B.1

Identification and Quantification Method for UCN2 by LC/MS/MS from Plasma/Serum from Mouse Disease Models

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Introduction: The identification of corticotropin-releasing hormone (CRH) has led to the discovery of a growing family of ligands and receptors. CRH receptor 1 (CRHR1) and CRHR2 are mammalian G-protein coupled receptors (GPCRs) with high affinity for CRH and the CRH family of peptides. CRHR1 is predominantly expressed in the brain and plays a vital role in the hypothalamic-pituitary-adrenal (HPA) axis stress responses by secreting adrenal corticotropic hormone (ACTH). CRHR2 is predominantly expressed in the periphery and urocortin 2 (UCN2), a CRHR2-specific ligand, has been involved in the regulation of glucose metabolism. Ucn-2, a 38 aa peptide, shows reasonable homology between rat and human CRH (~34%), Ucn-1 (43%) and Ucn-3 (37–40%). To measure the accurate concentration of UCN2 present in mouse plasma in our disease and intervention preclinical models, we have developed a novel LC/MS/MS method for UCN2 quantification. Methods: The biomarkers were analyzed by protein precipitation using organic extraction of 30 μL mouse/rat plasma, dried and reconstituted by adding 50 mM ammonium bicarbonate pH 8 and digested with trypsin. The tryptic peptides were then injected using nano-HPLC onto PepMap100 C18 pre-column (5mm $\times$ 300μm ID, 5 μm, 100 Å, Dionex) followed by chromatographic separation on a PepMap C18 RSLC nanocolumn (15 cm $\times$ 75 μm, 3 μm, 100 Å, Dionex). Mobile phase A and B were 0.1% formic acid in 2% ACN/ water and 0.1% formic acid in 90% ACN/water, respectively. The eluate was introduced into a Quantiva triple quadrupole mass spectrometer with EASY-Spray ion sources for optimum sensitivity. The LC-MS/MS data were analyzed and quantify by Skyline software. Data: In order to gain optimal sensitivity and efficiency as a quantitative biomarker a simple organic extraction was found to be the most advantageous method of sample prep which balances having a simple extraction method (as compared to SPE) but one that gave good overall recovery. Novel Aspect: We have developed sensitive and specific quantification methods by LC/MS/MS for the determination of UCN2 in endogenous levels across species in plasma/serum.

B.2

Integrating de novo sequencing with sequence database and spectral library search for in-depth analysis of DIA data with PEAKS

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In recent years, data-independent acquisition (DIA) Mass Spectrometry has become increasing popular due to its parallel nature of acquiring all fragment ions for all precursors within a selected m/z range. Recently, a new software release, PEAKS X+ allows for the searching of DIA data by both spectral library as well as direct database searching. This allows the user to benefit from the speed of a spectral library search when one is available but also the unique feature based detection and de novo algorithms inherent to PEAKS X. First, a library search is performed as the first pass of identification. Any spectra that are not matched to a library entry, are searched against a database, and then are de novo sequenced to determine the peptide sequence. Herein, we benchmark our PEAKS X+ library and database search using a publically available dataset against other DIA search software. Using the library search alone, PEAKS compares very well to the published numbers of identifications generated from Open SWATH, Skyline, Spectronaut, identifying ~900 more proteins than any other software. Similarly, when only using the direct database search of all spectra, PEAKS outperforms both Spectronaut and DIA-Umpire in terms of identifications observed by about ~500 protein identifications. Combined, the library and database search resulted in the highest amount of peptide and protein identifications. Taken together, this data suggests that PEAKS X+ will be a useful tool to researchers utilizing DIA Mass Spectrometry, looking to get the most identifications out of their data, regardless of the presence or absence of a spectral library. De novo sequencing provides identification of sequence variants and endogenous peptides.

B.3

High-Throughput PEAKS Workflow for Large Scale Quantitative Proteomics using PEAKS Online X

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In the last decade, we have seen unprecedented advancements in the field of proteomics. The recent advancement of instrumentation as well as software has led to an ever increasing number of large-scale quantitative proteomic studies. These projects, either research based or clinical based, often require quantitation of thousands of proteins, either by label free (LFQ) or by using isobaric tags such as TMT. PEAKS Online is a new high-throughput protein sequencing software solution that runs on a shared resource, is flexible to scale, and is fully parallelized with the ability to run on any cluster or multi-cluster CPU machine. Herein we describe the use of PEAKS online in analyzing two published data sets; one employing LFQ, and the other employing TMT. In both cases we demonstrate a drastic decrease in processing and search time using PEAKS online compared to other search software. Furthermore, because PEAKS online is based on the same proven de novo algorithms included in PEAKS Studio, we show an increase in the number of identifications observed in these datasets. In summary, PEAKS online provides an efficient, time-effective solution to search large-scale systems biology level proteomic experiments with unparalleled accuracy.

B.4

TAILS identifies candidate substrates and biomarkers of ADAMTS7, a therapeutic protease target in coronary artery disease

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Loss of function mutations in the secreted enzyme ADAMTS7 (a disintegrin and metalloproteinase with thrombospondin motifs 7) are associated with protection for coronary artery disease. Therefore, catalytic inhibition of ADAMTS7 has been proposed as a therapeutic strategy for treating cardiovascular disease. ADAMTS7 is a large secreted enzyme consisting of a metzincin catalytic domain and over 200 kDa of auxiliary domains with extensive post-translational modifications thought to target the enzyme to the extracellular matrix and hone in on proteolytic targets relevant to vascular function. Several proteins interacting with the carboxy terminal domain of ADAMTS7 have been proposed as catalytic substrates, however no verified substrate cleavage sites have been described. To identify ADAMTS7 extracellular substrates relevant to vascular disease, we have compared the secreted proteome of human umbilical vein endothelial cells (HUVEC) and human carotid artery vascular smooth muscle cells (HCA-SMC) expressing either a control luciferase, full-length mouse ADAMTS7 WT or full-length mouse ADAMTS7 E373Q catalytic mutant. Conditioned media from these cell types were collected, processed and concentrated for proteomic profiling of secretome and as input for TAILS. TAILS (terminal amine isotopic labeling of substrates, Kleifeld Nature Protocols 2011) is a method for identifying and distinguishing protease-generated neo–N termini from mature protein N termini. A TMT10 isobaric labeling strategy was employed to improve confidence of detection and quantification. Comparison of Luciferase/ADAMTS7 WT, Luciferase/ADAMTS7 E373Q and ADAMTS7 WT/ADAMTS7 E373Q results enabled us to identify auto-cleavage sites from the expressed mouse ADAMTS7 proteins and define a substrate consensus cleavage site. N-terminal cleavage sites from novel substrates significantly enriched in ADAMTS7 WT samples represent candidate substrates from relevant cardiovascular cell types and may offer biomarkers for ADAMTS7 catalytic activity.

B.5

Role of the Particle for Arrangement of Quaternary structure (PAQosome) in assembly/maturation of human protein complexes and networks

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Since its discovery a little over a decade ago, the Particle for Assembly of Quaternary structure (PAQosome, formerly RPAP3/R2TP/PFDL complex) has emerged as a critical organizer in the biogenesis of several protein complexes and networks such as protein assemblies involved in transcription, mRNA maturation, translation and nutrient-sensitive signalling pathways. Unsurprisingly, evidence is mounting that this chaperone machine may be involved in tumorigenesis, consistent with a role in regulating proliferation. We will present our most recent results that led to the identification of new client protein complexes and novel post-translational modification (PTM)-based modes of regulation of the PAQosome. Similar to what has recently been reported for axonemal dynein complexes involved in cilium motility, we now identify cytoplasmic dynein complexes that are responsible for cargo transport along microtubules as new clients of the PAQosome. We also identified a phosphorylation-dependent association of the PAQosome subunit RPAP3 with preribosome complexes. Additionally, we will report for the first time the identification of a small ORF-encoded PAQosome subunit, along with a possible role in the regulation of downstream gene, asparagine synthetase (ASNS) whose expression is linked to neurological disorders and response to asparaginase, a chemotherapeutic drug used in the treatment of acute lymphoblastic leukemia (ALL). These results define novel aspects of PAQosome function and regulation, some being associated with human diseases.

B.6

Comprehensive Comparative Immunopeptidome Analysis of I-Ab-bound peptides from Thymus, Splenic B cells and Dendritic cells in C57BL/6 mice

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Antigen presentation in the thymus is central to selection of CD4+ and CD8+ T cells. Despite postulated roles for differential central and peripheral antigen presentation pathways for MHC-I and MHC-II, comprehensive MHC Class II immunopeptidome analysis of the thymus has not been reported. Advances in mass spectrometry (MS) technology now allow analysis of the MHC-presented peptidomes of tissue samples containing small numbers of antigen-presenting cells. To map the immunopeptidome in the thymus of C57BL/6 mice presented by I-Ab, we used immunoaffinity coupled to MS. We identified >1000 peptides using a conventional data-dependent acquisition strategy (DDA). We compared the immunopeptidome of thymus to that of the splenic B cells and dendritic cells. We also identified >1000 peptides in splenic B cells and dendritic cells. Some of the core epitopes were unique to thymus or splenic B cells or dendritic cells, although most were shared. We observed differences in the general characteristics of the peptidomes presented by thymus as compared to splenic B cells and dendritic cells, including length distribution and hydrophobicity. To help understand the differences, we selected 57 peptides representative of thymic-derived, splenic B-derived, or shared peptidomes, and studied their binding affinity to I-Ab. Results suggest that the pool of peptides presented by I-Ab in thymus has lower MHC binding affinities than does the pool presented by splenic B cells. We are further mapping the immunopeptidome of TEC's to understand the peptide length differences in thymus peptidome and if they are regulated by differential DM mediated editing. As per our understanding about thymic epithelial cells, we do see many peptides derived from tissue restricted antigens or Aire-regulated genes. Also, many of the antigens are dominated by brain specific proteins. We are also exploring a data-independent acquisition strategy (DIA) for quantitative comparison of peptides identified in thymus, splenic B cells and dendritic cells to further characterize antigen presentation differences in thymus and periphery.

B.7

Diversity of the MHC-II immunopeptidome is modulated by the non-classical MHC proteins HLA-DM and HLA-DO and controls thymic selection of CD4+ and regulatory T cells

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Comprehensive analysis of the full spectrum of peptides presented by MHC-II molecules expressed by DO-sufficient and DO-deficient antigen-presenting cells in vivo and in vitro using quantitative mass spectrometry approaches shows that HLA-DO controls the diversity of the MHC-II peptide repertoire. HLA-DO expression leads to presentation of a broader distribution of peptides, with many low-abundance epitopes presented only in the presence of HLA-DO. HLA-DO is thought to regulate antigen presentation through inhibition of the catalytic peptide exchange factor HLA-DM. Peptides presented uniquely in the presence of HLA-DO were sensitive to HLA-DM-mediated exchange, suggesting that decreased DM editing of tight-binding peptides was responsible for the increased diversity in the presence of HLA-DO. Cell-type specific expression of HLA-DO is regulated differently from the coordinate regulation of other MHC-II antigen processing components, with prominent expression in medullary thymic epithelial cells, suggesting a potential role in shaping the repertoire of T cells as they develop in the thymus. Mice lacking H2-O, the HLA-DO ortholog, have reduced diversity in the MHC-II peptide repertoire presented by peripheral and thymic antigen-presenting cells, as well as Treg populations with altered TCR repertoire and function. To evaluate the role of DO in regulating T cell development, we compared the TCR sequences and functional characteristics of T cells from H2-O-/-and wild-type littermate controls. Conventional CD8 and CD4 populations were not significantly altered, but Treg populations were increased and more activated in H2-O-/-mice than in littermate controls, and exhibited greater suppression activity in vivo. We found decreased diversity in the TCR repertoire of Tregs but not conventional CD4 or CD8 T cells in H2-O-/-mice, with higher CDR3 hydrophobicity. These results suggest that Treg selection in the thymus is highly sensitive to immunopeptidome antigen density, and highlight an unexpected for MHC class II antigen presentation in controlling regulatory T cell populations.

B.8

Size exclusion chromatography-based proteomics to identify novel components of the HSP90-dependent proteome following pharmacologic inhibition in colon cancer cells

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The molecular chaperone heat shock protein 90 (HSP90) works in concert with its co-chaperones to stabilize its client proteins, which include several drivers of oncogenesis and malignant progression. Pharmocologic inhibitors of HSP90 are proposed to trigger widespread remodeling of cellular protein complexes, including dissociation of co-chaperones from HSP90, disruption of client protein signaling networks, and recruitment of the protein ubiquitination and degradation machinery. However, proteomic studies to date have focused on inhibitor-induced changes in total protein levels, often employing inhibitor concentrations and time-points unsuitable for detecting protein complex alterations. Here, we use size-exclusion chromatography in combination with mass spectrometry (SEC-MS) to characterize the changes in native protein complexes following treatment with the HSP90 inhibitor 17-AAG in the HT29 colon cancer cell line. Subunits of known protein complexes displayed similar SEC-MS elution profiles. After confirming that we could detect the signature cellular response to HSP90 inhibition (e.g., induction of heat shock proteins, decreased total levels of client proteins), we were surprised to find only modest perturbations to the global distribution of protein elution profiles in inhibitor-treated cells. Similarly, co-chaperones that co-eluted with HSP90 displayed no clear difference between control and treated conditions. However, analysis at an individual fraction level identified 119 "hits" ($p < 0.05$ and fold change $+/- 2$ between conditions), which included several known parts of the HSP90 proteome, as well as numerous proteins and pathways with no previous links to HSP90. We illustrate the utility of this approach by characterizing two novel components—previously implicated in malignancy—that would not have been identified with bulk proteomics. We expect SEC-MS to provide a fruitful avenue for similar pharmacology-based comparative proteomics in the future.

B.9

Disruption-Compensation (DisCo) analysis of RNA polymerase II Protein Interactome

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RNAPII transcription elongation is intricately regulated through dynamic protein-protein interactions (PPIs). While genetic screens have been fruitful in identification and characterization of many complexes involved in transcription, many of their biochemical mechanisms remain elusive. To interrogate how genetic perturbations cause changes in the RNAPII interactome, we have developed an affinity-purification mass spectrometry (AP-MS) based method, termed disruption-compensation (DisCo) network analysis. analysis. RNAPII complexes are affinity purified from either temperature sensitive or deletion RNAPII-elongation factor mutant yeast. The affinity-purified complexes are then isotopically labeled, multiplexed and quantitatively analyzed via MS^2 and MS^3.

DisCo analysis measures specific alterations in the RNAPII interactome that provide insights into the wide variety of biochemical changes that occur, because of genetic perturbation, at the level of PPIs. Data suggests that upon perturbation of individual RNAPII elongation factors, there are distinct changes in the PPIs of RNAPII with other canonical or situational transcription regulators. Interestingly, these changes are observed to occur both in proteins that have been previously shown to have a relationship with the perturbed elongation factor and in proteins with no established connection. Furthermore, the observed differences in interaction dynamics within the RNAPII interactome show both decreases and increases in abundance. These decreases in interaction abundance suggest distinct disruptions in RNAPII PPIs due to individual elongation factor perturbation, which provides insight into protein complex dynamics and the mechanism of transcription regulation through PPIs. The observed increases in interaction abundance suggest attempts at compensation by other proteins at the site of transcription during elongation factor perturbation. This data provides valuable insight into the regulatory pathways that occur at the sight of transcription, and add a level of functional understanding to genetic screening data.

In summary, DisCo network analysis harnesses the utility of genetic perturbation and the power of mass spectrometry to provide a quantitative method for interrogating the functional mechanism of individual proteins and their interactions within proteins complexes, regulatory pathways, and cellular processes. While our application of DisCo network analysis has been focused on RNAPII transcription and related protein complexes, the technique is generally applicable to study protein interaction dynamics in a highly quantitative fashion.

B.10

Plasma proteome profiling to detect and avoid sample-related biases in biomarker studies

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In the face of several challenges, mass spectrometry (MS)-based proteomics is now starting to live up to its initial promise as a generic technology for the discovery and quantification of proteins that reflect an individual's health or disease state (1). We developed a 'Plasma Proteome Profiling' pipeline that has proven to be very robust and capable of the rapid identification of more than 500 proteins in undepleted plasma and to monitor physiological changes (2–4). Here, we applied this pipeline to investigate potential contamination sources of plasma samples and establish tools for the detection of systematic bias in clinical studies (5). We acquire deep reference proteomes of erythrocytes, platelets, plasma and whole blood of 20 individuals (>6000 proteins). This resulted in three panels of contamination markers, reflecting the contamination with platelets and erythrocytes and partial coagulation events. These contamination marker panels can assess the quality of each sample, potential systematic bias in an entire study and the likelihood that individual biomarker candidates belong to the contaminant proteomes. As a showcase, we successfully applied the contamination marker panels to our own biomarker studies and the analysis is instantly available with an online platform at www.plasmaproteomeprofiling.org. Having examined our own studies in detail, we set out to survey the extent to which probably contamination proteins are reported as biomarker candidates in the literature. We revisited 210 published clinical studies and more than 50% reported proteins of the contamination marker panel. We provide recommendations for controlled and reproducible handling of blood samples and how to assess the quality of studies with the hope to improve outcomes of future studies and ultimately to easy the application of novel protein biomarkers in clinical application.

B.11

Production and Generation of Proteogenomics Databases using PyPGATK

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Proteogenomics approaches have recently been applied in various studies bridging the gap between proteomics and genomics. In a typical proteogenomics analysis, a customized database is built from genomic sequences and DNA variants for identifying novel and variant peptides from mass spectrometry–based proteomic data. Utilization of the existing genomic resources for proteogenomics analysis enhances peptide identification and generates protein-level evidence. We developed PyPGATK which is a *Python* package to generate customized protein databases. It enables the generation of protein sequence databases from ENSEMBL and cBioPortal as well as user-provided genomic sequences and DNA variant sets. A web-based interface is also implemented within the PRIDE framework to enable generation of customized protein databases through a clickable view. Additionally, a collection of protein sequence databases is made readily-available for download in *fasta* format. The collection contains 1) protein databases from noncoding transcripts including ncRNAs and pseudogenes that is in addition to alternative open-reading frames of canonical proteins for various species. 2) cancer-type specific protein databases from catalogues of somatic mutations provided by large cancer cohorts within COSMIC and cBioPortal. 3) variant protein databases are generated based on single nucleotide polymorphisms that are detected in normal samples from the 1000 Genomes project as well as all normal samples provided in gnomAD. These databases enable correction of current gene annotations and detection of novel protein-coding genes, detection of cancer-associated variant peptides, and detection of variant peptides expressed in normal samples. This work contributes to future proteogenomics studies by providing methods and resources to utilize genomics in analyses of mass-spectrometry datasets.

B.12

Multi-omic profiling reveals differential kinase activity in neuronal differentiation

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Functionally integrated neurons are generated during the dynamic process of continuous rewiring of genetic architecture and protein expression. Apart from regulation at the transcriptional and RNA level, neuronal differentiation is extensively controlled by post-translational regulation and modifications. Thus, a significant challenge in cell biology is to understand how these complex conceptually distinct regulatory events are organized and coordinated at the system level. Simultaneous profiling of the gene expression, global protein abundance and phosphorylation is an essential step in addressing this goal. Here, to study neural differentiation, we use a multi-omic approach in which RNA-sequencing and mass-spectrometry are employed to profile RNA, protein and phosphorylation abundance. We confirm that neuronal differentiation results in massive change in global protein abundance and phosphorylation. Preliminary network propagation analysis revealed differential kinase activity during this dynamic process. We expect that in the future this project will reveal new protein subnetworks and cellular processes coordinating neuronal differentiation and provide mechanistic insights into how genetic perturbation accompany neurodevelopmental diseases.

B.13

A novel HLA-peptide profiling workflow called MAPTACTM (Mono-Allelic-Purification-with-Tagged-Allele-Constructs) leverages mass spectrometry to improve neoantigen prediction

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T cell responses are mediated by the recognition of non-self antigens presented by human leukocyte antigen (HLA) heterodimers. A resurgence of interest in understanding antigen processing has been driven by the realization that antigens containing somatic mutations (neoantigens) can enable T cell responses against tumors. Personalized neoantigen vaccines attempt to identify and therapeutically target the subset of mutations that are most likely to yield HLA-presented neoantigens. To improve neoantigen prediction methods, we have developed a high-throughput, mono-allelic HLA profiling workflow called MAPTACTM (Mono-Allelic Purification with Tagged Allele Constructs) that utilizes LC-MS/MS. Our novel mono-allelic HLA-ligandomics approach, which does not require biased antibody immunopurification or HLA-null cell lines, enabled us to train highly accurate machine learning models for neoantigen prediction. Until recently, antigen prediction was based on biochemical assays that measure the binding between putative peptide antigens and specific HLA heterodimers. A limitation of this approach is that it does not account for events upstream of peptide binding such as protein-to-peptide processing or chaperone-mediated transport and loading. Therefore, studies using LC-MS/MS to directly identify peptides presented on living cells have accelerated antigen prediction improvements by alleviating this shortcoming. Our novel MAPTACTM approach enabled mono-allelic profiling without the use of biased antibodies or HLA-null cell lines that were previously required for mono-allelic cell line generation. Additionally, the use of MAPTACTM in multiple cell lines increased the depth of HLA-sampling by providing a high-throughput method to sample endogenously processed and presented peptides from diverse proteomes that are known to bind to a single HLA heterodimer. We further demonstrated the fidelity of MAPTACTM using a head-to-head comparison between conventional antibody-based HLA-peptide isolation methods and showed no detectable bias introduced by affinity-tagged HLA heterodimers. Finally, we utilized MAPTACTM to generate a large mono-allelic dataset that was leveraged to train and validate highly accurate machine learning models for neoantigen prediction.

B.14

Selective capture and release of nascent proteins for translatome profiling

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The rapid reprogramming of mRNA translation is a critical component of the cellular response to specific stimuli, such as stress factors, inhibitors, or environmental cues. To facilitate the study of dynamic translatomes, we have reported the use of the cell permeable O-propargyl-puromycin (OPP), an aminonucleoside tRNA mimic, to tag nascent polypeptide chains undergoing elongation in cultured cells. In our initial "OPP-ID" protocol [1], after a two-hour OPP treatment, we conjugated OPP-tagged polypeptides in cell lysates to biotin-azide using copper(I)-catalyzed azide-alkyne cycloaddition (click chemistry), captured the products by affinity chromatography using streptavidin magnetic beads, and performed on-bead tryptic digestion prior to LC-MS/MS analysis and label-free quantification. While robust for starting lysates containing as little as ~400 μg of protein, the inevitable problem of non-specific binding of proteins to the streptavidin beads left room for improvements to the method to minimize background and permit the analysis of smaller sized samples. In the present study, we have explored the use of three biotin-azide reagents with cleavable linkers: N-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) biotin-azide, 2-(4'-carbamoyl phenylazo)phenol (Diazo) biotin-azide, and 2-(4'-hydroxy-2'-alkoxy phenylazo)benzoic acid (HAZA) biotin-azide. Using these reagents, nascent polypeptides were selectively released from streptavidin agarose beads using chemical treatments appropriate for each cleavable linker. SDS-PAGE with silver staining was used to visualize the efficacy of the release treatments. We show that selective release of nascent polypeptides using cleavable biotin-azides dramatically minimizes the level of background proteins detected in the analysis as compared to samples generated by on-bead digestion, and thus generates superior preparations of nascent proteins for LC-MS/MS analysis. This improvement in the selective recovery of OPP-tagged nascent polypeptides will enable the analysis of low-level samples from challenging systems such as neuronal systems.

[1] Forester et al., *Proc Natl Acad Sci USA* 115:2353–2358 (2018).

B.15

Targeting a subset of the membrane proteome - the proteolipids that extract into chloroform, the proteolipidome

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The membrane proteome comprises at least 30% of the total proteome and includes many proteins of vital cellular function. Bottom-up proteomics protocols now give essentially complete coverage of the membrane proteome based largely upon hydrophilic segments of the target proteins. The proteolipids are named because they have the physical properties of lipids, that is they partition into the chloroform-enriched phase of an aqueous-organic phase separation just as lipids do. The chloroform-enriched phase of an aqueous/organic phase partition yields a unique set of proteins that we call the proteolipidome. The problem faced is the extreme contamination of the protein by a vast mixture of lipids including fats. Size-exclusion chromatography (SEC) in an appropriate solvent provides a potential means to separate the proteins that go down to approximately 3.5 kDa from lipids that go up to about 1.5 kDa. We used SEC in chloroform/methanol/1% aqueous formic acid (4/4/1; v/v) on a silica SEC column to investigate separation of proteolipids from lipids. On the column used, maximized recovery of protein lead to substantial lipid contamination so a compromise cut off position was chosen based upon online positive-ion low-resolution electrospray ionization mass spectrometry. A soon as the mass spectrum was dominated by the earliest eluting lipids collection of protein was terminated. Thus the protein fraction was substantially depleted of lipids. Top-down mass spectrometry experiments were performed on these fractions but were plagued by plasticizer contamination if the fractions were collected in plastic tubes or sodium adducts if collected in glass. Therefore the fractions were dried down and separated by reverse-phase liquid chromatography (RP-LC) after dissolution in 70% formic acid. RP-LC used a polymeric column at elevated temperature (40 °C) with the organic buffer supplemented with 50% isopropanol to enhance membrane protein elution, as previously described. The proteolipidome of mouse brain samples yielded large amounts of the c-subunit of the mitochondrial ATP synthase (7649 Da) and the equivalent subunit of the vacuolar ATPase (15717 Da).

B.16

Allosteric HSP70 inhibitors overcome proteasome inhibitor resistance by perturbing mitochondrial proteostasis

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Proteasome Inhibitor (PI) resistance remains a key clinical challenge in multiple myeloma, with multiple studies identifying upregulation of heat shock protein 70 (HSP70) chaperones as a mechanism of overcoming PI-induced proteotoxic stress. Here, we utilize various mass spectrometry methodologies to profile the novel "JG" class of allosteric HSP70 inhibitors as anti-myeloma therapeutics. We find that "JG" compounds exhibit increased efficacy against both derived and innate PI-resistant myeloma models, a favorable therapeutic index against bone marrow stromal cells, and in vivo anti-myeloma toxicity. Interestingly, we find that synergy and antagonism between the best-characterized analog JG98 and inhibitors targeting central proteostasis regulators HSP90, VCP/p97, and the proteasome are cell line dependent, suggesting differential protein homeostasis network wiring among various myeloma models. Employing miniaturized TMT-multiplexed proteomics, we find that JG98 leads to conserved destabilization of 55S mitochondrial ribosome subunits. This phenotype is not seen under HSP90, VCP/p97, or proteasome inhibition, illustrating that 55S depletion is specific to HSP70 inhibition. Employing multiplexed TMT-pulsed SILAC mass spectrometry and RNA-sequencing, we find that destabilization is caused by loss of nascent 55S subunit production rather than destabilization of existing 55S subunits. MRPS21 is identified as post-transcriptionally dependent on HSP70 for nascent production, illustrating potential chaperone-client interactions between HSP70 and specific 55S subunits. Finally, we utilize N-glycoproteomics to study the effect of HSP70 inhibition on cell surface protein expression in myeloma. Treatment with analog JG342 leads to downregulation of myeloma immunotherapy targets BCMA and ITGB7 and, surprisingly, upregulation of non-myeloma tumor markers CD166 and MUC16, pointing to a potential role for HSP70 in maintenance of highly expressed cell identity markers and suppression of non-identity markers.

B.17

Comparative Proteomic in Multiple Brain Regions of the Naked Mole-Rats and Mice

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Since first described, the African naked-mole rat has become one of the most sought-after study subjects across biological sciences due to their compelling physiological characteristics, including the ability to survive low-oxygen conditions, extended lifespan, and resistance to pain and tumorigenesis. To understand the mechanisms behind the unique traits of the naked-mole rat, many studies have been carried out targeting individual proteins and pathways. However, these studies mostly focused on the potential pathways and proteins responsible for the apparent features of the naked-mole rats. In this study, we analyzed the differential proteome of the cerebral cortex, hippocampus, and cerebellum for regional information and evaluate the possible uniqueness in the development of the naked-mole rat, compared to mice. Additionally, we evaluated varied ages of animals from each group. To do this, we performed standard-flow, label-free, differential proteomic analysis. A total of 2226 ± 228 unique proteins were identified across all samples. We observed no significant and compelling differences between the two age groups within each species. Gene ontology (GO) enrichment analysis suggests that most differential proteins belong to functions including neurotransmitter transport activity, oxidoreductase pathways, and lipid metabolism. Specifically, the expression of cold-inducible RNA-binding protein (CIRBP) and fatty acid-binding protein 5 (FABP5) were evaluated using an orthogonal method. CIRBP, which has been shown to inhibit hypoxia-induced neuronal apoptosis, was found to be increased in the naked-mole rat. This up-regulation, therefore, may have contribute to the adaptive lifestyle of low-oxygen living conditions. On the other hand, FABP5 was down regulated, suggesting that fatty acid transport is altered. We hypothesize that the naked-mole rat, unlike other animals develops their neuroprotection mechanism more prominently than neurogenesis.

B.18

Development and Application of a Targeted immuno-MRM-MS Assay to Identify the Mechanism of Activity and Resistance to Thalidomide Analogs

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Pharmacologic agents such as thalidomide and its analogs are highly active agents used in the treatment of a number of hematologic malignancies and are a cornerstone of treatment for multiple myeloma (MM) and myelodysplastic syndrome (MDS). These drugs function by increasing the affinity of the CUL4-RBX1-DDB1-CRBN (CRL4^{CRBN}) E3 ubiquitin ligase for a small set of protein substrates, leading to rapid degradation of disease-relevant proteins by the proteasome. To date, there is a limited understanding of the determinants of activity of these agents and how resistance develops. We have developed and utilized a novel quantitative, targeted mass spectrometry (MS) assay to determine the relative activities, kinetics, and cell-type specificity of thalidomide and four analogs, all but one of which are currently in clinical use or in clinical trials for hematologic malignancies. Each of these thalidomide analogs bind the CRL4^{CRBN} ubiquitin ligase and induce degradation of particular proteins as all five compounds studied demonstrated distinct patterns of substrate specificity that likely underlie the clinical activity and toxicities of each drug.

This fit-for-purpose Immuno-MRM assay was rapidly constructed and optimized for 22 peptides representing 10 protein substrates utilizing 20 polyclonal anti-peptide antibodies. Final deployment of the assay consists of plate-based automated enzymatic digestion and desalting as well as magnetic bead-based capture and elution. Antibody performance was improved by chemical crosslinking resulting in reduced interferences and improved capture efficiency. Response curves were performed to determine the linear range and LLOD/LLOQ for each target in two common multiple myeloma cell cultures, MM1S and KG-1. Finally, assay was applied to dose curve and time course studies of thalidomide and four of the analogs in common KG-1 cell line.

Our results demonstrate that the activity of each compound depends on the strength of ligase-substrate interaction in the presence of drug, the levels of the ubiquitin ligase, and the expression level of competing substrates thereby suggesting a novel mechanism of resistance mediated by competition between substrates for access to a limiting pool of the ubiquitin ligase. These studies provide general rules that govern drug-dependent substrate degradation and key differences between thalidomide analog activity in vitro and in vivo.

B.19

ITRAQ Proteomic Profiling of Tissue Interstitial Fluid from Paired High Grade Serous Ovarian Cancer and Fallopian Tube Samples Reveals Novel Biomarker Candidates

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High-grade serous ovarian cancer (HGSOC) is the most common type of ovarian cancer, with a generally poor prognosis resulting largely from advanced-stage diagnoses. Earlier detection of the disease leads to better outcomes and improved long-term survival, supporting the need to identify reliable early-stage biomarkers. The cancer secretome (proteins secreted, shed or leaked from cancer cells) provides a plausible source of biomarkers candidates, as these proteins are accessible to lymphatic drainage and may ultimately be detectable in blood and other biofluids.

Tissue interstitial fluid (TIF)—the fluid that bathes tumor and normal cells and constitutes a key component of the cellular microenvironment—comprises a sort of endogenous secretome, providing access to the dynamic complement of proteins expressed both normally and, more significantly, aberrantly by cancer cells. TIF studies can lead to an improved understanding of the tumor and normal tissue milieu, provide insight into tumor biology, and importantly, help identify novel candidate biomarkers amenable to downstream verification experiments. Here we describe deep proteomic characterization of a unique set of five HGSOC TIF samples and paired TIF from contralateral, histopathologically uninvolved Fallopian tube (FT), now widely considered to be the generative tissue for HGSOC. Tissue samples were obtained in the pathology suite within minutes of surgical resection and TIF was extracted within an hour under physiological conditions. Using ITRAQ isobaric labeling for precise relative quantification, over 5000 protein groups were confidently identified in all HGSOC / FT TIF pairs, of which approximately 10% were significantly differentially expressed by a moderated T-test. Top candidates recapitulate previously established HGSOC biomarkers, including CA125 and HE4, and provide a large number of novel biomarker candidates. Immunohistochemistry analysis of novel candidates verify robust expression differences between HGSOC and FT tissues. Preliminary targeted proteomic measurements of time-of-diagnosis plasma samples from ovarian cancer patients and matched controls show novel candidates to be detectable and differentially expressed. Collectively, these data demonstrate the power of TIF-based proteomics and illustrate a discovery-to-verification pipeline amenable to biomarker discovery in any solid tumor.

B.20

Application of Quantitative Phosphoproteomics in the Study of Plant Mechanosignaling

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Being sessile organisms, plants must regulate their growth and development in order to respond to a variety of internal and external stimuli during their life cycles. Mechanical forces, such as touch and wind, trigger developmental changes, called "thigmomorphogenesis," in plants, substantiating the ability of plants to perceive and respond to such stimuli. However, the signaling components responsible for mechanosignaling are largely unknown. A major thigmomorphogenetic response in *Arabidopsis* delayed bolting, *i.e.*, the emergence of the flowering stem. Here, we performed a quantitative phosphoproteomics approach based on SILIA (stable isotope labeling in *Arabidopsis*) to profile the changes in protein phosphorylation resulting from 40 seconds of force stimulation in *Arabidopsis thaliana*. Our analysis resulted in 24 touch-responsive phosphopeptides, of which many were derived from kinases, phosphatases, cytoskeleton proteins, membrane proteins, and ion transporters. Among these phosphorylation events, the phosphorylation of S625 residue of the previously uncharacterized protein TOUCH-REGULATED PHOSPHOPROTEIN1 (TREPH1) increased rapidly in touch-stimulated plants, as confirmed by immunoblots. Morphological and transcriptomic studies showed that TREPH1 is required for the touch-induced delay of bolting and changes of gene expression, respectively. Furthermore, the transgenic wild type TREPH1, but not a non-phosphorylatable site-specific isoform of TREPH1 (S625A), restored touch-induced flowering delay of *treph1–1* loss-of-function mutant, indicating the necessity of the residue for TREPH1 function. Taken together, these observations identify TREPH1, a phosphoprotein player in thigmomorphogenesis regulation of *Arabidopsis*, and substantiate that TREPH1 and its 625th serine residue may play a role in delayed bolting of *Arabidopsis* thigmomorphogenesis.

Reference:
Wang, K., Yang, Z., Qing, D., Ren, F., Liu, S., Zheng, Q., Liu, J., Zhang, W., Dai, C., Wu, M., Chehab, E.W., Braam, J., and Li, N. (2018) Functional Phosphoproteomics Reveal Key Roles for MKK2 and TREPH1 in *Arabidopsis* Touch Responses. *Proc Natl Acad Sci U S A* 115(43):E10265–E10274.

B.21

Deep Label-Free Quant: Deciphering the MS1 Hieroglyph with IT and AI

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In tandem mass spectrometry (MSMS), 90%+ of the information resides in MS1 m/z data because relatively few precursors get fragmented. Yet conventional data analysis focuses primarily on MS2 spectra and sequence statistics, with MS1 data treated as an afterthought. This backward approach only scratches the surface of what's possible.

"Deep" label-free quantitation (DLFQ) instead focuses on identifying/quantifying MS1 precursor ions using MS2-identified peptides as a starting guide. This is like interpreting Egyptian hieroglyphs as an integrated story, instead of a collection of unconnected sentences, guided by a Rosetta Stone. It allows significantly more peptides and proteins to be characterized, particularly those of low abundance or with post-translational modifications (PTM) of clinical relevance.

Instead of looking at a multi-hour MSMS run as a single experiment, deeper analysis is possible by analyzing it as thousands of one-second mini-experiments. Here, every m/z data-point is standalone evidence of an ion even if it appears only once or twice.

For low abundance peptides, thousands of anonymous ones are manifested as a single pair of isotopic MS1 data-points. That's enough to determine charge, precursor mass, retention time, and rough quantity (i.e. apex intensity). But without a way to infer sequence, they are typically discarded as noise. We show how powerful IT can uncover mass interrelationships that imply sequence information.

Co-eluded peptides confuse ion assignments and cause irreproducible quantitation. AI can in principle combat this, but most techniques require extensive annotated datasets that proteomics lacks. We discovered that using an AI image processing technique, computer vision (CV), can be applied without annotated training data to the extent that data can be interpreted as an image. We show how CV's Hough Transform for finding lines can detect co-eluded peptides.

DLFQ can uniquely identify and quantify low-abundance, modified peptides otherwise out of reach.

B.22

Quantitative proteomics of MPK4 phosphorylation dynamics and interacting proteins

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It has been reported that Arabidopsis MAP kinase 4 (MPK4) was a negative regulator in plant immunity, and it is activated by pathogen-associated molecular patterns (PAMPs), such as flg22. The molecular mechanisms by which MPK4 is activated and regulates plant defense remain elusive. Here we investigated Arabidopsis defense against a bacterial pathogen Pseudomonas syringae pv. tomato (Pst) DC3000 when Brassica napus MPK4 (BnMPK4) is overexpressed (OE). We showed that the OE plants have increased sensitivity to flg22-triggered reactive oxygen species(ROS) burst in guard cells, which resulted in enhanced stomatal closure compared to wild-type (WT). During flg22 activation, dynamic phosphorylation events within and outside of the conserved TEY activation loop were observed. To elucidate how BnMPK4 functions during the defense response, we used immunoprecipitation coupled with label-free quantitative proteomics to identify BnMPK4 interacting proteins in the absence and presence of flg22. Using kinase assay, we showed evidence that some of the interacting proteins were potential MPK4 substrates. Our results led to generation of MPK4-associated protein network, and insight into the MPK4 molecular functions.

B.23

Composition of the myddosome during the innate immune response

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Critical for the innate immune response to PAMPs, including viral RNA, the myddosome is a complex of proteins characterized by the presence of myeloid differentiation factor 88 (Myd88). The myddosome acts to transfer signals from the toll-like receptor (TLR) proteins to TNF receptor-associated factor 6 (TRAF6). In addition to Myd88, the myddosome contains multiple copies of the interleukin-1 receptor associated kinases (IRAK) 2 and 4 and is theorized to form following stimulation of the TLR proteins. Using affinity purification – mass spectrometry analysis (AP-MS), we identified the Myd88-associated proteins in mouse immortalized bone marrow-derived macrophages before and after lipopolysaccharide (LPS) treatment. Prior to LPS treatment, we found the stable association of Myd88 to IRAK4, TRAF6, and other proteins related to the response to LPS. Following LPS treatment, Myd88 associated to additional downstream effector and inhibitor proteins, including IRAK1, IRAK2, IRAK3, and Tnip1. Using stable isotope labeling by amino acids in cell culture (SILAC), we found that the association of Myd88 to the IRAK proteins changes as the LPS response progresses, with IRAK1 showing strong association initially but then being replaced by IRAK2 as time progressed. Because of this, we are examining the mechanism(s) that regulate the activity of the myddosome. We are currently using AP-MS followed by phosphopeptide enrichment with MS/MS analysis to identify possible phosphorylation sites. Using these data, we will evaluate the role of phosphorylation on myddosome activity.

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B.24

Thermal profiling as a novel tool to analyze the impacts of missense mutants on the proteome

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Thousands of missense mutations have been associated with disease, ~60% of which have been predicted to affect protein stability and/or protein-protein interactions (PPIs). Changes in mRNA have shown to explain only ~40% of protein level changes, thus, a full understanding of disease relies not only on genomic data, but also on defining the proteome. Many disease-causing proteins have existing structural information, but these data do not provide a full picture of how protein function or PPIs are affected. Current proteomic methods of studying effects of mutations have focused on measures of protein abundance or PTMs and often require much time and some level of expertise. High-throughput methodology to evaluate how mutations in a single protein could affect PPI networks would help streamline the characterization of global mutant proteins and aid in the prediction of phenotypic outcomes resulting from genomic mutations. Towards this goal, we adapted cellular thermal shift assay (CETSA) coupled with mass spectrometry (MS) for the study of the effects of missense mutations on protein thermal stability on a global proteome level, which we have named Temperature sensitive Mutant Proteome Profiling (TeMPP). TeMPP and quantitative global proteomics were performed on temperature sensitive mutant yeast strains to characterize global effects of missense mutants within proteins of the ubiquitin proteasome system and the transcription machinery. Our current data suggests TeMPP to be highly specific to proteins associated with the mutated protein of interest and capable of differentiating effects between two proteins in the same complex.Overall, TeMPP provides an efficient and highly specific approach to profile global effects of missense mutations without the need for large amounts of starting material or genetic manipulation of the system of interest. Use of this method along with other complementary-omics approaches will help build a foundation for the characterization of how missense mutations can affect cellular protein homeostasis and thereby lead to disease phenotypes.

B.25

Quantitative Proteomic Analysis to Identify Developmentally Regulated Proteins in Leishmania Major Isolate

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Background: The proteomes of two consecutive developmental stages (procyclic and metacyclic promastigotes) from Iranian Leishmania (L.) major isolates were investigated using SWATH-MS quantitative proteomics approach.

Methods: Isolated proteins from procyclic and metacyclic lysate were digested, fractionated and subjected to SWATH-MS. Proteins significantly different in abundance were analyzed via gene ontology (GO) and protein-protein interaction network (PPIN).

Results: Our study showed that 52 proteins were changed in abundance between the two consecutive developmental stages. Proteins significantly different in abundance were classified into nine categories by GO analysis. Significant modulations in translation, antioxidant and stress-related defenses, energy metabolism, structural and motility-related proteins were observed between procyclic and metacyclic forms. We found that elongation factor-2 and various structural constituents of ribosome were down-regulated during metacyclogenesis, while motility related proteins including ADP-ribosylation factor-3, paraflegellar rod protein-2C and tubulin alpha-chain were up-regulated. According to Network analysis, ENOL has been introduced as main hub-bottleneck protein and EF-1b, Hsp60 and GDH have been determined as seed proteins.

Conclusions: Our results show that significant proteins in abundance are crucial features of metacyclogenesis in L. major. The protein function analysis illustrated that synthetic pathway involved proteins were down-regulated in metacyclic, which is in agreement with the stationary phase of parasite growth, while up-regulation of motility and energy metabolism related proteins is consistent with infective feature of metacyclic. Also, our data proved the possibility of SWATH-MS as viable approach to quickly identify novel stage-specific proteins in Leishmania and further studies are required for the validation of the results.

Keywords: Cutaneous Leishmaniasis, L. major, Metacyclogenesis, MS Spectrometry, Quantitative Proteomic.

B.26

SC-514 Loaded PLGA Particle Formed by Single Emulsion Method

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Poly (lactic-co-glycolic acid) (PLGA) is a biocompatible member of the aliphatic polyester family of biodegradable polymers. PLGA has long been an established option for drug delivery applications, predominantly because it has been FDA-approved for use in humans as a form of resorbable sutures. SC-514 is a relatively new hydrophobic dug, which has been shown to have anti-cancer effects via inhibition of NF-KB dependent gene expression in cancer cells. SC-514 was encapsulated in PLGA nanoparticles via single-emulsion method. The SC-514 loaded PLGA nanoparticles (diameter=49.4nm) synthesized has the potential to increase the bioavailability of SC-514 drug in prostate cancer treatment. Hence, increasing the therapeutic effect of SC-514 in prostate cancer treatment.

B.27

Purification and Proteomic Analysis of the Golgi Outpost – an Organelle that Nucleates Microtubules in Oligodendrocytes

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Canonically, microtubules form or nucleate off of the centrosome in animal cells. In recent years, acentrosomal microtubule-organizing centers (MTOCs) have also been shown to nucleate microtubules. For example, the Golgi outpost acts as a local source of microtubule nucleation in Drosophila neuronal dendrites (Jan et al., 2013). Muscle cells, which have grid-like microtubules, also contain Golgi outposts positioned at microtubule intersections (Ralston et al., 2013). We now find Golgi outposts positioned in oligodendrocytes along processes extending toward axons and along myelin sheaths. We identify a marker for this mysterious organelle by screening our lab's RNA-seq database for microtubule-associated proteins that are both highly and specifically expressed in oligodendrocytes. TPPP (tubulin polymerization promoting protein) selectively localizes to Golgi outposts but not to cell body Golgi; its identification as the first specific Golgi outpost marker allows us to biochemically purify Golgi outposts. We adapted a classic differential sucrose gradient centrifugation protocol to purify Golgi (Warren et al., 2006) from neonatal rat brains; Golgi outposts were further separated by anti-TPPP immunoprecipitation. In immuno-EM images, Golgi labeled by anti-TPPP gold beads associate with microtubules. Mass spectrometry of TPPP-positive Golgi yielded 118 proteins that are enriched relative to Golgi input. Surprisingly, many hits are signaling proteins. We validate the hit MYO18A, a myosin motor previously shown to mediate Golgi dispersion and bind to the Golgi protein GOLPH3.

To further determine the function of TPPP, we show in cell-free assays that recombinant TPPP protein robustly nucleates new microtubules. To interrogate the function of Golgi outposts, we cultured oligodendrocytes from TPPP knockout mice, which have aberrant branching and mixed microtubule polarity (in contrast to uniform polarity in wildtype cells). Both in vitro in 3D cell cultures and in vivo with confocal microscopy of brains, TPPP loss results in myelin sheaths with about half the length of wildtype sheaths. Physiologically, TPPP knockout mice display hypomyelination and motor coordination deficits. Together, our data demonstrate that microtubule nucleation by the Golgi outpost protein TPPP is critical for myelination. Though there is much to learn about how Golgi outposts are regulated, we hope our proteome will help yield future insights.

B.28

Autoimmune regulator dependent alterations in proteome profiles in spermatogonial cells

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Autoimmune Regulator (AIRE) is a gene associated with Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). AIRE is expressed heavily in the thymic epithelial cells and is involved in maintaining self tolerance through regulating the expression of tissue specific antigens. Testis is the most predominant extra-thymic location where a heavy expression of AIRE is reported. Homozygous Aire-deficient male mice were infertile, possibly due to impaired spermatogenesis, deregulated germ cell apoptosis or autoimmunity. We report that AIRE is expressed in the testis of neonatal, adolescent and adult mice. AIRE expression was detected in GFRα+ (spermatogonia), GFRα-/SCP3+ (meiotic) and GFRα-/PGK2+ (post-meiotic) germ cells in mouse testis. GC1-spg, a germ cell derived cell line, did not express AIRE. Retinoic acid induced AIRE expression in GC1-spg cells. Ectopic expression of AIRE in GC1-spg cells using label-free LC-MS/MS identified a total of 371 proteins which were differentially expressed. 100 proteins were up regulated and 271 proteins were down regulated. Data are available via ProteomeXchange with identifier PXD002511. Functional analysis of the differentially expressed proteins showed increased levels of various nucleic acid binding proteins and transcription factors and a decreased level of various cytoskeletal and structural proteins in the Aire over expressing cells as compared to the empty vector transfected controls. The transcripts of a select set of the upregulated proteins were also elevated. However, there was no corresponding decrease in the mRNA levels of the downregulated set of proteins. Molecular function network analysis indicated that AIRE influenced gene expression in GC1-spg cells by acting at multiple levels including transcription, translation, RNA processing, protein transport, protein localization and protein degradation, thus setting the foundation in understanding the functional role of AIRE in germ cell biology.

B.29

Molecular bases of ferroptosis induction by endoperoxides

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Ferroptosis is a novel type of regulated cell death with well-defined biochemical and morphological features. In contrast to apoptosis or necrosis, ferroptotic cell death is dependent on intracellular labile iron and is mediated by the accumulation of reactive oxygen species and lipid hydroperoxides. Ferroptosis has been implicated in cancer cell death as well as in neural degeneration in nervous system pathologies and trauma (Alzheimer and Parkinson's disease, ischemic and hemorrhagic stroke, spinal cord injury). Triggering ferroptosis with small molecules is thus an attractive strategy in cancer, while anti-ferroptotic small molecules may find utility in neurodegenerative disease. It has been well-validated that ferroptosis can be driven by inhibition of cysteine uptake and inactivation of glutathione peroxidase 4 (GPX4), a lipid repair enzyme. Among several classes of ferroptosis inducers is the small molecule endoperoxide FINO2. Recent studies suggest that FINO2 acts via a mechanism distinct from that of other inducers, but its molecular pharmacology remains obscure. We synthesized analogs of FINO2 and designed a probe (Ad-FINO3) wherein activation by ferrous iron produces a reactive intermediate capable of cross-linking nearby proteins. We then used Tandem Mass Tag (TMT) to identify proteins that associate with Ad-FINO3 in a saturable (competeable) fashion. A number of proteins involved in regulation of the redox state of the cells emerged from these experiments as possible candidates for direct targets of endoperoxide mediated ferroptosis initiation. This work was supported by the Dr. Miriam And Sheldon G. Adelson Medical Research Foundation (AMRF), NIH grant R01AI105106 and the UCSF Program for Breakthrough Biomedical Research (PBBR).

B.30

Systematic identification of fat-derived secreted factors acting on the brain

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Organs have highly specialized functions, yet these are closely interconnected to preserve homeostasis within an organism. Organ secreted factors, including proteins, are a means of inter-organ communication and coordinate functions across the organism. Some mammalian secreted factors have been identified, such as the adipose-derived metabolic regulators leptin and adiponectin. However, the identification approaches have not been systematic, and many observations of functional associations across organs remain poorly defined. For example, obesity is a major risk factor for the development of neurological disorders such as dementia, suggesting a close connection between fat tissue and the brain. Nevertheless, signaling mechanisms between these two organs are not well explored. Our aim is to investigate fat-derived factors that may modulate the functions of the nervous system.

Enzyme-catalyzed proximity labeling allows the spatial-proximal protein labeling via a promiscuous enzyme, for subsequent enrichment and identification by mass spectrometry (MS)-based proteomics. We used an evolved highly active biotin ligase, TurboG3, for efficient biotinylation within whole organisms. Drosophila melanogaster strains were engineered to produce TurboG3 localized to the endoplasmic reticulum lumen (TurboG3-kdel) within the fat body. This allows biotinylation of conventionally secreted proteins from this tissue, which would then reach the blood stream and other organs. Drosophila brains were dissected and biotinylated proteins enriched using streptavidin-coated magnetic beads followed by on-bead digestion. Eluted peptide were labeled with tandem mass tags (TMT) for quantitative MS-based proteomics (Q Exactive HF-X). After stringent filtering criteria using unbiotinylated controls, we identified ~100 proteins in the fly brain that are expected to be derived from the fat body. Many of these proteins are uncharacterized, of small size, carry signal peptides, and 70% are evolutionarily conserved. Importantly, cross-referencing this list with RNA-seq data show that the majority of these genes are exclusively expressed in the fly fat body.

Our results revealed fat-derived secreted factors with candidate functions in the Drosophila nervous system. Functional characterization of these proteins will better define the cross-talk between these organs. Importantly, factors conserved in humans could be used for therapeutic interventions and will be critical in future studies aiming to understand the associations between obesity and neurological disorders.

B.31

Improved HLA peptide sequencing accuracy and sensitivity via optimized scoring of database search results and de novo interpretations

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Adaptive immune responses rely partially on cytotoxic T cells to eliminate cells displaying disease-specific peptide antigens on human leukocyte antigen (HLA) class I molecules. If an antigen contains a basic residue, it may be in any position, rather than always at the C-terminus as with tryptic peptides. The binding specificity of each class I HLA allele leads to datasets of peptides of length 8–12 with sequence motifs containing conserved anchor residues, at positions 2 and the C-terminus, that are characteristic of each allele. Our collection of >90 mono-allelic LC-MS/MS datasets, generated with high-resolution HCD fragmentation, includes motifs with anchor residues that are basic, acidic, hydrophobic, or proline. Through study of MS/MS fragmentation in these datasets, scoring in the Spectrum Mill DB-search and de novo MS/MS interpretation algorithms has been optimized to increase the ID rate for DB-searches of individual alleles by 5–80% relative to scoring intended for tryptic peptides. The greatest improvements are for spectra that are rich in internal ions and b-ion series. Revised scoring yields more peptides with similar sequence logos indicating that no anchor motifs are lost and some outliers, likely false-positive IDs, are eliminated. Applying a PSM quality threshold (backbone cleavage score) to require > 4–5 residues of unambiguous sequence substantially helps suppress false-positive ID's. Improved scoring enables maintaining sequencing accuracy and sensitivity in the large search spaces involved in identifying peptides from novel unannotated open reading frames (nuORFs) in the human transcriptome.

B.32

Plasma proteome profiling discovers potential biomarkers of non-alcoholic fatty liver disease

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Non-alcoholic fatty liver disease (NAFLD), defined by accumulation of fat in the liver, affects 25% of the global population. NAFLD is generally asymptomatic, and therefore can gradually progress to cirrhosis, marked by inflammation and tissue scarring of the liver. At present, the transition to this point is not well understood or easily diagnosed. The crucial secretory function of the liver provides the window to monitor protein changes in the blood plasma that reflect disease status. In this study, using our streamlined workflow of plasma proteome profiling, we provide an unbiased and hypothesis-free overview of the total protein changes in the plasma during the progression of NAFLD to cirrhosis in several human cohorts. We investigate global plasma proteome changes in 48 patients with cirrhosis, NAFLD with normal glucose tolerance or NAFLD with diabetes. To obtain in-depth proteome measurements, we coupled our workflow with the recently developed "Boxcar" method. Our results revealed a dramatic shift in the quantitative proteome composition in liver cirrhosis compared to controls, reflected by 57 significantly regulated proteins. These changes reflect dysregulation of proteins associated with thrombosis, immune system regulation, inflammation and homeostasis. In the two NAFLD cohorts, eight proteins were significantly regulated, two of which had already been suggested as potential biomarkers for NAFLD – providing positive controls. Polymeric immunoglobulin receptor (PIGR) and Fructose-bisphosphate aldolase B (ALDOB) were significantly elevated in both the human NAFLD and cirrhosis cohorts, as well as in mouse models, making them potential biomarker candidates of liver damage. Furthermore, our data strongly associates dipeptidyl peptidase 4 (DPP4), a protease which targets glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), to NAFLD and cirrhosis. Inhibitors of DPP4 are among the top selling drugs word wide. Our data also implicate other peptidases in liver disease. Blocking the functions of these proteins may have effects on the metabolic or fibrotic process in NAFLD progression, thus making them interesting targets for therapeutic ramifications. In summary, we found changes in the plasma proteome of patients with NAFLD and cirrhosis using novel scan modes, and report a panel of potential biomarkers for these liver diseases.

B.33

Discovery of Mitochondrial Protease ClpP as a Target for the Anticancer Compounds ONC201 and Related Analogs

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Abstract ONC201 is a first-in-class imipridone molecule currently in clinical trials for the treatment of multiple cancers. Despite enormous clinical potential, the mechanism of action remained unknown until recently. We examined a series of novel ONC201 analogs (TR compounds, Madera Therapeutics) for effects on cell viability and stress signaling in breast and other cancer models. These compounds were found to be $\sim$50–100 times more potent at inhibiting cell proliferation and inducing the integrated stress response (ISR) protein ATF4 than ONC201. Proteomics experiments demonstrated that both ONC201 and TR-57 induced the loss of a large number of mitochondrial proteins. To identify the potential target for ONC201 and TR-57, we immobilized an analog containing an amine linker (TR-80) to agarose beads. Using immobilized TR-80, we identified the human mitochondrial caseinolytic protease P (ClpP) as a specific binding protein by mass spectrometry. Affinity chromatography/drug competition assays showed that the TR compounds bound ClpP with $\sim$10 fold higher affinity than ONC201. Importantly, the peptidase and protease activity of recombinant ClpP was strongly activated by ONC201 and the TR compounds in a dose- and time-dependent manner, with the TR compounds displaying a $\sim$10–100 fold increase in potency than ONC201. SiRNA knockdown of ClpP in SUM159 cells reduced the response to ONC201 and the TR compounds including induction of CHOP, loss of the mitochondrial proteins (TFAM, TUFM) and the cytostatic effects of these compounds. Thus, we report that ClpP directly binds ONC201 and the related TR compounds, and is an important biological target for this class of molecules. Moreover, these studies provide for the first time, a biochemical basis for the difference in efficacy between ONC201 and the TR compounds.

B.34

Functional Versatility of Mycobacterium marinum Type III Polyketide Synthases

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Mycobacterial pathogenesis is hallmarked by lipidic polyketides that decorate the cell envelope and mediate infection. However, factors mediating persistence remain largely unknown. Dynamic cell wall remodeling could facilitate the different pathogenic phases. Recent studies have implicated type III polyketide synthases (PKSs) in cell wall alterations in several bacteria. Comparative genome analysis revealed several type III pks genes in mycobacteria. Mycobacterium marinum genome harbors four type III pkss that largely group into three pks genomic clusters. mmar_2470 and mmar_2474 form a cluster with other type I pkss, while mmar_2190 and mmar_4313 are grouped independently with genes for several polyketide modifiers. Interestingly, two of these unique pks genomic clusters are conserved exclusively in pathogenic species. Cell-free reconstitution assays and high-resolution mass spectrometric analyses revealed the capability of these proteins to accept various monocarboxyl-CoA substrates and extend with dicarboxyl-CoA extender units to biosynthesize a palette of polyketide metabolites. MMAR_2470 and MMAR_2474 proteins utilized two different extenders to biosynthesize methylated polyketide products. Notably, MMAR_2474 proteins displayed unprecedented functional flexibility to biosynthesize alkyl-resorcinol and acyl-phloroglucinol products from the same catalytic core. Structure-based homology modeling, product docking, and mutational studies identified residues that could facilitate the distinctive catalysis of these proteins. Functional investigations in heterologous mycobacterial strain implicated MMAR_2474 protein to be vital for mycobacterial survival in stationary biofilms. Our study thus provides new insights on functional importance of type III PKSs conserved in pathogenic mycobacterial species and delineates mechanistically crucial residue positions that can be modulated to generate a repertoire of unusual biologically active type III polyketides.

B.35

Characterizing and Targeting the Cell Surface Proteome of Hypoxic Pancreatic Cancer

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Low oxygen in tumors, termed hypoxia, leads to poorer patient prognoses. In response to hypoxia, cancer cells activate highly regulated cellular pathways and gene programs, promoting survival, migration, immune privilege, and increased mortality for patients. These hypoxic phenotypes are often present in cancer types with particularly high mortality rates, with pancreatic cancer exhibiting the most severe hypoxia. These hypoxia-specific effects suggest that tumor hypoxia could be leveraged for early stage diagnostics, as well as selective targeting of hypoxia-induced antigens in tumors. To elucidate the effects of hypoxia on the cell surface proteome of pancreatic cancer cells, Panc-1 and Capan-1 cell lines were expanded in heavy and light isotopically labeled SILAC media to allow for quantitative comparison via tryptic LC-MS/MS. We utilized a glycoprotein labeling biotin-hydrazide strategy for selective cell surface protein isolation to identify novel hypoxic cell surface proteins in pancreatic cancer. Within this dataset, we discovered a novel hypoxia-induced pancreatic cancer target, vasorin (VASN). Furthermore, we found that knockdown of VASN decreases the ability of pancreatic cancer cells to survive and proliferate under hypoxia, suggesting VASN serves as an essential protein for pancreatic cancer cell adaptation in hypoxic environments. Through the use of phage-display, we isolated antibody clones against the ectodomain of VASN, and showed that these clones did not bind to the surface of VASN expressing cells. However, immunoprecipitation of the filtered media with our biotinylated antibody clones, and subsequent western blotting with commercial antibodies, revealed our in-house antibody clones recognized a shed portion of the VASN ectodomain. Together, these findings suggest that VASN plays a significant role in the adaptation of pancreatic cancer to hypoxic stress, and that its proteolysis may alter the signaling in hypoxic solid tumors. Identification and characterization of this cleaved form of VASN is critical to understand the role of cleavage in altering signaling, as well as whether this cleavage event is necessary for imparting a growth advantage to cells expressing VASN under hypoxia. Furthermore, identification of cleaved VASN will contribute to the development of diagnostic and therapeutic options for patients with pancreatic cancer.

B.36

Automated Quality Assessment of Chromatographic Peaks in Targeted Proteomics Data using Machine Learning

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Targeted proteomics using mass spectrometry (MS) is a powerful technology used to detect proteins of interest with high quantitative accuracy and reproducibility. Recent advances in the field, have significantly focused on increasing sensitivity and multiplexing capacity, resulting in an increased rate of data production. It is evident that novel analytical tools are necessary to meet the demand for data processing, especially when considering that development and validation requires manual inspection of chromatographic peaks. Such analysis can be time-consuming, prone to inter- and intra-operator variability and limits the throughput of analysis. To address this challenge, we previously developed TargetedMSQC, an R package that facilitates quality control and verification of proteomic peaks in targeted proteomic experiments. The package calculates quality control metrics that describe several aspects of a chromatographic peak, and takes advantage of supervised machine learning algorithms to predict poor chromatography based on training set of peaks annotated by an expert analyst. TargetedMSQC has improved the current quality assessment workflow in our lab. In the latest version, we have enabled additional features to tackle some of the limitations of the original tool. We have extended TargetedMSQC to support integration with Skyline, while further automating the pipeline and improving accuracy of predictions. Using unsupervised clustering we can simplify and expedite the annotation process for creating a training set by extending a single annotation to clusters of similar peaks, thus enhancing quality of user experience. Additionally, heuristic based labeling of data has been included to facilitate annotation of peaks for training. Data engineering is used to further enhance the size and diversity of the training set for improving the model performance. Ultimately, automating the assessment of peak quality offers a more objective and systematic approach for the analysis of targeted mass spectrometry experiments and extends the pipeline towards more robust and accurate analysis.

B.37

Application of TurboID-mediated proximity labeling for mapping a GSK3 kinase signaling network in Arabidopsis

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Protein kinases play important roles in signal transduction and cellular regulation by phosphorylating specific cellular proteins. Identification of these substrate proteins of kinases is crucial for understanding regulatory pathways, but is technically challenging. To catalyze phosphorylation effectively, the interactions between kinases and their substrates are often transient and dynamic, which is a challenge for traditional methods of analyzing protein-protein interactions (PPIs). Recently, proximity labeling (PL) dependent biotin identification approach such as TurboID has been developed to capture both stable and transient PPI in animal system. In this study, we applied TurboID in plant tissues to identify transient and stable interactors of the BIN2 kinase, a GSK3-like kinase acting as a key component of the brassinosteroid (BR) signaling pathway. We generated transgenic Arabidopsis plants expressing a BIN2-YFP-TurboID fusion protein, or a YFP-YFP-TurboID as a control. The sample and control plants were metabolically labelledwith stable isotope 14N and 15N (the order reversed in replicates). After incubating tissueswith 50 μM biotin for 3 h, proteins were extracted from the sample and control plants mixed together at equal amount, and the biotinylated proteins were enriched with streptavidin beads, digested into tryptic peptides and analyzed by LC-MS/MS. For in vivo phosphoproteomics analysis, plants were treated with or without bikinin, a GSK3 inhibitor, to suppress BIN2 activity in plants. Phosphopeptides were enriched using Fe-IMAC StageTip and analyzed by LC-MS/MS. We identified 310 BIN2 interactors including several known BIN2 interactors such as BZR1, BES1, YODA, and HSP90 from BIN2-TurboID experiment. Then, the phosphoproteomic analysis of bikinin-treated plants identified 1705 phosphoproteins that were down-regulated after bikinin treatment. These include 126 of the 310 identified in BIN2-TurboID experiment, which are considered potential BIN2 direct substrates. Our study demonstrates that TurboID coupled with phosphoproteomics study is a powerful tool for identifying kinase-substrate relationship and dissecting regulatory networks in plants.

B.38

MHC-TreASUre_Hunt: a computational pipeline for the identification of MHC-I peptides from mass spectrometry

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The identification of tumor presented MHC-I ligands are integral for the development of T cell-based cancer immunotherapeutics. However, the lack of specific enzymatic cleavage sites, the prevalence of variable post translational modifications and diversity of source proteins makes characterizations of tumor MHC-I peptidomes by mass spectrometry a significant technical challenge. We have developed MHC-TreASUre_Hunt, a computational pipeline purposely designed for the rapid and accurate identification of MHC-I peptides. This is a command line application that performs fast database searches on raw MS spectra. In its default configuration, MHC-TreASUre_Hunt is powered by the ultrafast database search engine, MSFragger, and performs target-decoy analysis using a random forest algorithm trained on spectral features, peptide motifs, and expression data. It processes spectra from both data-dependent (DDA) and data-independent acquisition (DIA) schemes, and has a modular design permitting simplified integration of different database search engines and post processing algorithms for user flexibility. The performance of MHC-treASUre_Hunt was benchmarked on publicly available DIA spectra of the HEK293(HLA-A*03:01, HLA-B*07:01, HLA-C*07:01) and Maver-1(HLA-A*24:02, HLA-A*26:01, HLA-B*38:01, HLA-B*44:02, HLA-C*05:01, HLA-C*12:03) MHC-I peptidome. In both cases, the majority of peptides previously identified were confirmed (57% Maver-1, 65% HEK293). Furthermore, MHC-TreASUre_Hunt made an additional 1,979 novel peptide identifications in the HEK dataset and 2,048 in the Maver-1 dataset. Logo plot analysis of the peptides identified by MHC-TreASUre_Hunt show that they adhere to the expected binding motifs, and a vast majority are predicted to bind to at least one of the expressed HLA alleles (87% Maver-1, 88% HEK293). MHC-TreASUre_Hunt is easily deployed on computational clusters allowing for expansive searches to be completed in as little as 30 minutes.

B.39

Identification of spatiotemporally resolved GPCR protein interaction networks regulating receptor function

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G protein-coupled receptors (GPCRs) represent the largest family of signaling receptors and drug targets. Following ligand-induced activation of GPCRs signal transduction is mediated by protein interaction networks operating on short timescales and across multiple cellular locations. While temporal dynamics of protein interactions have been previously characterized, a major challenge remains largely unmet: how to interrogate the protein interaction networks engaged by GPCRs while capturing both their spatial and temporal context.

Here, we developed a novel analytical approach combining APEX-based proximity labeling with global and targeted, quantitative proteomics and a system of spatial references, which delivers, with sub-minute temporal resolution, protein interaction networks and subcellular location for a target protein of interest.

We applied this approach to interrogate how protein interaction networks engaged by GPCRs respond to ligand-induced activation. We not only validated capture of proteins known to interact with the receptors, including those with transient or low affinity interactions, but demonstrated that our pipeline can be used to discover new network components regulating receptor function. Specifically, we identified TOM1 and WWP2 as members of a previously unrecognized ubiquitin network that controls homeostatic down-regulation of the delta opioid receptors. Recently, we extended this approach to examine the protein interaction networks engaged by the mu-type opioid (MOR) after stimulation with full, partial, and G protein functional selective agonists. The proximity labeling data did not only provide information about the interaction networks engaged by MOR depending on the agonist, but also how the different agonists influence intracellular trafficking of the receptor.

In summary, we described and validated a methodology that extends the utility of APEX-mediated proximity labeling to achieve spatiotemporally resolved protein network interrogation in intact cells.

B.40

Accelerating DIA Studies to Extend Workflow Utility, Using Fast Microflow LC Gradients

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Proteomics has typically been done using nanoflow LC for sensitivity but the time to results slow. With higher flow rates, sample can be loaded faster, trap/column can be washed and equilibrated faster, and gradients are formed faster, allowing much faster run times to be achieved. Microflow LC has been used increasing in quantitative proteomics in combination with SWATH® Acquisition, to provide better robustness and higher throughput when measuring larger sample cohorts. Here, the sensitivity impact of microflow as well as the impact of gradient length on protein quantitation results with DIA was explored. Nanoflow and microflow LC was performed on the SCIEX TripleTOF® 6600 System with OptiFlow® Source using the nanoLC™ 425 system. Trap-elute workflow was used and a range of gradient lengths were explored, and key acquisition parameters for SWATH Acquisition were varied to optimize for the much faster run times. Data was processed with SWATH 2.0 microapp in PeakView® Software 2.2 and OneOmics™ in SCIEX Cloud. Using complex digested cell lysates, SWATH Acquisition experiments were performed using gradient lengths ranging from 5–45 mins for microflow and 1 and 2 hour gradients for nanoflow and protein quantitation results were assessed. Moving to microflow, similar quantitative results were obtained when ~4x more protein was used confirming the feasibility of using microflow for many proteomics applications. Fast MS/MS acquisition rates were found to be critical because this enabled more, smaller variable Q1 windows to improve S/N for quantitation. Even with the fastest microflow gradients, methods with 60–100 windows with very fast accumulation times of 15 msec improved results. Optimization results will be presented. Optimized methods were then used to compare quality of quantitation between long and shortened gradients and similar fold change values were measured confirming accelerated gradients can be used for industrialized quantitative proteomics.

B.41

eIF4E selective control for metabolic fitness and healthspan

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Health- and life-span are coupled to nutrient availability and dietary composition. From yeast to mammals, the mechanistic target of rapamycin (mTOR) pathway tightly regulates the coordination of nutrient and energy availability for proper translational control. An outstanding question is how translational cues allow for physiological adaptations to nutrient availability in cell-autonomous and non-autonomous manners to maintain metabolic fitness and healthspan in vivo. Previously, we identified that the major cap-binding protein for mRNA translation downstream of mTOR in complex 1, eIF4E, is maintained at excessive levels for normal growth requirements and mammalian development. This begs the question, as to which cellular processes and selective transcripts may rely on eIF4E at 100% expression levels. As nutrient abundance drives anabolic processes, including protein synthesis, we set out to test how eIF4E levels influence metabolic homeostasis linked to diet and the cellular environment. We find that mice with a 50% loss of eIF4E expression show resistance to diet-induced obesity with improved glucose tolerance and a decrease in hepatic steatosis. We further demonstrate tissue specific roles for eIF4E activity in regulating full-body metabolic adaptations to diet and thermogenic stress. Currently, we are identifying translational targets that may rely on eIF4E dosage responsible for rewiring metabolic pathways observed in eIF4E haploinsufficient mice. Together, our data reveal that diminished eIF4E levels can promote enhanced metabolic fitness and may be a potential therapeutic target for the treatment of obesity-related disease.

B.42

Profiling of phosphorylation sites of Arabidopsis microsomes with PolyMAC-Ti nanoparticle bead enrichment and mass spectrometry

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Protein phosphorylation plays important roles in signal transduction of cells in response to internal or external stimulus. Identification of phosphopeptides and phosphorylation sites is therefore critical in the functional characterization of protein phosphorylation catalyzed by specific kinases on specific amino acid residues. We previously utilized TiO_2 and $Zr4+$ to enrich phosphopeptides from membrane-shaved leaf microsomes of Arabidopsis followed by mass spectrometry (MS) analysis yield identification of 18 sites of 15 proteins. In order to identify more phosphopeptides, PolyMAC-Ti (TYMORA) beads was introduced for phosphopeptide enrichment. Arabidopsis seedlings were suspension-cultured. Plant samples were harvested and subjected to microsome isolation. Membrane shaving of isolated microsomes was carried out by trypsin digestion. Phosphopeptides were enriched using PolyMAC-Ti beads followed by Orbitrap LC-MS/MS (Thermo Scientific) analysis. By using PolyMAC-Ti bead for enrichment, we identified some previously identified phosphopeptides, and some newly identified ones (i.e. ribosomal proteins).

B.43

Characterizing the role of ribosomal protein phosphorylation in control of gene regulation and cell fate

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A central question in cell biology is how gene expression is temporally and spatially regulated in response to external signals. Historically, the ribosome has been viewed as a mere participant in gene regulation; however, recent work has suggested that "specialized ribosomes" with unique composition or activity may regulate gene expression by selectively translating subsets of mRNAs[1–3]. It remains unknown whether post-translational modifications of ribosomal proteins (RPs) may provide an additional layer of heterogeneity that allows for dynamic regulation of ribosomal activity, particularly downstream of external cell signaling cues. To address this question, I will present my work developing a targeted, kinase-centric approach and an unbiased, substrate-centric approach to study and functionally characterize RP phosphorylation during cellular differentiation. For the former approach, we have assembled a list of candidate kinases using our lab's recently mapped "ribo-interactome": all proteins that interact with mammalian ribosomes[3]. We have confirmed that several of these kinases from the ribo-interactome bind directly to the ribosome. Next, we are employing a technology pioneered by the Shokat lab to engineer kinases to use bulky adenosine triphosphate (ATP) analogs that will allow us to identify specific substrates of the kinase of interest and the precise sites of modification by mass spectrometry[4]. For the substrate-centric approach, we have employed mass spectrometry-based proteomics to develop a global map of all RP phosphorylation sites. Presently, we have identified 18 phosphosites in RPs from mouse embryonic stem cell ribosomes, and we are following these sites to determine whether they change during differentiation along selective cellular lineages. This work will provide insight into the dynamics of ribosome activity mediated by post-translational modification of the ribosome and its functional consequences for cell differentiation, signaling, and gene expression.

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B.44

HYPERsol: flash-frozen results from archival FFPE samples for clinical proteomics

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● Formalin fixed paraffin embedding (FFPE) is a decades-old sample preparation technique common in experimental research and medicine. FFPE samples can be stored indefinitely at room temperature, resulting in an exceptionally large and rich worldwide collection. Despite its potential to significantly impact medicine, proteomic analysis of FFPE samples has lagged. Traditionally, samples are first laboriously deparaffinized with often-toxic organic solvents. Subsequent protein extraction is extremely critical but no consensus has been reached as to an optimal protocol.

● Here we present High-Yield Protein Extraction and Recovery by direct SOLubilization (HYPERsol), a standardized one-pot workflow for FFPE proteomics. The HYPERsol workflow combines the HYPERsol buffer (5% SDS), Covaris AFA ultrasonication and ProtiFi S-Trap sample processing. HYPERsol eliminates deparaffinization and exhaustively solubilizes entire FFPE samples, including the paraffin, yielding ID rates and quantification values very similar to flash-frozen (R = 0.94).

● To benchmark HYPERsol performance, we compared tissue treated in parallel either by flash-freezing or FFPE according to standard histopathology procedures. All samples were extracted with SDS using probe sonication or Covaris AFA ultrasonication. SDS was removed by standard precipitation or S-Traps. Protein identification rates and reproducibility were evaluated after analysis on a Thermo QE HF-X or Fusion mass spectrometer.

● Compared to standard procedures, the use of S-Traps resulted in significant increases in peptide ($\geq$30%) and protein identification rate ($\geq$20% increase) with greater reproducibility. The use of AFA decreased hands-on time, increased ID rates an additional 6%–8% and significantly increased protein yield from FFPE samples (80%–$\geq$200%). The HYPERsol combination yielded ID rates comparable to those obtained from fresh frozen tissue (101%/ 97% ID rate for peptides/proteins) while eliminating toxic xylene and saving approximately 5–6 hrs in sample processing by eliminating organic deparaffinization.

● HYPERsol solves the problem of extraction bias and achieves the goal of reproducible standardized protein recovery from FFPE samples in a workflow suited to automated, high-throughput analyses. We anticipate this workflow will assist to usher in a new era of clinical proteomics.

B.45

Tag-free rapid enrichment of ribosome-associated proteins across cell types, tissues, and species.

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The ribosome has traditionally been viewed as a compositionally constant molecular machine that passively translates genetic information into functional proteins. However, recent work from our lab has shown that there are specialized ribosomes that are tailored to translate specific transcripts, allowing for gene regulation at the translation level. Such specialization can be achieved through compositional differences in the translation machinery, such as through differences in ribosome-associated proteins (RAPs).

Previously, our lab developed an endogenous FLAG-IP based ribosome pulldown method to identify hundreds of RAPs from different functional classes, which potentially link translation directly to specific cellular processes, such as metabolism. However, this method is limited to samples that are amenable to genetic editing to introduce an epitope tag.

We developed a tag-free method called RAP IDentification by Affinity to SulfHydryl-charged resin (RAPIDASH) to isolate RAPs based on the biophysical properties of the ribosome. We combined density ultracentrifugation with affinity chromatography, selecting for high molecular weight, RNA containing complexes (i.e. mostly ribosomes), while preserving the interacting proteins. Using this method, we have found potential tissue-specific RAPs from the forebrain, limbs, and liver of wild type mouse embryos. This method will enable the identification of RAPs from various biological samples and enable new insight into ribosome specialization across evolution.