POSTER SESSION

POSTER CONTEST FINALISTS

(1) Site-Specific Reactivity of Nonenzymatic Lysine Acetylation

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Protein acetylation of lysine ε-amino groups is abundant in cells, particularly within mitochondria. The contribution of enzyme-catalyzed and nonenzymatic acetylation in mitochondria remains unresolved. Here, we utilize a newly developed approach to measure site-specific, nonenzymatic acetylation rates for 90 sites in eight native purified proteins. Lysine reactivity (as second-order rate constants) with acetyl-phosphate and acetyl-CoA ranged over 3 orders of magnitude, and higher chemical reactivity tracked with likelihood of dynamic modification *in vivo*, providing evidence that enzyme-catalyzed acylation might not be necessary to explain the prevalence of acetylation in mitochondria. Structural analysis revealed that many highly reactive sites exist within clusters of basic residues, whereas lysines that show low reactivity are engaged in strong attractive electrostatic interactions with acidic residues. Lysine clusters are predicted to be high-affinity substrates of mitochondrial deacetylase SIRT3 both *in vitro* and *in vivo*. Our analysis describing rate determination of lysine acetylation is directly applicable to investigate targeted and proteome-wide acetylation, whether or not the reaction is enzyme catalyzed.

(2) MASH Suite Pro: a Comprehensive Tool for Top-down Proteomics

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Top-down mass spectrometry (MS)-based proteomics is arguably a disruptive technology for the comprehensive analysis of all proteoforms arising from post-translational modifications (PTMs) and genetic variations. However, the complexity of top-down high-resolution mass spectra presents significant challenges for data analysis, hindering identification and characterization of the PTMs, and quantification of the proteoforms with various PTMs. In contrast to the well-developed software packages for data analysis in bottom-up proteomics, the data analysis tools in top-down proteomics remain under-developed. Moreover, despite recent efforts to develop algorithms and tools for top-down high-resolution mass spectral processing and analysis, a multifunctional software platform, which allows for the identification and characterization of PTMs, especially unexpected PTMs, and quantitation of the proteoforms, is still lacking. Herein, we have developed MASH Suite Pro, a comprehensive software tool with multifaceted functionality for comprehensive analysis of protein PTMs. MASH Suite Pro is capable of processing high-resolution MS and MS/MS data using multiple deconvolution algorithms. In addition, MASH Suite Pro allows for the identification and characterization of proteins with expected and unexpected PTMs and sequence variations, as well as the relative quantitation of different proteoforms in different experimental conditions. The program also provides various visualization components for validation of computational output. Furthermore, MASH Suite Pro facilitates data reporting and presentation via direct output of the graphics. Thus, MASH Suite Pro significantly simplifies and accelerates the processing and analysis of top-down proteomics data for the identification and characterization of protein PTMs. We envision that MASH Suite Pro will be able to greatly advance the field of PTM analysis in top-down proteomics.

(3) Top-down Quantitative Proteomics Reveals Novel Mechanistic Insights in Acute Myocardial Infarction

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Heart failure (HF) afflicts approximately 5 million Americans and is frequently precipitated by myocardial infarction (MI); however, the mechanisms underlying impaired cardiac function early after acute MI (AMI) remain unclear. Sarcomeric proteins, which include myofilament and Z-disc proteins, mediate cardiac contraction and play a central role in cardiac signal reception and transduction. Myofilaments are the target of numerous intracellular signals whereas the Z-disc is becoming increasingly recognized as a nodal point in cardiac signaling. Herein, we aim to gain a thorough understanding of alterations in sarcomeric protein phosphorylation and signaling pathways in the early stage after AMI towards the identification of novel therapeutic targets to prevent the onset of HF.

We recently uncovered a significant concerted reduction in the phosphorylation of cardiac troponin I (cTnI) and myosin light chain 2 (MLC2), two important myofilament proteins, as well as enigma homolog isoform 2 (ENH2), a Z-disc protein not previously known to be phosphorylated, in the myocardium of AMI swine using top-down proteomics. These findings implicate, for the first time, Z-disc protein phosphorylation in contractile dysfunction after MI. Tandem MS analysis localized the sites of decreasing phosphorylation in cTnI, MLC2, and ENH2 to Ser22/Ser23, Ser14, and Ser118, respectively. The phosphorylation of cTnI-Ser22/Ser23 is primarily mediated by PKA downstream of β-adrenergic receptors in the heart; however, preliminary signaling pathway analysis showed that the expression of PKA was unchanged following AMI. Interestingly, we found that there is a significant reduction in the levels of cAMP, the second messenger responsible for PKA activation, suggesting that PKA activity may be impaired post-AMI. Additionally, we identified a significant increase in the expression of PP2A, a key phosphatase in the heart, which may contribute to the observed reduction in sarcomeric protein phosphorylation following AMI. We have also begun analyzing tissue bordering the infarct [the infarct border zone (BZ)] as well as tissue distal to the infarct [the remote zone (RZ)] and preliminary data shows that cTnI phosphorylation is also decreased in the BZ and, to a lesser extent, in the RZ; a finding that may have implications for limiting infarct expansion and salvaging functional myocardium after MI.

(4) Identification of O-GlcNAc Modification Targets in Mouse Retinal Pericytes: Implication of p53 in Pathogenesis of Diabetic Retinopathy

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Diabetic retinopathy (DR) is a major cause of blindness in middle age people, which is closely linked to the chronic exposure to high levels of glucose in diabetes. Hyperglycemic conditions have detrimental effects on many tissues and cell types, especially the retinal vascular cells including early loss of pericytes (PC). However, the mechanisms behind this selective sensitivity of retinal PC to hyperglycemia are undefined. Hyperglycemia activates several metabolic pathways, including the Hexosamine Biosynthetic Pathway (HBP). Uridine diphosphate N-acetylglucosamine (GlcNAc) is the product of the HBP and the substrate for O-linked β-N-acetylglucosamine (O-GlcNAc) modification. This modification affects a wide range of proteins by altering their stability, activity and/or protein interactions. However, its contribution to the development and progression of diabetic retinopathy remains unexplored. We have recently demonstrated that the level of O-GlcNAc modification in response to high glucose is variable in various retinal vascular cells. Retinal PC responded with the highest increase in O-GlcNAc modification compared to retinal endothelial cells and astrocytes. Here we show that these differences translated into functional changes, with an increase in apoptosis and decrease in migration of retinal PC, not just under high glucose but also under treatment with O-GlcNAc modification inducers, PUGNAc and Thiamet-G. To gain insight into the molecular mechanisms involved, we have used click-It chemistry and LC-MS analysis and identified 431 target proteins of O-GlcNAc modification in retinal PC using an alkynyl-modified GlcNAc analog (GlcNAlk). Among

the O-GlcNAc target proteins identified here 115 of them were not previously reported to be target of O-GlcNAc modification. We have identified at least 34 of these proteins with important roles in various aspects of cell death processes. Our results indicated that increased O-GlcNAc modification of p53 was associated with an increase in its protein levels in retinal PC. Together our results suggest that post-translational O-GlcNAc modification of p53 and its increased levels may contribute to selective early loss of PC during diabetes. Thus, modulation of O-GlcNAc modification may provide a novel treatment strategy to prevent the initiation and progression of DR.

(5) Combining DiLeu Isobaric Labeling and Label-free Approaches for Metabolite Quantification and Biomarker Discovery of Lower Urinary Tract Symptoms

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Lower urinary tract symptoms (LUTS) commonly afflict aging males, including a range of urinary irritative and obstructive symptoms. Current treatment is mostly based on patient-reported symptoms, and understanding the molecular mechanisms of LUTS is challenging because of the lack of adequate biomarkers. This study aims to develop mass spectrometry-based methods for metabolite quantification and candidate metabolite biomarker discovery of LUTS in human urine. Machine learning algorithms were applied to feature selection and classification, resulting in a sensitive and specific predicative model to classify patients into diseased or non-diseased categories. Forty six urine samples were collected from LUTS patients and controls. Urinary metabolite and protein fractions were separated and investigated through different workflows. An accurate relative quantification method for amine-containing metabolites was established using our lab's custom synthesized 4-plex DiLeu isobaric labeling reagents.1 DiLeu labeling improved the throughput of quantitative analysis, the electrospray ionization efficiency, and the detection sensitivities of metabolites in both CE-ESI-MS/MS and LC-ESI-MS/MS platforms. A comprehensive workflow for metabolite biomarker discovery of LUTS was developed. It integrated urine sample preparation, instrumental analysis, data processing, biomarker selection and identification, classification model construction, pathway analysis, and biomarker validation. Combining untargeted label-free and DiLeu labeling quantification methods allowed the identification and validation of candidate metabolic biomarkers of LUTS in human urine. The panel of candidate biomarkers and the established classification model have great potential to be used for targeted mechanistic studies, therapeutic assessment, and objective and non-invasive diagnosis for LUTS in the future.

Reference: 1. Hao,L. et al. Analyst. 2015, 140: 467-475.

(6) Functionalized Magnetic Nanoparticles with Multivalent Ligands for Top-down Phosphoproteomics

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Reversible protein phosphorylation plays a pivotal role in the control of many important biological processes, such as cell growth, division, and the dysregulation of the phosphorylation-mediated signaling pathways has been linked to many human diseases. Therefore, the ability to effectively capture, separate, and comprehensive analyze phosphoproteins from complex biological samples is crucial but remains a challenge due to the low abundance of phosphoproteins and the low stoichiometry of phosphorylation. To address the challenge, we have developed nanoparticle reagents to capture phosphoproteins globally out of complex mixtures followed by top-down proteomics analysis of the captured whole phosphoproteins.

We synthesized superparamagnetic Fe_3O_4 nanoparticles (NPs) functionalized with dinuclear Zn (II)-dipicolylamine (Zn-DPA) ligands that are linked via glutaric acid (hereafter referred to as GAPT). The enrichment performance of the Fe_3O_4-GAPT-Zn NPs was tested using a simple protein mixture and then cell and tissue lysates, two highly complex protein mixtures. The SDS-PAGE analysis with Pro-Q diamond and SyproRuby staining clearly indicated

that the Fe_3O_4-GAPT-Zn NPs can enrich phosphoproteins from complex biological samples with high affinity and efficiency. We also have examined the intact proteins present in the complex swine heart tissue extracts before and after enrichment by LC-MS. Remarkably, the top-down MS data clearly showed that phosphoproteins in swine heart tissue extracts were enriched by the NPs, even in the presence of highly abundant blood proteins.

(7) Quantification of Histone PTMs in SAHA-treated MCF-7 Breast Cancer Cells Using Data-Independent Acquisition Mass Spectrometry

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Histone post-translational modifications (PTMs) are important regulators of chromatin structure, gene expression, and genome maintenance. Quantitative analysis of histone PTMs by mass spectrometry remains challenging due to the complex and combinatorial nature of histone PTMs. The most commonly used mass spectrometry-based method for high-throughput histone PTM analysis is data-dependent acquisition (DDA). However, stochastic precursor selection and dependence on MS1 ions for quantification impede comprehensive interrogation of histone PTM states using DDA methods. To overcome these limitations, we utilized a data-independent acquisition (DIA) workflow that provides superior run-to-run consistency and post acquisition flexibility in comparison to DDA methods. In addition, we developed a novel DIA-based methodology to quantify isobaric, co-eluting histone peptides that lack unique MS2 transitions. Our method enabled deconvolution and quantification of histone PTMs that are otherwise refractory to quantitation, including the heavily acetylated tail of histone H4. Using this workflow, we investigated the effects of the pharmaceutical histone deacetylase inhibitor SAHA (suberoylanilide hydroxamic acid, Vorinostat) on the global histone PTM state of human breast cancer MCF-7 cells. A total of 62 unique histone PTMs were quantified, revealing novel SAHA-induced changes in acetylation and methylation of histones H2A, H3, and H4. This unique workflow has yielded valuable mechanistic insight into the effects of SAHA on histone modifications.

(8) New Electron Transfer Dissociation Technologies for Characterizing Post-Translational Modifications

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Electron transfer dissociation (ETD) is an enabling technology for mass spectrometry-based protein sequence analysis and characterization of post-translational modifications (PTMs). ETD promotes extensive cleavage of peptide backbone bonds while preserving labile PTMs on the residues they modify, facilitating site-specific determination of a vast array of modifications (*e.g.,* phosphorylation and glycosylation). Even with the advances ETD has afforded in the past decade, however, the full potential of ETD technologies remains untapped. We have explored two avenues to further the impact ETD can provide for proteome and PTM analyses: 1) improve ETD fragmentation of intact proteins for top down approaches and 2) implement a robust platform for characterization of the acidic proteome and acidic PTMs that are otherwise intractable with current methods. To achieve both of these goals, we have focused on improving the dissociation efficiencies of ETD reactions for both cationic and anionic precursors using concurrent infrared (IR) photoactivation, termed activated ion ETD (AI-ETD). The dependence of ETD reactions on precursor charge density limits its effectiveness; as charge density decreases, non-dissociative electron transfer (ETnoD) becomes more likely due to non-covalent gas-phase interactions, reducing the production of sequence-informative product ions. AI-ETD leverages additional energetic activation from IR photons (10.6 μm) concomitant with the ETD reaction to disrupt secondary gas-phase structure, significantly improving precursor-to-product ion conversion. Here we show that the activated ion approach, *i.e.*, concurrent IR photoactivation, enhances the efficacy of both ETD reactions on precursor cations and also negative ETD (NETD) reactions on precursor anions. AI-ETD and AI-NETD enable more robust PTM analyses, especially for

phosphorylation, sulfation, and glycosylation, and here we highlight how this technology is providing insight into the mechanisms of virulence in *Acinetobacter* species, opportunistic pathogens that are one of the leading causes of nosocomial (hospital-acquired) infections worldwide.

(9) Cardiomyocyte Cell Surface Proteins – Applications & Emerging Technologies

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Cell surface proteins are transmembrane, GPI-anchored, and extracellular matrix proteins that include receptors, enzymes, and transporters. Proteins at the cell surface participate in inter- and intracellular communication, cellular structure, and adhesion. More than 60% of drug therapies target membrane proteins and 38% of disease related proteins are membrane associated. Cell surface proteins can be exploited as accessible markers of live cells for targeted drug delivery and cell sorting without requiring genetic modifications. Despite their critical importance to biology and disease, cell surface proteins have been historically understudied because of the limited availability of reliable antibodies suitable for quantitation and the inability of transcriptomic approaches to inform localization. However, recent powerful discovery-driven mass spectrometry approaches are beginning to address these issues. In this study, we have applied an innovative chemoproteomic strategy termed Cell Surface Capture (CSC) to generate unique views of pluripotent stem cells and their cardiac derivatives for the purpose of identifying new cell surface proteins for basic science and clinical applications. Results to date include: (1) quantitative profiling of 6 proteins over 100 days of differentiation, establishing that cell surface proteins are sufficiently dynamic to support long-term efforts of immunophenotyping maturation stage specific cardiomyocytes; (2) identification of a novel cell surface marker for cardiomyocytes, including new monoclonal antibodies against this protein; (3) evidence of our novel cardiomyocyte marker in human tissue and diseased patient serum. Ongoing efforts are focused on employing automated sample processing and new bioconjugation chemistries to enable quantitative discovery of cell surface proteins, discrimination between proteoforms, and analyses on small numbers (1E6) of human primary cells. These improvements will be harnessed to establish a streamlined process facilitating translation of CSC discovery efforts to serum biomarker studies.

(10) A Novel Three Dimensional Liquid Chromatography Platform for Top-down Proteomics

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Top-down mass spectrometry (MS)-based proteomics has unique advantages for the analysis of proteoforms including post-translational modifications, and has great potential for the elucidation of disease mechanisms and clinical diagnostics. Despite recent advancements in protein separation, the extreme complexity of the proteome remains challenging; necessitating multiple separation strategies prior to MS. However, few multi-dimensional liquid chromatography (MDLC) approaches have been developed, hindering protein identification; hence, novel MDLC approaches are desired to tackle this problem. Recently, we identified a new MS-compatible salt for hydrophobic interaction chromatography (HIC). In this work, we have coupled HIC with ion exchange chromatography (IEC) and reverse-phase chromatography (RPC) for top-down proteomics and showed that this novel 3DLC approach significantly enhanced protein identification compared to a conventional 2DLC (IEC/RPC) approach.

Initially, we demonstrated the different selectivity between HIC and IEC on two sets of selected standard protein mixtures. Based on our previous investigation as well as literature reports, there exists orthogonality between HIC and RPC as well as IEC and RPC. Hence, this mutual orthogonality among IEC, HIC and RPC promises that their combination is supposed to lead to enhanced separation capability.

Next, we showed that a 3D (IEC/HIC/RPC) approach greatly outperforms the conventional 2D IEC-RPC approach, utilizing crude HEK 293 cell lysate samples. According to the UV chromatograms, HIC provided a fine and further

fractionation after IEC in 3DLC, significantly improving the resolution power of the separation. For the same IEC fraction out of 35 fractions, 640 proteins were identified in the 3D approach (corresponding to 201 non-redundant proteins), as compared to 47 in the 2D approach. We also show that simply prolonging the gradients in RPC in the 2D approach, leading to minimal improvement in protein separation and identifications. Therefore, this novel 3DLC method can effectively separate intact proteins for deep proteome coverage in top-down proteomics.

GENERAL POSTER SESSION

(11) The Epigenetic Modulation of Aberrant Synaptic Plasticity in Tuberous Sclerosis Complex

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Tuberous Sclerosis Complex (TSC) is an autosomal dominant, multi-system spectrum disorder that affects approximately 1 in 6,000 people. The disorder is characterized by the formation of benign growths that most commonly develop in the brain, kidney, heart, lungs, eyes and skin (Curatolo, P., 2002; Crino, P.B. 2006 & 2013). Patients with TSC display developmental delays, cognitive defects, autism and epilepsy. This disease is caused by a loss of function mutation in either the *TSC1* or *TSC2* genes, resulting in the disinhibition of mammalian Target of Rapamycin (mTOR), a key regulator of cell survival and proliferation, protein synthesis and metabolism (van Slegtenhorst, 1997; Tang, S.J., 2002; Hou, L., 2004). Current treatment involves the modulation of hyperactive mTOR activity, but chronic mTOR inhibition may have adverse effects on patient health (Schindler, C.E., 2014; Carracedo, A., 2008; Kusne, Y., 2014). There is a need to find a TSC specific treatment that does not present with harmful side effects.

Acute hippocampal slices from adult male heterozygous TSC2 mutant mice ($TSC2^{+/-}$) display abnormal long term potentiation (LTP) and long term depression (LTD). A 1X theta burst stimulation elicits short term potentiation in adult male wild type (WT) hippocampi, but this paradigm induces LTP in hippocampi obtained from gender and age matched $TSC2^{+/-}$ mice. In contrast to adult male WT mice, the $TSC2^{+/-}$ mice display a rapamycin insensitive form of metabotropic glutamate receptor (mGluR) mediated LTD.

We have shown that adult $TSC2^{+/-}$ mice have increased Erk1/2 signaling that bypasses the canonical mTOR signaling cascade to drive atypical synaptic plasticity (Potter, W., 2013). A whole genome expression analysis of cortical samples resected from human TSC and non-TSC patients suggests that chromatin alterations around key genes encoding components of the Mek/Erk signaling pathway appear to drive the plasticity alteration upon loss of a TSC allele. We find that the modification of chromatin structure through the pharmacological inhibition of histone deacetylation and demethylation attenuates Erk1/2 signaling and restores normal LTP and LTD in adult male $TSC2^{+/-}$ mice. This study is the first to describe epigenetic mechanisms influencing synaptic plasticity alterations in TSC and can unearth a novel therapeutic option for TSC patients.

(12) Online Hydrophobic Interaction Chromatography - Mass Spectrometry

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The recent rapid development in top-down proteomics has led to a high demand for mass spectrometry (MS) friendly chromatographic techniques for separation of intact proteins. Conventional hydrophobic interaction chromatography (HIC) preserves native structure of proteins and separates proteins with high resolution. However, it requires high concentrations of nonvolatile salts, precluding direct analysis of the eluents by a mass spectrometer. Here we have developed a new series of more hydrophobic HIC column materials that can retain proteins using MS-friendly volatile salt, ammonium acetate. Subsequently, we have evaluated and characterized these newly synthesized HIC materials. With the more hydrophobic stationary phases, the function of the salt seems to be to preserve protein structure rather than to promote retention. More importantly, we report here the first successful demonstration of online HIC-MS for top-down proteomics using individual standard proteins and protein mixtures. Thus this new HIC appears to be a promising MS-friendly separation technique that can minimize protein denaturation.

(13) High Resolution and High Mass Accuracy Multiply Charged MALDI Technique for *in situ* Protein Characterization – Sequencing, Identification and Visualization

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MALDI-LTQ-Orbitrap mass spectrometer is a hybrid instrument equipped with linear ion trap and orbitrap as mass analyzers. With mostly singly charged ions generated in the MALDI source, the maximum *m/z* of 4000 limits the analytes to small-medium size molecules. Herein, we present the first study of multiply charged MALDI MS in combination with *in situ* enzymatic digestion on an orbitrap platform using 2-nitrophloroglucinol (2-NPG) as matrix for large molecule analysis.

The conditions for generating multiply charged signals and enzymatic digestion were optimized. Direct tissue analysis revealed a large amount of multiply charged ions along with singly charged neuropeptides. With ultrahigh mass accuracy, several rat brain neuropeptides and proteins were identified by accurate mass matching with sub ppm mass error. We also identified several proteins in the enzymatically digested tissue via peptide mass fingerprint searching. Tandem MS fragmentation analysis was used to sequence a doubly charged ion. Spatial distributions of both singly charged neuropeptides and doubly charged proteins were mapped and imaged.

For the first time, multiply charged MALDI full MS, tandem MS and MSI analyses have been achieved on a commercially available, ultra high performance MALDI-LTQ-Orbitrap instrument. Molecules as large as 14930.36 Da have been detected with up to five positive charges at 100k mass resolution. The finding of this study is crucial for performing high resolution and high mass accuracy protein analysis directly from tissue surface without homogenization and protein extraction.

(14) Evidence for Differential Structural Preferences of the Leu7Pro Mutant Neuropeptide Y Signal Peptide Probed by Ion Mobility-Mass Spectrometry

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Neuropeptide Y (NPY) is an important neuromodulator that regulates food intake, vasoconstriction, hormone release, kidney function, lipolysis and angiogenesis. The T1128C polymorphism in the NPY gene changes results in the seventh amino acid in the signal peptide of NPY from leucine to proline (Leu7Pro). Studies have shown that the mutant signal peptide leads to more efficient production and release of NPY. Using IM-MS assisted by MD, structures of wild-type and mutant signal peptides can be determined from experimentally derived collision cross section (CCS) values, which reflect the conformation adopted by the ions in the gas phase under defined

experimental conditions. Molecular dynamics simulation employs empirical force fields to explore the structure and dynamics of molecular models, generating the theoretical CCS for each conformation. By comparing the theoretical CCS with experimental CCS, conformation dynamics of wild-type and mutant signal peptide can be elucidated. Dissolved in 50% methanol water, the ions of wild-type and mutant signal peptide at charge state 2, 3 and 4 can be detected. At charge state 2, both wild-type and mutant signal peptides have only one conformation, whereas their conformations differ from each other. At charge state 3, wild-type and mutant-type have one common conformation, whereas wild-type has one additional unfolded conformation. At charge state 4, wild-type and mutant signal peptide have two common conformations, whereas the mutant-type has one additional conformation. Upon activation, the compact conformations for charge states +3 and +4 of mutant-type and wild-type ions can be activated to more unfolded conformations, whereas the conformations for charge state +2 of mutant-type and wild-type ions remain unchanged.Each peak in the ion mobility profile for wild-type and mutant signal peptides were assigned different conformation based on the MD simulation results.

(15) Identification of Proteoforms and Proteoform Families Using Measurements of Intact Mass and Lysine Count

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Proteoforms are the different forms of proteins that arise from genetic variations, splice variations, RNA editing, and PTMs. Knowledge of the proteoforms, rather than just the proteins, is essential for thoroughly understanding a biological system. We present a new strategy for proteoform identification that uses only measurements of accurate intact mass and lysine count. This process is enabled by NeuCode SILAC labeling and novel data processing steps, and it does not require any fragmentation spectra.

NeuCode SILAC-labeled yeast was mixed in a 2:1 ratio of the “light” and “heavy” forms. Twelve GELFREE fractions of the lysate were analyzed by LC-MS (Orbitrap, full MS scans, R=100,000). Mass spectra were deconvoluted and deisotoped to yield a list of accurate intact masses. Lysine counts were calculated using the mass difference between co-eluting NeuCode pairs, given the mass shift of 0.036 Da per lysine. Each intact mass and lysine count combination was compared to a list created in silico from the Uniprot database. Exact matches, or matches that differed by the mass of a PTM (or PTM combination), were considered proteoform identifications.

We identified nearly 1500 proteoforms belonging to about 200 proteoform families. We are in the process of validating the proteoform identifications using both bottom-up and top-down proteomics data from the same sample. Future work will extend this proteoform identification strategy to include quantification by using the NeuCode SILAC peaks for both lysine counting and relative abundance measurement.

(16) Integration and Application of Separation Strategies to Multiplex Imaging Mass Spectrometry for Analysis of Complex Biological Samples

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MALDI mass spectrometry has been widely used in bio-omics studies for its higher tolerance to impurities, flexibility for re-analysis and the ability of on-target chemical reactions. Their couplings with separation dimension are usually achieved by offline sample collection followed by mass spectral profiling, which is labor-intensive and loses temporal resolution. To address these limitations, we developed an effective platform to couple separation dimension with imaging mass spectrometry. As two of the most predominant separation techniques LC and CE, we explore their coupling to MALDI-Orbitrap imaging for sensitive and automated analysis of tryptic peptides and extracted neuropeptide mixtures.

LC/CE flow is collected directly on the customized MALDI plate and then covered by matrix with an automated sprayer so that highly homogenous traces can be formed with significantly improved reproducibility. Uniform matrix layers results in greatly enhanced MS signal-to-noise ratios compared with regular LC/CE-MALDI-MS systems. A top 10 data dependant acquisition had been applied on mass spectrometric imaging (MSI) experiment using mouse serum trypsin digest and the data was further processed through database search by PEAKS and

Proteome Discoverer. The LC-MALDI imaging platform yielded 108 proteins matched in total and LC-ESI data showed 141 protein groups identified, indicating similar results in terms of peptide identification and sequence coverage when compared to LC-ESI-Orbitrap. This platform has also been applied to the characterization of neuropeptides extracted from crustacean hemolymph. With the power of multiplexed imaging capability, we set tandem MS experiments in parallel to full MS scan. Thirty-six most abundant neuropeptides belonging to diverse families were manually selected and confirmed by accurate mass matching. Analytes of interest are shown as colored region with ImageQuest software. With 2D and 3D imaging, low-abundance peptides that are suppressed in TIC can be easily extracted and observed, illustrating an efficient MSI platform featuring enhanced throughput and sensitivity.

(17) Characterization of Tropomyosin Proteoforms in Skeletal Muscle by Top-Down Mass Spectrometry

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Introduction

Tropomyosin (Tm) is a coiled-coil protein, which plays an essential role in regulating muscle contraction. Skeletal muscle Tm presents a high degree of complexity with a number of proteoforms arising from multiple encoding genes, alternative splicing, and post-translational modifications (PTMs). However, a comprehensive characterization of skeletal muscle Tm proteoforms is still lacking. Herein, we have employed top-down mass spectrometry (MS) to decipher all Tm proteoforms present in various types of skeletal muscles in multiple species to have a better understanding of their distinct functions.

Methods

Tm was extracted from skeletal muscle (~8 mg) by homogenizing tissues in 80 µL HEPES and TFA buffers consecutively. Reverse-phase chromatography was utilized to separate myofilament proteins and Tm fractions were collected simultaneously. High-resolution MS analysis of purified Tm was then conducted in a linear ion trap/Fourier transform ion cyclotron resonance (FT-ICR) MS. To collect MS/MS spectrum, the precursor ions were fragmented by electron capture dissociation (ECD) and collisionally activated dissociation (CAD). In-house developed MASH Suite software was used to analyze MS and MS/MS data.

Preliminary Data

High-resolution MS and MS/MS data revealed comprehensive sequence and PTMs of pig Tm isoforms. Two major pig Tm isoforms with molecular weight (MW) 32736.07 (α-Tm) and MW 32877.98 (β-Tm) were observed. Moreover, mono-phosphorylated α-Tm ($_p$α-Tm, MW = 32816.06) was identified with a mass increment of 79.99 Da compared with mass of α-Tm.

MS/MS was performed on α-Tm, $_p$α-Tm and β-Tm, respectively. By analyzing ECD fragments of α-Tm, two amino acid polymorphisms, Arg38→Gln38 and Pro64→Leu64 were identified. Moreover, with the consideration of acetylation on the first amino acid (42.01Da) at the N-terminus, the calculated MW 32736.07 matches exactly with the experimental MW 32735.75. Meanwhile, the ECD data of β-Tm also suggested an acetylation on the first amino acid at the N-terminus. The modified sequences of three different proteoforms matched well with the ECD fragments data.

In summary, comprehensive sequences of major Tm forms (α-Tm, $_p$α-Tm and β-Tm) in pig skeletal muscle were confirmed in this study, which provides a better understanding of functional role of Tm in skeletal muscle cell. Our future work includes top-down MS characterization of Tm proteoforms from various types of rat and human skeletal muscles.

(18) Epigenetic Control of Epileptogenesis

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Epilepsy is the third most prevalent neurological disorder with an incidence of 1 in 26 individuals. While a number of anti-convulsants exist to treat single seizure episodes, no anti-epileptogenic drugs are currently available to stop the progression of the disease. Epileptogenesis, or the process by which a normal human brain is reprogrammed to become epileptic, is associated with numerous pathogical changes, including alterations in synaptic plasticity, cell death, inflammation, and a reduction in seizure threshold. To investigate what transcription factors and nuclear proteins are driving gene expression changes associated with these pathological changes, we have used a novel bioinformatics tool developed by our laboratory to analyze genome expression datasets generated by the Epilepsy Microarray Consortium. These datasets consist of expression profiles of dentate granule cells laser captured after Status Epilepticus in four different rat seizure models: pilocarpine, kainic acid, self-sustained Status Epilepticus and electrical kindling. We have found increased histone methyltransferase function was the principal driver of gene expression changes during epileptogenesis in all epilepsy models. This is significant because histone methyltransferases play an important role during development, where they epigenetically and stably silence genes. Aberrant silencing by these enzymes has also been observed in cancer. In the future, we intend to test the hypothesis enhanced histone methyltransferase activity is responsible for pathogenic alterations observed in epilepsy. We plan to take pharmacological, biochemical, and electrophysiological approaches to discern whether the observed increase in methylation is pro or anti-epileptogenic. We intend to use the convulsant Kainic Acid to induce Status Epilepticus and test whether small molecule methyltransferase inhibitors can alter seizure propensity and aberrant silencing. Our goal for this project is to be one of the first to characterize a role for a well-known epigenetic regulator in a new disease model, and determine whether targeting histone methyltransferases via small molecule intervention can prevent the development of one of the most common neurological disorders affecting the American population today.

(19) Selective Elimination of Human Pluripotent Stem Cells through Inhibition of an NAD^+ Synthesis Pathway

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Human pluripotent stem cells (hPSC) are a leading candidate for cellular therapies as they are capable of continual self-renewal and can differentiate into every cell type in the human body. In recent years, significant progress has been made in developing protocols for efficient differentiation. Despite increased efficiency, the tumorigenic potential of hPSC is a major limitation to the implementation of cellular therapies as remnant hPSC in cells destined for transplant can form tumors. Thus, methods to selectively eliminate remnant hPSC from their differentiated progeny are required before the widespread clinical application of this technology. Metabolic inhibition is one potential approach to selectively target hPSC, as hPSC are characterized by a reliance on aerobic glycolysis (Warburg effect) and decreased mitochondrial ATP production. Proteomic analysis of cell surface proteins on hPSC and differentiated cells identified Glucose transporter 1 (GLUT1) as a potential target for hPSC glycolytic metabolism. This study tested GLUT1 inhibitors as a method for the selective elimination of hPSC.

One reported GLUT1 inhibitor, STF-31, is selectively toxic to hPSC across a broad range of culture conditions and can be used to selectively eliminate hPSC from their differentiated progeny. However, studies show the mechanism of cell death in hPSC is through inhibition of nicotinamide phosphoribosyltransferase (NAMPT), an enzyme that mediates NAD^+ synthesis. NAMPT inhibition leads to rapid depletion of NAD^+ in hPSC followed by inhibition of hPSC metabolism and loss of ATP. Inhibition of NAMPT in differentiated cells indicate that ATP levels are maintained, likely though maintenance of mitochondrial metabolism. These findings demonstrate the importance of the NAMPT mediated NAD^+ synthesis pathway in hPSC biology and describe a novel strategy that is selectively toxic to hPSC. Future studies will continue to define the mechanism of selective toxicity, including, use of metabolomics to determine how mitochondrial metabolism is maintained after NAMPT inhibition.

(20) Mass Spectral Measurement of Feeding-related Neuropeptide Secretion in Crustacean ia *in vivo* Microdialysis

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Mass spectrometry (MS)-based methods have revolutionized the field of neuropeptidomic research in various organisms. The ability to measure dynamic changes of signaling molecules, such as neuropeptides (NPs), associated with behavior and physiological manipulation is in great demand in order to decipher potential functions of these compounds. Microdialysis (MD) sampling technique has shown great utility in the field of neuroscience for its ability to sample secreted neurotransmitters continuously from living animal with defined temporal resolution. Coupling MD sampling with MS detection provides a unique opportunity to correlate neurochemical content with behavior thus offering additional insights. Here, we take advantage of these utilities by *in vivo* MD sampling to study feeding-related NP secretion in a simple crustacean nervous system.

Here, in order to get a comprehensive list of NPs for further quantitative measurement we first examined the collection time required while maintaining temporal resolution. Collectively, more than 50 NPs from 9 different NP families were identified from 6 h collection. Label free quantitation of fed and unfed dialysate was also conducted with different temporal resolution. Preliminary experiments showed a general increasing trend for most of identified NPs after feeding. Fewer NPs were detected as the collection time was shorter; however, a much bigger fold change for several NPs was observed. One member of the crustacean hyperglycemic hormone precursor-related peptide (CPRP) was observed a modest fold change in 6 h (1.3) and 2 h dialysate (1.6). However, a much more dramatic fold changes, greater than 20-fold, was observed with 1 h dialysate. Such results indicated that shorter duration time is critical for accurate reflection of *in vivo* NP level changes upon feeding. In the meantime, more sensitive detection that enables better temporal resolution or sample enrichment technique would be needed for probing dynamic changes occurring at a faster time scale.

(21) The Dynamic Phosphoproteome of Peripheral Nerve Injury and Chronic Pain

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The molecular mechanisms of chronic neuropathic pain are poorly understood. Following nerve injury, peripheral glial cells and macrophages soon become activated to provide an immune response, initiating a cascade to up-stream pain centers in the dorsal root ganglion (DRG) and spinal cord (SC). The spreading inflammation is accompanied with synaptic changes and maladaptive neuroplasticity, transforming the acute condition into chronic pain. Our study aims to elucidate the dynamic expression and phosphorylation of DRG and SC proteins that are key to the establishment of chronic pain, as well as protein dynamics following pulsed radiofrequency (PRF), a clinical treatment for neuropathic pain. Through quantitative mass spectrometry, we provide a glimpse of the chronic pain proteome with unparalleled depth and scale.

Initial experiments were aimed at the difference in protein expression dynamics between the SNI and SHAM groups. To our knowledge, it is the first large-scale protein analysis in both the DRG and SC of a model neuropathic pain specimen. Interestingly, while the downregulation of periaxin and COL3A1 in the DRGs suggested neuron degeneration and demyelination, both of these proteins were highly upregulated in the SC along with basement membrane proteins LAMB1, LAMA2, and LAMC1. We hypothesize the increased myelination and tissue membrane buildup was a protective response as the encroaching inflammation reached the SC.

Several highly upregulated and downregulated proteins from our initial experiments were kinases and phosphatases, suggesting a strong role for phosphorylation in neuropathic pain. We were particularly interested in the remedial protein phosphorylation changes induced by PRF treatment. DRG and SC tissues from PRF rats will be directly compared to SNI tissues for both expression and phosphorylation dynamics. Initial results from SC samples have identified and quantified nearly 10,000 distinct phosphorylated proteoforms. Phosphoproteins of interest will be further validated via western blots with an additional SHAM group for three-way comparisons.

(22) An Integrated and High Throughput Approach for *in situ* Protein Digestion, Peptide Imaging and Sequence Verification

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Imaging mass spectrometry (IMS) is a powerful technique. Unlike histochemical techniques, IMS does not require labeling of molecules and it can differentiate numerous modifications. Due to tissue heterogeneity, molecules with small mass differences often exhibit completely different distributions; therefore, high mass accuracy and high resolution measurement capability is required. Herein, we describe an effective method that performs untargeted protein and peptide mapping followed by tandem MS on the same tissue and searched against a home-built database for identification and verification.

Mouse pituitary was dissected and cut into 12µm thick sections. Slices were rinsed with ethanol and trypsin was applied by TM sprayer, followed by DHB application. Crabs were cold-anesthetized and pericardial organs were harvested. The tissue was rinsed in doubly distilled water and mounted onto glass slide. TM sprayer was used to coat the tissues with DHB. Samples were analyzed by MALDI Orbitrap. Ion images were generated by ImageQuest and sequences were verified via PEAKS against a home-built database.

For mouse pituitary samples, more than 40 tryptic peptides from 9 proteins were identified and verified directly from pituitary using direct tissue profiling coupled with data dependent acquisition (DDA) on the same tissue. In addition to detection of relatively high abundance protein, peptides with low abundance were also detected with high mass accuracy and sequenced with substantial fragmentation and coverage. Several tryptic peptides from somatotropin were detected with mass error below 1.6 ppm and exhibited colocalization patterns in the anterior lobe. For neuropeptidomic study of the pericardial organ isolated from crustacean, 55 neuropeptides from 9 peptide families were identified by direct tissue profiling and accurate mass measurement. The sequence of 10 neuropeptides from 5 peptide families were generated by data dependent acquisition and verified by de novo sequencing, all of which have been reported to be related to feeding behavior.

(23) Structural Analysis of Monomeric and Dimeric Neuropeptide Y (NPY) with IM-MS, HDX MS, and MD Simulations

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Neuropeptide Y (NPY) is an important neuropeptide that regulates diverse physiological processes, which is amidated in the C-terminus. NPY dimerization at high concentration is believed to store the peptide in an inactive form and to allow a slow release of the active monomer. The structure of NPY monomer was resolved by NMR at relatively high concentration (4-5mM). Thus far, the structure of NPY monomer at biological condition (at nanomolar concentration) remains ambiguous and the dynamic process of dimerization is not well understood. We employ a multi-pronged approach incorporating ion mobility MS (IM-MS), molecular dynamics (MD) simulations, and hydrogen/deuterium exchange (HDX) MS, to investigate on the conformations of the NPY monomer and dimer and their dynamic conversions.

We studied the CCS of NPY monomer and NPY dimer. The arrival time distribution suggested that the NPY monomer has two major conformations that are relatively stable, while the NPY dimer may have more conformations, and some of them are metastable or interconvertable. Both gas phase and solution phase MD simulations were performed on NPY monomer and dimer, respectively. Their theoretical CCS values were calculated by MOBCAL. The theoretical CCS of NPY monomer shows good agreement with the experimental CCS, while the CCS of NPY dimer shows different distribution with the experimental CCS. This discrepancy might be caused by the different initial state we generated for the NPY dimer from the real gas phase conformation in IM-MS. The HDX MS suggested the folding/unfolding process of the NPY dimer is rapid, within 2 minutes. We are using a continuous-flow mixing micro-reactor to characterize the short-lived NPY dimer intermediates by pulsed HDX. The dimerization sites discovered with HDX MS will be used to improve our MD model.

(24) Quantitative Analysis of AKT/mTOR Pathway using Immunoprecipitation and Targeted Mass Spectrometry

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Introduction and Objectives

The PI3K/AKT/mTOR pathway plays a central role in tumor progression and anti-cancer drug resistance. Quantitative measurement of AKT/mTOR pathway protein expression and post-translational modification (PTM) status is necessary for classifying disease states, monitoring cancer progression and determining treatment response. Immunoprecipitation mass spectrometry (IP-MS) is increasingly used to enrich, detect and quantify low abundant proteins and PTMs. Here, we used an improved IP-MS workflow to enrich single or multiple AKT/mTOR pathway proteins simultaneously for targeted MS quantitation.

Methods:

Serum starved A549 and HCT116 cells were stimulated with EGF or IGF-1 to activate AKT/mTOR pathway signaling. Pathway targets were enriched via an improved IP workflow using Pierce Protein A/G and Streptavidin magnetic beads and eluates were processed using in-solution digestion for LC-MS analysis. Targeted SRM MS assays were developed for quantitation of AKT/mTOR pathway target peptides including EGFR, IR, IGF1R, IRS1, AKT2, AKT1, PTEN, PIK3CA, PIK3R1, mTOR and p70S6K. Multiple targets were also immunoprecipitated simultaneously and quantitated by a multiplexed targeted SRM assay.

Results and Discussion

In this study, we validated numerous antibodies for IP-MS with both Pierce Protein A/G and Streptavidin magnetic beads. Enrichment of total and phosphorylated forms of EGFR, AKT1, AKT2, mTOR, GSK3beta, p70S6K and PTEN resulted in quantitation of low to sub nanogram target levels in two cell lysates by targeted MS. We also combined target antibodies to enrich multiple AKT/mTOR pathway protein targets for a single IP-MS analysis. This multiplex enrichment and targeted assay can be used for simultaneous detection and quantitation of AKT/mTOR pathway proteins in other cancer cell lines or tissue samples.

Conclusion

Improved IP-MS workflow enables multiplexed enrichment, detection, and quantitation of PI3K/AKT/mTOR pathway proteins and PTMs at sub to low ng/mL concentrations.

(25) Global Discovery of Protein Post-translational Modifications

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Simultaneous discovery of many known or even unknown post-translational modifications (PTMs) in a biological sample has been a great challenge to the proteomics community, especially with the prevalence of datasets acquired with in-depth proteome coverage. We report here a global PTM discovery strategy, named G-PTM-D, that is able to discover a variety of modifications (including PTMs, chemical derivatives, metal adducts, amino acid variants, etc.) with high confidence in highly complex unenriched samples. This approach takes advantage of the curated UniProt PTM database, combined with potential modification information obtained from a search with an ultra-wide precursor mass tolerance. G-PTM-D places virtually no limit on the number of modifications per peptide, does not require the presence of unmodified counterparts, and displays higher sensitivity and confidence compared with established modification search tools. A major revelation of this investigation is the degree of post-translational modification on a proteome-wide scale in unenriched samples. We identified a total of 249,223 PSMs in human from 7,306 proteins at 1% FDR. Of these PSMs, 18.3% (45,687) were for modified peptides. The software package is open-source, runs on the Windows operating system, and thus will be easily applicable to any high-resolution proteomic datasets.

(26) High pH Reversed-Phase Peptide Fractionation in a Convenient Spin-Column Format

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Introduction and Objectives

Many biologically relevant changes in the proteome occur at the mid-to-low range of the protein abundance scale. Off-line fractionation of complex peptide mixtures from sample digests enables deeper proteome sequencing through increased protein identifications and sequence coverage. High pH reversed-phase fractionation enables orthogonal peptide fractionation to low pH reversed-phased separation and does not require desalting. In this study, we assessed peptide/protein identification numbers, fractional resolution and reproducibility of high pH reversed-phase fractionation in a spin column format.

Methods

Protein extracts from HeLa lysates were digested sequentially with Lys-C and trypsin and peptide quantitation was performed using a Thermo Scientific Pierce™ Colorimetric Peptide Quantitation Assay. Portions of the digested samples were labeled with Thermo Scientific Tandem Mass Tag™ (TMT™) reagents. Pierce™ High pH Reversed-Phase Fractionation Kits were used to fractionate both native and TMT-labeled digest samples into eight fractions by an increasing acetonitrile step-gradient elution. Fractions were dried in a vacuum centrifuge and re-suspended in 0.1% formic acid prior to LC/MS analysis on Thermo Scientific Orbitrap Fusion™ Tribrid™ mass spectrometer. All fractionations were performed in triplicate, with sample loads ranging from 10-100µg, as determined by quantitative peptide assays.

Results and Discussion

We assessed different column matrices, fill amounts and step gradients for column-to-column fractionation reproducibility, peptide fractional profiles, peptide fractionation resolution, and unique peptide/protein identification numbers. Prior to LC/MS analysis, injected sample volumes were adjusted to deliver 1µg of sample material on-column. With the optimized format and fractionation protocol, we routinely identified ~4,300 protein groups using a single two-hour LC gradient which increased to ~7,500 protein groups upon combining the data from all eight fractions (two-hour LC runs each). This resulted in greater than 100% increase in unique peptide identifications. This increase in identifications was observed for 10-100µg sample amounts of both native and TMT-labeled samples.

(27) DRIPPER Mass Spectrometry: A new directed method provides insights into the SUMO/ubiquitin network cooperation with the Slx5/8 protein complex to maintain genome stability

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The evolutionarily conserved replication protein, Mcm10 (minichromosome maintenance protein 10) participates in origin activation, coordinates DNA synthesis and protects genome integrity. Synthetic genetic array analysis suggested that the SUMO-targeted E3 ubiquitin ligase complex Slx5/8 supported the survival of *mcm10-1* mutants by alleviating replication stress. The Slx5/8 complex ubiquitinates sumoylated proteins via the RING domain and promotes DNA repair. Indeed, the RING domain of Slx5 was necessary to rescue the synthetic sickness of *mcm10-1 slx5Δ* mutants. To understand how the SUMO/ubiquitin network and Slx5/8 cooperate to maintain genome stability, we sought to profile differentially abundant SUMO targets in mcm10-1 mutants. In response, we developed a new directed mass spectrometry method (Directed RIPPER or DRIPPER for short). DRIPPER performs a two-stage analysis: 1) label-free relative quantification, and, 2) inclusion list directed mass spectrometry. We first ran triplicate label-free analyses (MS1 only) of background (3 x *mcm10-1* cells and 3 x WT cells) and His-tagged (3 x *mcm10-1* cells and 3 x WT cells). Next, we created an inclusion list of differentially abundant His-tagged *mcm10-1* and WT cells not measured in the background runs at $p < 0.001$ (student's t-test) with a retention time windows of ± 2 minutes. We then used resulting inclusion list, ~2,700 analytes, to direct the MS2 analysis for identification of

the differentially abundant analytes. The DRIPPER method outperformed a comparable DDA method revealing ~100 SUMO conjugates differently abundant in *mcm10-1* cells compared to WT. Importantly, 30-40% of these proteins participate in pathways contributing to genome stability. We also determined that Slx5/8 regulates the stability of sumoylated Bir1 and Sli15. We are currently investigating if Slx5/8-regulation of CPC subunits affects the mitotic checkpoint. Together, our data provide insights into how Slx5/8 and sumoylation maintain genome stability in response to endogenous replication stress.

(28) Mass Spectrometric Investigation of Neuropeptidomic Alteration in Food Intake

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Feeding behavior is critical for animal survival and energy homeostasis. The rising prevalence of obesity and anorexia nervosa further highlights the needs for a thorough understanding of the mechanism involved in the regulation and signaling pathways of food intake and energy homeostasis. For NP identification and quantification, half of 24 adult male rats were food-deprived while a measured amount of food was given to the other half. They were sacrificed by decapitation 2.5 hr after food is given. Nucleus accumbens (Acb) were removed with a 2 mm micro-punch and extracted by ice-cold acidified methanol (90:10:1 / MeOH: water: acetic acid) and filtered by 3kDa molecular weight cutoff devices. After desalting, the brain tissue extracts were subjected to MS analysis using ESI –Orbitrap coupled with NanoLC. Peptide quantification was performed by SIEVE using internal standards. By this method, 52 neuropeptides have shown more than 2-fold increase or decrease after feeding. Some previously known feeding-related neuropeptides like enkephalins, neuropeptide Y, substance P, and MCH have shown a decline in fed rats to different extent. More intriguingly, different proteolytic isoforms of ProSAAS preprohormone have shown different trends after feeding. Among them, big LEN exhibited 2-fold elevation; PEN was increased by 2.5 times; little SAAS increased 1.7 times. Intra-Acb shell infusion of big LEN (N=3, 10 µg/side) and PEN (N=3, 10 µg/side) produced a small but significant increase of food-intake in satiated rats, whereas infusion of little SAAS neuropeptide (10 µg/side) did not alter food intake. In follow-up analyses, additional observational measure, locomotion, rearing, eating, drinking and grooming, were scored and subjected to ANOVA statistical analysis.

(29) Novel iDiLeu Labeling Coupled with High-Resolution Mass Spectrometry for Absolute Quantification of Candidate Biomarkers in Preclinical Alzheimer's Disease

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The pathophysiological process of Alzheimer's disease (AD) begins many years before the diagnosis of AD dementia, so it is important to determine biomarkers and their threshold that best predicts progression from the preclinical to the clinical stage of AD dementia. Our proteomics analysis of cerebrospinal fluid (CSF) from participants with preclinical AD revealed 7 candidate protein biomarkers. To confirm and accurately quantify these potential biomarker candidates, the in-house developed isotopic dimethylated leucine (iDiLeu) reagents were employed for absolute quantification. Each of the 5-plex mass difference iDiLeu reagents was comprised of an isotopic *N,N*-dimethyl leucine which selectively labels the N-terminus of peptide as well as lysine side chains via an amine reactive triazine ester moiety. By labeling target peptides with different iDiLeu channels, a four-point calibration curve was constructed to allow for determination of the absolute amount of target analytes in a single LC-MS run.

Apolipoprotein E (ApoE) was one of the potential biomarker candidates, and was utilized to perform proof-of-principle experiment with the iDiLeu strategy. Plotting the actual iDiLeu channel ratios against the theoretical iDiLeu ratios revealed a linear response comprising the ApoE peptides with R^2 around 0.990. The limit of quantification for ApoE standard was 0.1 fmol/µL. To quantify ApoE in preclinical AD subjects, ApoE tryptic peptides labeled with d3, d6, d9, and d12 were mixed at the concentration of 1, 10, 100, 1000 fmol/µL, and then

spiked into d0 labeled CSF samples. The calibration curve showed high linearity with an R^2 of 0.9999, and high accuracy with a relative standard deviation less than 15% based on two technical replicates.

The novel iDiLeu-enabled standard curve approach makes the absolute quantification of candidate biomarker proteins in preclinical AD subjects possible. This strategy could be further applied to simultaneously quantify more candidate biomarkers in CSF samples and other biofluids.

(30) Quantitative Analysis of Glycans Labeled with Multiplex Carbonyl-Reactive Tandem Mass Tags Using Capillary Electrophoresis-Electrospray Ionization-Mass Spectrometry

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Glycosylation of proteins is one of the most important post-translational modifications. The recently developed sixplex carbonyl-reactive aminoxy tandem mass tag (aminoxyTMT) reagents enable multiplexed characterization and quantitative comparison of structurally complex glycans between different biological samples. The tertiary amine functionality in the reporter region of the aminoxyTMT labels leads to increased ionization efficiency of the labeled glycans thus improving electrospray ionization-mass spectrometry (ESI-MS) detection sensitivity. The aminoxyTMT labeling also makes electrophoretic separation of the labeled neutral and acidic glycans feasible. In this work, we characterized the ESI and collision induced dissociation (CID) behavior of the aminoxyTMT-labeled neutral and sialylated glycans. For the high-mannose N-glycans and small sialylated oligosaccharides, CID fragmentation of $[M+Na+H]^{2+}$ precursor ion provides the most informative MS^2 spectra for both quantitative and qualitative analysis. For complex N-glycans, MS^3 of the protonated Y1(H) ion can be used for relative quantification without interference from the HexNAc fragments. Online capillary electrophoresis (CE)-ESI-MS/MS analyses of multiplexed aminoxyTMT-labeled human milk oligosaccharides (HMO) and different types of N-glycans released from glycoprotein standards were demonstrated. Improved resolution and quantification accuracy of the labeled HMO isomers was achieved by coupling CE with traveling wave ion mobility (TWIM)-CID-MS/MS. N-Glycans released from human serum protein digests were labeled with six-plex aminoxyTMT and subjected to CE-ESI-MS/pseudo-MS^3 analysis, which demonstrated the potential utility of this relative quantification platform for glycan analysis of more complex biological samples.