## C.1

# Purification and Proteomic Mapping of Murine Liver 19S Proteasome Complexes

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Proteasome complexes play an indispensable role in maintaining cell homeostasis. 19S proteasome complexes, also known as the regulatory particles, are critical components of the 26S proteasome system by governing substrates entry and tuning the catalytic activities of the 20S proteasomes. Multiple studies on proteasome functions reported accumulatively a total of 22 mammalian 19S subunits forming two sub-complexes, the base and the lid. Unfortunately, a comprehensive proteomic blueprint of mammalian 19S complexes remains scarce; which has made it difficult to advance our understanding of the dynamics of proteasome function and substrate specificity. A key limitation is the technology challenges encountered in order to obtain purified 19S proteasome complexes. Accordingly, we conducted a study to establish a 19S proteasome purification strategy from murine liver using multidimensional chromatography. DEAE-Sepharose ion-exchange chromatography was showed as a key step in purification and significant efforts have been made to optimize this procedure. Through numerous trial-and-error pilot studies, the elution condition was finalized to a 14 column volume linear NaCl gradient from 0% buffer B to 55% buffer B (buffer A: 20 mM Tris, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, 5% glycerol; buffer B: 20 mM Tris, 1 mM $MgCl_2$, 450 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, 5% glycerol); and the19S proteasome concentrated fractions were recovered at 35% buffer B with a conductivity of 18.5 mS/cm. These improvements empowered an enhanced enrichment factor by at least 5 fold of what is obtained using a method that is previously reported. The structural intactness and purity of the 19S complexes were verified by immunoblotting with antibodies against subunit components of the base (Rpt4), the lid (Rpn7) and the 20S proteasomes ($\alpha$3), respectively. The functional viability of the 19S proteasome complex was assured with its ability to boost 20S peptidase activities by 3 fold. With LC-MS/MS analysis, 21 out of the 22 reported mammalian 19S subunits were identified with high confidence. Rpn14 was not identified in this study indicating its substoichiometric assembly. The heterogeneity of the 19S was preserved during the purification. This documents the first proteomic map of the liver 19S complexes.

## C.2

# Complementary Fragmentations of Peptides from Lys-C and Lys-N Digests

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Tryptic digest is the most commonly used method to cleave proteins into smaller peptides amiable for online LCMSMS. MSMS by collision-induced dissociation (CID) of the tryptic peptides produce many y-type C-terminal containing product ions. To sequence peptides and to localize possible posttranslational modifications on peptides, observation of complete peptide bond cleavages are desirable. However, due to the nature of CID fragmentation, many sequence-information carrying ions are not detected in tryptic peptides. In electron capture dissociation (ECD) or electron transfer dissociation (ETD) experiments, a larger peptide capable of carrying 3 or more charges is preferred and tryptic peptides with lower charge states produce many z-type of ions due to the charge retention at the C-terminus. We have investigated the fragmentation behavior of peptides produced by Lys-C and Lys-N digest. Lys-C peptides are larger than tryptic peptides with a Lysine at the C-terminus, while Lysine residues are at N-terminus for Lys-N peptides. Preliminary data show the equivalent Lys-N peptides produce more b-ions in CID and more c-ions in ECD than that for Lys-C peptides. By carrying out both Lys-C and Lys-C digests, we expect to obtain more peptide/protein identification and better PTM site assignments. In-gel Lys-N digest method will also be developed.

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## C.3

# Construction of a Peptide Database and Its Applications for LCMS Based Proteomics

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Proteomic discoveries using LCMS based methods are often hindered by redundant peptide identifications as well as analytical difficulties that prevent the identification of low abundant and interfering peptides. In order to circumvent these limitations, we developed a method that matches features in an LCMS map using [m/z, tR, z] values to previously identified peptides stored in a database.

In this presentation, we demonstrate a workflow for building a peptide database containing [m/z, tR, normalized tR, z] with corresponding Mascot search results using the human breast carcinoma cell line T47D as a model system. Subsequently, from this database a reference map with a format of [theoretical m/z, normalized tR, z, peptide sequence, protein ID] was generated. Peptide identification in LCMS maps occurs by aligning the features found in the LCMS maps to their identical features found in the reference map within an m/z and tR window (40 ppm, 3 minutes), and then extracting the peptide information. This strategy for peptide identification was validated by aligning the reference map to an IDA precursor map of T47D peptides with 98.3% of the features from the IDA data aligning correctly to the reference map. Additionally, when the reference map was aligned to the LCMS map for T47D peptides, the peptide identification efficiency increased 3fold in comparison to the IDA experiment for the same sample (474 identified peptides for IDA vs. 1509 for reference map). Finally, this approach was successfully applied to identify and quantify differentially spiked nonhuman proteins in T47D samples. The demonstrated advantages of using a reference map in this study include: identification of peptides without sequencing, identification of low abundant and interfering peptides, and facilitation of quantitative analysis by selectively extracting peptides of interest.

## C.4

# Seasonal and Stage-Specific Protein Expression in Liver of Golden-Mantled Ground Squirrel, a Large-Scale Quantitative Analysis

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Studies of hibernation in the patterns of mRNA and protein expression in golden-mantled ground squirrel to date demonstrate that a minority of all gene products alter seasonally for the animals to achieve, maintain, and survive the hibernation season. A previous study at the mRNA level revealed that changes were almost exclusively seasonal. We later performed a proteomic study to compare ~900 2D gel protein spots from 9 summer livers with those of 9 animals entering torpor, and found that less than 10% of the protein complement underwent a change in seasonal steady-state level, indicating that most of the animals' normal biochemical function is maintained, and a minor portion of the proteome contributes to a seasonal transition that results in a profoundly protected winter phenotype. But, in addition to summer to winter changes in protein patterns, the dynamic and repeated winter season cycling between torpid and aroused states suggests a separate set of changes, possibly at the more acute level of post-translational modification of proteins. A current method in quantitative proteomics, DIGE (Difference Gel Electrophoresis), enables our investigation of changes in abundance and/or modification of the most abundant and soluble protein gene products in a given sample, separated by 2D gel electrophoresis. We compared proteins in liver extracts taken from ground squirrels in each of 6 stages in the hibernator's year, *i.e.*, summer (SA) and 5 stages of the winter: entrance into torpor (Ent), early torpor (ET), late torpor (LT), arousing from torpor (Ar), and interbout aroused (IBA) to examine and quantify the predicted changes both seasonally and among the winter stages for 2000–2500 separable protein spots, about 1600 of which we estimate to be useful for quantitative analysis. The DIGE method allows for analysis of larger proteomic sample sizes than previously possible and consequently more powerful statistical discernment. Sample sizes were as follows: $n = 12$ for SA, Ent, ET, and IBA; $n = 6$ for LT and Ar. Hundreds of spots that demonstrated large and/or significant fold changes (*i.e.*, $>2$ with p value after FDR $<0.05$ and any fold change with FDR $<0.02$) were robotically picked, digested with trypsin and analyzed for identification by LC-MS/MS. The results of this experiment and their functional implications for the hibernating phenotype will be discussed.

## C.5

# Protein N-Myristoylation and Prenylation in an Insect Cell-Free Protein Synthesis System and Their Identification by Mass Spectrometry

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Protein N-myristoylation and prenylation are the important lipid modifications of proteins, and they play crucial roles in regulating reversible protein-membrane and protein-protein interactions. Metabolic labeling is an effective strategy for analysis of these modifications. However, it cannot be used to identify the exact location of the modification. In this study, we tried to establish a novel and effective strategy to analyze these protein modifications by combining a cell-free protein synthesis system from *Spodoptera frugiperda* insect cells, Transdirect *insect cell*, with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and MALDI-quadrupole ion trap (QIT)-TOF MS.

Epitope-tagged truncated human gelsolin (tGelsolin) and human rhoC, which are natural N-myristoylated and geranylgeranylated protein respectively, were synthesized using the insect cell-free protein synthesis system with or without addition of a specific substrate for each protein modification, such as myristoyl-CoA, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate. The affinity-purified proteins were reduced and alkylated and then digested with trypsin or lysyl endopeptidase.

In the case of the tGelsolin, when myristoyl-CoA was added to the translation reaction mixture, a peak corresponding to the N-myristoylated tryptic peptide was clearly observed, whereas this peak was hardly detected when myristoyl-CoA was not added. The peptide peak was identified as the N-myristoylated peptide fragment by tandem MS analysis using MALDI-QIT-TOF MS. We also constructed the Gly-2 to Ala (G2A) mutant, in which the N-myristoylation motif was disrupted, and analyzed by the same strategy. N-myristoylation did not occur on the G2A mutant, whereas this mutant was found to be N-acetylated after removal of the initiating Met.

In the case of the rhoC, it was found that it could serve as a substrate for both prenyltransferases in the presence of either farnesyl or geranylgeranyl pyrophosphate, whereas geranylgeranylation was exclusively observed when both prenyl pyrophosphates were added to the translation reaction mixture. These results indicated that the rhoC protein was a better substrate for geranylgeranyltransferase I than for farnesyltransferase.

These results indicate that the insect cell-free protein synthesis system, as is the case with the rabbit reticulocyte lysate system, possesses N-myristoyltransferases and both prenyltransferases. In conclusion, a combination of the cell-free protein synthesis system with MS is an effective strategy to analyze protein N-myristoylation and prenylation.

## C.6

# Protein Sulfation in *Drosophila* Kc Cells: Which Amino Acids and Which Proteins?

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In terms of studying covalent modifications, phosphorylation is traditionally the most studied post translational modification. It is believed that most regulatory processes are controlled to some extent by covalent modifications.

In recent years other modifications have become of greater interest among the biological sciences. The differential modifications on histones, such as acetylations and methylations seem to become a field on its own, pushing the limits of current instrumentation and software. The discovery of the ubiquitin system, a small protein that covalently modifies proteins, was awarded the Nobel Prize in 2004, which shows the importance of emerging PTM in understanding the underlying biological mechanisms.

Modification repositories such as Unimod, report over 500 modifications both naturally occurring and user induced. Some of these include the sulfation of tyrosine, serine and threonine. Sulfation, the covalent labeling of a sulfate group to an hydroxy group, has been explored to some extent, with a focus on tyrosine. Tyrosine sulfation was first described in 1954, but little is known about the overall role or importance of this modification as compared to phosphorylation for instance.

The latest generation mass spectrometers with their high accuracy and fast acquisition rates are very suitable to explore peptide and protein modifications. Sulfation of serine and threonine has been first described in this context in 2004.

Previously we reported the identification of sulfated peptides as part of a large phosphopeptide study in *Drosophila* Kc cells. These studies showed that like phosphorylation, serine is the 'preferred' amino acid for sulfation.

This work described here will not only deal with type of peptides purified as well as strategies how to distinguish between phosphorylation and sulfation, but also with the preliminary study in the sulfated protein identification reveal many interesting findings. Initial indications show that overwhelmingly, these proteins are involved in binding processes to both proteins and nucleic acids.

## C.7

# Dissecting the Role of 24S-Hydroxycholesterol in Cholesterol Homeostasis in Neurons

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24S-Hydroxycholesterol (24OHC) is a metabolite of cholesterol uniquely formed in brain, usually in neurons, in a reaction catalyzed by the brain specific cytochrome P450 (CYP), 46A1. 24OHC is a biologically active molecule which behaves as a ligand to the liver X receptor (LXR), the $\beta$ form of which is also expressed in neurons, and as an antagonist to SREBPs. SREBP2 is a transcription factors which activate all the genes required to produce cholesterol, while SREBP1c activates genes required to convert acetyl CoA to long chain fatty acids. The interplay between 24OHC, SREBPs and LXR is effected by LXR being a transcription factor for SREBPs. In the current study we have sought to investigate the relationship between 24OHC, LXR, SREBPs and cholesterol homeostasis using a proteomic approach.

Cortical neurons 108 cells/plate were treated with (a) 24OHC using (2-hydroxypropyl)-$\beta$-cyclodextrin (HPBCD) as vehicle, (b) cholesterol with HPBCD, (c) HPBCD alone, and (d) T1317, an LXR agonist. Cells were lysed, proteins digested and peptides isotopically labeled using iTRAQ reagents. The labeled peptides were then combined and analyzed by offline 2D LCMS/MS. Data was searched and relative quantification information on protein abundance determined using Mascot from Matrix Science.

Like 24OHC, cholesterol is an antagonist to SREBPs, but is not an LXR ligand. On the other hand, T1317 is a LXR ligand, but not an antagonist to SREBPs. Thus, by monitoring the changes in protein expression induced by these small molecules we can decipher the competing effects of 24OHC on SREBPs and LXR.

To summarize the results, 24OHC was found to downregulate the expression of enzymes of the cholesterol synthesis pathway *i.e.*, SREBP2 regulation, but upregulate the expression of apoE (LXR regulation), while the expression of enzymes in the fatty acid synthesis pathway were not effected (SREBP1c regulation). Thus in times of cholesterol excess, cholesterol will become metabolised to 24OHC which will, through SREBP2 regulation, reduce cholesterol synthesis, while upregulate apoE synthesis via LXR activation leading to cholesterol storage, and thereby restoration of cholesterol balance.

## C.8

# Combinations of Post-Translational Modifications of Human Histone H3 Revealed by Tandem FTICR/ECD Mass Spectrometry

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It has been suggested that chromatin-involved epigenetic mechanisms affect the accessibility of effector proteins and regulate important downstream cellular activities via modifications of chromatin structure resulting from combinatorial covalent modifications of histones. Despite its significance, comprehensive characterization of modifications on human histone H3 remains a challenge for both traditional peptide-based and recently developed intact protein based mass spectrometry because of the overwhelming number of modifications on it.

We characterized human histone H3 by enzymatically digesting it into large peptides between 3K and 6K Dalton. Individual peptides and their modification variants were isolated by a combination of chromatography and ion selection in the mass spectrometer. The isolated ions were subjected to electron capture dissociation (ECD). An interactive algorithm was designed and employed to elucidate the PTM combinations from several thousands of mathematically possible assignments. The combinations of modifications and their corresponding occupancies were obtained. Such information provides a solid foundation for further comprehensive studies on biological function of chromatin. This study also demonstrates the advantages of our strategy to use large peptides: (1) ECD is capable of dissociating them to generate nearly complete sequence coverage and elucidating very complicated combinations of modifications. (2) The sensitivity at large peptide level is much higher than that for the whole intact protein of H3.

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## C.9

# Proteomics of Chondrosarcoma: Identification of Key-Proteins

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Articular cartilage consists exclusively of chondrocytes embedded into a dense extracellular matrix, mostly made of collagens and proteoglycans (aggrecan). These macromolecules have so far impaired the detailed two-dimensional electrophoresis-based proteomic analysis of this tissue. A reliable method for a direct proteomic characterization of human cartilage has been recently developed in our laboratory. The material was minced into fine tissue sheets, proteins were extracted at high ionic strength and proteoglycans were precipitated with 1% (w/v) cetylpyridinium chloride. Following chloroform/methanol precipitation, the pellet was resuspended in a urea, thiourea, CHAPS and Triton X-100 mixture and subjected to two-dimensional electrophoresis analysis (first dimension, IPG strip of pH range 4.5–8.0; second dimension 12% w/v acrylamide). Under these conditions, more than 600 protein states were reproducibly separated after silver staining from 500 mg articular cartilage. The selected proteins were digested by trypsin, analyzed by MALDI-TOF mass spectrometry and identified from databases (MSDB, Swiss-Prot). This approach has been used to investigate benign and cancerous cartilage tumors, chondroma and chondrosarcoma, respectively. Although chondrosarcoma is a relatively rare disease, it is a very aggressive pathology, with a 5-year survival rate as low as 10% due to a very high metastatic activity. Therefore it is necessary to discover protein markers to early diagnose the disease and to better understand the molecular basis of the cancer process. Samples from articular cartilage, 8 chondroma, 18 conventional chondrosarcoma and 7 dedifferentiated or unconventional chondrosarcoma were obtained from local hospitals and subjected to proteomic comparison. Three protein spots (MQ1, MQ2, MQ3) were found to vary quantitatively from one group of tissue to another and were identified by peptide mass spectrometry after trypsic digestion. Further quantitation was performed by western blot after antibody generation. MQ1 was found to be over-expressed in benign tumours and low grade conventional chondrosarcoma, compared to articular cartilage, but lost in unconventional or dedifferentiated chondrosarcoma. We are currently studying a possible role of MQ1 in the control of chondrocyte phenotype.

MQ2 and MQ3 correspond to two very closely related polypeptides exhibiting 83% sequence homology. However, MQ2 was found mostly in healthy cartilage and chondroma (8/8 chondroma, 3/18 conventional chondrosarcoma), while MQ3 was detected specifically in cancerous tumours (1/8 chondroma, 23/25 cancerous tumours). Therefore, MQ2 and MQ3 might emerge as useful tools for diagnosis of conventional chondrosarcoma. The functional involvement of MQ2 and MQ3 in angiogenesis and cell migration is now investigated.

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## C.10

# Neuroproteomics of Chromaffin Secretory Vesicles Reveals Protein Systems Utilized for Peptide Neurotransmitter Production and Secretion

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Regulated secretory vesicles produce, store, and secrete active peptide neurotransmitters that function in cell-cell communication. To gain knowledge of the protein systems involved in secretory vesicle functions for neurotransmission, we analyzed proteins in the soluble and membrane fractions of these dense core secretory vesicles purified from neuroendocrine chromaffin cells. Chromaffin secretory vesicles have been investigated as an excellent model for understanding neurotransmitter synthesizing enzymes in brain. In the first phase of this study, soluble and membrane fractions of these vesicles were subjected to SDS-PAGE separation, and proteins from systematically sectioned gel lanes were identified by microcapillary LC-MS/MS ($\mu$LC-MS/MS) of tryptic peptides. The identified proteins revealed functional categories of prohormones, proteases, catecholamine neurotransmitter metabolism, protein folding, redox regulation, ATPases, calcium regulation, signaling components, exocytotic mechanisms, and related functions. Chromogranin A, B, and C represent the most highly abundant proteins in both soluble and membrane components of the secretory vesicles. In the second phase of this study, we removed abundant chromogranins from both soluble and membrane fractions of isolated secretory vesicles to allow the analysis of lower abundant proteins. The soluble protein fraction was separated by gel filtration into several fractions that were subjected to trypsin digestion and triplicate nano-LC-MS/MS analyses. The membrane fraction was extracted and subjected to 1-D and 2-D nano-LC-MS/MS analysis. In addition, we isolated low molecular weight peptides (less than 10kDa) for nano-LC-MS/MS. These proteins were analyzed by nano-LC-MS/MS of tryptic peptides with bioinformatic evaluations appropriate for neuropeptides. The results of these studies assisted in the identification of low abundance proteins previously unidentified in these vesicles as well as neuropeptides from the low molecular weight pool. Combined results demonstrate the intravesicular protein environment that support the biosynthesis and secretion of active peptide neurotransmitters.

## C.11

# Bioinformatic Determination of Protein Isoform Representation of Tryptic Peptides from Mass Spectrometry Analysis: Designation of RAB Isoforms in Secretory Vesicles

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Determination of isoform members of a designated protein family Identification of peptides from mass spectrometry data involves direct comparisons between the experimentally observed fragment ions and predicted fragments based on known current protein sequence database analyses. However, tryptic peptide fragments representing homologous domains of protein isoforms are not sufficient to define specific isoforms. Only tryptic peptide fragment sequences derived from unique domains of the protein isoform can indicate the specific isoform. In contrast, tryptic peptides derived from homologous domains of protein members can predict the protein family, but not the specific isoform. It is, therefore, critical for current bioinformatic programs to analyze tryptic peptides in a manner that can distinguish predictions for specific protein isoforms, compared to predictions for homologous members of a protein family based on tryptic peptide data from shared domains. A challenge currently exists to determine isoform identity of related members of one protein family. While common domains can indicate the presence of that family, it may be insufficient to distinguish between isoforms. Current bioinformatic tools available for protein identification do not distinguish such isoforms. For these reasons, we have developed as part of identification pipeline, a method to positively identify unique isoforms. Our proteomic studies of secretory vesicle proteins (from bovine adrenal medulla) initially suggested numerous isoforms of Rab proteins from Sequest based analyses. However, these analyses provided prediction for specific Rab isoforms, when, in fact, the tryptic peptide data represented homologous domains of multiple Rab members. To define the specific Rab isoform, compared to multiple candidate Rab members, we subjected the tryptic peptide sequence data to analyses by clustering methods using BLASTCLUST and performed comparisons against UniProt90 to groups of proteins identified from the same or closely related families. The peptides used to identify these proteins were aligned back to the predicted full-length Rab protein members. Through this alignment, we are able to extract the peptides necessary to uniquely identify a particular Rab isoform. We subsequently selected the minimum set of peptides that can uniquely identify one Rab isoform from another. This process continued so that tryptic peptides were organized to define specific Rab isoforms and to define subgroups of Rab isoforms in the sample. This approach for isoform analyses of parent proteins from which tryptic peptides are derived for mass spectrometry-based identification will facilitate identification of specific protein isoforms and homologous members of designated protein families.

## C.12

## Comparison of In-House and Commercial Software for Analysis of MALDI-TOF-MS Spectra of Whole Cell Lysates for Bacterial Identification

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We created a MySQL program to analyze MALDI-TOF data of whole cell lysates of hundreds of mostly pathogenic strains of *Escherichia coli*, *Salmonella enterica* and species of *Campylobacter* and created a database for easy retrieval and additional analyses. The web interface created for the database provides a means for any operating system (Windows, Unix and *nix systems, Macs) to access and analyze the data easily. Data were generated by methods reported previously. A Boolean approach to dataset comparison was developed to indicate similarities in datasets for bacterial species identification. Data generated with two different MALDI-TOF instruments (Bruker Reflex II and Bruker Microflex) were compared. The same datasets were analyzed also by a commercial software package for species differentiation (Bruker Biotyper). Relevance to available online protein identification will be discussed.

## C.13

## Apolipoprotein L I mediates IFN-$\gamma$ Stimulation of Normal Human Bronchial Epithelial Cells: A Proteomics Approach

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Airway epithelial cell functions not only as a physical barrier, but also a regulator of airway pathophysiology. Chronic obstructive pulmonary disease and acute lung injury are associated with bronchial epithelial cell activation and cytokine production. The present study investigated the effects of IFN-$\gamma$ on normal human bronchial epithelial cells using proteomics approach. Epithelial cells stimulated with IFN-$\gamma$ lost their polygonal shape and appeared as drawn-out cells with long extensions. We studied differential protein expression profiles of epithelial cells with and without IFN-$\gamma$ treatment. Total proteins from control and IFN-$\gamma$-stimulated epithelial cells were separated by two-dimensional gel electrophoresis, and proteins with significant changes in expression levels were identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS/MS). IFN-$\gamma$ markedly altered the levels of many proteins including IFN-induced 35 kDa protein (IFP-35), IFP-53, serpin B1, N-myc downstream-regulated gene-1 (NDRG-1), tapasin isoform 1, apolipoprotein L (ApoL) II, gamma-actin, keratin 10, and tropomyosin 3. Interestingly, we found that the whole ApoL family (ApoL I–VI, except ApoL V) was dramatically up-regulated by IFN-$\gamma$ using RT-PCR analysis. ApoL I siRNA pretreatment of epithelial cells blocked IFN-$\gamma$-induced morphological changes, suggesting a role of ApoL I in human epithelial cell cytoskeleton rearrangement. These results reveal that IFN-$\gamma$ can markedly alter the proteome of human bronchial epithelial cells, and ApoL I may be involved in bronchial epithelial cell activation and airway inflammation.

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## C.14

## Targeted Mass Spectrometric Strategy for Global Mapping of Ubiquitination on Proteins

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Protein modification by ubiquitin is an important event that represents a highly versatile means for regulation of protein function, including targeting for degradation, proteolytic activation and intracellular localization. Hence, mapping of protein ubiquitination sites is crucial for elucidation of its mechanisms yielding insights into cell regulation. Recently, global mass spectrometry approaches have been employed for determination of ubiquitination sites, however multiple analysis are often required to obtain more complete characterization of modification sites. Even targeting modified peptides by accurate mass inclusion lists do not always detect all sites. Presented here is a high-throughput targeted mass spectrometric approach based on Multiple Reaction Monitoring (MRM) which enabled complete mapping of ubiquitination sites on a protein.

## C.15

# Phosphoprotein Identification and Phosphorylation Site Assignment Using Different Mass Spectrometers and Search Engines

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Phosphorylation is probably one of the most frequent posttranslational modifications of proteins. In a cell, about 30% of the total proteins are phosphorylated in any given condition. Protein phosphorylation-dephosphorylation events play a primordial role in almost all aspects of cell function including signal transduction, cell cycle or apoptosis.

The activation of the immune response mediated by T lymphocytes also carries different protein phosphorylation-dephosphorylation events. In order to understand the functionality of lymphocytes in an immune response we need to study the phosphorylation profiles that occur in these cells in response to activation.

Considering the complexity of a whole cell lysate and the low amount of phosphorylated peptides versus the non phosphorylated ones, a robust and effective method for the analysis of the phosphoproteome is of the highest importance in the field of proteomics.

The aim of this study is to obtain the best technical approach for protein identification and phosphorylation site assignment in whole T lymphocyte lysate, by the use of different instruments and database search engines.

Digested lysate of rested T lymphocytes was first enriched in phosphopeptides by performing an ion metal affinity chromatography (IMAC). After, strong cation exchange (SCX) chromatography was used to further fractionate the phosphopeptide-enriched lysate.

Some of the fractions containing a high amount of phosphopeptides were selected to investigate the effectiveness of protein identification and phosphorylation site assignment with quadrupole-TOF and quadrupole ion trap based instruments. Comparative study was also carried out for protein identification and assignment of phosphorylation sites using Mascot and Protein Prospector search algorithms.

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## C.16

# Detection of Intrasubunit Cross-linking Interactions in the Regulatory $\beta$ Subunit of Phosphorylase Kinase Suggest a Possible Flip-Flop Mechanism of Activation by Phosphorylation

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Phosphorylase kinase (PhK), an $(\alpha,\beta,\gamma,\delta)_4$ complex, regulates glycogenolysis. Its activity, catalyzed by the $\gamma$ subunit, is tightly controlled by phosphorylation and activators acting through allosteric sites on its regulatory $\alpha$, $\beta$, and $\Delta$ subunits. Several lines of evidence indicate that coupled conformational changes occur in the $\beta$ and $\gamma$ subunits in response to such activators. The conformational change induced in the $\beta$ subunit, characterized by positive $\beta$-$\beta$ interactions in yeast two-hybrid assays and increased cross-linking of the $\beta$ subunits in the phosphorylated PhK complex by 1,5-diflouro-2,4-dinitrobenzene (DFDNB), is mediated by the N-terminal phosphorylatable region of $\beta$ ($\beta$1–31). Spectroscopic and *in-silico* analyses indicate for $\beta$ that it has an $\alpha/\beta$ fold and that it contains several stretches (residues 42–62, 969–1009 and 1032–1047) that have coiled-coil forming propensities, with the latter region having the greatest calculated propensity (0.8 for a theoretical maximum of 1) and potential for promoting $\beta$ self-association. Recently we demonstrated that transmission of the activating phosphorylation signal in PhK involves both $\beta$1–31 and the C-terminal regulatory domain of $\gamma$ ($\gamma$CRD), an allosteric switch in PhK. Both domains were shown to be proximal to one another in the active phosphorylated enzyme complex by isolating and identifying a conjugate corresponding to chemical crosslinking between K303 and R18 in the $\gamma$CRD and $\beta$1–31 domains, respectively. Based on the findings above, we have proposed a model for $\beta$ subunit interactions in PhK, in which phosphorylation of the $\beta$1–31 region disrupts intramolecular $\beta$ contacts, exposing a dimerization domain and concomitantly promoting interactions between the $\beta$1–31 and $\gamma$CRD domains. In support of this model, we have identified two pairs of cross-linked peptides from digests of intramolecularly cross-linked $\beta$ subunits in the phosphorylated PhK complex that together correspond to cross-linking between regions of $\beta$ comprising residues 19–23 and 319–331 using a combination of DFDNB chemical cross-linking, mass spectrometric (MS) and data-based search methods. Analysis of the $\beta$319–331 region indicates that it is a reverse polar analog of the $\gamma$CRD region ($^{298}$RHFSPRGKFKVI) shown to be crosslinked to $\beta$1–31 in our previous study, in that a small peptide stretch (RKFGYKGKLKRI) directly flanking the residues of $\beta$319–331 crosslinked to $\beta$1–31 (progressing from the C-terminus to the N-terminus) is 50% identical with the corresponding peptide stretch in the $\gamma$CRD. The results suggest a possible flip-flop interaction between the phosphorylatable $\beta$1–31 region and the $\beta$319–331 and $\gamma$CRD domains.

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## C.17

# Statistical Similarities between Transcriptomics and Quantitative Shotgun Proteomics Data

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Label-free shotgun proteomics methods, such as the multi-dimensional protein identification technology (MudPIT), have proven to provide quantitative insights into cellular mechanisms. If the large collection of microarray-specific statistical tools was applicable to the analysis of these datasets, it would certainly foster an important advancement of proteomics research. Here, we analyze two large MudPIT datasets—one containing 8 replicates of the soluble fraction of a yeast whole-cell lysate, one containing 9 replicates of a human immuno-precipitate—to test whether normalized spectral abundance factor (NSAF) values share with Affymetrix GeneChip transcript abundance values substantially similar statistical properties. First, we show similar dynamic range and distribution properties of these two types of numeric values. Next, we observe that the standard deviation (SD) of a protein's NSAF values is dependent on the average NSAF value of the protein itself, following a power law. This relationship can be modeled by a power law global error model (PLGEM), initially developed to describe the variance-versus-mean dependence that exists in GeneChip data. PLGEM parameters obtained from MudPIT datasets prove to be surprisingly similar to the typical parameters observed in GeneChip datasets. The most important common feature identified by this approach is that, although in absolute terms the SD of replicated abundance values increases as a function of increasing average abundance, the coefficient of variation—a relative measure of variability—becomes progressively smaller under the same conditions. We next show that PLGEM parameters are reasonably stable to decreasing numbers of replicates. We finally illustrate one possible application of PLGEM in the identification of differentially abundant proteins, which might potentially outperform standard statistical tests. In summary, we believe that this body of work lays the foundation for the application of microarray-specific tools in the analysis of shotgun proteomics data.

## C.18

# Huntington's Disease Protein Contributes to RNAi through Association with Argonaute and P Bodies

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Huntington's disease (HD) is a dominant autosomal neurodegenerative disorder caused by an expanded poly-glutamine stretch within the Huntingtin (Htt) protein. Analysis of postmortem HD brains reveals intracellular aggregates and striatal atrophy that appear to be the consequence of excitotoxicity, apoptosis, mitochondrial defects, and altered mRNA levels. Poly-Q expansion alters Htt function and likely causes aberrant interactions with other cellular proteins; with this premise in mind we adopted a novel biochemical approach, purifying epitope tagged wild type and poly-Q expanded Htt (HttMut) with endogenous interacting proteins. Semi-quantitative mass spectrometrometic analysis of purified proteins identified Argonaute (Ago) proteins that were more abundant in the wt Htt purifications and confirmed reported protein interactions with CA150 and TPR. Ago proteins are central in post-transcriptional gene silencing (PTGS) pathways and Ago-2 is sufficient for RNA interference (RNAi) *in vitro*. We detected distinct peptides for two of the four Ago isoforms: Ago-1 and Ago-2. To confirm the interaction between Ago-2 and Htt, we over expressed Flag-Ago-2 with Htt or HttMut. Both Htt and HttMut co-immunopreciptated with an anti-Flag antibody and recovery was unaffected by RNase treatment. Further, immunofluorescence of endogenous and over expressed proteins revealed Htt and Ago-2 co-localize (more so for Htt than HttMut) in perinuclear foci in several cell lines. We next speculated and proved that Htt is present in P bodies –the subcellular location of mRNA degradation –by Htt co-localization with P-body marker DCP-1. Cells transfected with Htt siRNAs showed a significant two fold reduction in the ability to assemble P bodies compared to control cells transfected with luciferase siRNAs and demonstrated a 3 fold reduction in RNAi comparable to cells depleted of Ago-2 which showed a 5 fold reduction relative to control cells, suggesting Htt functions as an Ago-2 accessory factor necessary for efficient RNAi. Of interest, neuronal cell lines from a HD-mouse model exhibit impaired siRNA dependent PTGS. We identified Ago-2 as a novel Htt interacting partner, showed Htt to be present at P bodies, demonstrated a functional relationship between Htt protein levels and RNAi efficiency, and described a novel pathway perturbed in a HD model. These data lead us to suggest that the reported changes in mRNA levels observed in HD may be in part attributed to HttMut's role in PTGS.

## C.19

# Proteomics Analysis of Aurora Kinases: Mitosis and the RNAi Machinery

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Aurora kinases are critical for progression of eukaryotic cells through mitosis. Improper function of Aurora kinases results in genomic instability, and they are overexpressed in a variety of human cancers. In order to better understand their biochemical functions, a tandem affinity purification strategy was used to isolate multiprotein complexes containing human Auroras A and B. Proteins in each complex were separated by gel electrophoresis and bands were excised from top to bottom of the gel. Each band was digested in situ with trypsin and the resulting digests analyzed by microcapillary reverse-phase LC-ESI-MS/MS and protein database search.

A protein network map was constructed consisting of Aurora kinases and known interactors, several additional mitotic regulators, SWI/SNF chromatin remodeling factors, and, interestingly, core components of RNAi machinery argonaute 1 (Ago1) and argonaute 2 (Ago2). We find that Ago1 co-localizes with Aurora A to the centrosomes during early mitosis and with Aurora B to the central spindle and midbody by late mitosis. Ago1 knockdown results in severe chromosome segregation defects and Aurora B mislocalization, suggesting a new role for Ago1 as a regulator of Aurora B function. Accordingly, the kinase activity of Aurora B appears to be selectively impaired. In contrast, Ago1 overexpression causes binucleation and an increase in centrosome numbers that resembles Aurora A overexpression phenotypes. Taken together, these findings suggest a functional link between Aurora kinases and the RNAi machinery in the regulation of mitotic progression.

## C.20

# Global Profiling of Proteolysis in Apoptosis

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Although proteases were initially characterized as mediators of nonspecific protein degradation, it is now understood that many of these enzymes are highly selective and play pivotal regulatory roles in many biological processes. Proteases can activate or inactivate protein substrates through specific and limited proteolysis to elicit a wide diversity of phenotypes in health and disease. Unfortunately, current methods for monitoring proteolytic events in complex samples suffer from limitations and lag far behind those used to study other posttranslational modifications such as phosphorylation, ubiquitination, and glycosylation. We have recently developed a novel strategy for proteomic profiling of proteolysis in complex biochemical samples that is based on selective enzymatic labeling of protein N-termini, the formation of which is a chemical hallmark of proteolysis. Selective labeling of protein N-termini is achieved using an engineered peptide ligase termed subtiligase, a variant of the serine protease subtilisin. Subtiligase-mediated labeling of proteins, tryptic digestion of labeled protein mixtures, affinity purification of labeled N-terminal peptides, and sequencing of recovered peptides by tandem mass spectrometry permits cataloguing of all N-termini in a given sample. N-termini mapping to internal protein sequences are indicative of proteolytic processing of the corresponding proteins, and enrichment of such N-termini in stimulated experimental samples relative to control samples is indicative of stimulus-dependent proteolytic processing. We have utilized this method to profile the proteolysis carried out by caspases during apoptosis (programmed cell death). Based on diverse studies in different organisms and cell types, there are currently approximately 400 known caspase substrates. Using a single experimental system of one cell type and one apoptotic inducer, our studies have thus far resulted in identification of approximately 50 reported caspase substrates and an additional 200 previously unreported caspase substrates. In addition to identifying targets of proteolysis, our method also permits assignment of cleavage sites, facilitating evaluation of the functional consequences of proteolytic processing in each case.

## C.21

# Methods for Utilizing MS3 Data in Peptide Identification Strategies

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Improvements in ion trap instrumentation have made n-dimensional mass spectrometry more accessible. The faster acquisition times of newer linear trap instruments such as the LTQ provide the option of collecting MS3 spectra of abundant MS2 fragment ions with overall cycle times similar, if not better, to those of normal MS/MS2 cycles on older 3D trap instruments. As a result, a number of researchers are choosing to collect MS3 spectra during LC-MS/MS runs. This is considered particularly useful in the case of proteins identified by single peptides and for the analysis of phosphopeptides, the spectra of which are frequently dominated by a major fragment ion representing neutral loss of the phosphate group from the precursor peptide.

Our research investigates alternative methods for utilizing the MS3 spectra, with the goal of selecting an optimal computational strategy. The first overall strategy is based on combining MS2 and MS3 spectra prior to database searching. Different mechanisms for merging MS2 and MS3 peak lists prior to database searching are discussed: namely, a summation methodology which, following intensity normalization, involves adding MS3 peaks to the MS2 spectra to generate a more comprehensive spectrum, as well as an intersection methodology which aims to construct a simplified spectrum composed of only one ion type (1). The second overall strategy involves separate searches of MS2 and MS3 spectra. Resulting peptide assignments to mass spectra are then analyzed using a statistical model that translates the additional information obtained by coupling consecutive MS2 and MS3 search results, based on consecutive scan numbers, into an adjusted probability score for peptide identifications. Results demonstrate that the post-database search adjustment model provides a significant increase in discriminating power between correct and incorrect peptide identifications.

Three datasets were utilized in this study to evaluate the performance of the different peptide identification strategies. The first dataset was derived from a mixture of nine purified proteins. Acquired mass spectra were searched using SEQUEST against a database consisting of the nine protein sequences and common contaminants appended to a reversed copy of the human IPI database. In this manner, true hits could easily be distinguished from false positives. The second dataset consisted of 278 individually purified and analyzed human proteins. To examine performance of the methods on a complex dataset, a drosophila whole-cell lysate, enriched for phosphopeptides using the IMAC procedure, was also utilized (2). The false positive rate for this dataset was measured using the reverse database method. All spectra in this study were generated on LTQ or LTQ Orbitrap mass spectrometers.

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## C.22

# Liver Histidine Kinase Activity: Mass Spectrometric Detection of Histone H4 Histidine Phosphorylation

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Kinases that phosphorylate substrate proteins on tyrosine, serine and threonine (*i.e.*, phosphohydroxyamino (P-O) bond), play critical roles in all aspects of mammalian cell homeostasis. They are extensively studied and their importance in cell biology is unquestioned. Not so histidine kinases. Unlike these common kinases, histidine kinases (HK) phosphorylate proteins on the imidazole nitrogens of histidine, which are acid-labile and therefore easily hydrolysed. Hence, experimental conditions used to study serine, threonine and tyrosine phosphorylation are not suitable for the study of the phosphoramidate (P–N) bond of phosphohistidine.

Various reports of histidine kinase activity and its products have been demonstrated in eukaryotes (1,2). Biochemically, histidine kinase activity has been definitively shown in mammalian cells to correlate with cellular proliferation (3). This research has been greatly supported by the development of techniques specific for the study of histidine kinase activity and methods to detect proteins containing phosphohistidine (4,5).

Using these techniques that are specific to the study of histidine phosphorylation, we have partially purified a liver histidine kinase that phosphorylates histone H4 on the histidine 18 residue. This was confirmed by histidine kinase specific kinase assay and by assessing proteolytic digests of histone H4 via mass spectrometry (6) to identify the presence of histidine phosphorylation. In addition, we also sought to initially characterise the kinase activity of the enzyme by assessing the effects of pH and kinase inhibitors on the histidine phosphorylation of histone H4.

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## C.23

# Identification of the Missing Components of a Receptor Kinase-mediated Steroid Signal Transduction Pathway Using Quantitative Proteomic Profiling

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Brassinosteroid (BR) is a steroid hormone that plays essential roles in plant growth and development. BR is perceived by the cell surface receptor-like kinase BRI1, and downstream signal transduction involves another receptor-like kinase (BAK1), a GSK3-like kinase (BIN2), and nuclear transcription factors (BZR1 and BZR2/BES1). BR binds to the extracellular domain of BRI1 to induce BRI1-BAK1 dimerization and receptor kinase activation, leading to inhibition of BIN2 and dephosphorylation of the BZR1 and BZR2/BES1 transcription factors. When BR levels are low, BIN2 phosphorylates BZR1 and BZR2/BES1 to inhibit their nuclear accumulation and DNA binding. How the receptor kinases regulate BIN2 activity remains the only major gap in our understanding of the pathway. In order to identify additional proteins involved in BR signal transduction and BR-regulated physiological response, we used two-dimensional difference gel electrophoresis (2-D DIGE) to identify BR-regulated proteins in total protein, plasma membrane (PM), and phosphoprotein fractions. While proteomic changes were detected in total protein samples only after 3 hr of BR treatment, obvious responses were observed in PM and phosphoprotein fractions after a 0.5 hr BR treatment. Using tandem mass spectrometry (LC-MS/MS), we identified over 65 BR-regulated proteins. While none of the known BR signaling proteins were identified in total protein samples, BAK1 and BZR1 were identified in the PM and phosphoprotein fractions, respectively, as proteins that show BR-induced phosphorylation changes (spot shift along the IEF dimension). In addition, several novel proteins were identified and shown to function in BR response. Among these are two novel kinases that showed BR-induced phosphorylation changes. These two kinases are closest homologs of each other in the *Arabidopsis* genome, and we named them BR-regulated kinase 1 and 2 (BRK1 and BRK2). To understand the function of BRK1 and BRK2 in BR signaling, we have shown that overexpression of BRK2 and its homologs suppresses the receptor mutant bri1, suggesting that BRK1 represents a family of positive regulators of BR signaling. Preliminary results indicate that BRK1 and BRK2 interact with both BRI1 and BIN2 *in vivo*, and are phosphorylated by BRI1 *in vitro*, suggesting that BRK1 and BRK2 mediate signal transduction from the receptor kinase to BIN2. Therefore, it is likely that our proteomic study has closed the last major gap in the BR signaling pathway, establishing the BR pathway as the first complete signal transduction pathway from cell surface receptor kinases to the nucleus in plants as well as for steroid hormones.

## C.24

# A Novel Stable Isotope Labeling Approach for Quantitative Proteomics

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Quantitative analysis of protein expression levels is a critical issue in proteomics. Elegant stable isotope based approaches including ICAT, SILAC and iTRAQ have been developed and utilized in many studies. Hereby we describe a simple and cost effective method using acetone to modify amine groups at both N-terminus and lysine residues. We demonstrate that this reductive amination reaction specifically generates an isopropyl addition to the amine groups. We also show that the reaction can be utilized to modify both peptides and proteins. At the peptide level, the reaction occurs with over 95% yield in 10 minutes with high specificity. At the proteins level, the reaction reaches equilibrium in approximately 2 hours. Using acetone (d0-acetone) and deuterium labeled acetone (d6-acetone), this reaction introduces 6 Da mass split between the d0-isopropyl and d6-isopropyl adducts, which is ideally suited for quantitative analysis. When this approach is employed to modify tryptic peptides and subsequently quantify relative protein abundances, the linear range is at least 2 orders of magnitude. When this approach is utilized to modify proteins prior to trypsin digestion, a linear range of 2 orders of magnitude is also demonstrated. In addition, the isopropyl modification can also increase electrospray ionization efficiency of peptides, improve trypsin digestion efficacy by partially unfolding proteins, and generating overlapping tryptic digestion maps. These features all contribute to achieving better sequence coverage for protein identification. In summary, the reductive amination using acetone is a simple, effective and inexpensive approach that can improve protein identification and enables a novel binary protein quantitative analysis.

## C.25

# Phosphorylation Dictates Proteolytic Function of Mammalian 20S Proteasome Complexes: Insights Gained via Phospho-proteome Analysis

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The 20S proteasome complexes play a critical role in intracellular protein quality control. Posttranslational modification based heterogeneity of the 20S proteasome subunits was evidenced by the pattern they display on a 2D electrophoresis. Phosphorylation contributed to this diversity, which was demonstrated by immunoblotting, phospho-protein dye, and mass spectrometry analysis. We tested the hypothesis that phosphorylation mediates 20S proteasomes function. To gain insights into the molecular mechanisms of proteasome regulation, we conducted a comprehensive proteomic survey of the phosphor-proteome in mammalian 20S proteasomes complexes. This study used four experimental strategies in parallel to systemically screen the phospho-proteome of murine cardiac and liver 20S proteasomes. The use of $TiO_2$ resin was a critical step to enrich the substoichiometric phospho-peptides. The combination of differential prefractionation strategies prevented low abundant phospho-peptides from being overwhelmed by the dominant ones. Our approach has been approved effective; a total of 11 phosphorylation sites were identified from the endogenous murine cardiac 20S proteasomes as the outcome of this systematic characterization; nine of them were novel identifications. According to the crystallized 3D structure of the bovine liver 20S proteasomes, nine phosphorylation sites were exposed to the exterior of the 20S proteasomes subunits, while the exposure of the other two symbolized a major structural rearrangement. With trypsin as the endo-protease, liver 20S proteasomes were found to possess nine kinase regulated sites. Seven phosphorylation sites were observed in both endogenous purified cardiac and liver 20S proteasomes. Using spectra counts as a relative quantitative measure, only $\alpha$7 serine-250 residue was phosphorylated at a percentage higher than 50% in both liver and heart, suggesting a possibility of a constitutive phosphorylation site. Furthermore, PKA has been reported as cardiac 20S proteasomes associating kinase. Incubation of proteasomes with PKA increased both liver and cardiac 20S proteasomes activities, which correlated to novel phosphorylation site identifications in $\alpha$2, $\alpha$3, $\alpha$5, $\beta$6 subunits of the cardiac 20S proteasomes and $\beta$1, $\beta$2 subunits of the liver 20S proteasomes. This indicates certain 20S proteasome phosphorylation sites can only be detected after activating the corresponding kinases. To our knowledge, this represents the very first study delineating a phospho-proteome map of mammalian proteasome complexes; we report that the murine 20S proteasome phospho-proteome contain at least 23 phosphorylation sites. Phosphorylation is demonstrated as a key regulatory event for proteolytic function of these mammalian 20S proteasome complexes.

## C.26

# Proteome Profiling Using an Integrated Top-down and Bottom-up Strategy

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Mass spectrometry has become one of the most powerful analytical tools for proteomics and systems biology research. In principle, there are two complementary strategies for mass spectrometry-based protein characterization: bottom-up and top-down, each with its own strengths and weaknesses. We present an integrated top-down and bottom-up approach facilitated by concurrent liquid chromatography-mass spectrometry (LC-MS) analysis and fraction collection for comprehensive high-throughput intact protein profiling. The approach employs high resolution reversed phase LC separations coupled on-line with a 12T Fourier transform ion cyclotron resonance spectrometer to tentatively identify modified proteins, using detected intact protein masses in conjunction with bare protein identifications from the bottom-up data of the same fraction. These identifications are incorporated into a target ion list for subsequent offline gas phase fragmentation (CAD and ECD) that uses only an aliquot of the original fraction used for bottom-up analysis. Decoupling LC-MS and MS/MS analysis eliminates the time constraint associated with on-line LC-MS/MS and enables averaging over multiple tandem mass spectra to improve overall sequence coverage. Additionally, by limiting bottom-up analyses to individual fractions, the complexity of the protein mixture undergoing analysis is reduced, which enables confident assignments of intact proteins and their gas phase fragments.

We demonstrated the feasibility of this novel comprehensive strategy by identifying protein modifications and isoforms from standard protein mixtures. For example, we identified three phosphorylated isoforms of $\beta$-casein. These isoforms differed in mass by 40 Da and 109 Da, and were identified as SNP mutants with a Pro to His switch and an additional Ser to Arg replacement, respectively. A bottom-up approach alone, even with 100% sequence coverage, cannot validate the presence of three distinctive isoforms (*i.e.*, distinguish between one isoform with two SNPs and two isoforms each with a single SNP). The integrated dataset revealed additional SNPs, heme-containing proteins, and posttranslational modifications such as acetylations and phosphorylations. We are further applying this integrated strategy to generate an intact protein level view of the viral proteome (*e.g.*, vaccinia virus).