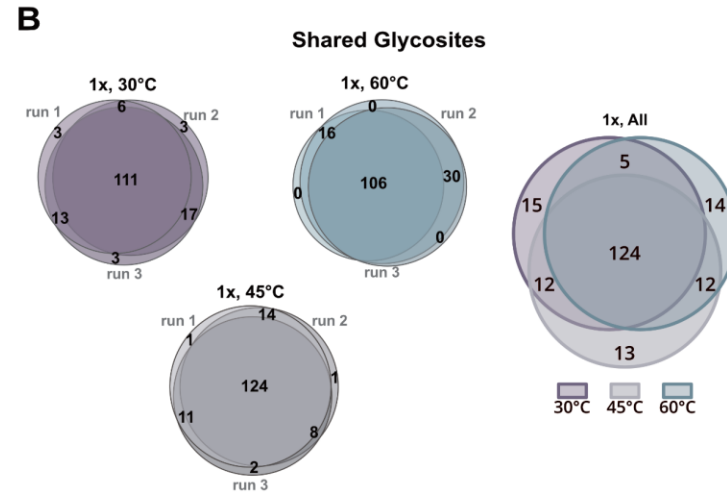
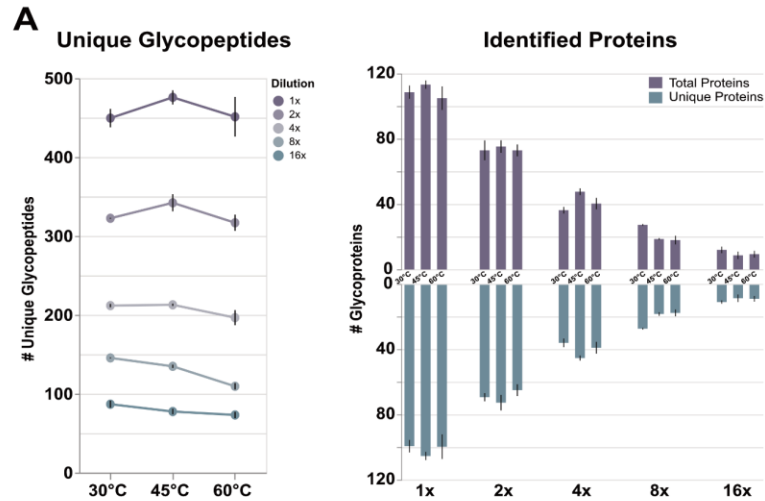


Investigating Temperature-based Aberrations



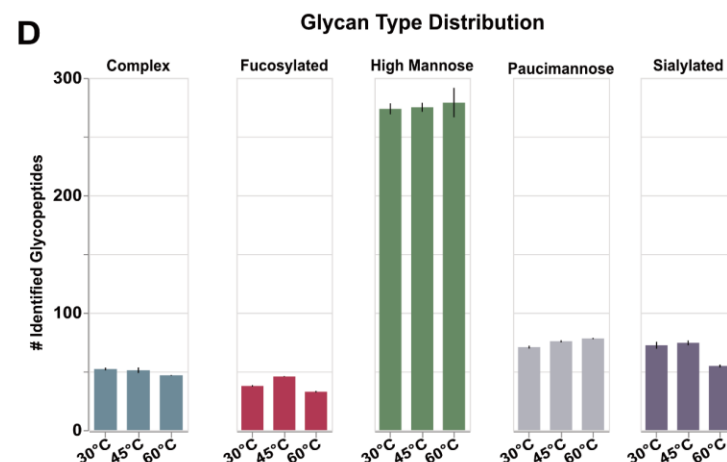
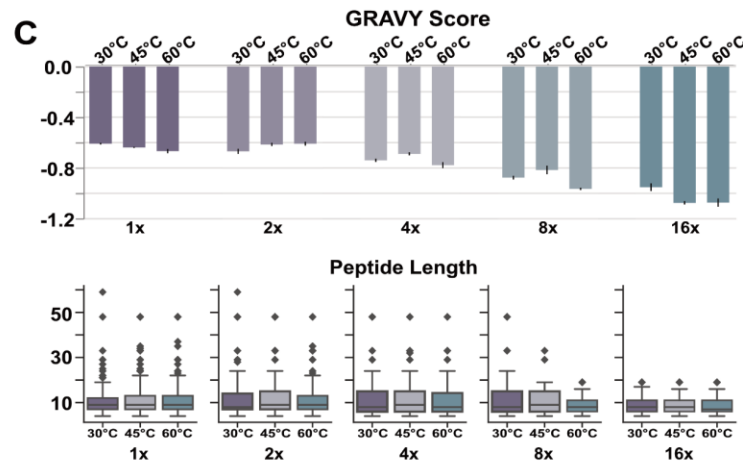


Investigating Temperature-based Aberrations



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

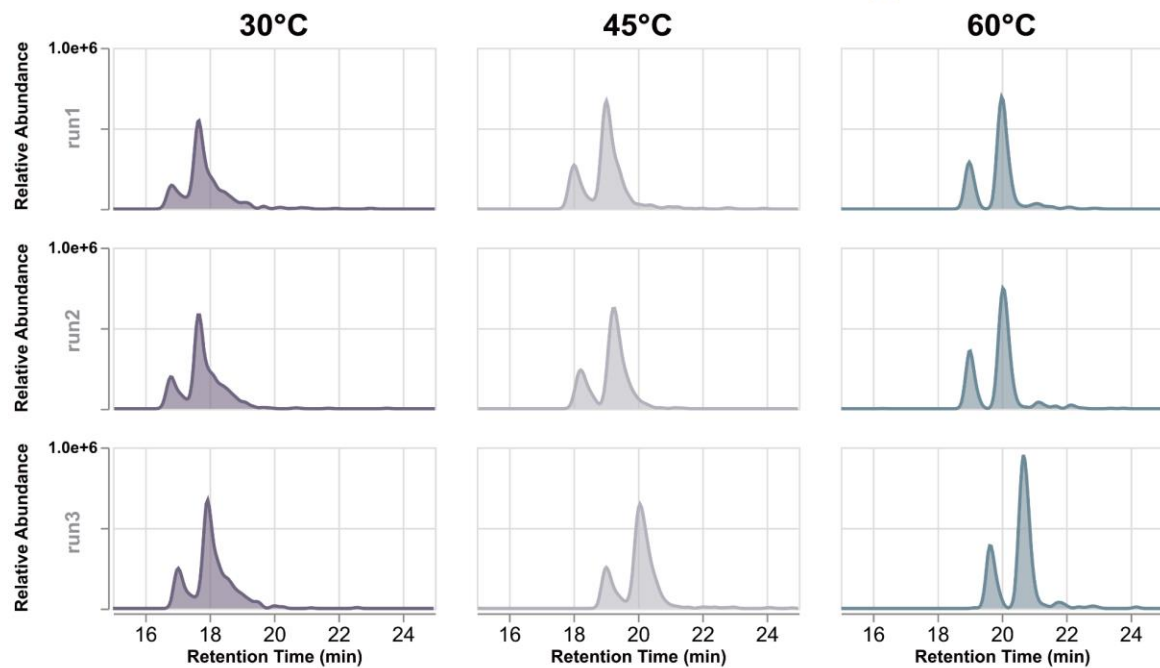




Temperature Inspired Chromatographic Changes

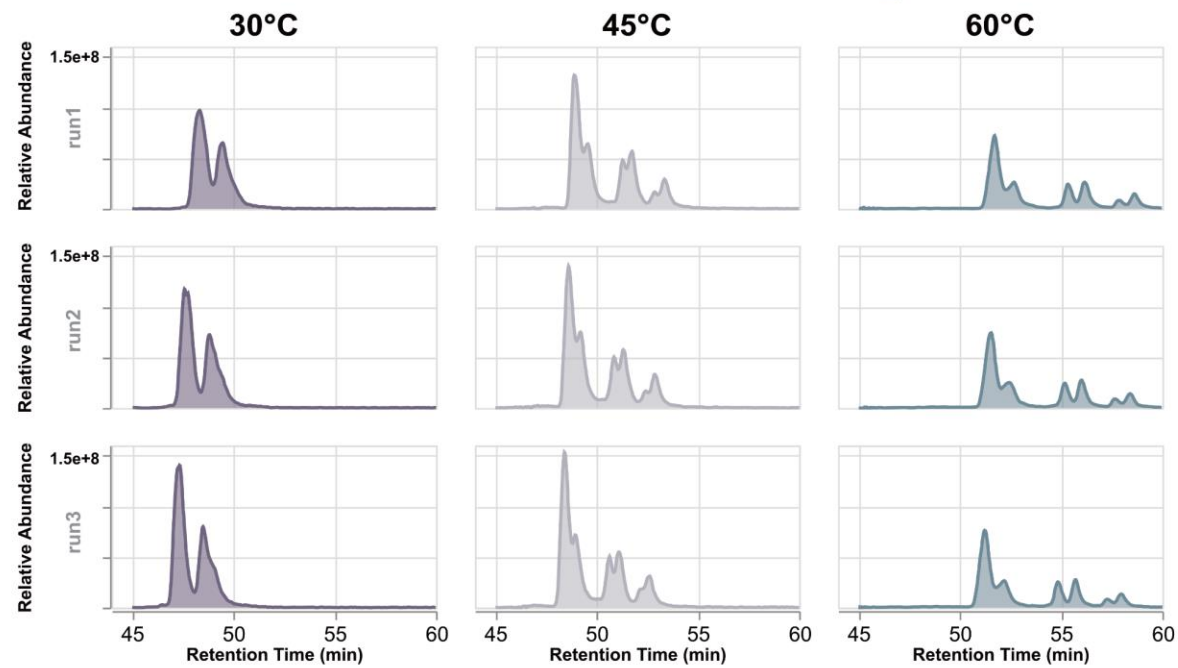
A

AEPPLNASASDQGEK +



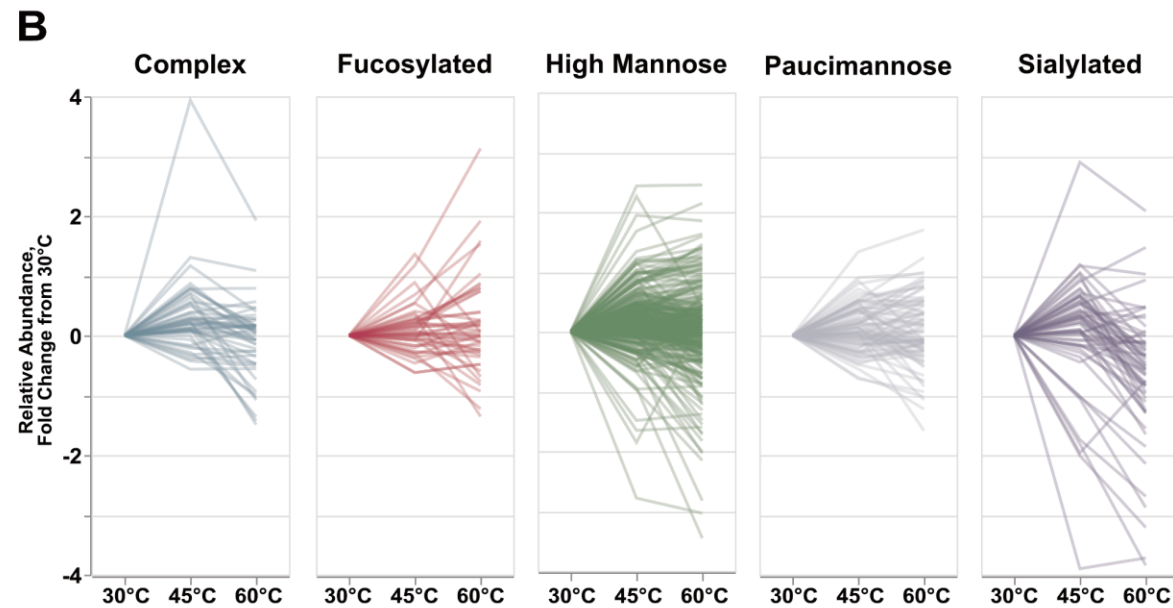
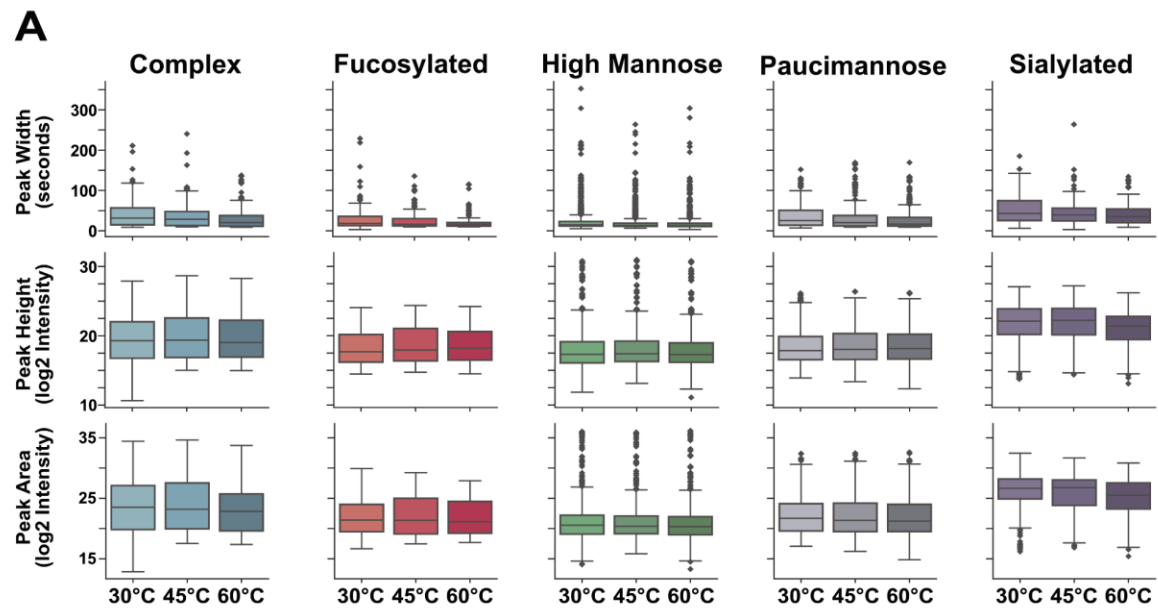
B

LCPDCPLLAPLNDSR +



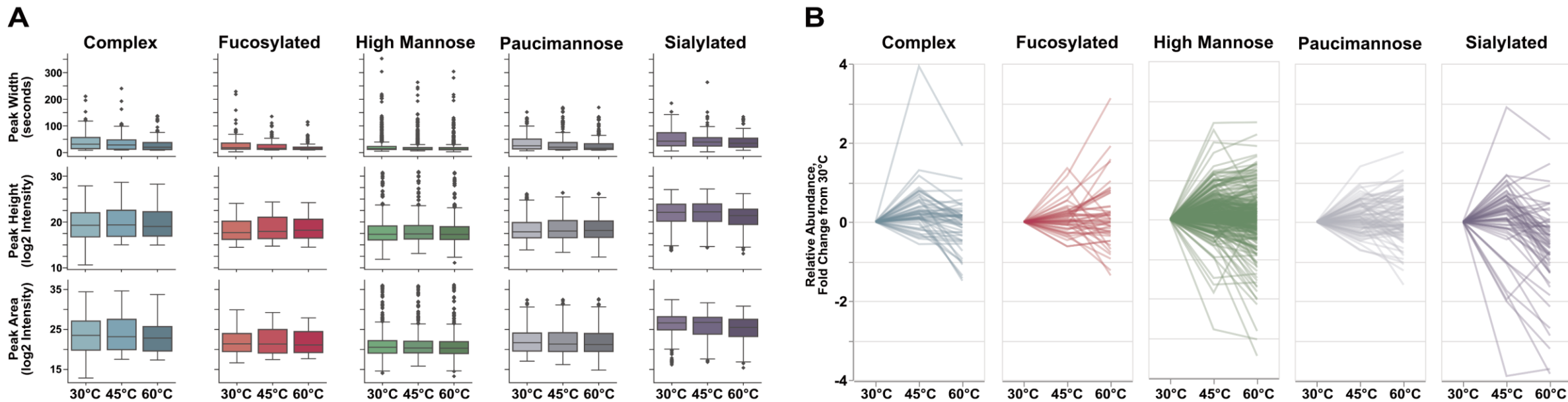


Resolution vs. Signal Intensity





Resolution vs. Signal Intensity



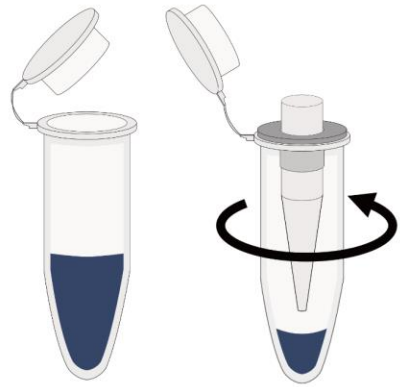
Conclusions

- 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

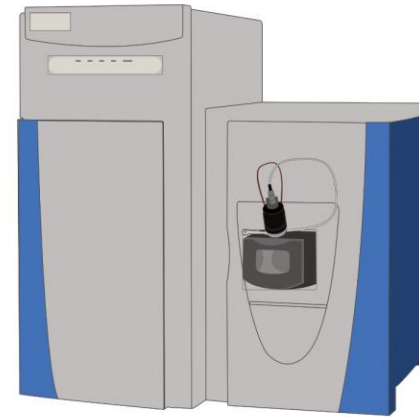
Preparation



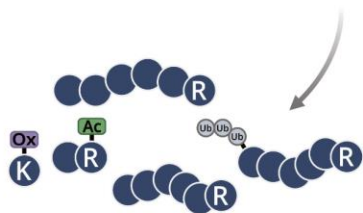
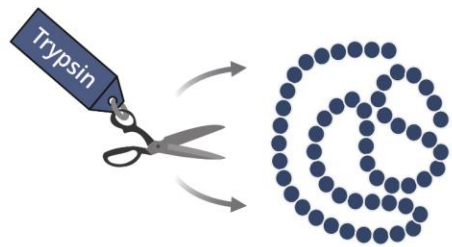
Separation



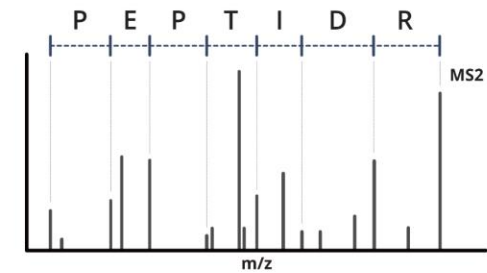
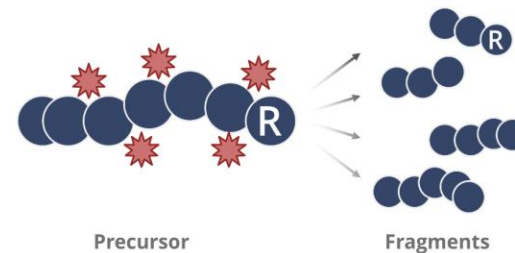
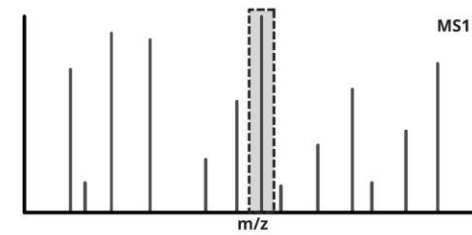
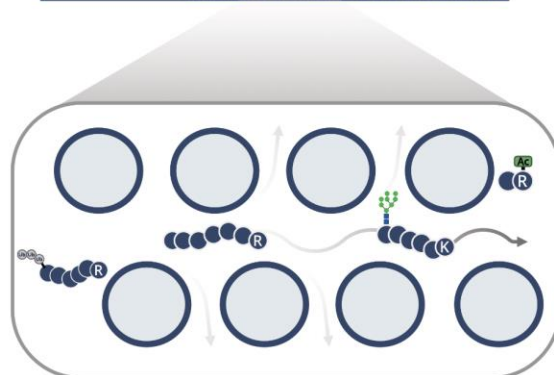
Detection



Analysis



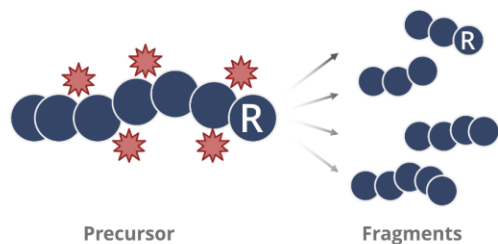
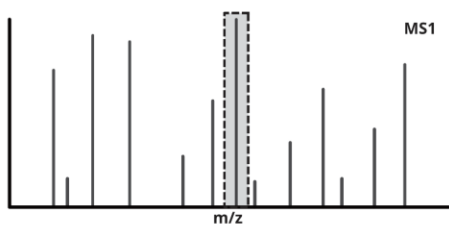
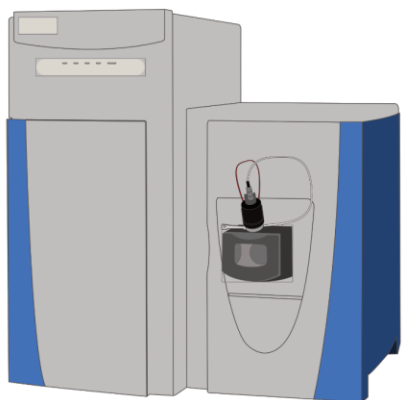
RPLC



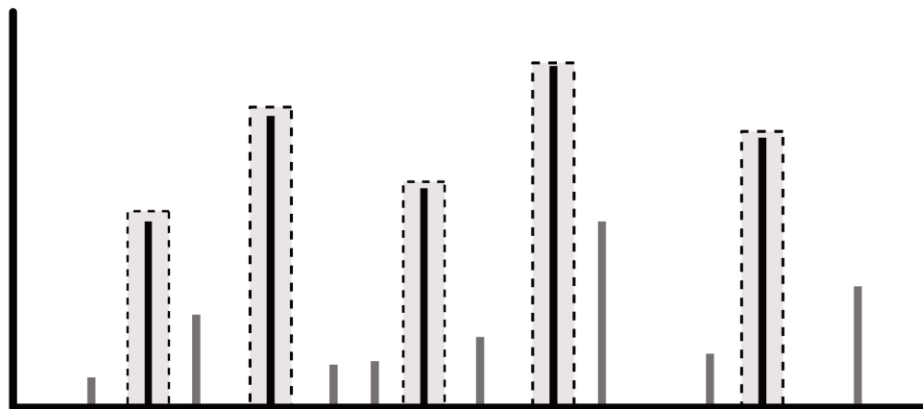


Dependent vs. Independent Acquisition

Detection

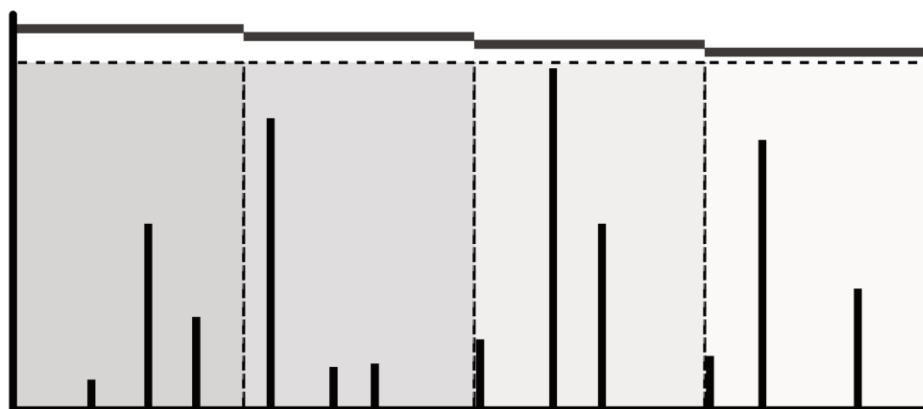


Data-Dependent Acquisition (DDA)



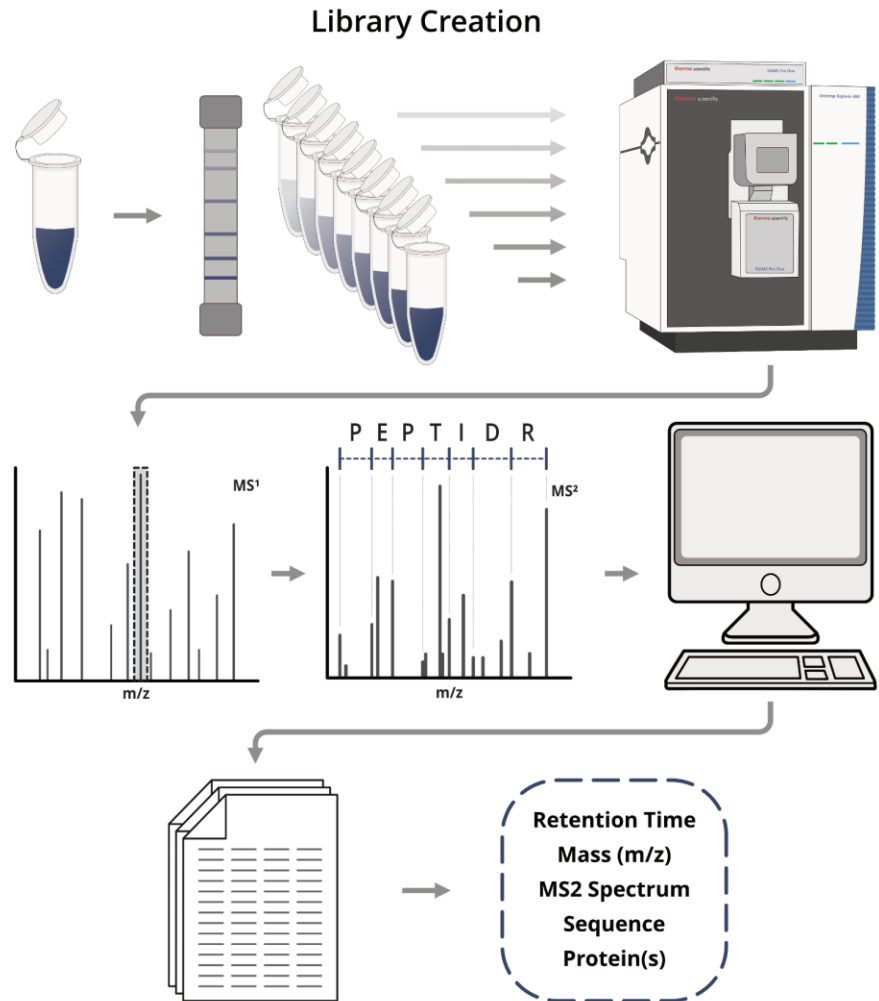
Confident and clean
but biased towards
highly-abundant
analytes

Data-Independent Acquisition (DIA)



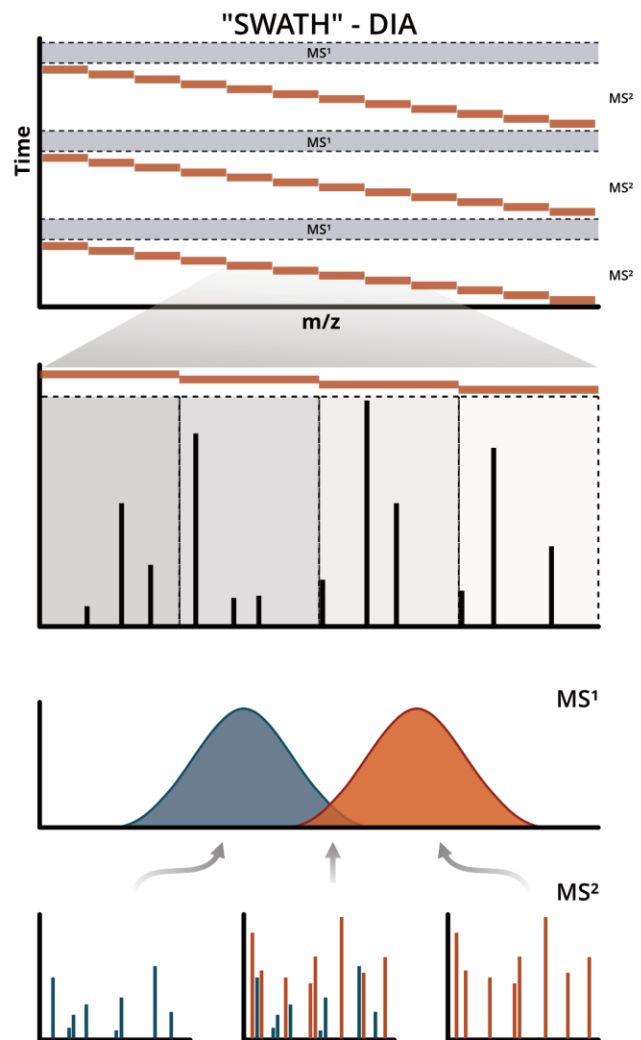
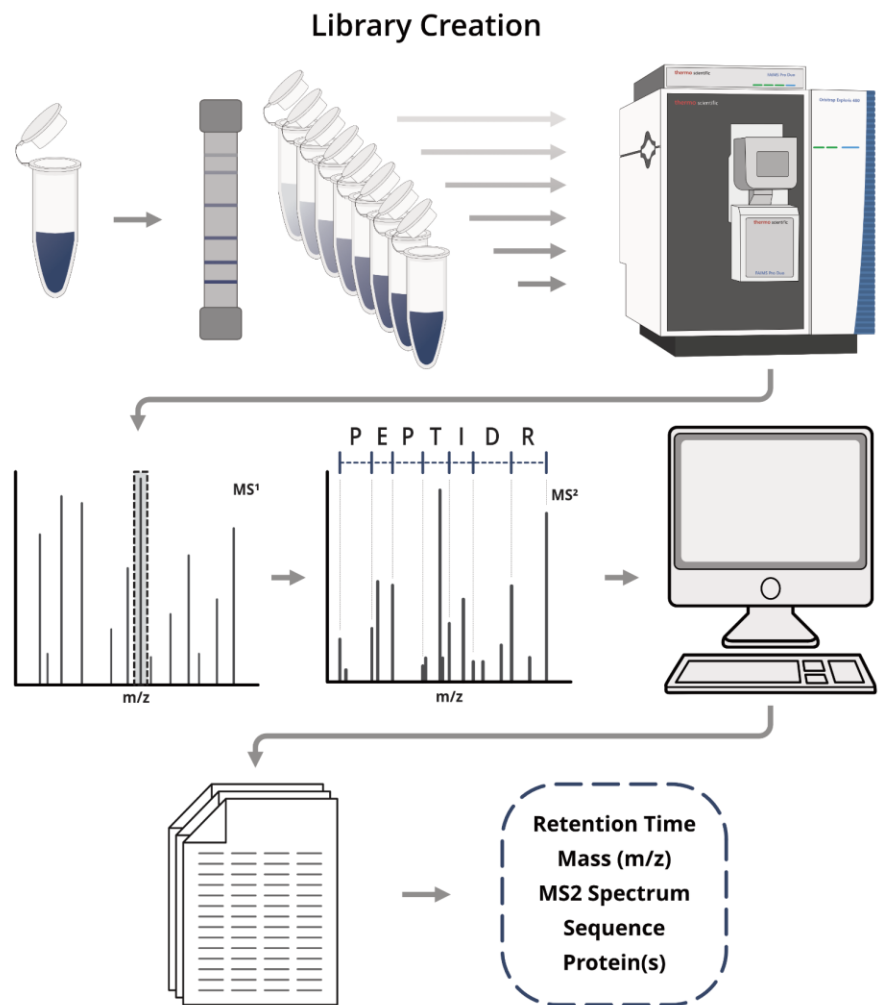
Unbiased and higher
throughput but data
processing is more
complex

DIA and Spectral Libraries





DIA and Spectral Libraries

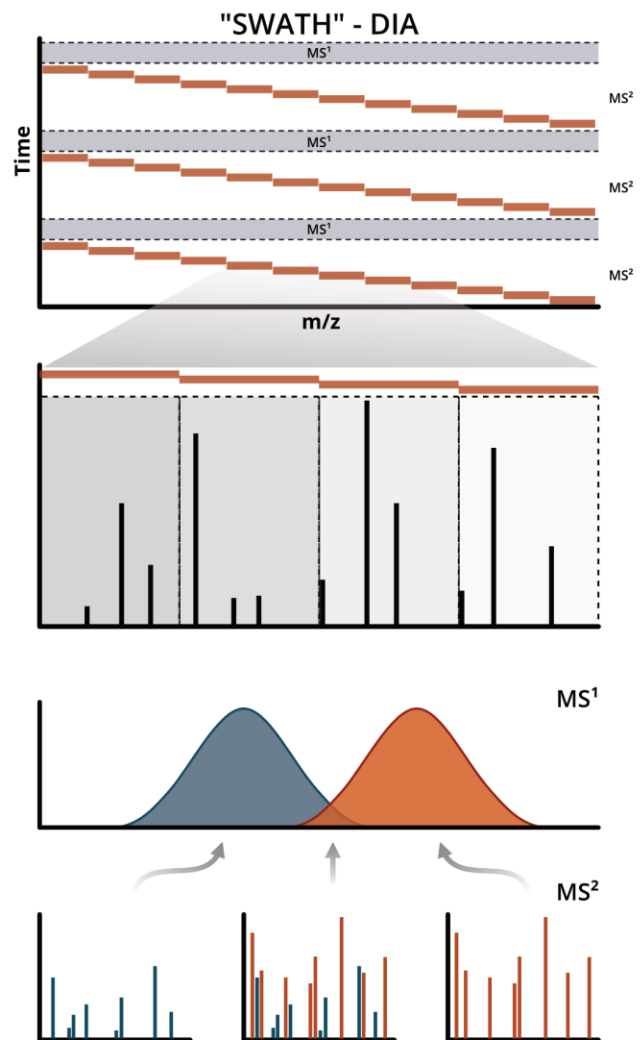
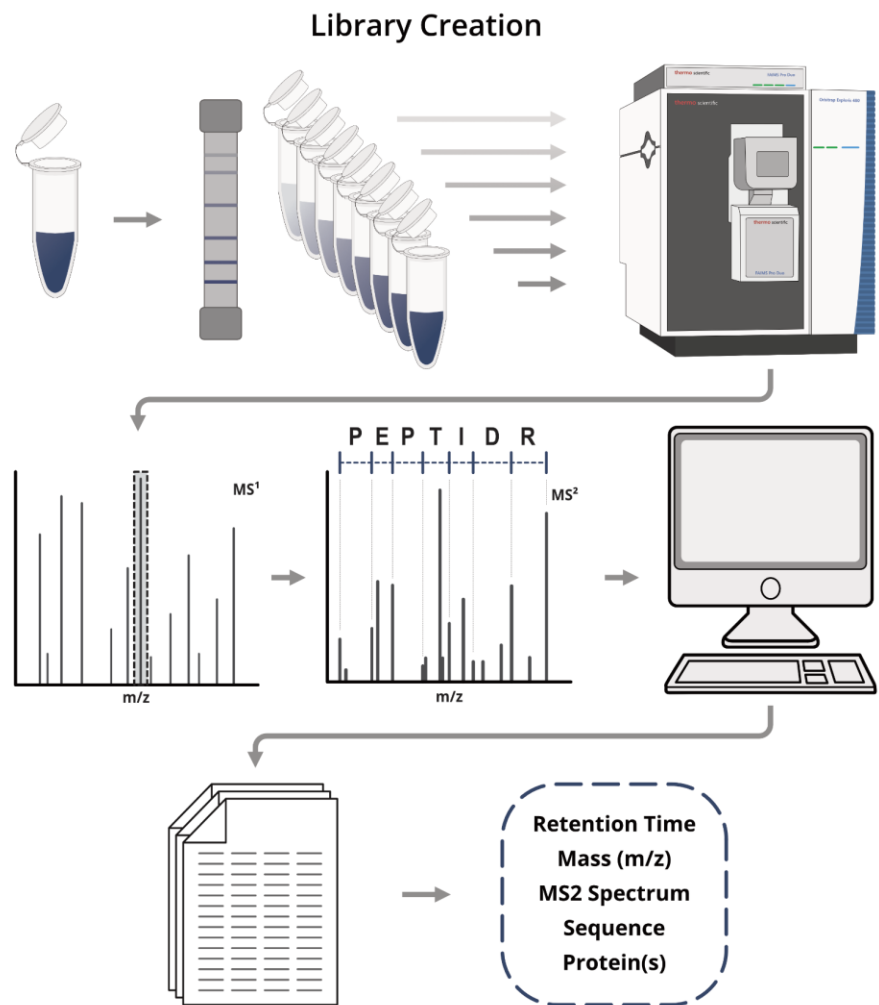


Spectral Libraries

Retention Time	MS ² Spectrum	Mass	Sequence	Protein
74.6 min		846.36	PEPTIDR	RNAS1
75.5 min		846.36	NLTKDR	A1AGP



DIA and Spectral Libraries



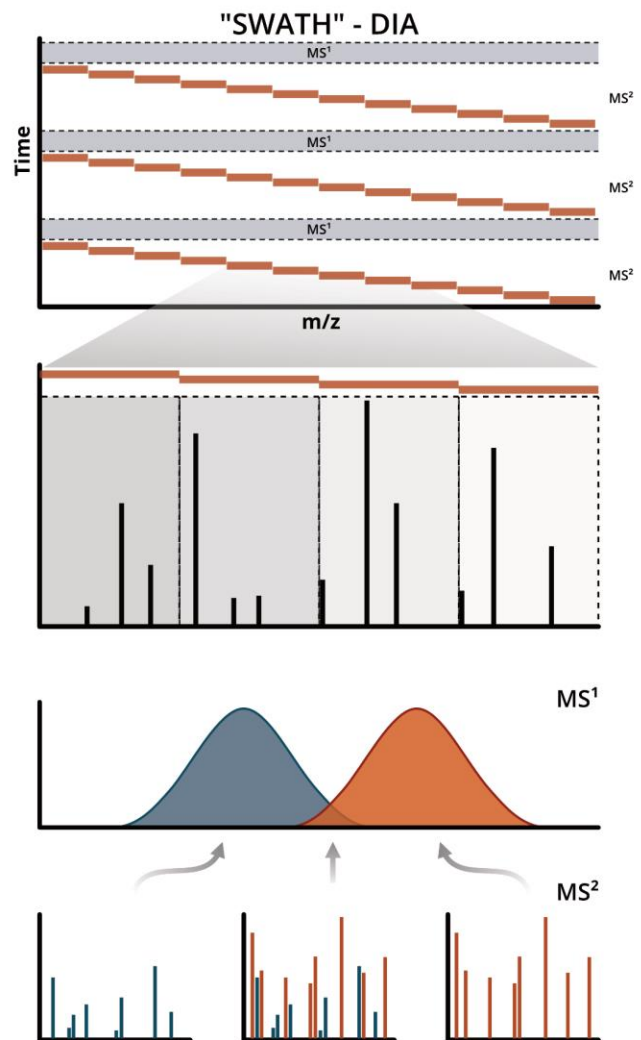
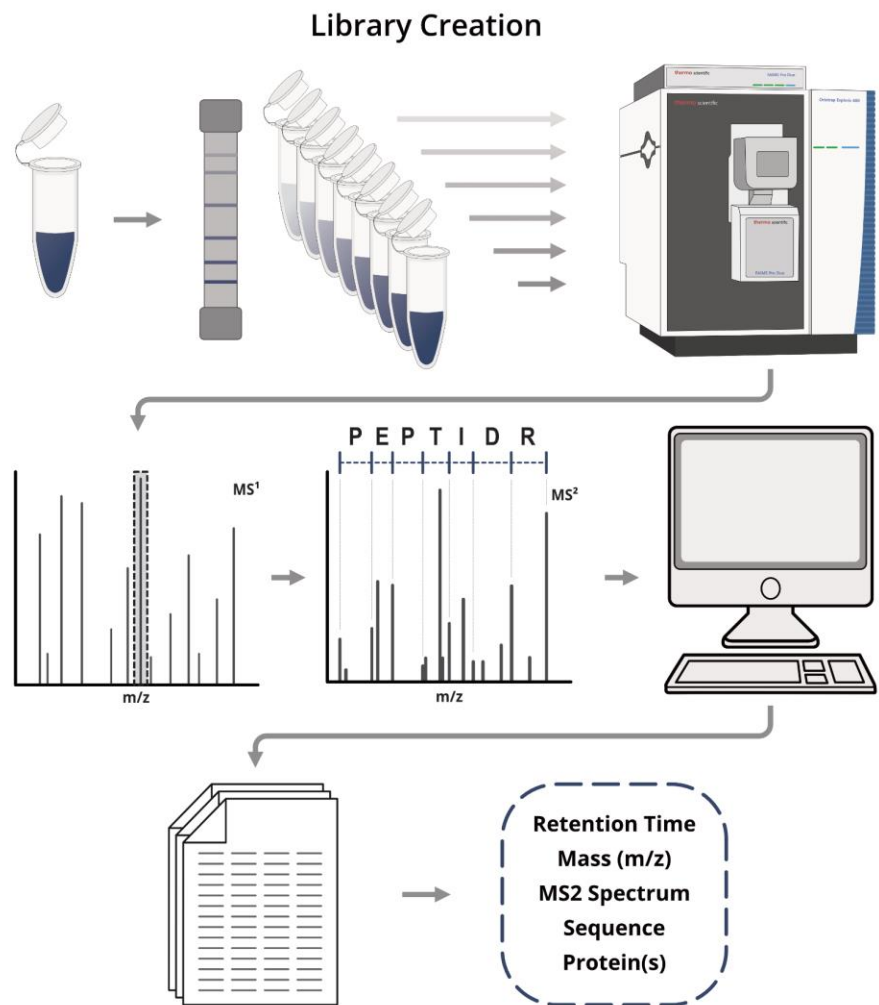
Spectral Libraries

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75.5 min		846.36	NLTKDR	A1AGP

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



DIA and Spectral Libraries



Spectral Libraries

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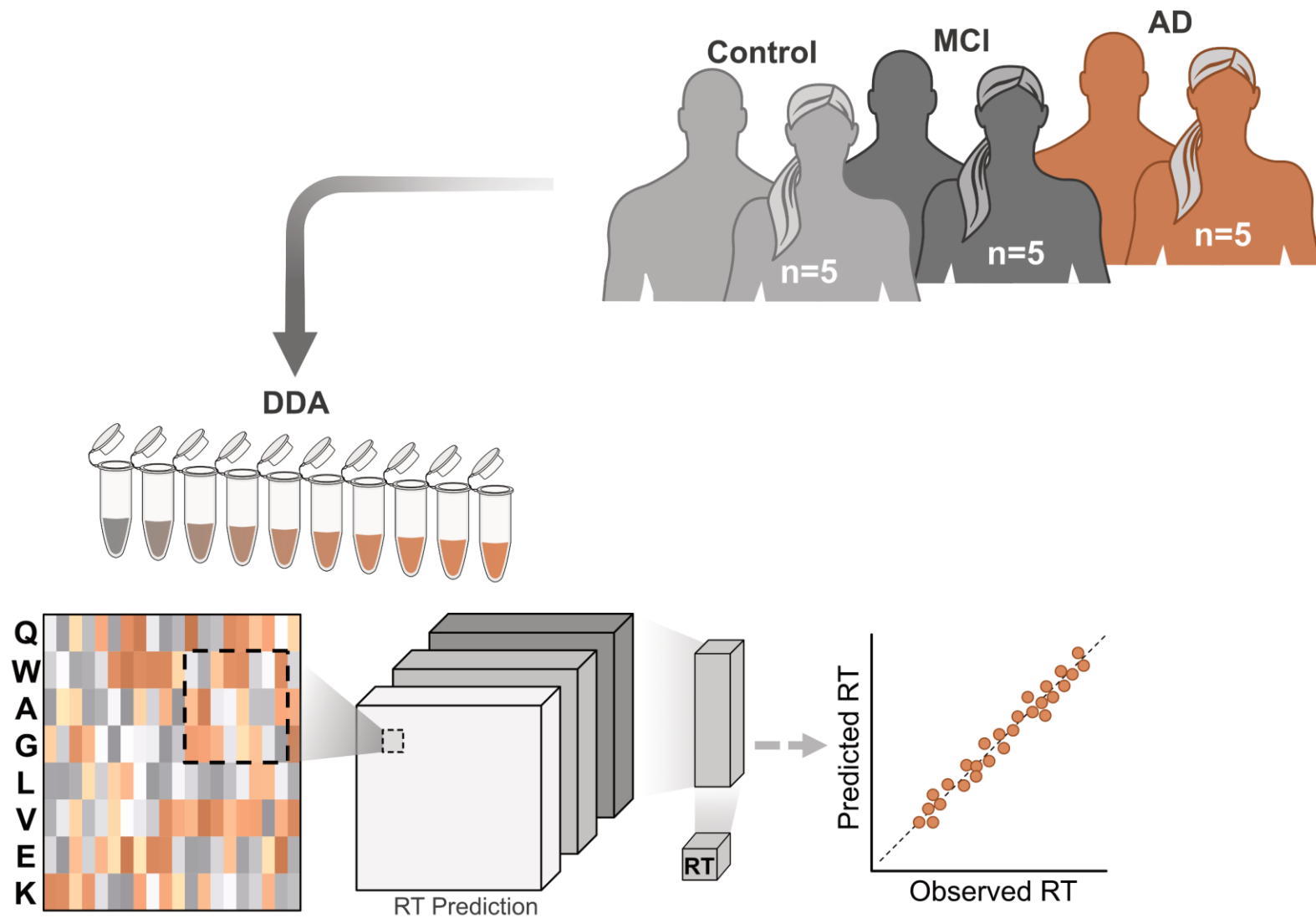
Is it possible to adapt spectral libraries from existing analyses to a new experiment?



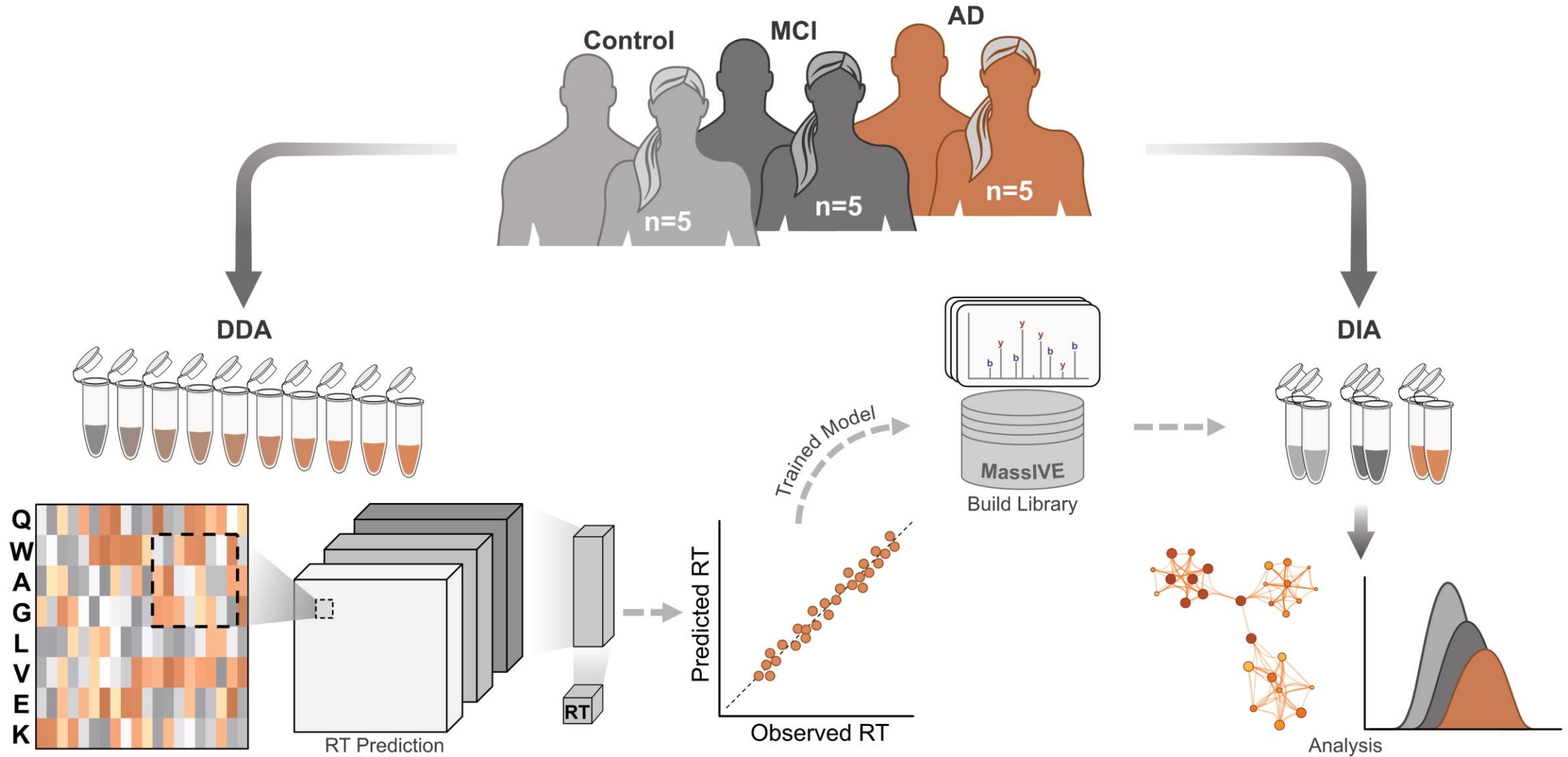
Questions and Experimental Goals

- 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

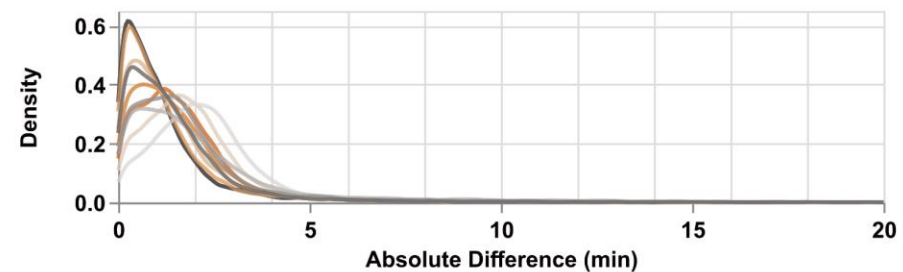
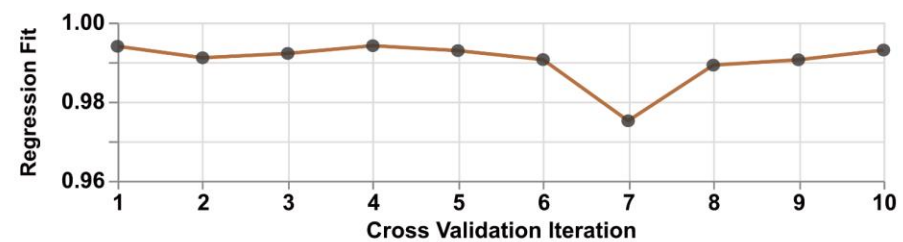
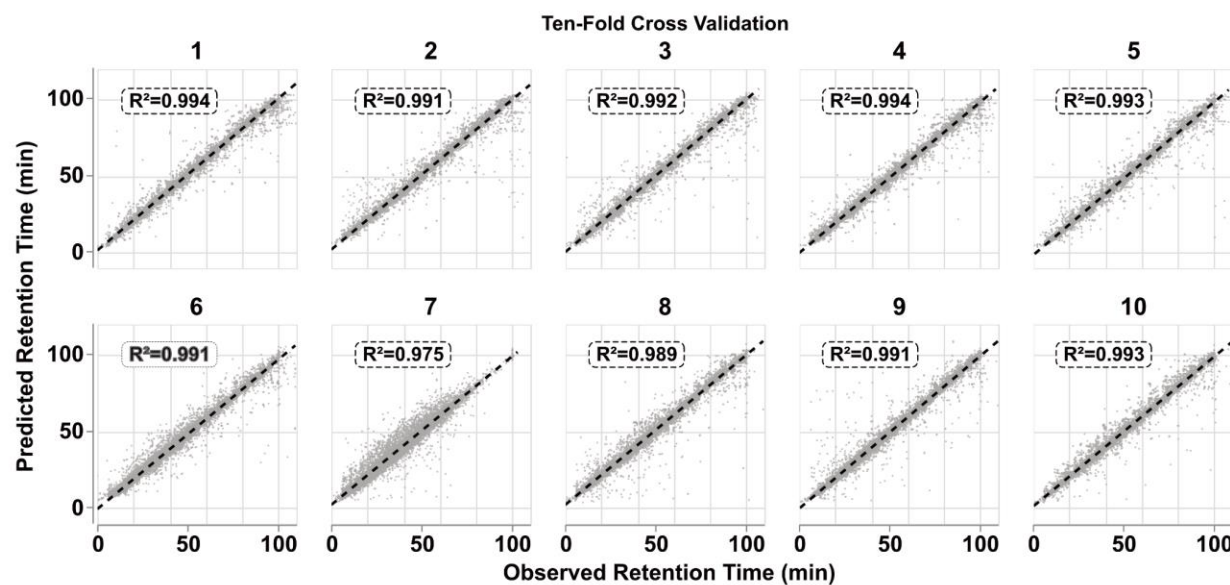
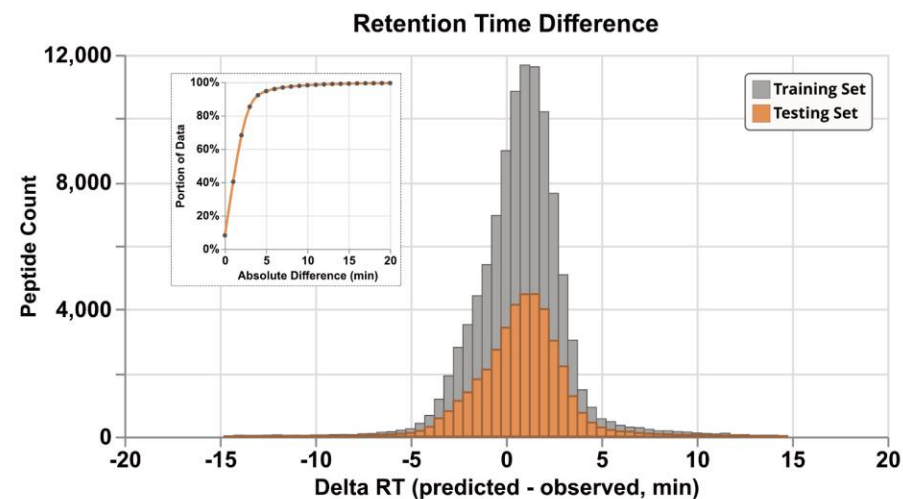
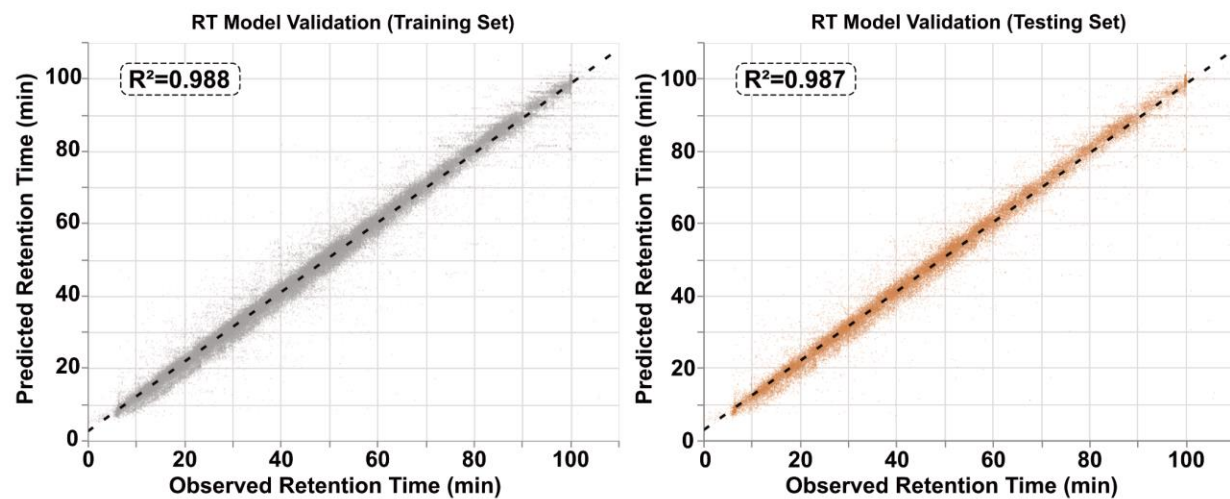


Parallel Workflow



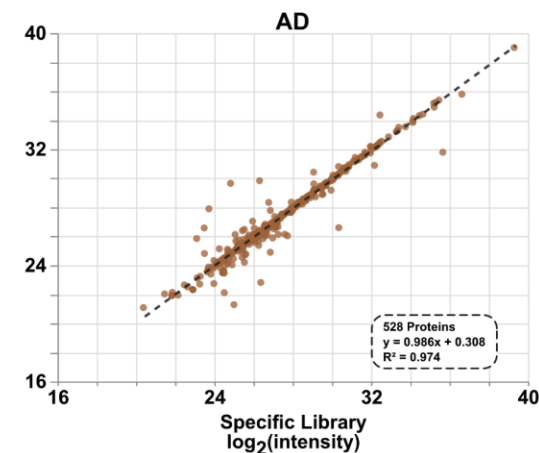
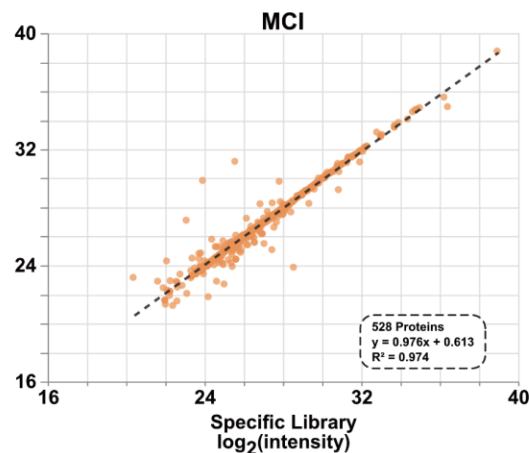
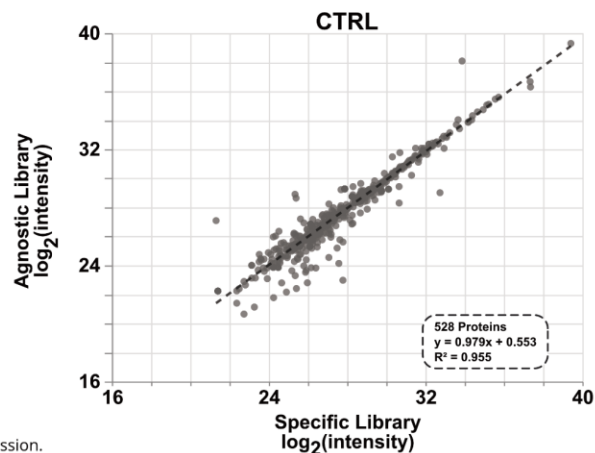
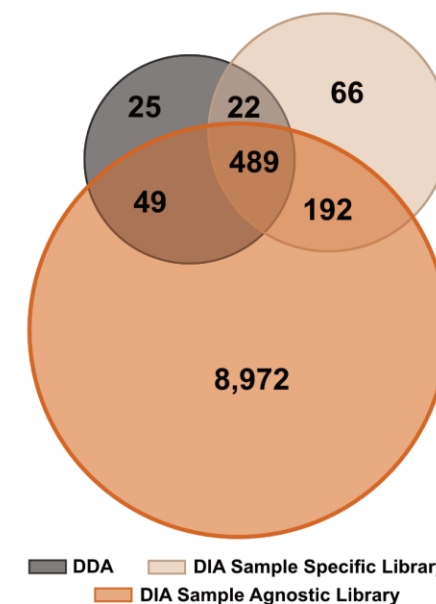
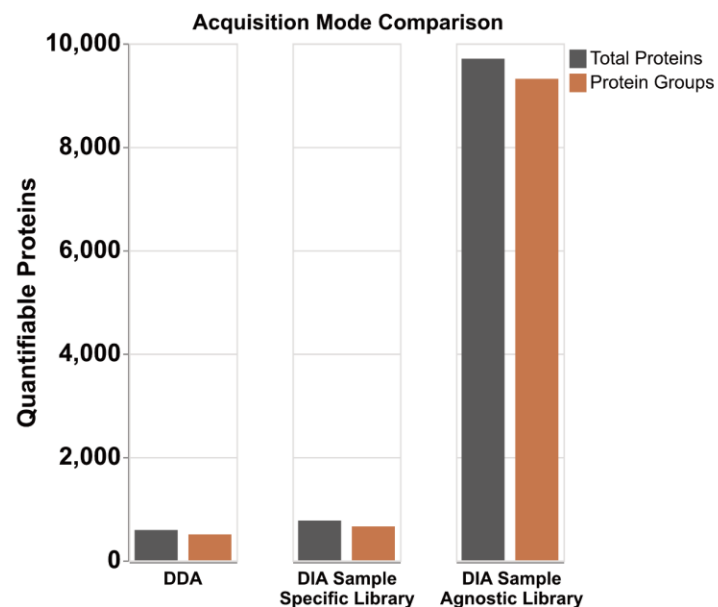
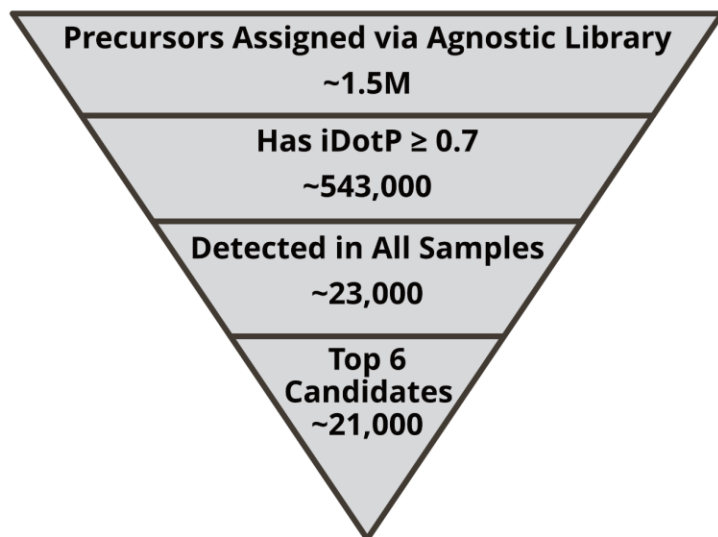


Model Validation

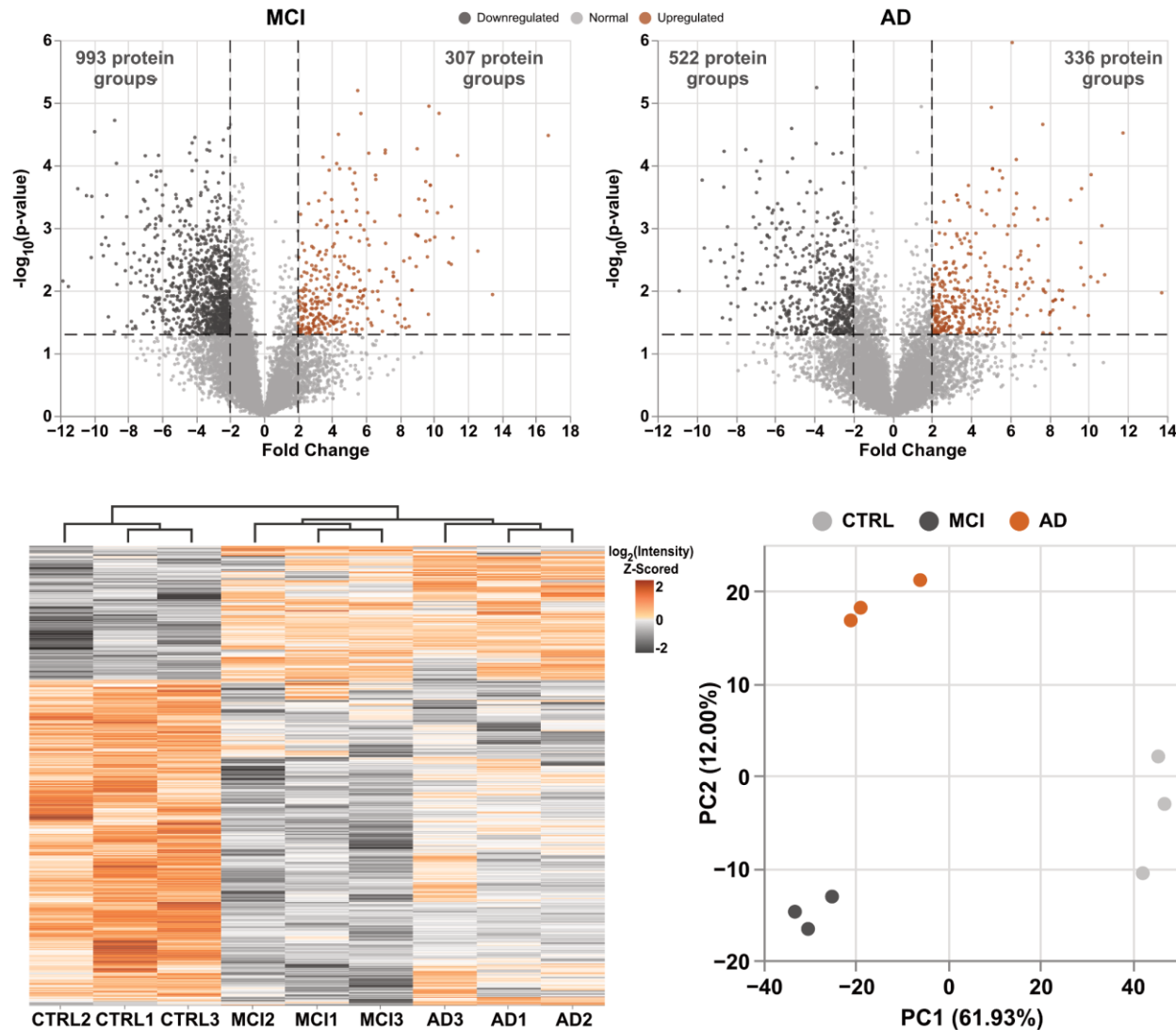




Quantitative Comparison



Disease State Discrimination

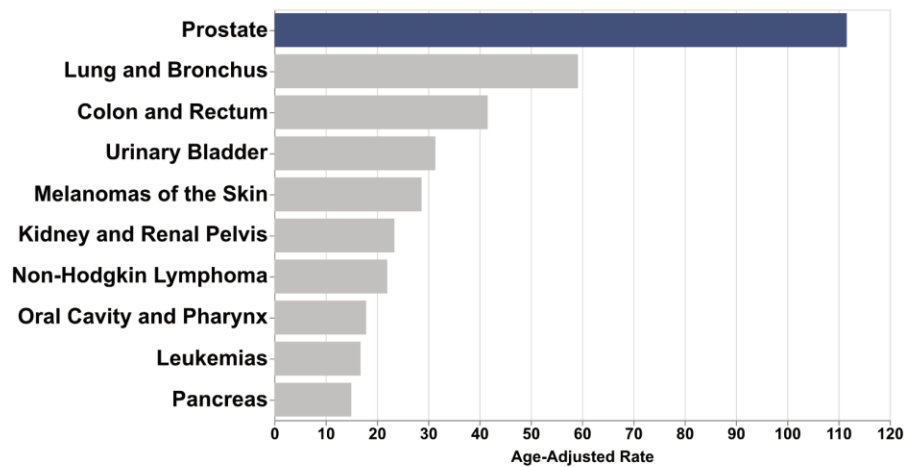
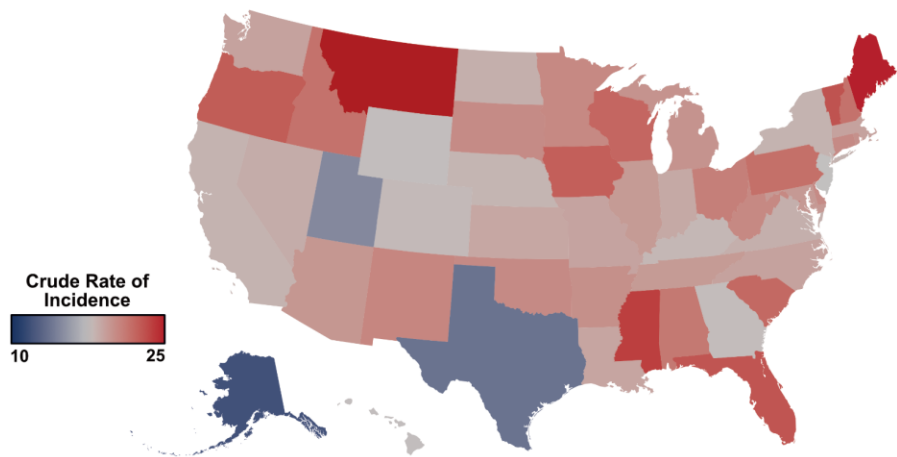


Outcomes

- 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 dysruption.
- 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.

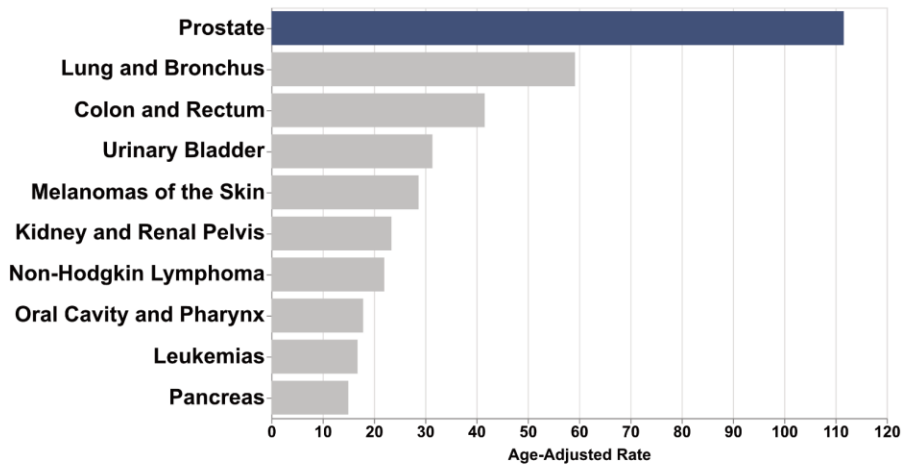
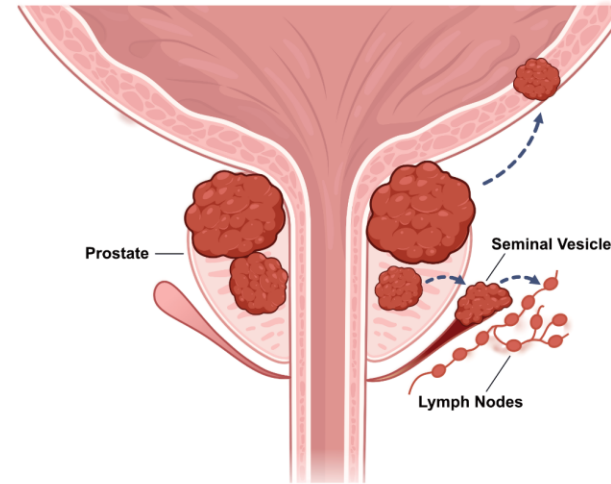
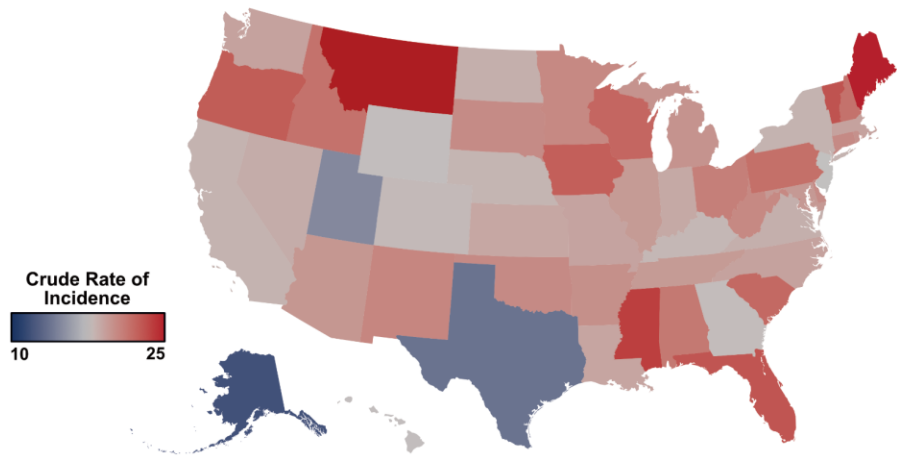


Prostate Cancer Incidence and Outlook



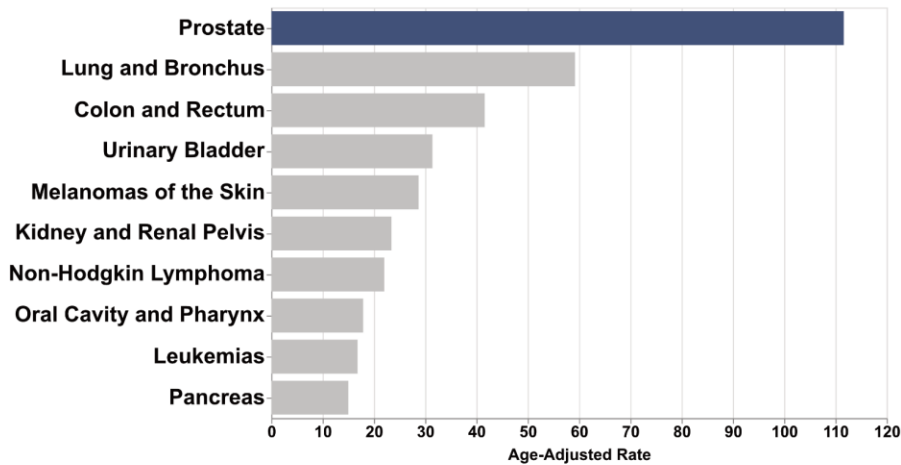
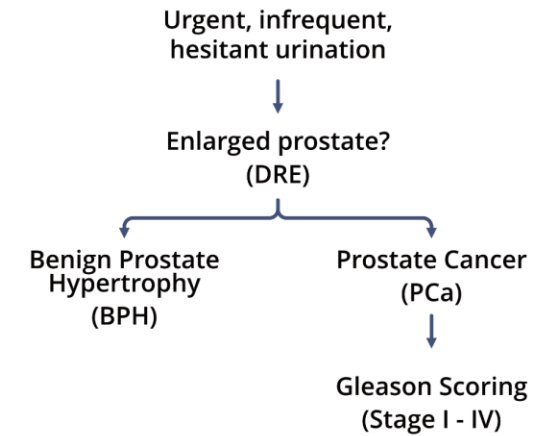
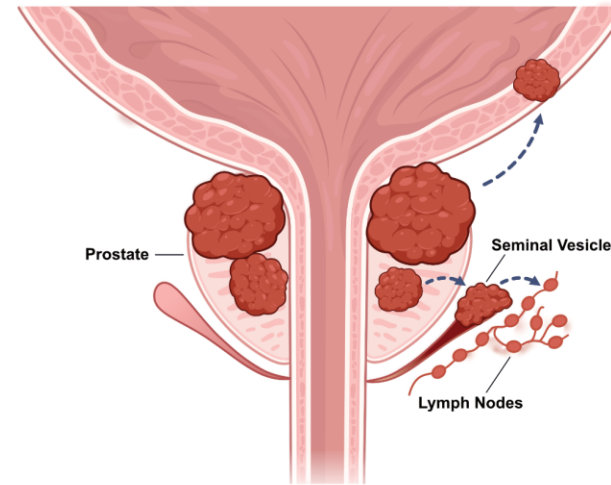
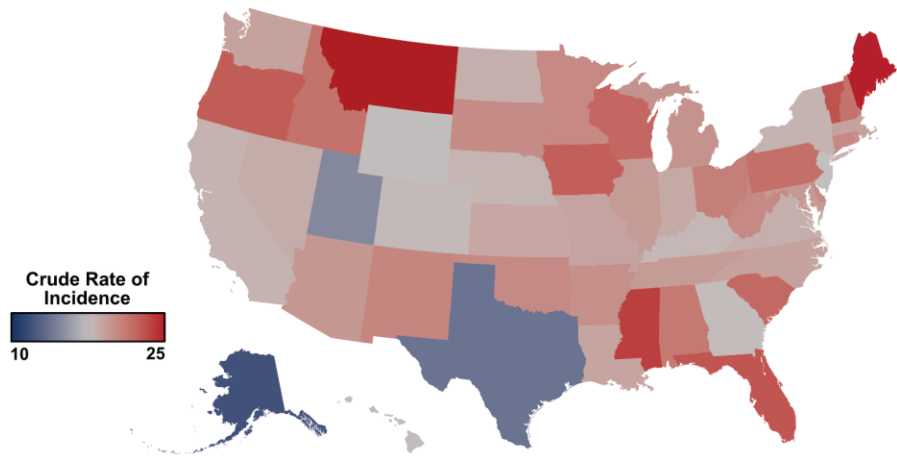


Prostate Cancer Incidence and Outlook



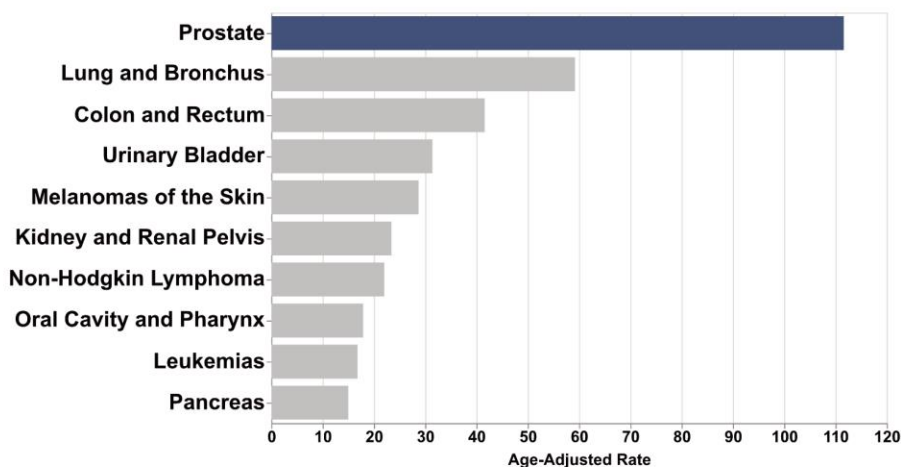
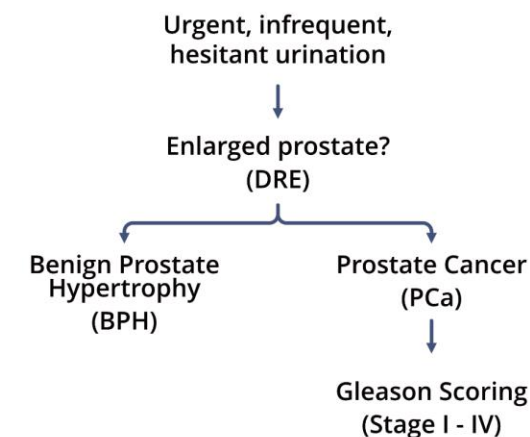
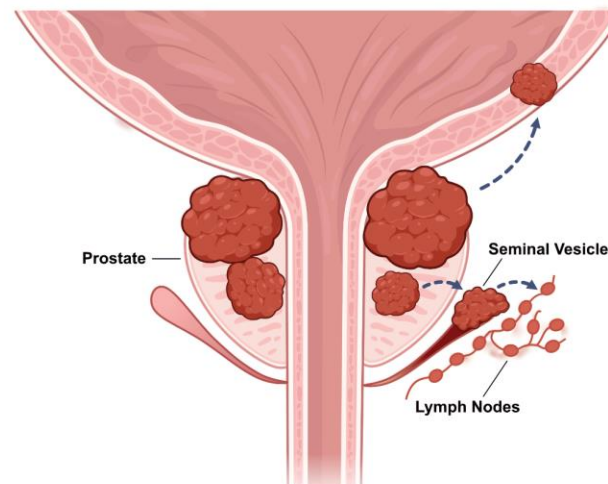
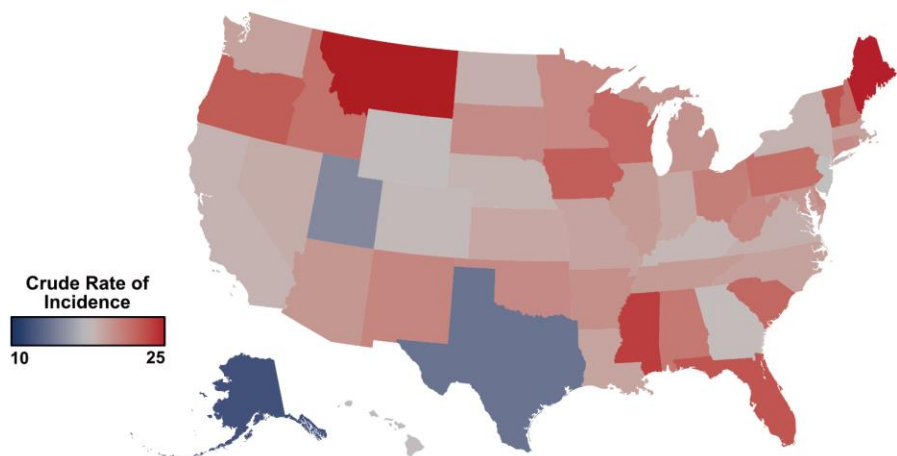


Prostate Cancer Incidence and Outlook



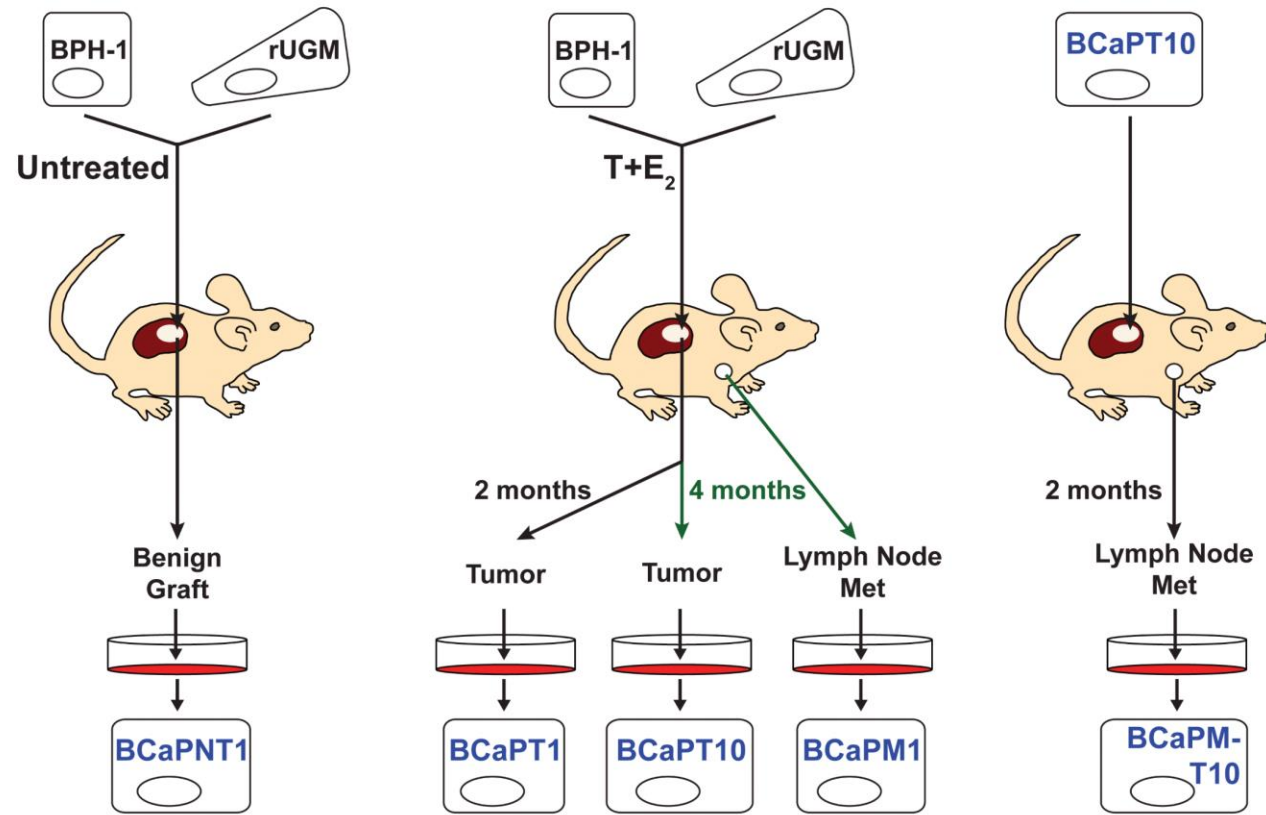


Prostate Cancer Incidence and Outlook



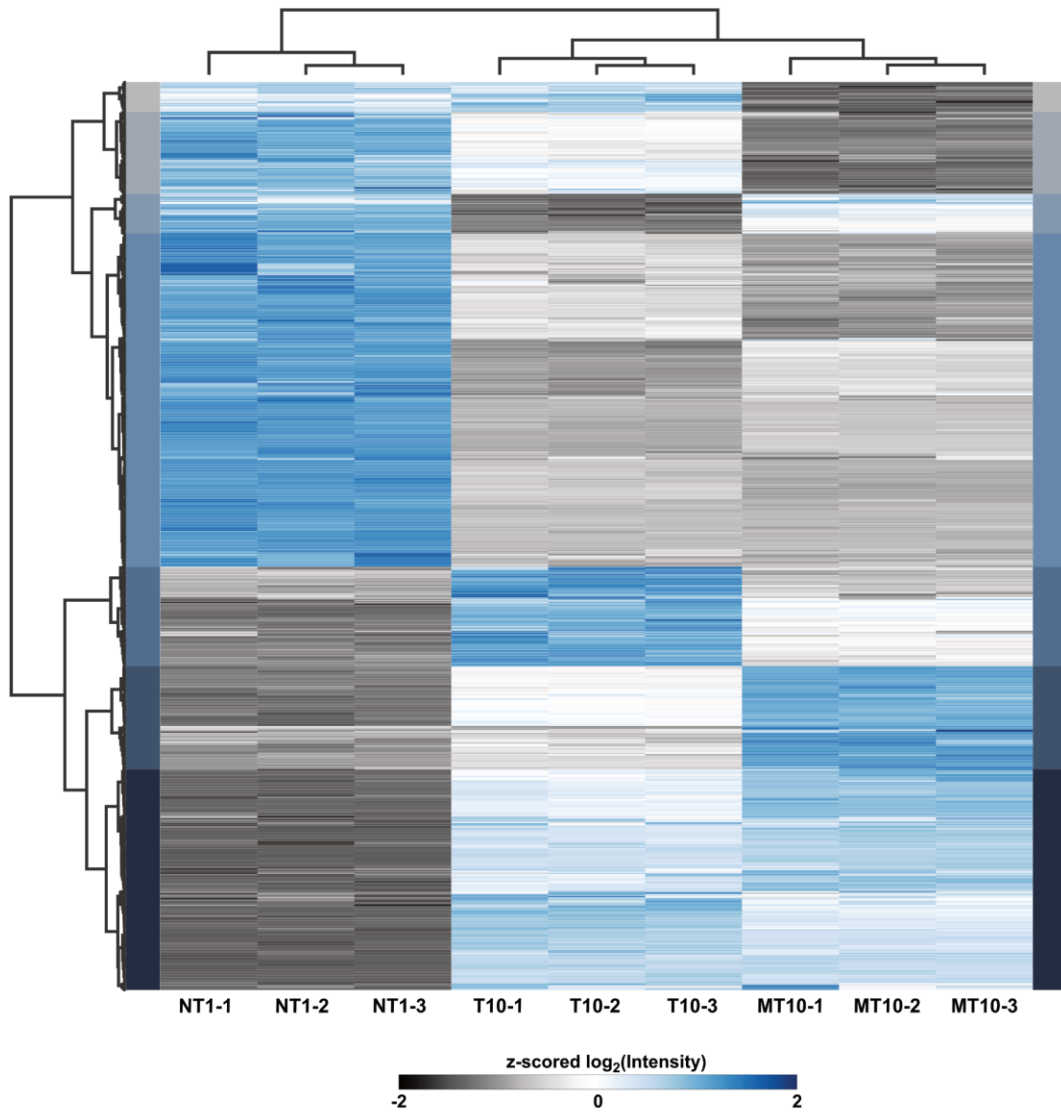
- Leading rates of incidence and mortality necessitate preventative screening
- Urological screening (e.g., DRE) remain the most direct means of PCa detection but still require biopsy and serological confirmation
- Therapeutic monitoring and PCa progression dependent on a single biomarker
- There is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

BPH-1-Derived Cancer Progression Cell Model

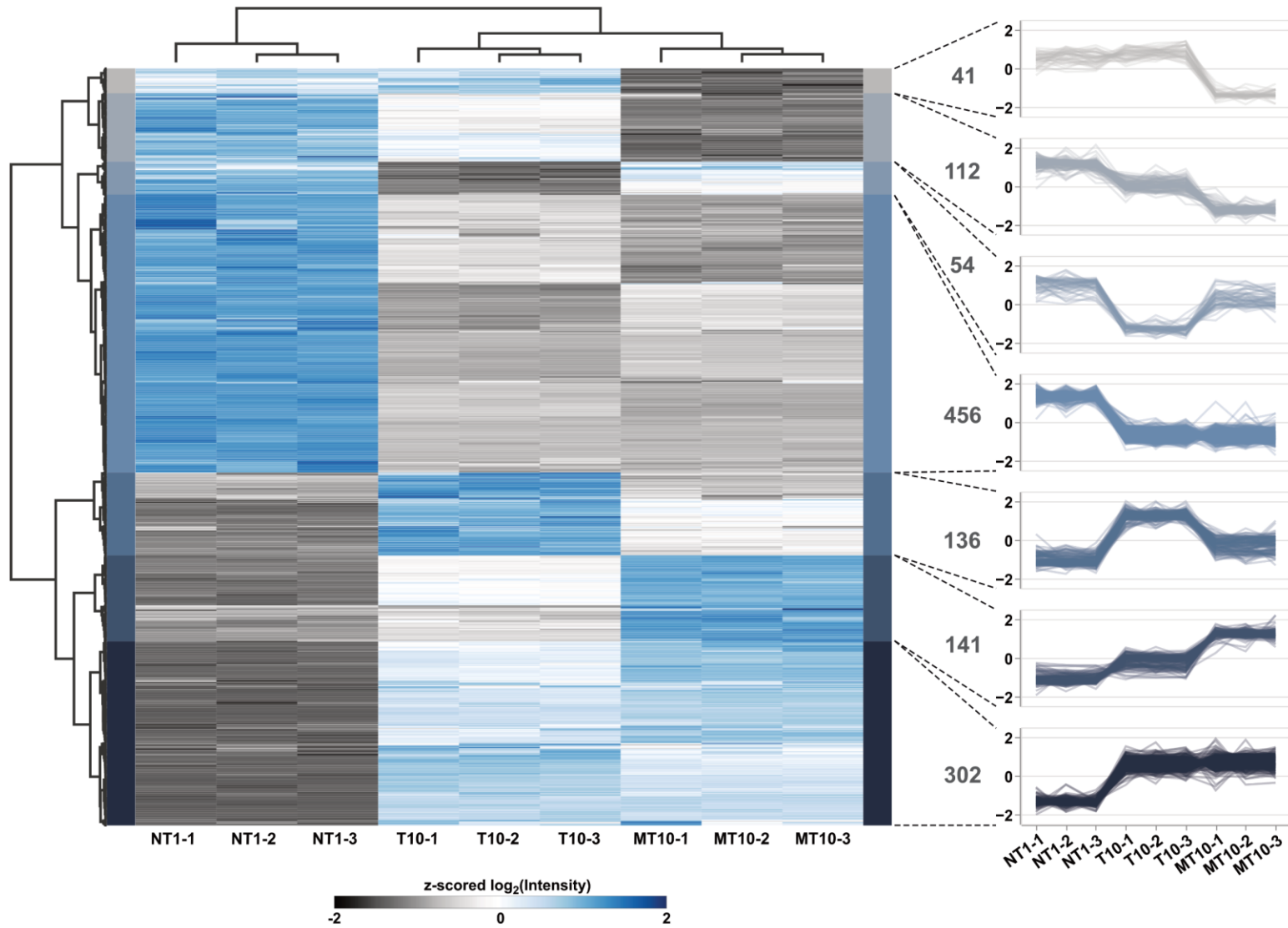


- Early investigations demonstrated Benign Prostatic Hypertrophy (BPH) can progress to cancer in presence of hormones
- Ricke and colleagues developed cell line model of cancer progression that mimics the genetic, phenotypic, and molecular characteristics of PCa
- Proteomic analyses of discrete phenotypes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

PCa Proteomic Fingerprinting

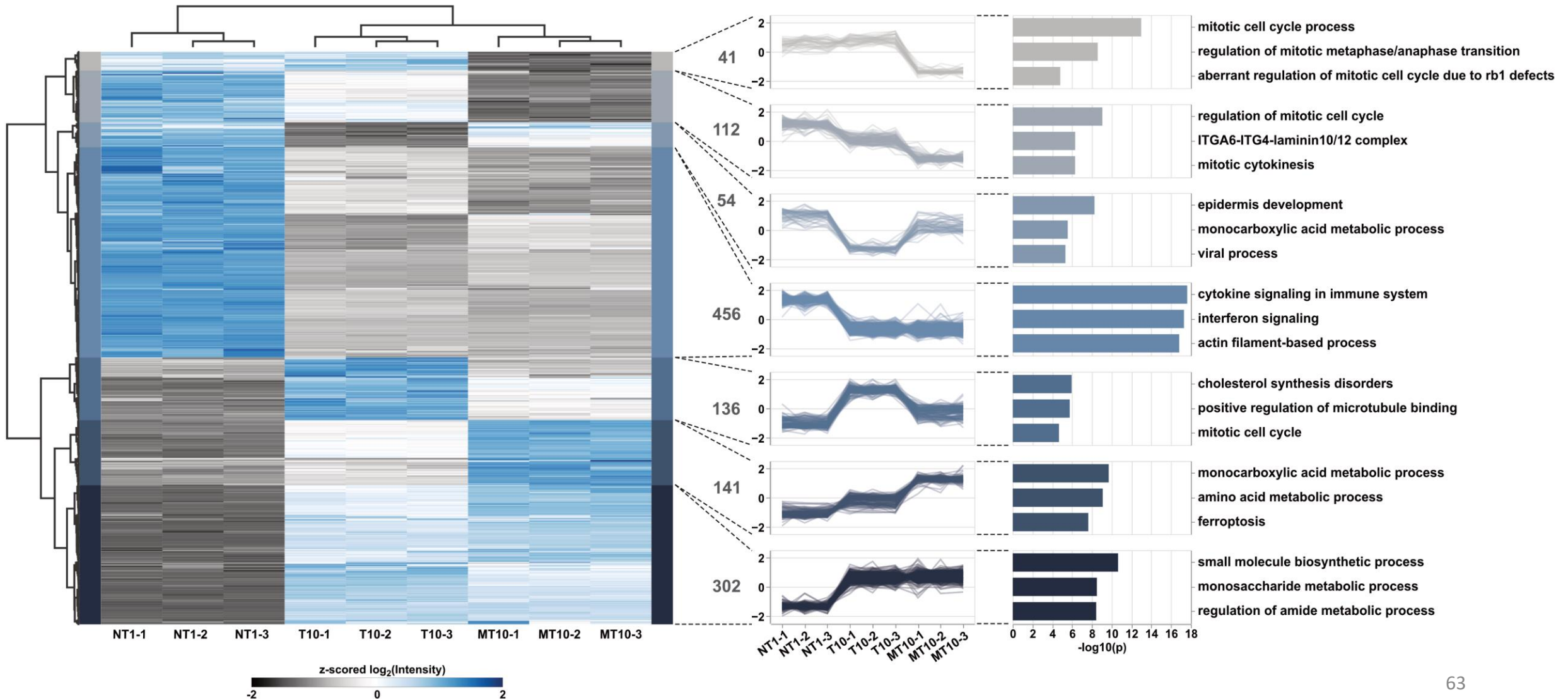


PCa Proteomic Fingerprinting



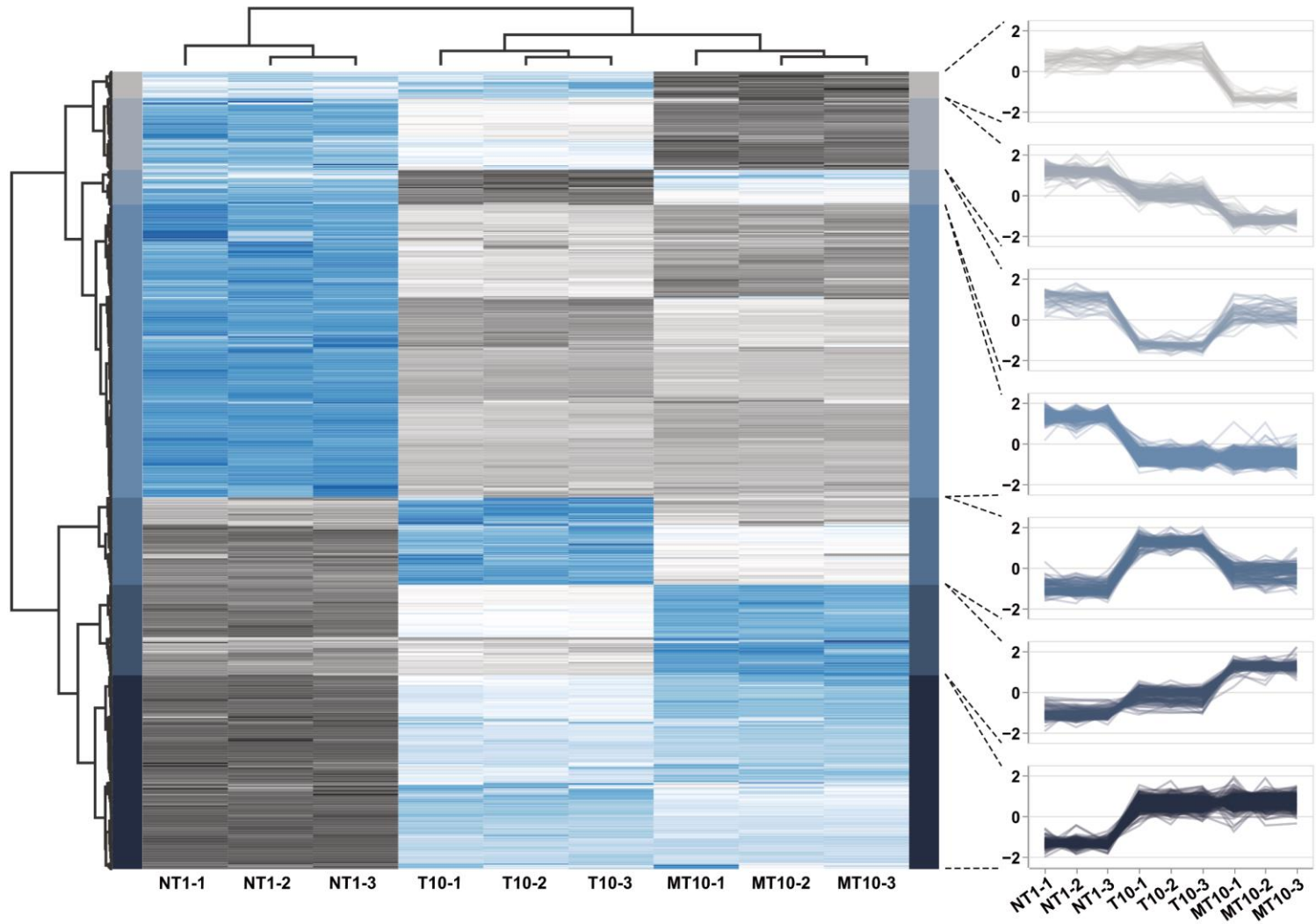


PCa Proteomic Fingerprinting





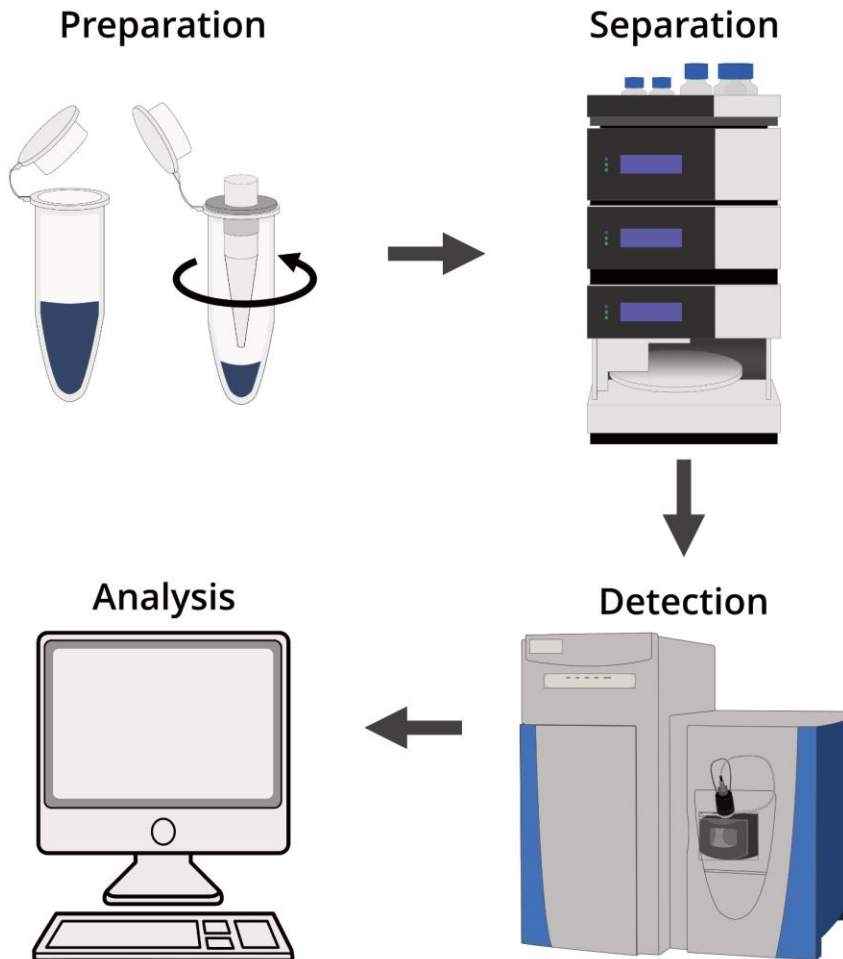
Future Directions



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.

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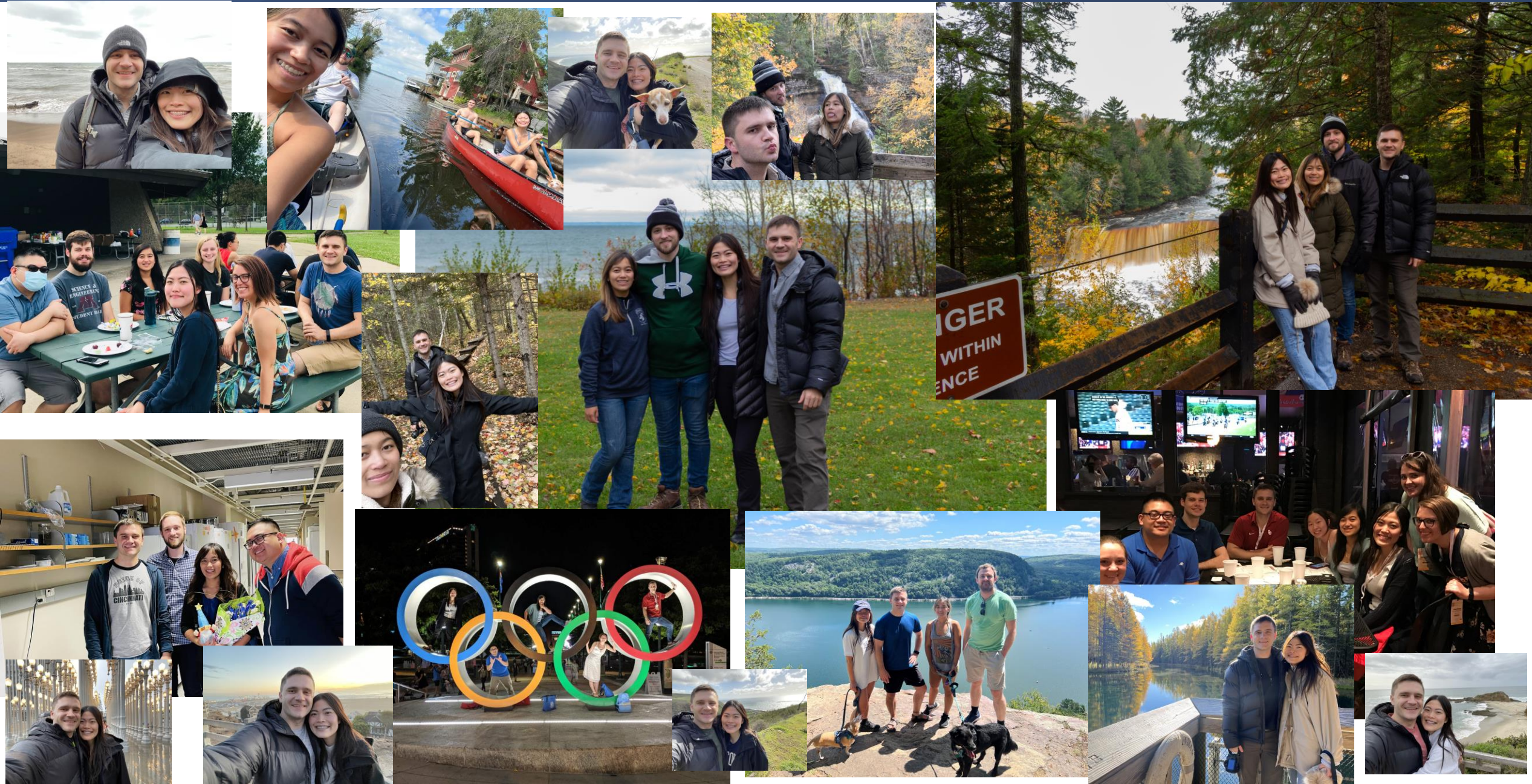
Ricke Lab

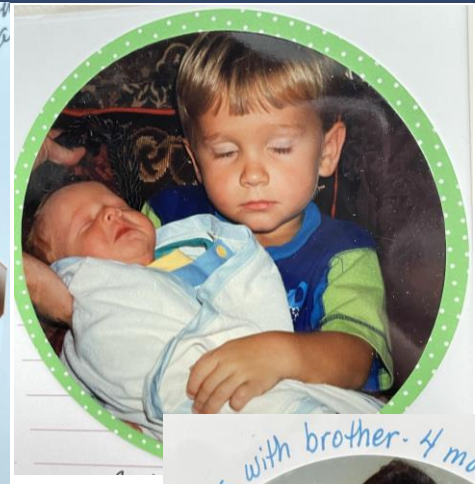
Dr. Will Ricke
Dr. Teresa Liu

Support

Dr. Cameron Scarlett
Molly Pelliteri-Hahn







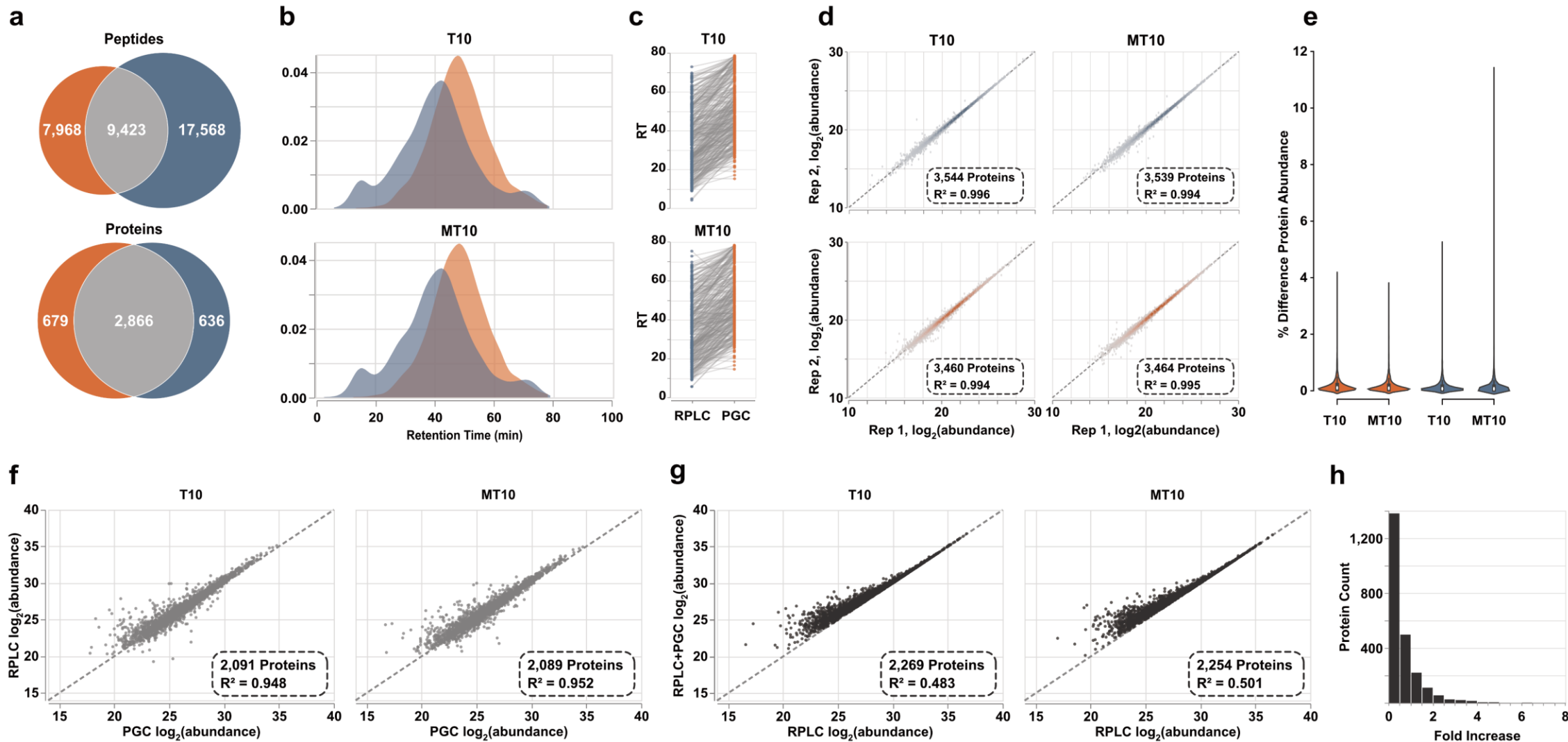
with brother - 4 months



Supplemental

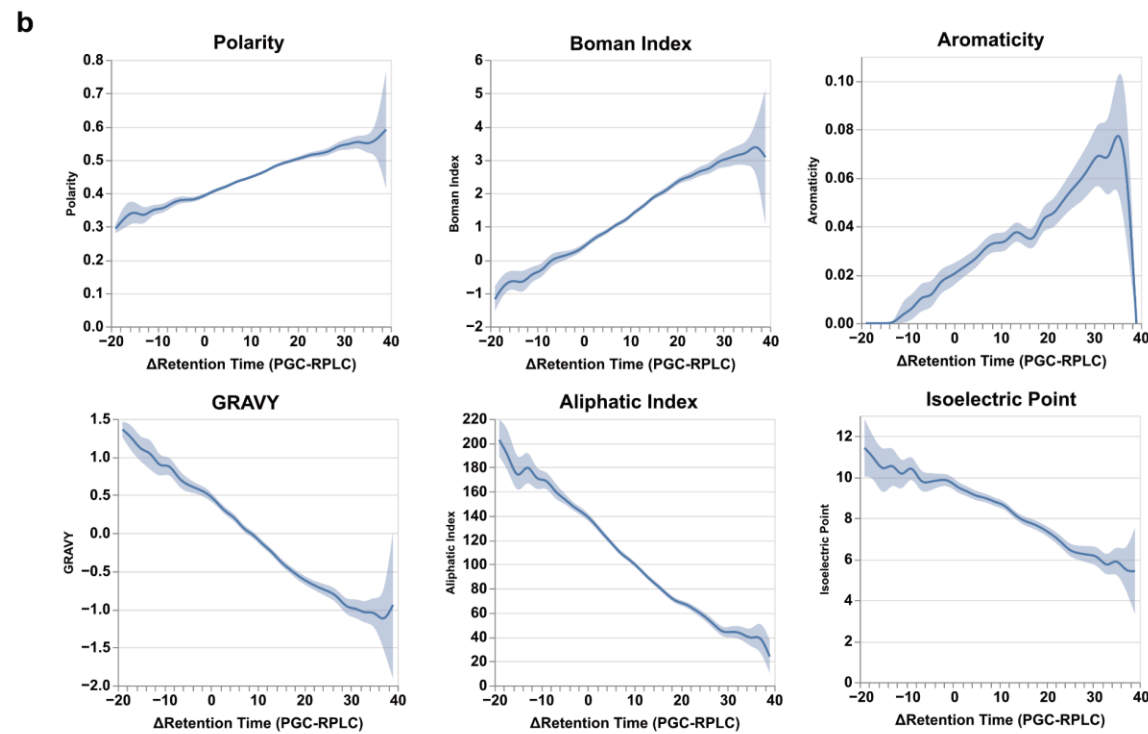
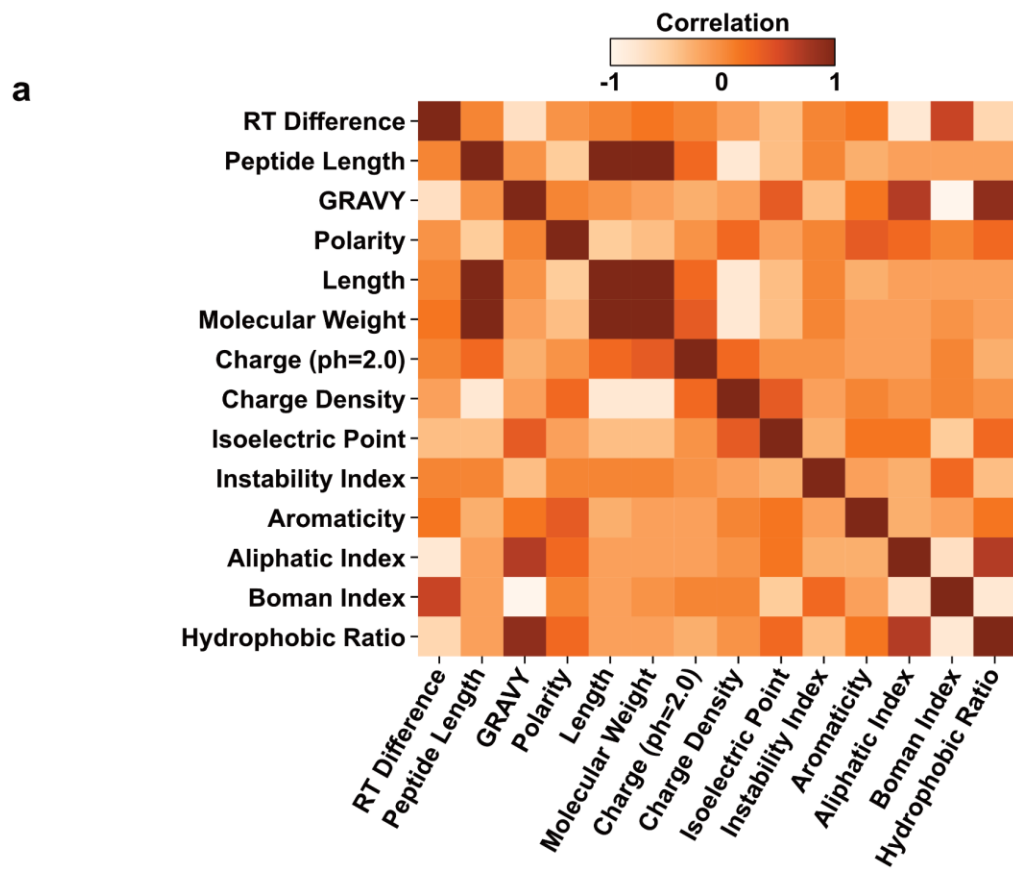


How PGC Separations Affect Proteomic Measurement



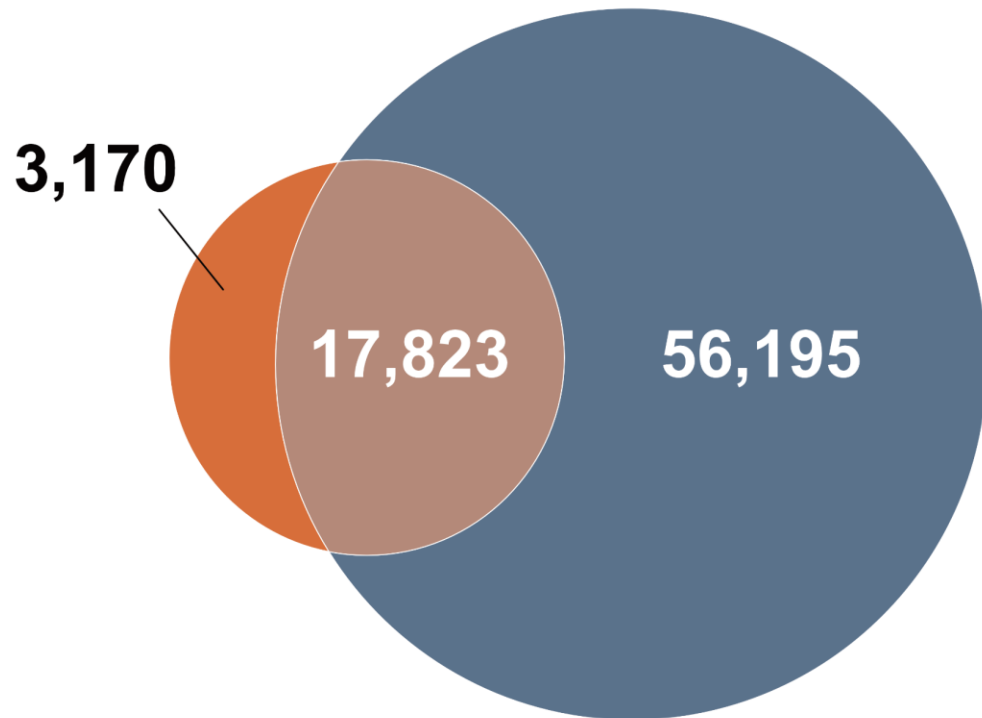


Peptide Character Affecting PGC Retention

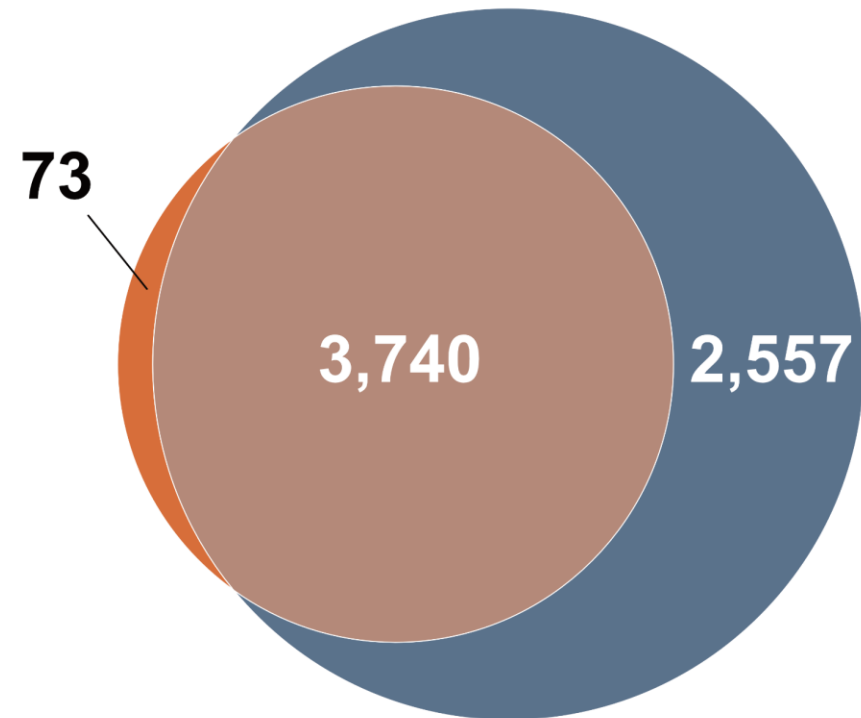


Library-Free Comparison, RPLC vs. PGC

Peptides

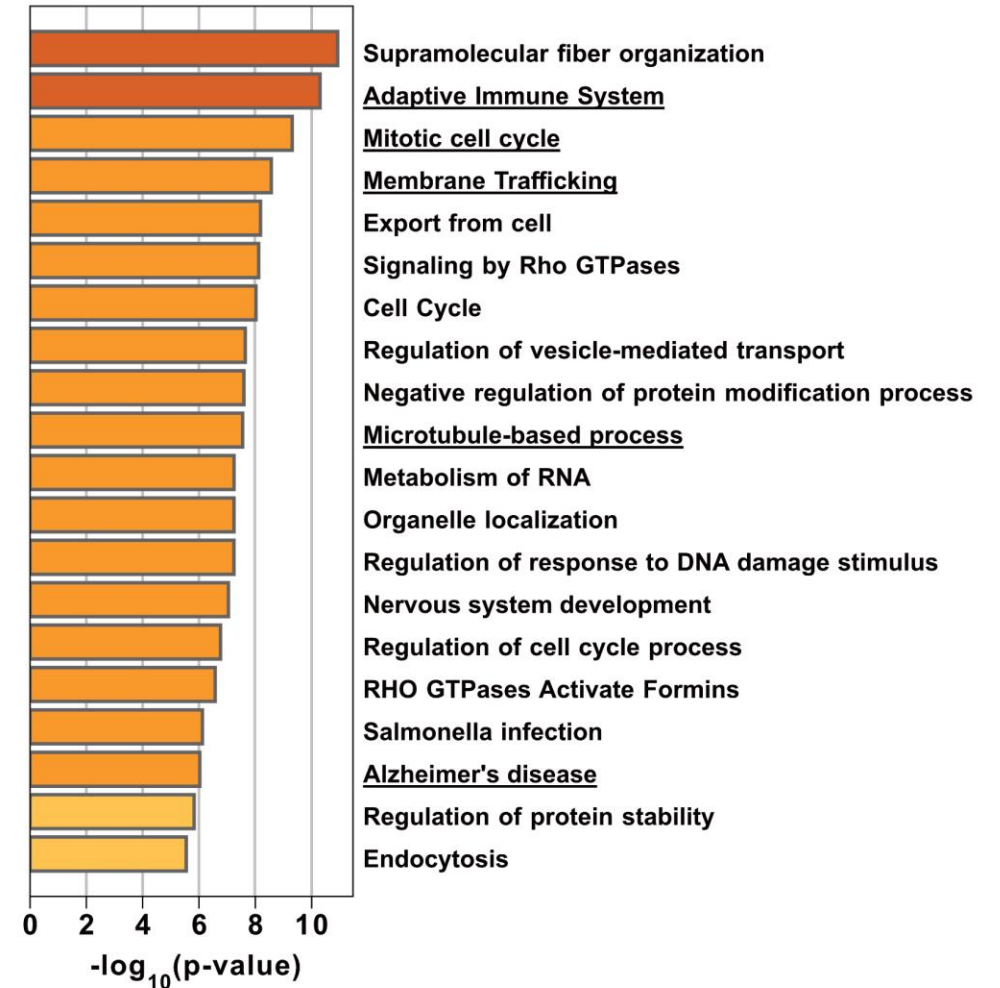
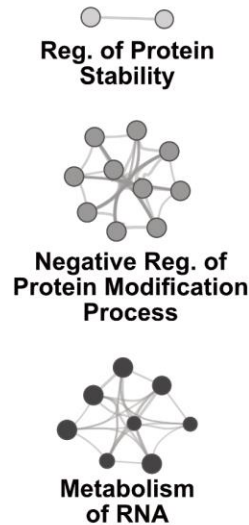
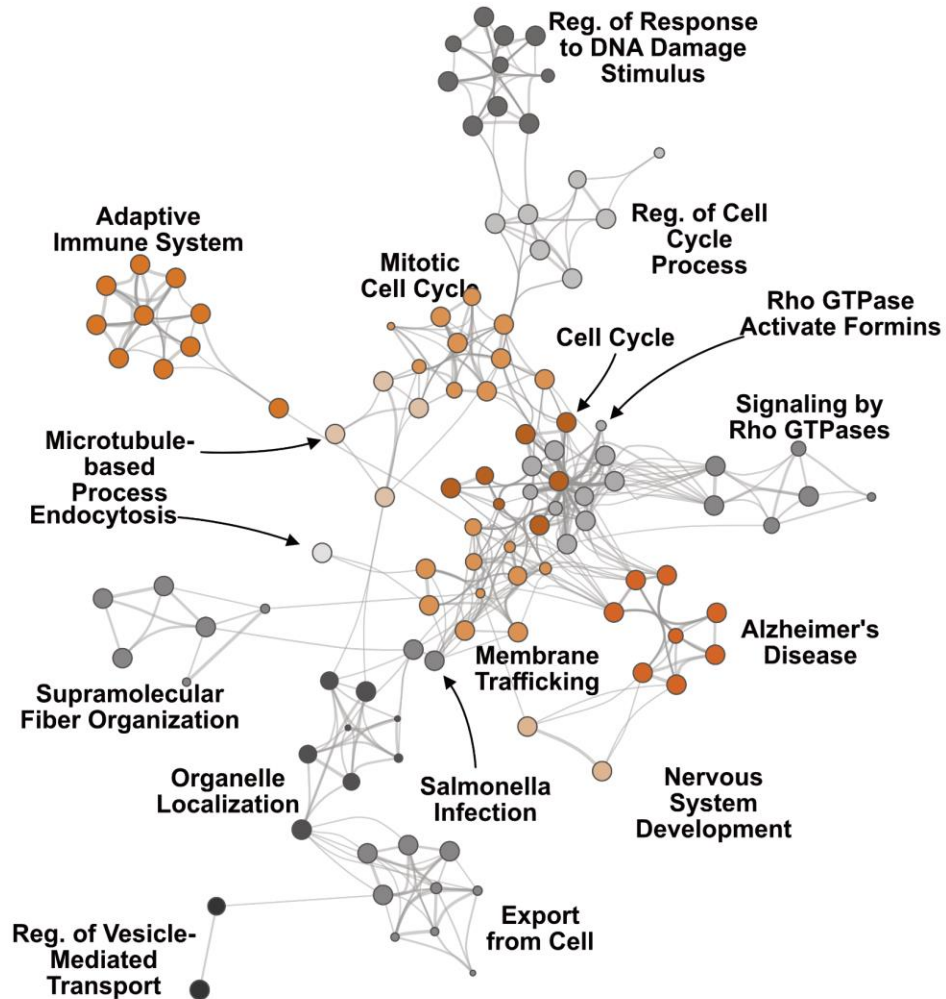


Proteins



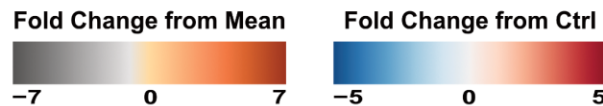
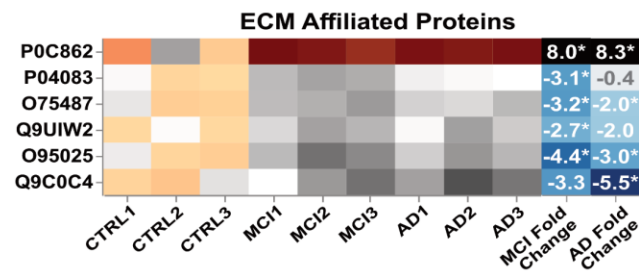
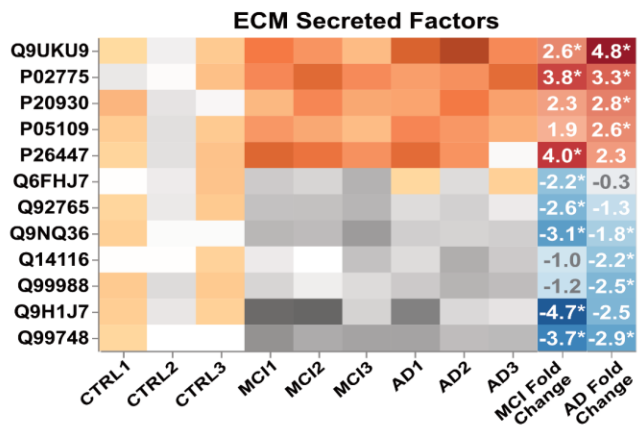
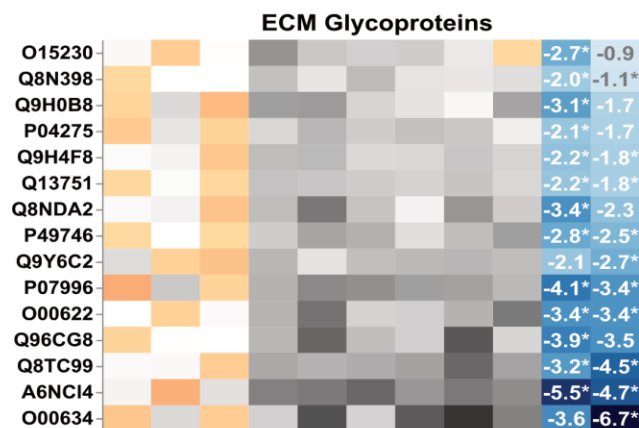
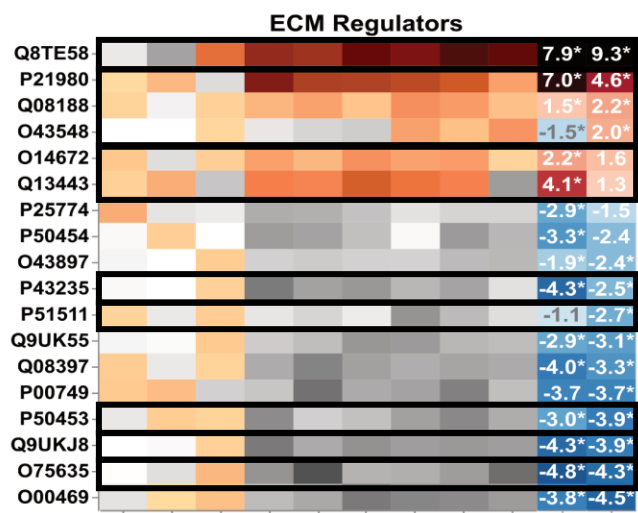


Dysregulated Pathways





ECM Reorganization

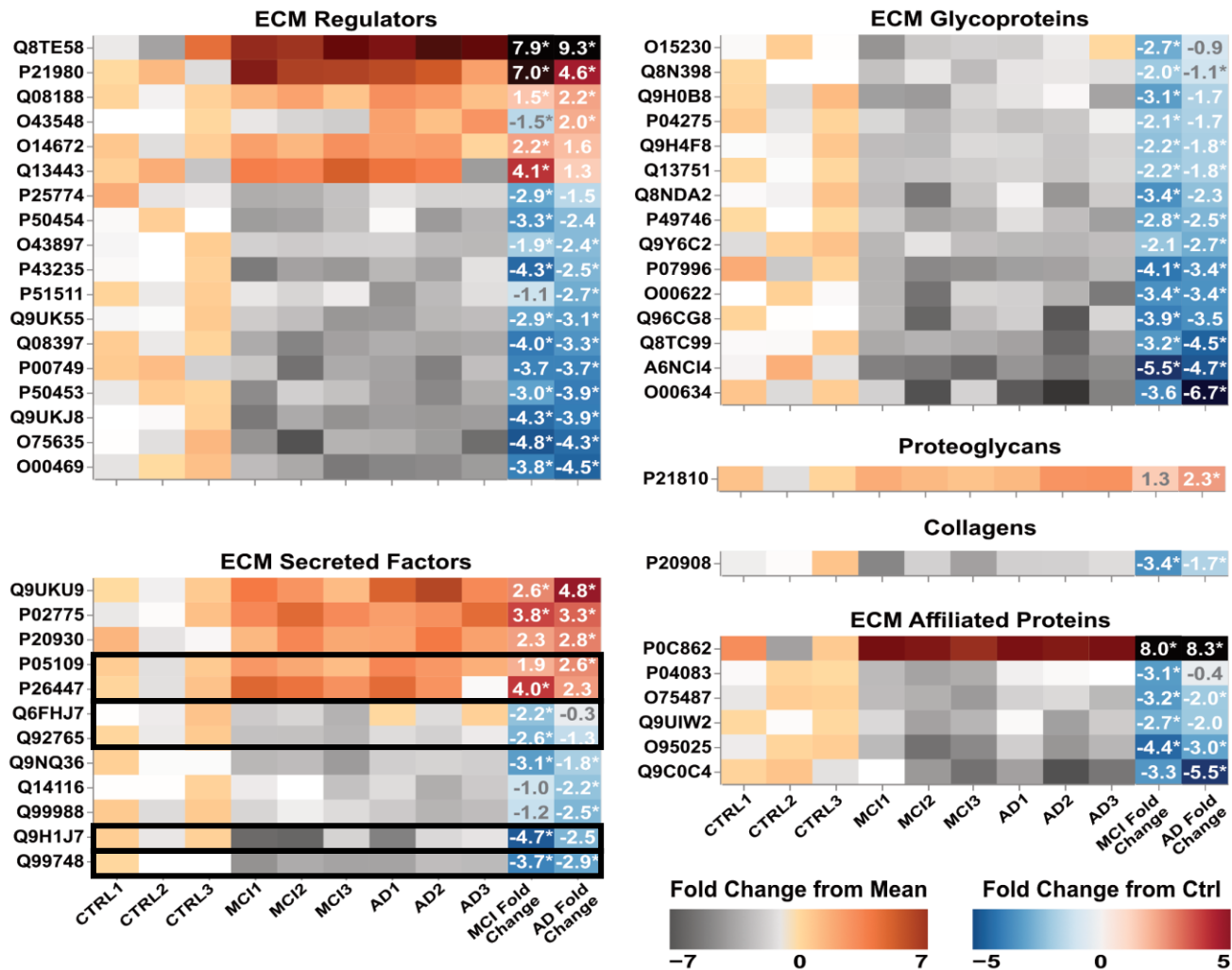


Dysregulated Proteins

- Disintegrin and metalloproteinases
- Gamma glutamyltransferases
- Serpins



ECM Reorganization

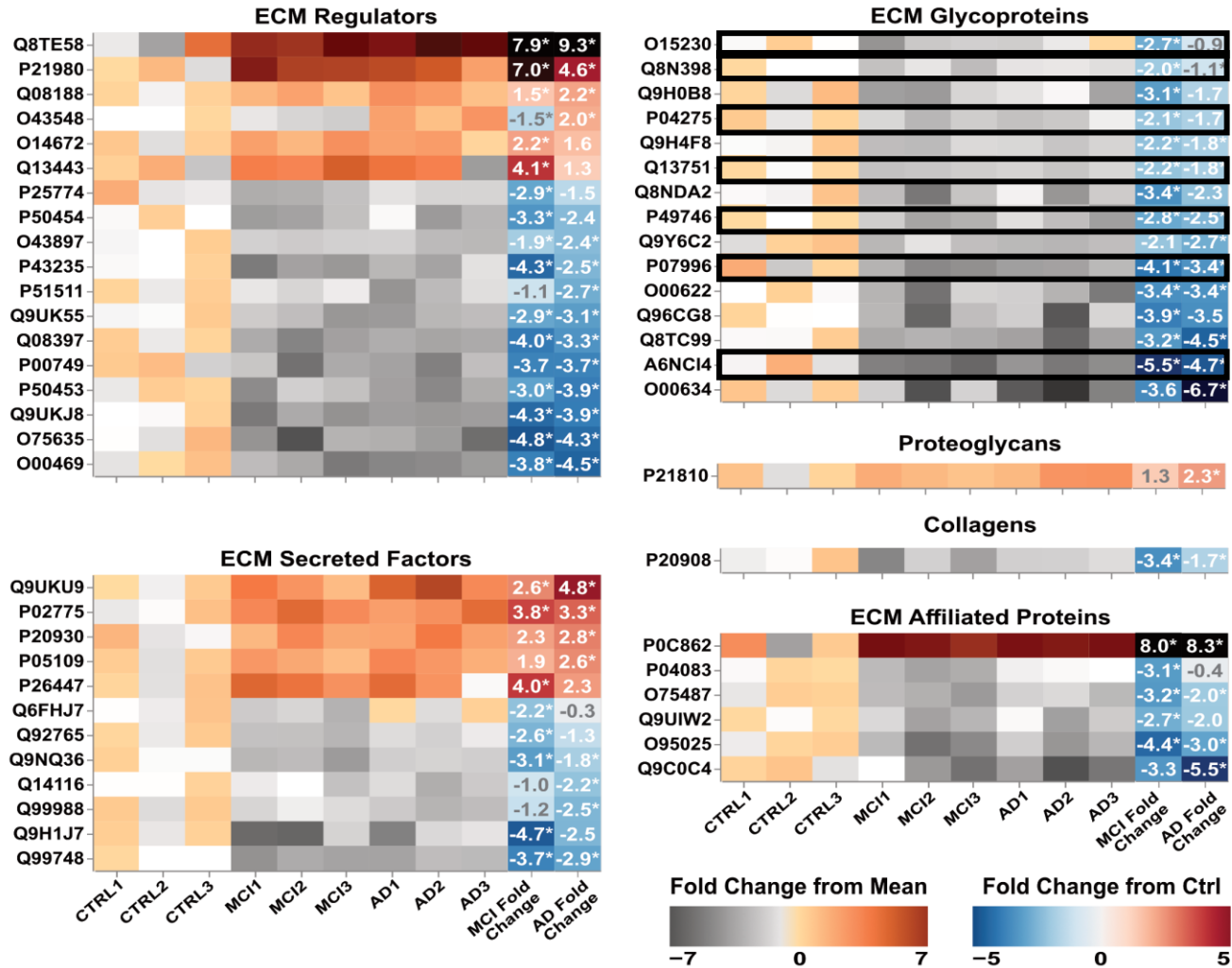


Dysregulated Proteins

- Disintegrin and metalloproteinases
- Gamma glutamyltransferases
- Serpins
- S100 Proteins
- Wnt Pathway Proteins
- Neurturin



ECM Reorganization

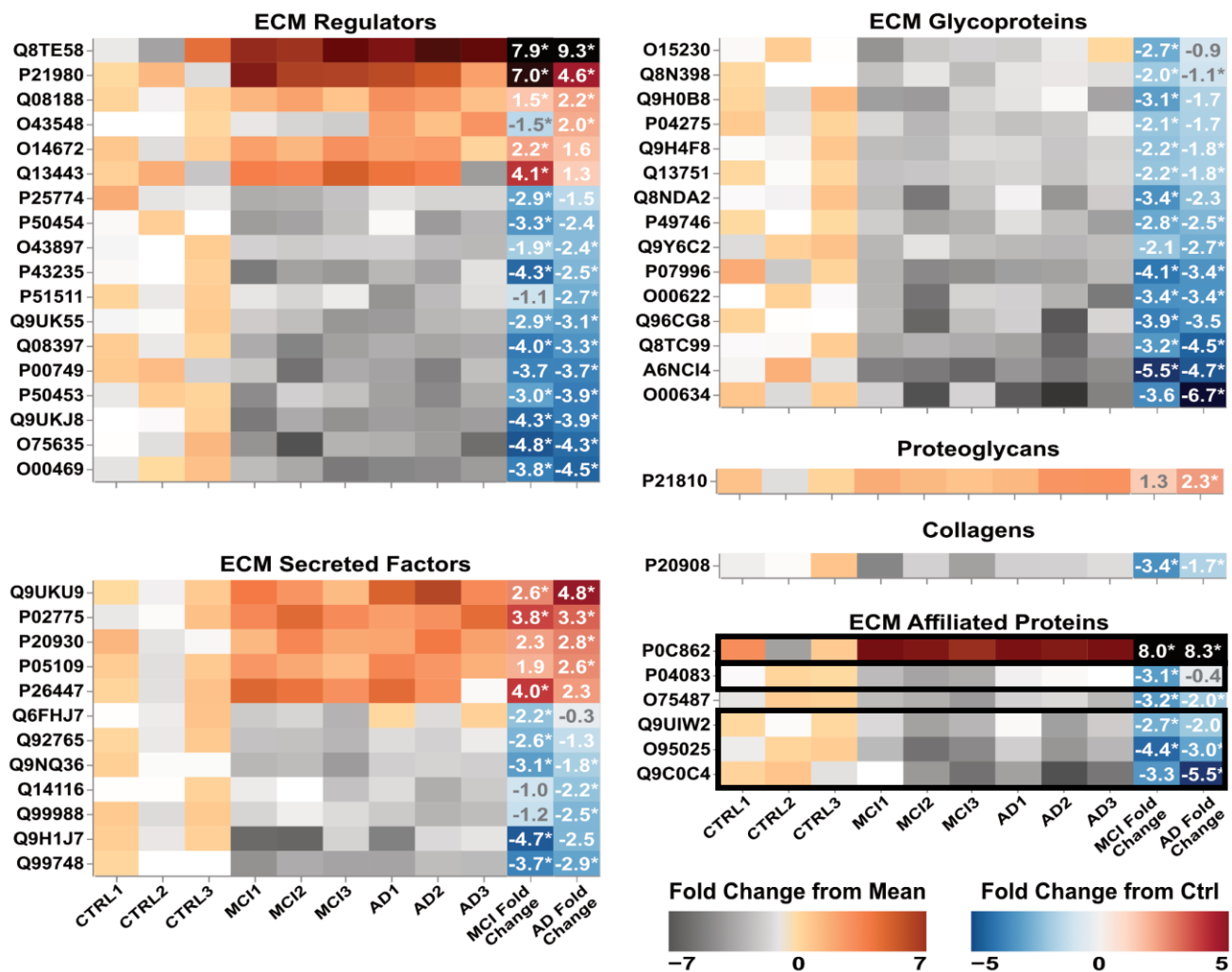


Dysregulated Proteins

- Disintegrin and metalloproteinases
- Gamma glutamyltransferases
- Serpins
- S100 Proteins
- Wnt Pathway Proteins
- Neurturin
- Von Willebrand Factors
- Laminin Subunits
- Thrombospondins



ECM Reorganization



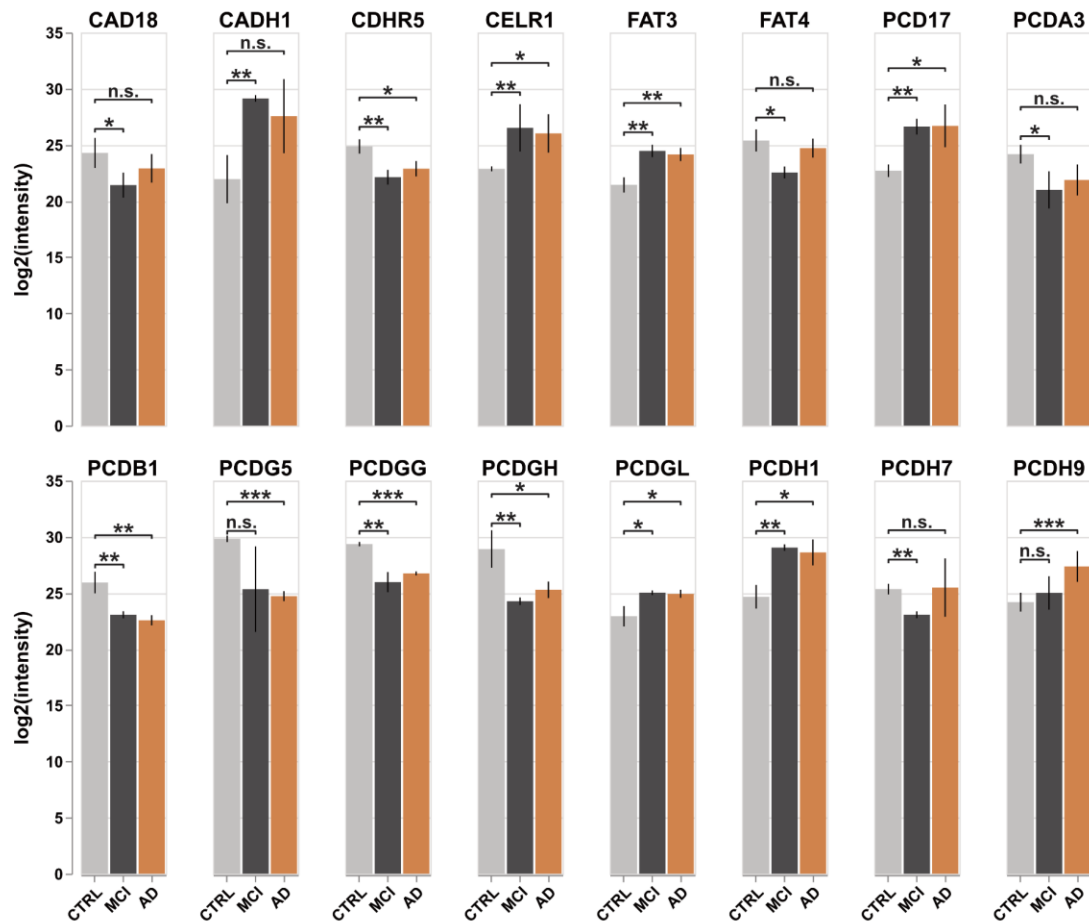
Dysregulated Proteins

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



Protein Class Dysregulation

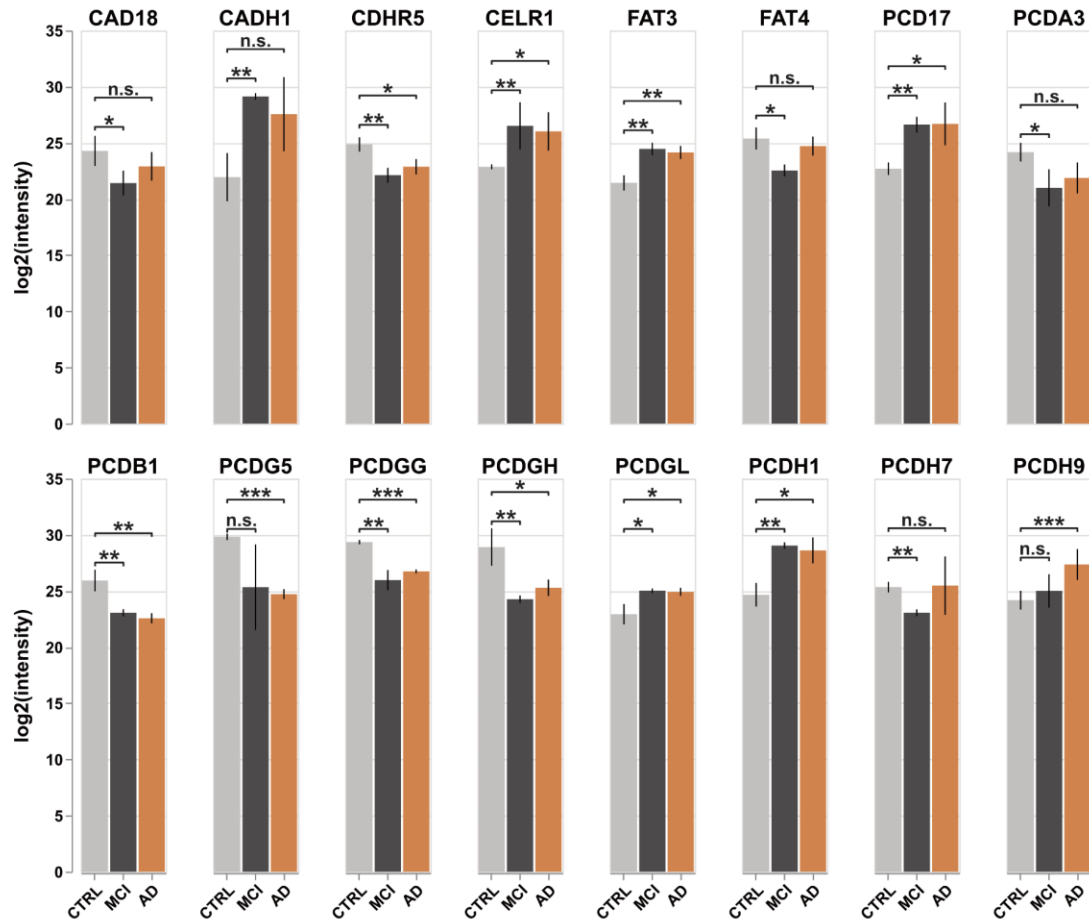
Cadherins & Protocadherins



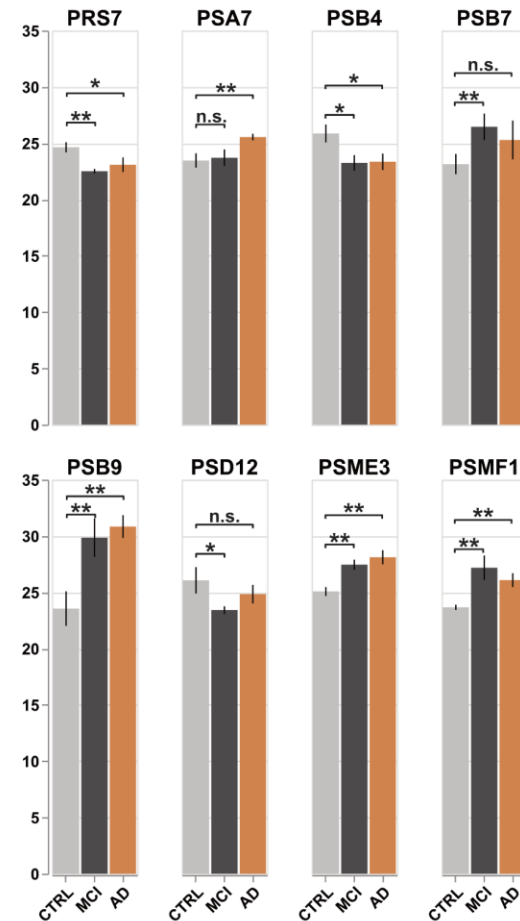


Protein Class Dysregulation

Cadherins & Protocadherins



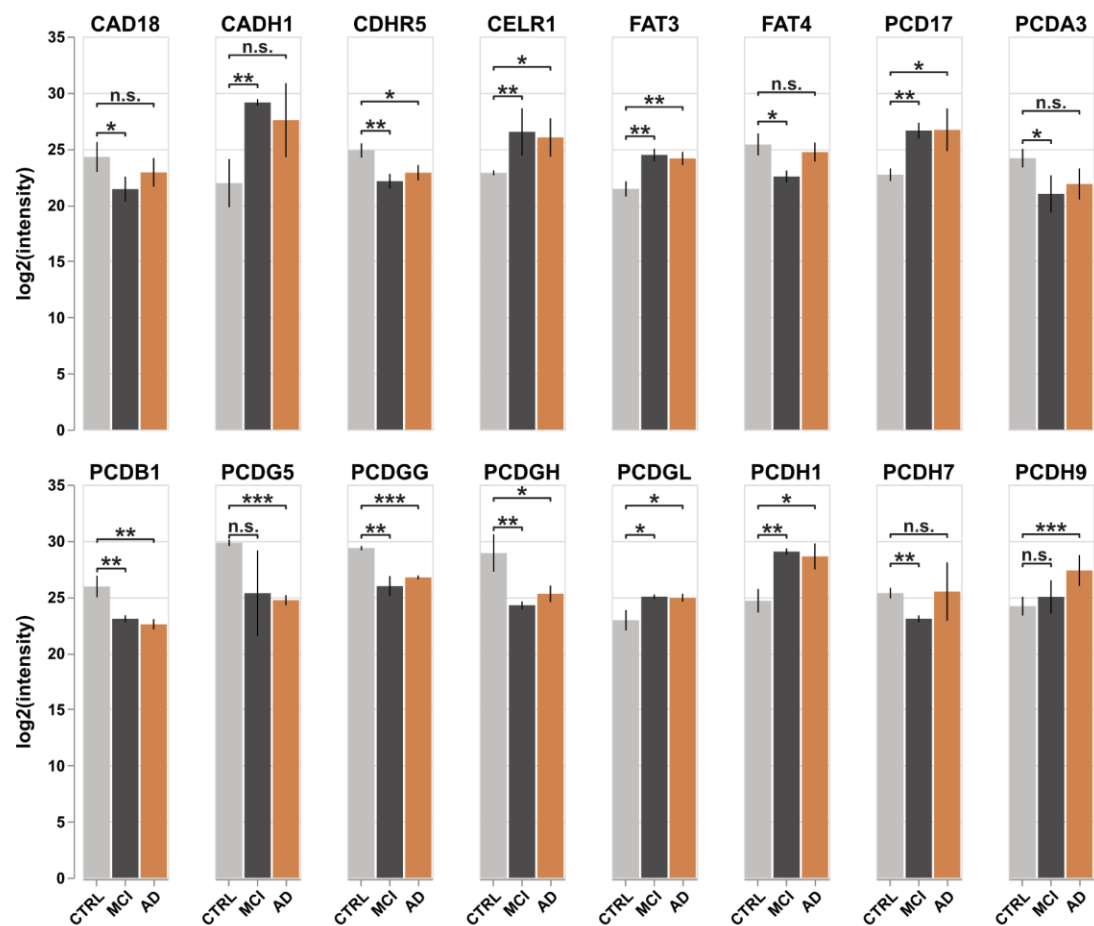
Proteasome



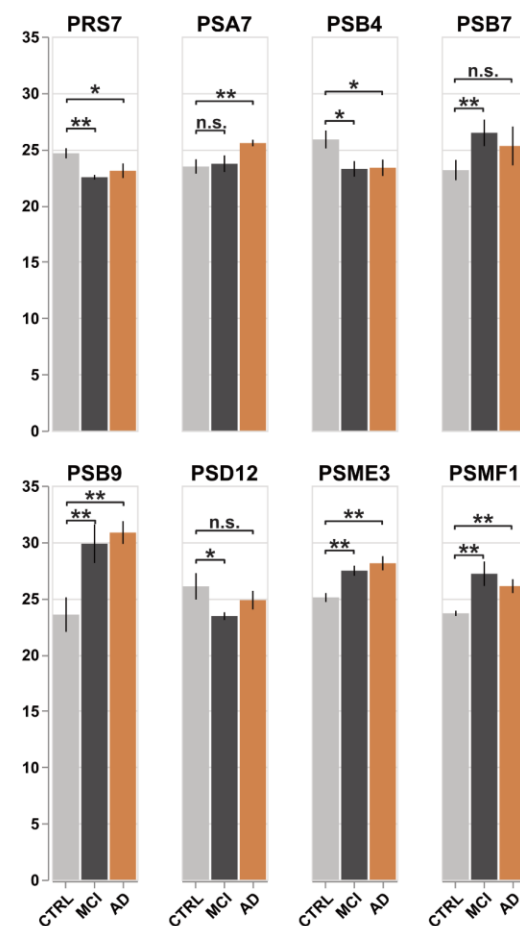


Protein Class Dysregulation

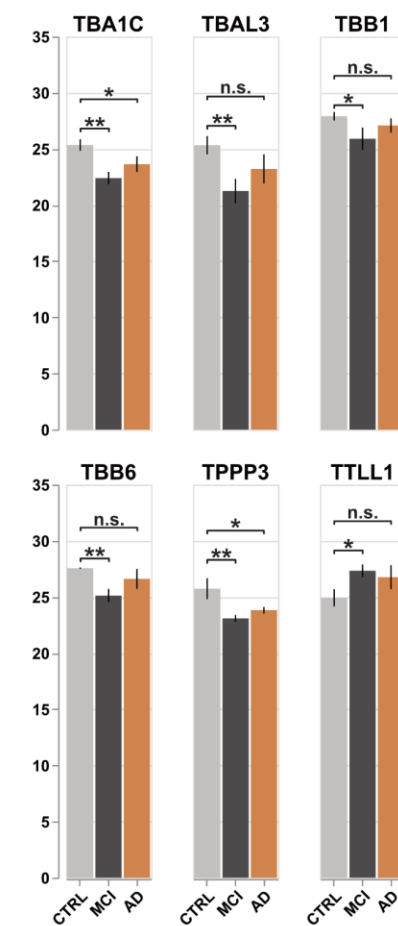
Cadherins & Protocadherins



Proteasome

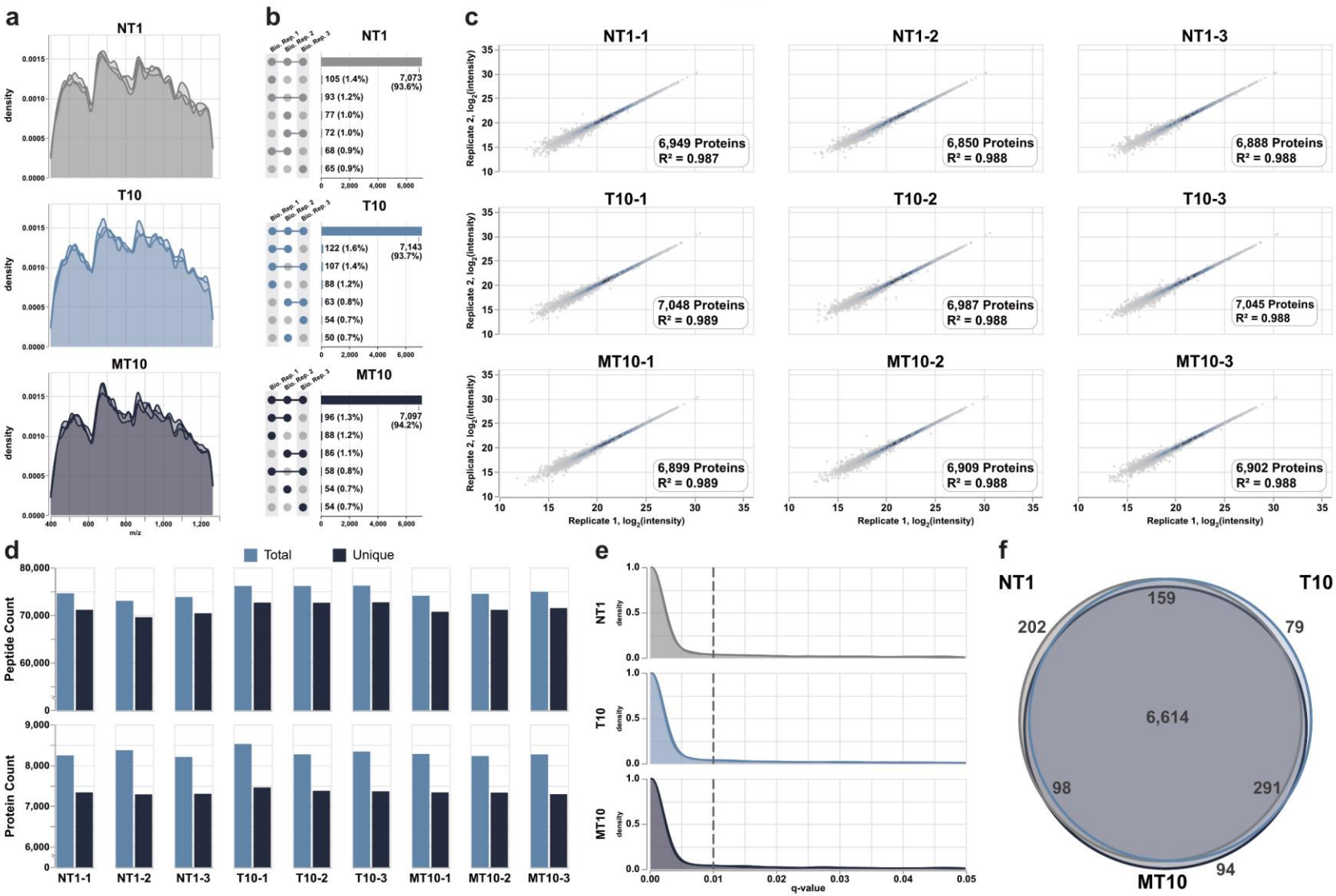


Tubulins



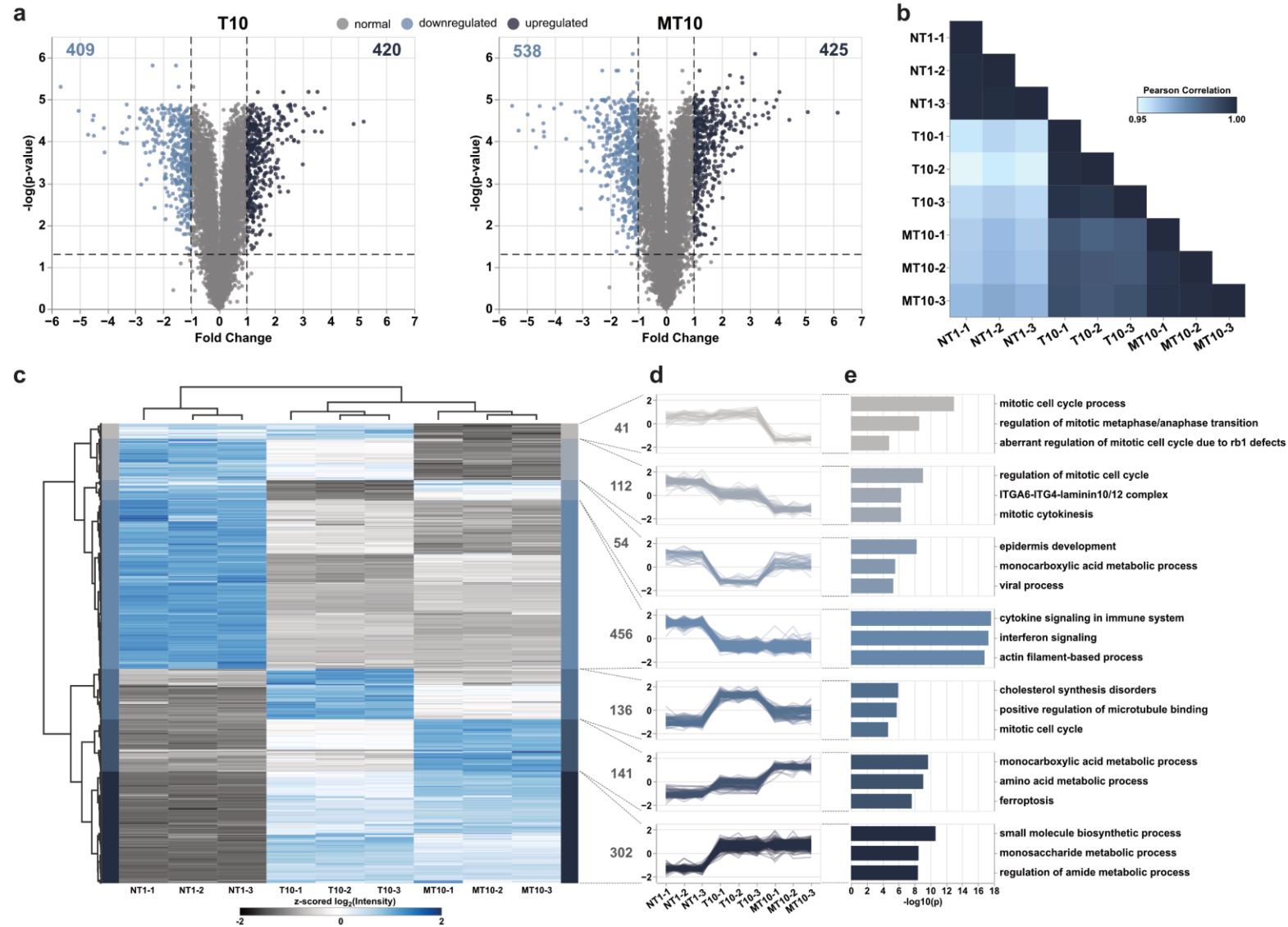


DIA Provides Extensive Profiling Access

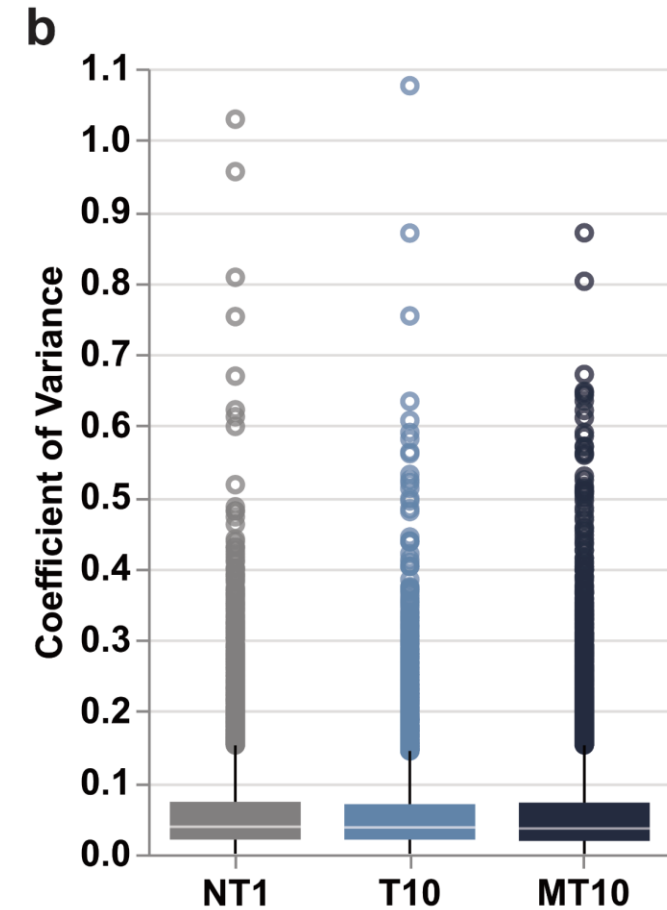
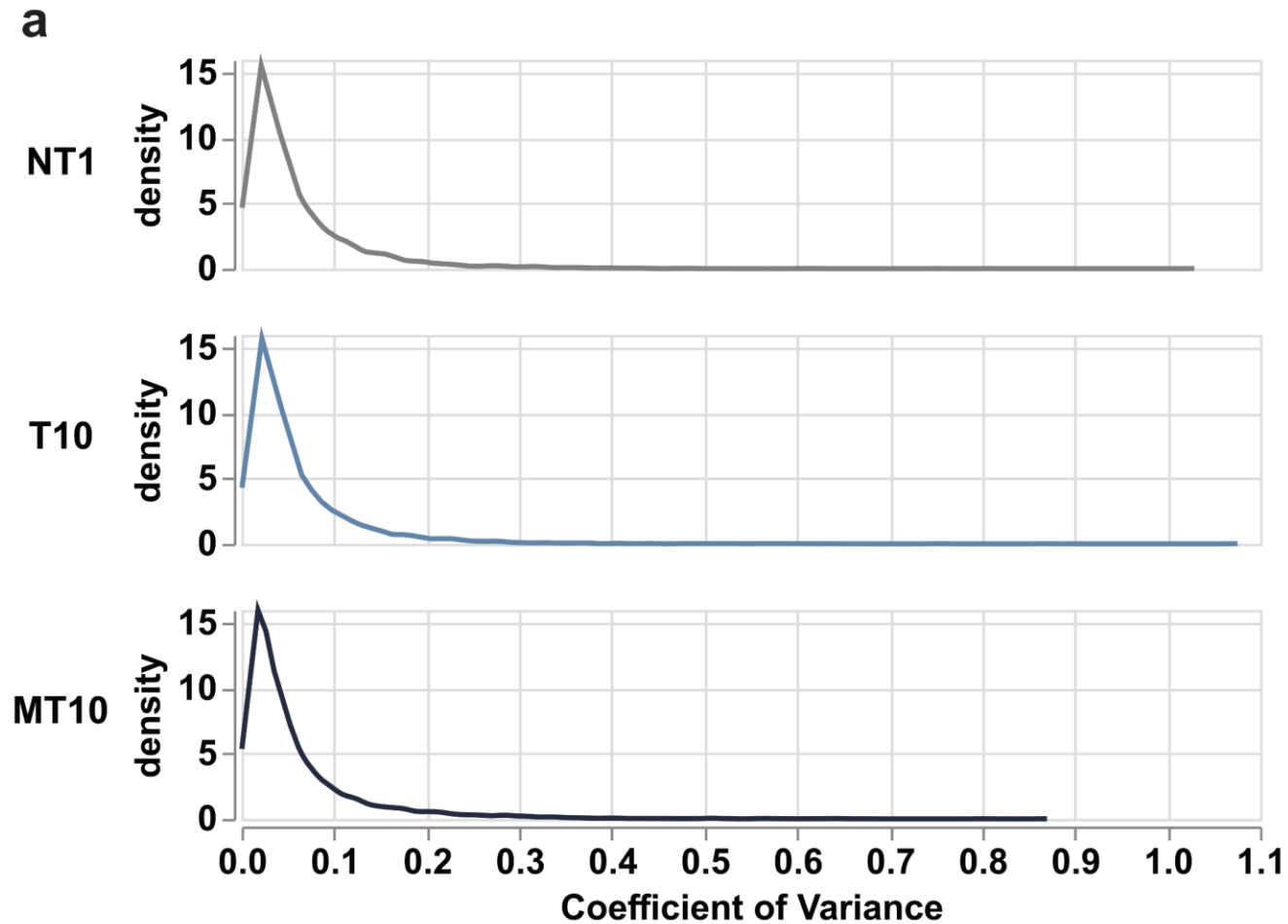




DIA Provides Extensive Profiling Access

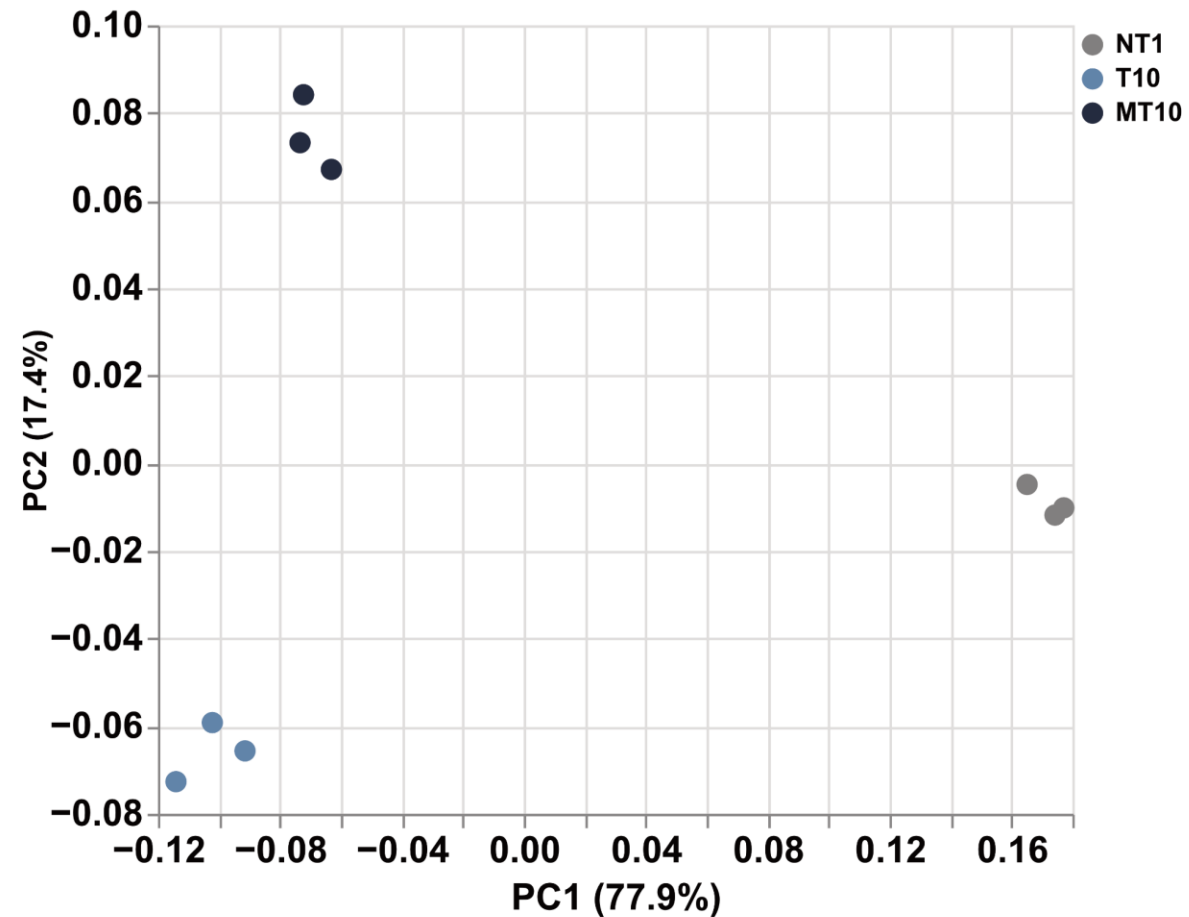
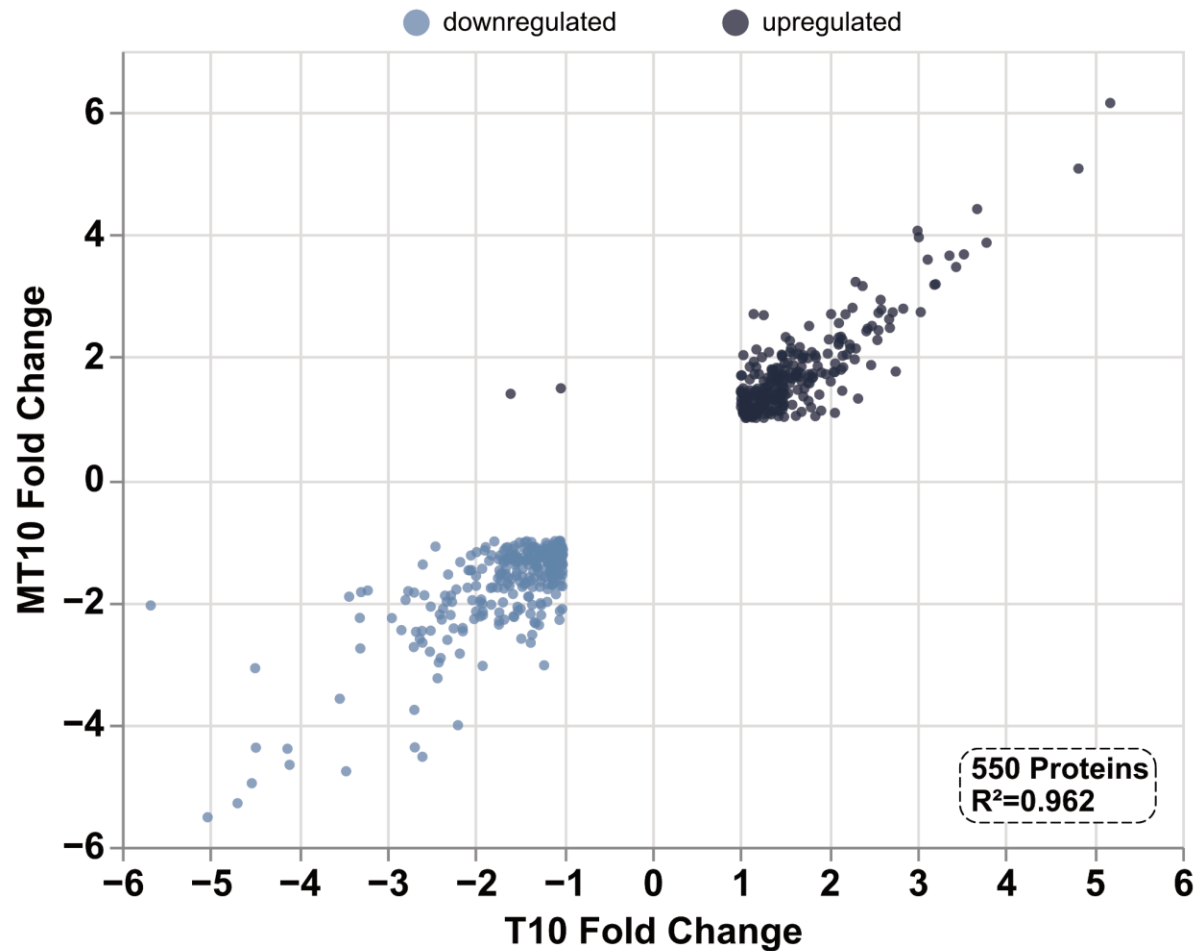


Variance in BCaP DIA Measurements

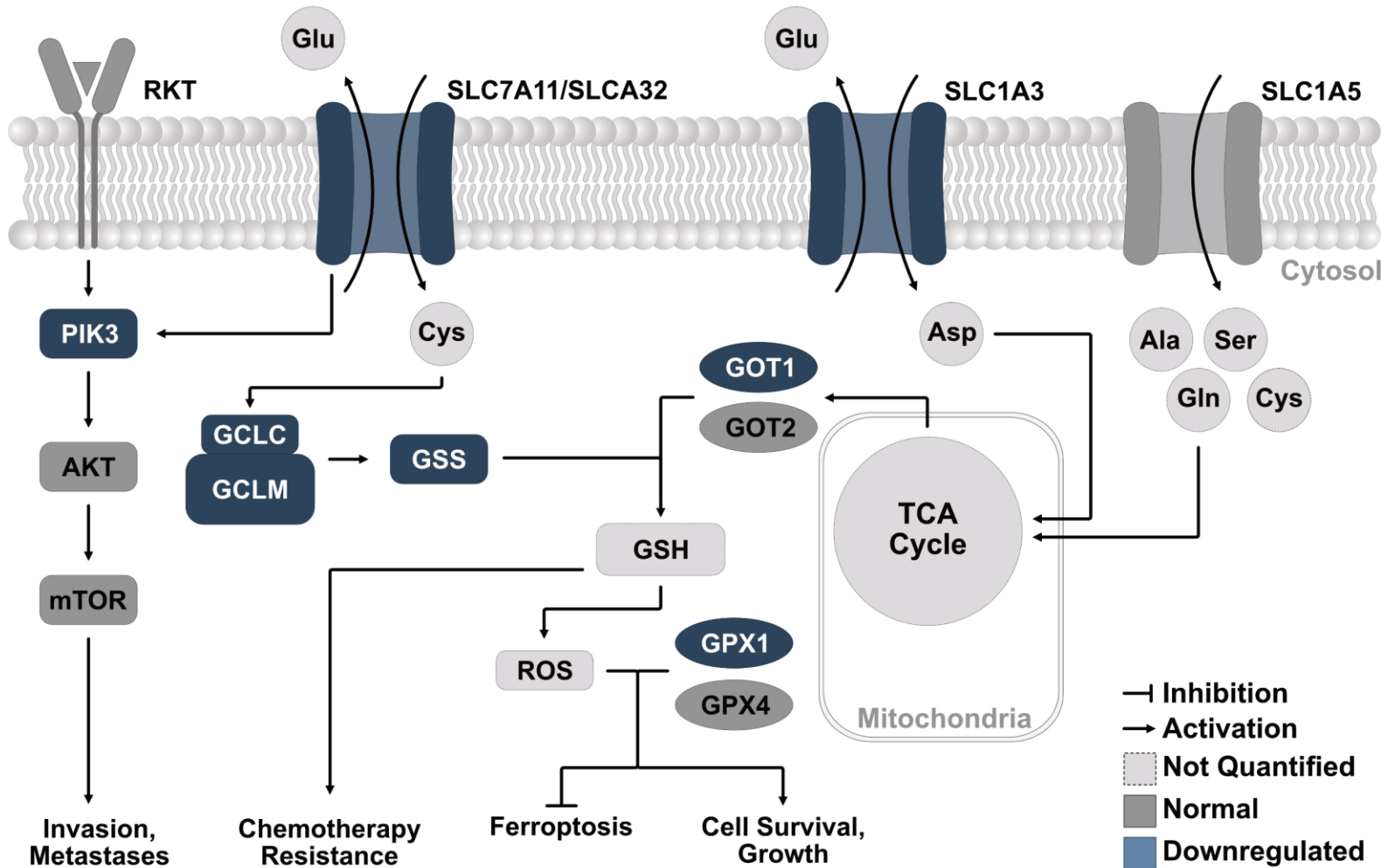




Correlation and Dimensional Reduction



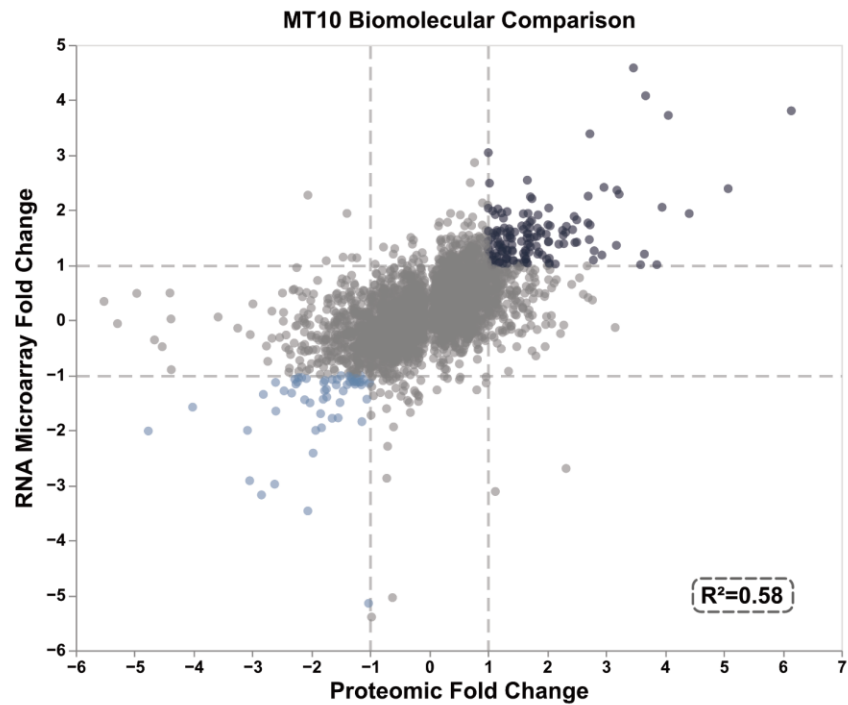
GSH Overproduction in PCa



- 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*

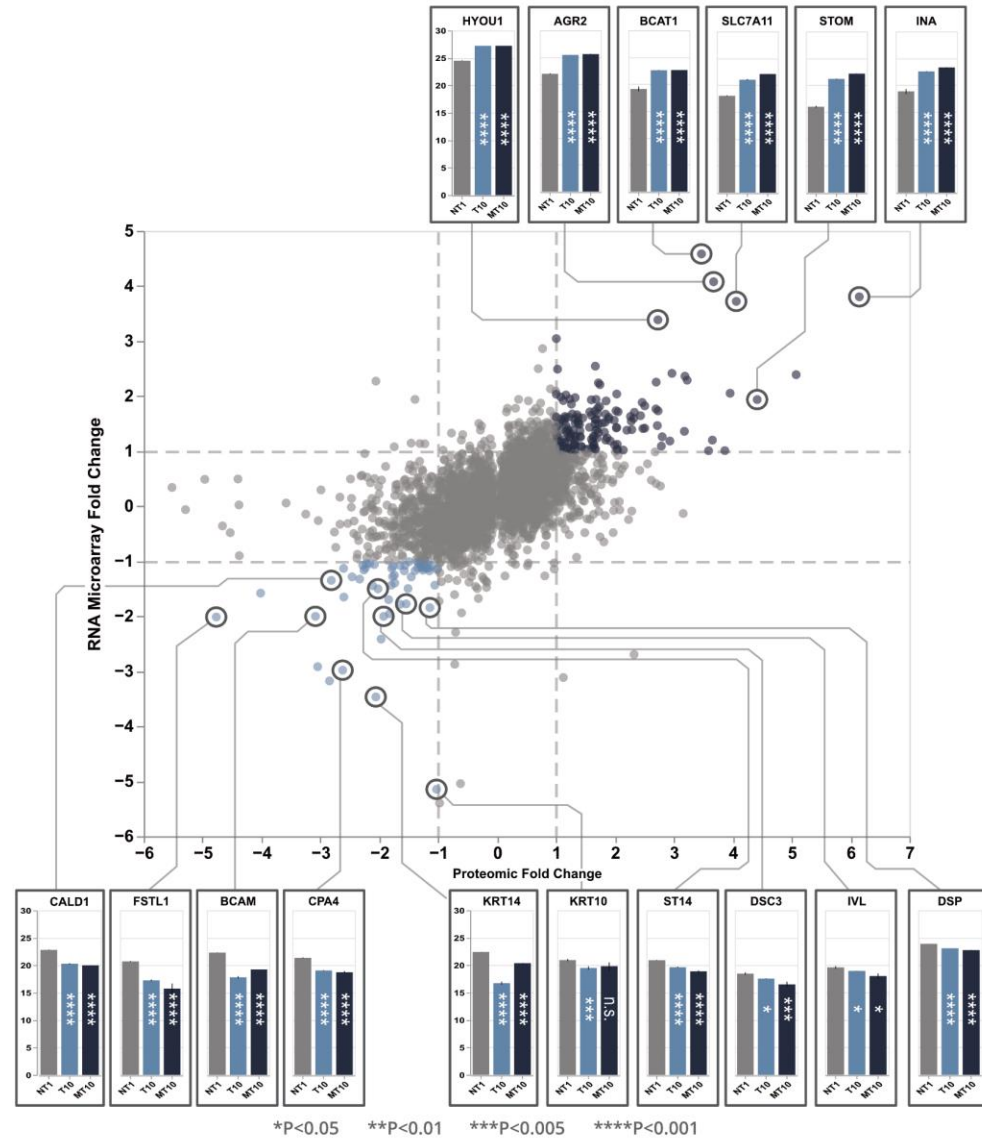


Illuminating Putative Biomarkers



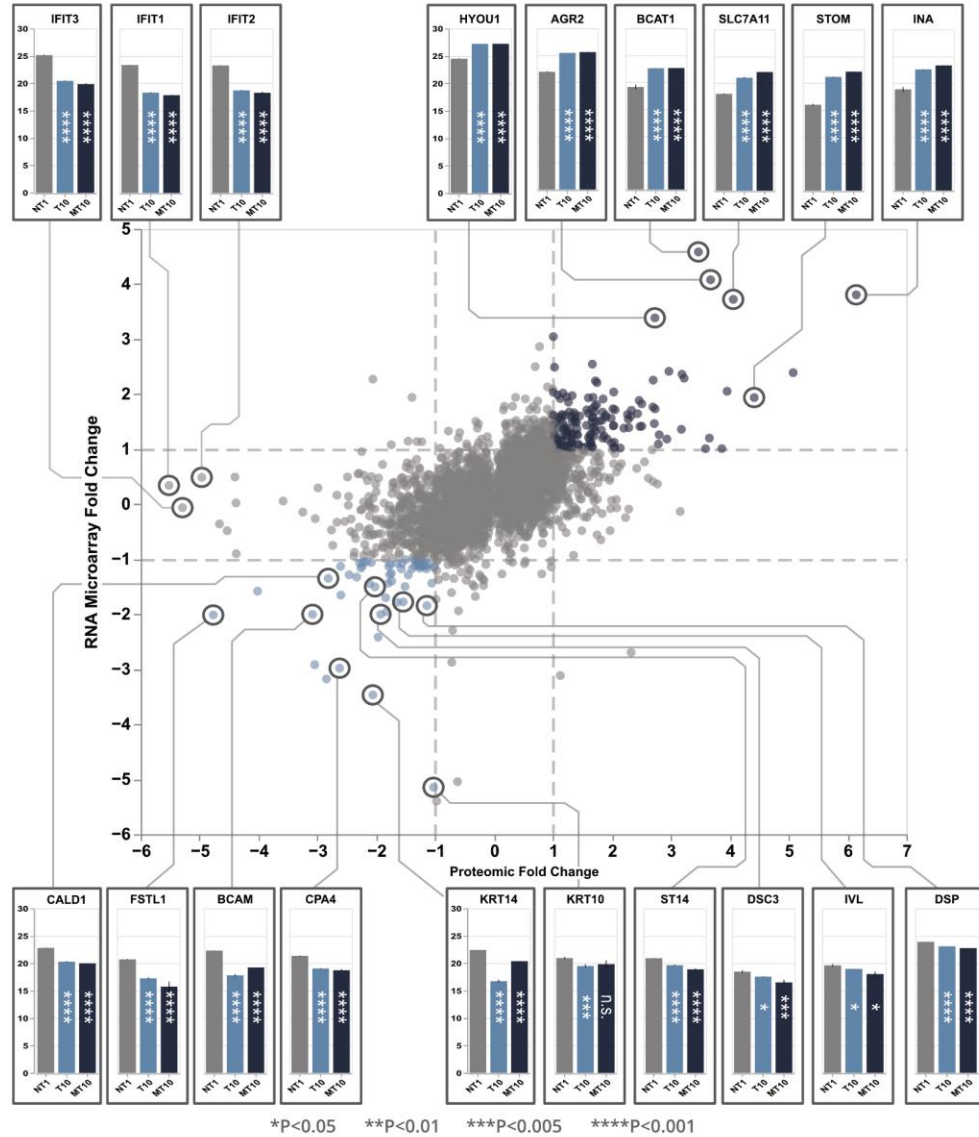


Illuminating Putative Biomarkers





Illuminating Putative Biomarkers





Illuminating Putative Biomarkers

