Gold for Ubiquitin in Vancouver
FIRST CONFERENCE ON PROTEOMICS OF PROTEIN DEGRADATION AND UBIQUITIN PATHWAYS HELD JUNE 6–8, 2010 IN VANCOUVER, UNIVERSITY OF BRITISH COLUMBIA, ORGANIZED BY LAN HUANG, THIBAULT MAYOR, AND PEIPEI PING*

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The rise of proteomics has had tremendous influence on analysis and understanding of the role of post-translational modifications in biological processes. The covalent attachment of small proteins like ubiquitin, SUMO,1 or other ubiquitin-like proteins (Ubls) is one class of post-translational modifications where proteomics has had notable impact. Various proteomics approaches, but in particular mass spectrometry-based analyses, have influenced the field and enabled significant advances over the past few years. The first meeting dedicated to proteomics of protein degradation and ubiquitin pathways showcased these advances and allowed a glimpse at future contributions of proteomics to this field. With its many attractive drug targets, the ubiquitin and proteasome system, as well as other proteolysis pathways, could offer new therapies for various human diseases including cancer and neurodegenerative disorders.

The covalent linkage of ubiquitin to other proteins is catalyzed by the E1-E2-E3 cascade of enzymatic reactions whereby the many different E3 ubiquitin ligases provide substrate specificity to the process of protein ubiquitylation (1). Ubiquitylation is best known for targeting proteins for degradation by the proteasome, but other functions for ubiquitylation independent of proteolysis are also known. Likewise, modifications with SUMO or other Ubls generally do not regulate protein degradation but instead control subcellular localization, protein interactions, or change protein conformation and activity (2).

The questions addressed by proteomics approaches to ubiquitylation and Ub1 modifications are plentiful. They range from very specific, e.g. determination of the modified residue in a substrate protein, to complex, such as protein dynamics in proteome-wide ubiquitin (or Ubl) modification profiles (3). In either case, the rapid technological advancements (particularly in mass spectrometry instrumentation as well as quantitation and separation technologies) have allowed impressive progress, which was evident in the First Conference on Proteomics of Protein Degradation and Ubiquitin Pathways in Vancouver (http://ppdup.org/) (Fig. 1). Molecular & Cellular Proteomics 10: 10.1074/mcp.R110.003863, 1–6, 2011.

KEYNOTE ADDRESSES
The two plenary lectures were delivered by Ray Deshaies (Pasadena, CA) and Dan Finley (Boston, MA), who were among the first to exploit mass spectrometry-based proteomics to address mechanistic questions of the ubiquitin-proteasome system (UPS). R. Deshaies presented work that analyzed the role of the AAA-ATPase Cdc48/p97/VCP in protein degradation (4). The yeast Cdc48 and its highly conserved mammalian homolog p97/VCP have long been connected with the UPS and are thought to deliver ubiquitylated proteins to the proteasome for degradation. Consistent with this role in proteasome targeting, the Deshaies group noticed that in yeast mutants carrying a temperature-sensitive allele of CDC48 or after p97 knockdown in mammalian cells levels of total ubiquitylated proteins are significantly increased. However, when proteasomes were purified from yeast cells, they found more ubiquitin chains bound to the proteasome in cdc48 mutants than in wild-type cells. This surprising result argues against the simple model for Cdc48 in substrate delivery to the proteasome, which predicted reduced levels of ubiquitin conjugates at the proteasome after Cdc48 inactivation. R. Deshaies then showed mass spectrometric analyses comparing proteasome-interacting proteins in wild-type cells and cdc48 mutants and found that many proteasome-interacting proteins were specific for cdc48 mutants, suggesting the intriguing possibility that degradation substrates accumulate at the proteasome in cells lacking Cdc48. One of these proteins, the RNA polymerase II subunit Rpb1, was analyzed in more detail. Rpb1 is ubiquitylated and degraded in response to UV radiation. cdc48 mutants accumulated ubiquitylated Rpb1 after UV radiation, and significant amounts were bound to proteasomes. These findings suggest that for Rpb1 the primary role for Cdc48 function appears to be late...
in the degradation process after substrate arrives at the proteasome rather than in an earlier targeting step. R. D'eshiaies also reported system-wide quantitative ubiquitin profiling experiments that compared ubiquitin profiles in wild-type and cdc48 mutants. They found that the levels of about 10% of ubiquitylated proteins were increased in cells lacking Cdc48 activity, further underscoring the importance of Cdc48 for the UPS.

Several other presentations highlighted the growing interest in the diverse roles of the AAA-ATPase Cdc48/p97. For example, Peter Kaiser (Irvine, CA) described a quantitative proteomics approach to understand how missense mutations in human p97/VCP cause the hereditary disease inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia (IBMPFD), a fatal form of inclusion body myopathy with no available cure. The SILAC-based MS strategy revealed dramatically increased binding of some p97 cofactors, in particular Npl4, to the disease-causing p97/VCP mutants. This gain of function behavior is consistent with the dominant character of IBMPFD and supports a recent report suggesting p97 cofactor imbalance as a cause for this human disease (5).

D. Finley presented interesting work on a new inhibitor of the USP14/Ubp6 deubiquitinating enzyme (DUB), which is associated to the proteasome. Although DUB activity at the proteasome is required to allow the degradation of ubiquitylated substrates (and permit their entrance in the 20 S chamber), it may also prevent the degradation of substrates by removing ubiquitin before their targeting for proteolysis. The Finley team reasoned that inhibition of such DUB activity may accelerate the degradation of a pool of substrates like misfolded proteins. They identified, in collaboration with the group of Randy King (Boston, MA), a new reversible inhibitor targeting USP14 and found that it increased the degradation of model substrates like cyclinB and Sic1PYp by the proteasome. Remarkably, the compound reduced the levels of several aggregation-prone proteins (e.g. TPD43) in mouse embryonic fibroblast cells expressing USP14 but not in USP14−/− control cells. In addition, after oxidative damage, fewer oxidized proteins accumulated in the presence of the
USP14 inhibitor, resulting in an increase of cell viability. This exciting work opens new possibilities in the field where proteasome-dependent degradation may be up-regulated by specific compounds. It also nicely illustrates how the proteasome itself plays an active role in regulating proteolysis by remodeling the ubiquitin chains of substrates.

The role of DUBs in the ubiquitin system was further illustrated in other presentations. Notably, the laboratory of Kathy Gould (Nashville, TN) performed a “global census” of the 20 DUBs in fission yeast by combining analyses of localization, binding partners (using mass spectrometry), and mutation-associated phenotypes. They assigned several new functions for DUBs based on their census profile. For instance, Ubp4 and Ubp5 were found to function at the endosome and Golgi, respectively. Remarkably, DUBs displayed an astonishing capacity to compensate for other DUB gene deletion. For instance, no less than five DUB deletions were required to obtain a strong defect in endocytosis. Richard Gardner (Seattle, WA) presented results on Ubp10 in budding yeast where new substrates were identified after comparing data obtained from two different proteomics approaches. In the first approach, Ubp10 binding partners were identified after pulldown following in vivo cross-linking. In the second approach, proteins more ubiquitylated in ubp10Δ cells (due to the absence of the DUB) were identified after purifying ubiquitylated proteins and comparing them with wild-type cells. These experiments confirmed the role of Ubp10 in gene silencing and suggested a new role in 40 S ribosome biogenesis.

UBIQUITIN LIGASES

E3 ubiquitin ligases are considered the key regulators of the UPS. Wade Harper (Boston, MA) presented a comprehensive proteomics approach to understand Cullin-RING ligases (CRLs) (6). CRLs are multisubunit ligases assembled on a cullin scaffold. Generally, they consist of two to three components forming the core ligase involved in E2 recruitment and activation and a substrate adapter for substrate selection. Hundreds of substrate adapters can dock to a few different core ligases, generating the substantial diversity of this ligase family. These adapter interactions can be key nodes for E3 regulatory processes. Studies in fission yeast suggested that the COP9 signalosome complex CSN as well as the protein CAND1, both tightly linked to the cullin neddylation cycle, regulate adapter subunit binding at various levels (7).

W. Harper’s team approached the dynamics of CRL regulation by constructing a quantitative proteomics platform to systematically examine CRL architecture as CRLs transition from active to inactive states using an inhibitor of the Nedd8 conjugation pathway (MLN4924). Utilizing a multiplex absolute quantitation by mass spectrometry (AQUA)-based quantitative MS approach, they were able to analyze not only the global composition of CRLs but also the absolute stoichiometry of the various CRL components. This approach can be broadly applied to examine other protein signaling networks and how their composition and post-translational modifications change in response to pathway perturbations. As an interesting technical note, W. Harper mentioned that a significant preservation of the neddylation status during purification is achieved by the metalloprotease inhibitor o-phenanthroline, which inhibits the zinc-dependent deneddylation activity of CSN.

Poul Sorensen (Vancouver, British Columbia, Canada) reported an interesting connection between the tumor suppressor Hace1 and CAND1. Genetic experiments by the Sorensen team suggest that Hace1 functions as an inhibitor of several CRLs. Using mass spectrometry, they identified CAND1 as a major interactor of Hace1 and found that CAND1 binding was significantly increased under nutritional stress or hypoxia. Under these same conditions, less CAND1 was bound to cullins, implying that Hace1 acts by CAND1 sequestration.

Millennium Pharmaceuticals has developed the neddylation inhibitor MLN4924 (used in the aforementioned work by W. Harper), which targets the Nedd8 activating enzyme and is in clinical trials in patients with advanced malignancies. Cullin modification by the ubiquitin-like protein Nedd8 is crucial for CRL activity, and MLN4924 thus functions indirectly as a potent CRL inhibitor (9). Stabilization of the DNA replication factor and CRL substrate Cdt1 and subsequent induction of apoptosis have been identified as the primary mode for cancer cell cytotoxicity of MLN4924. Eric Lightcap (Cambridge, MA) reported efforts to identify useful biomarkers and synergetic pathways for MLN4924 effects. Large scale SILAC experiments were used to identify over 650 proteins that were stabilized upon MLN4924 treatment in A375 melanoma cells. A chemical genetics-type strategy was then used to categorize the identified proteins based on the combined effect of their siRNA knockdown and MLN4924 treatment on A375 cell proliferation. Knockdown of Cdt1 or p53 suppressed, whereas knockdown of the CRL adapter Cdt2, p21, or cyclin E2 enhanced the effect of MLN4924. The presentation by E. Lightcap demonstrated the power of integrated approaches that combine proteomics with expression profiling and/or siRNA screens.

Ubiquitylation by CRLs has been thought to be primarily regulated at the level of substrate recruitment, but the dynamics of CRL complexes is clearly an important area that might also offer pharmacological points of intervention. P. Kaiser reported signal-induced, active disassembly of the yeast CRL SCFMet30. The Kaiser group showed that heavy metal stress induces autoubiquitylation of the adapter subunit Met30, which recruits Cdc48 that in turn actively dissociates Met30 from the core ligase, leading to ligase inactivation. A quantitative mass spectrometric survey of all SCF ligase complexes in yeast showed that SCFMet30 was selectively disassembled, demonstrating ligase-specific dissociation of adapter components as a novel mechanism for CRL regulation.
IDENTIFICATION OF UBIQUITYLATED PROTEINS

Various groups presented results obtained from mass spectrometric identification of ubiquitin conjugates after biochemical purification. Notably, the groups of R. Deshaies and Hugo Mayor (Derio, Spain) used tagged ubiquitin approaches, whereas others, like the laboratory of Manuel Rodriguez (Derio, Spain), favored enrichment with ubiquitin binding domains. As reminded by R. Gardner, each of these methods has its advantages and drawbacks. R. Deshaies showed how quantitative mass spectrometry can be used to estimate the efficacy and specificity of the enrichment by comparing proteins derived from cells, which do or do not express the tagged ubiquitin. The diverse strategies that have been developed for system-level ubiquitin profiling should be applicable to the identification of ubiquitin ligase substrates by comparing ubiquitin profiles of wild-type cells with ligase mutants. Given the immense number of ubiquitin ligases and the almost complete lack of substrate identities that can currently be associated with these ligases, such an approach would have tremendous impact on biology and biomedical research. So far, few effective methods for substrate identification have been reported, suggesting that further improvements of strategies and instrumentation are necessary. Thibault Mayor (Vancouver, British Columbia, Canada) used tagged ubiquitin and quantitative MS to show that heat shock caused a rapid increase of ubiquitylation of cytoplasmic proteins in budding yeast. HUL5, which encodes for a HECT ubiquitin ligase, was found to be involved in this stress response pathway. By comparing ubiquitylated proteins from hul5Δ and wild-type cells, his group was able to identify proteins that were more ubiquitylated in wild-type cells, suggesting that they may be HUL5 substrates. Alternative strategies for ligase substrate identification have been reported by Ben Major (Chapel Hill, NC) and James Wohlschlegel (Los Angeles, CA). B. Major reported that proteasome inhibition can be used to trap the substrate-ligase complex, and mass spectrometric identification of ligase bound proteins can thus reveal substrates. J. Wohlschlegel’s team uses CRL substrate adapter mutants that cannot associate with the core ligase as substrate baits in a mass spectrometric approach. A large variety of approaches will likely continue to be used, and researchers should consider possible bias introduced by each of these methods.

UBIQUITYLAION SITES AND CHAIN TOPOLOGY

The 114-dalton Gly-Gly peptide modification is the trademark signature found on lysine after trypsin digest. It is increasingly used to identify ubiquitylation sites as well as to characterize ubiquitin chain topology (10). There were several discussions on the confidence of these peptide identifications. Junmin Peng (Atlanta, GA) presented data illustrating how bona fide ubiquitylated peptides and chemically induced artifacts (e.g. by iodoacetamide) could be distinguished based on a specific neutral loss in the spectra of the ubiquitin peptide containing a modified Lys-48. J. Peng also presented data indicating that heating the sample significantly increases this artifact (even when using the alternate reagent chloroacetamide); samples should therefore be prepared accordingly. For a long time, it was not possible to use detection of modification remnants similar to the ubiquitin Gly-Gly modification for other Ubis (e.g. SUMO) that lack a trypsin cleavage site near their carboxyl termini. Alfred Verteagaal (Leiden, Netherlands) presented data using an engineered SUMO in which an arginine residue was introduced to allow the detection of SUMO-induced Gly-Gly signatures. A similar approach had been applied to identify Smt3 modifications in yeast (11). Brian Raught (Toronto, Ontario, Canada) presented an alternative approach to this problem in which a longer fragment of the Ubl carboxy-terminal remnant was used for identification. To overcome the substantial complexity of spectrum identification, the peptide spectra of the Ubl remnant signature are first detected prior to the identification of the modified peptide. Remarkably, due to the large length of these peptides, which typically have four or more charges, charge filtration can be applied during the sample analysis to enrich for tandem mass spectra of modified peptides. These new methods will potentially help us to increase the number of ubiquitylation site discoveries.

AQUA of ubiquitin linkages is performed by a growing number of laboratories. Michael Glickman (Haifa, Israel) presented AQUA data obtained after overexpressing a K0 ubiquitin mutant (in which all seven Lys are replaced by Ala). M. Glickman found that a high portion of conjugated ubiquitin is in the form of monoubiquitin or short chains. Affinity purification of chains attached to K0 ubiquitin combined with SILAC quantitation suggested that K0 is preferentially associated with Lys-63 chains; therefore, the Glickman group suggested that chain editing may occur preferentially on Lys-48 polyubiquitin chains to prevent the addition of a terminating ubiquitin mutant (i.e. K0).

The formation of a specific ubiquitin linkage is thought to be mediated by both the E2 and E3. Don Kirkpatrick (South San Francisco, CA) nicely illustrated how AQUA can be used to study ubiquitin conjugation. He showed that a mutation in the active site of UbcH5A E2-conjugating enzyme favored the synthesis of Lys-63 linkages (instead of Lys-11) when associated with the Murf1 E3 ligase but not when associated with Nedd4L. Intriguingly, the converse mutation in Ubc13 E2 provoked the opposite effect, namely increase of Lys-11 linkages. A better understanding of the factors influencing the chain topology will be a great challenge in the next years, and proteomics analysis will likely play an important role in this task.

One of the very exciting recent debates is which polyubiquitin chain linkages signal for proteasome degradation. Early work established that Lys-48-linked ubiquitin constitutes a degradation signal (12), but recent studies paint a more complex situation. J. Peng presented very nice results based on
AQUA of ubiquitin linkages in mammalian cells. Interestingly, inhibition of the proteasome first induced a rapid increase of all linkages besides Lys-63, which only rose after 10 h of inhibition. This result raises the possibility that a major remodeling of the chain topology may occur in the cell during proteotoxic stress. It also implies that all ubiquitin linkages but Lys-63 may constitute bona fide signals for degradation in the cell as had been suggested by previous proteomics studies (13). In agreement with this, Changwei Liu (Aurora, CO) showed that the proteasome is very active in trimming Lys-63 linkages via proteasome-bound DUBs, thereby preventing the efficient degradation of substrates modified with Lys-63-linked ubiquitin chains. There is now a growing consensus that other ubiquitin linkages, such as Lys-11, serve as potent proteasome degradation signals and that both Lys-63 and amino-terminal linkages are mainly involved in proteasome-independent signaling.

**THE PROTEASOME**

The proteasome is a large multiprotein complex that has been intensively scrutinized by mass spectrometry in yeast and mammalian cells. Rich Vierstra (Madison, WI) presented proteomics analysis of the plant proteasome. Intriguingly, by using a top-down approach, the Vierstra laboratory found that the 20 S subunit PBG1 was processed like the other three catalytic subunits, suggesting that the proteasome may harbor a novel proteolytic site.

Our impression of the proteasome has been dominated by its depiction as the wastebasket of the cell that is constitutionally active with most of the regulation occurring upstream at the ubiquitylation steps. A much more complicated picture with potentially many subpopulations of proteasome complexes and specific regulatory pathways is emerging, not the least due to proteomics approaches. The group of Peipei Ping (Los Angeles, CA) explored proteasome heterogeneity in cardiac tissues using free flow electrophoresis-based approaches and correlated 20 S activity in these fractions with complex composition and post-translational modifications of 20 S subunits identified by mass spectrometry. The Ping group is also working on establishing a spectral peptide database to accelerate the analysis of proteasome samples. In addition to post-translational modifications, regulation of the proteasome assembly has been recently more carefully analyzed. Lan Huang (Irvine, CA) presented exciting results showing how oxidative stress induces the dissociation of the 19 and 20 S subunits after performing a careful quantitative proteomics analysis of proteasome subunit composition in yeast and mammalian cells. 26 S proteasome disassembly required Ecm29, and deletion of ECM29 reduced the viability after oxidative stress. These data indicate that cells have developed specific signaling pathways to modulate proteasome function in response to environmental conditions and that such regulatory mechanisms at the level of the proteasome are important for cellular integrity.

Diversity of proteasome composition is found in the immune system where proteasomes are important for generation of peptides for surface presentation. The so-called immunoproteasome contains a different set of catalytic subunits. The group of Shigeo Murata (Tokyo, Japan) presented interesting insight into a potential role of a thymus-specific proteasome β-subunit. They compared peptides generated by immunoproteasomes and the thymic proteasome in vivo and detected significant differences. They suggest that the unique peptide repertoire generated by the thymic proteasome is key for positive selection of developing thymocytes.

**PROTEOLYSIS AND PEPTIDOMICS**

Although the ubiquitin field generally focuses on degradation by the proteasome, other proteases also dictate their will in (and out) of the cell. Jim Wells (San Francisco, CA) delivered an outstanding and entertaining presentation to describe a “battle of the Titans” between the proteasome and other apoptotic proteases. One major challenge is to understand how the different waves of caspase activities are regulated during apoptosis. The Wells group developed numerous proteomics approaches to identify which proteins are specifically cleaved by proteases like caspases. They identified about 1000 caspase targets, which are typically only cleaved once and which are often part of larger protein complexes including the proteasome. Remarkably, the proteasome and caspase appear in a reciprocal negative feedback loop where caspases get ubiquitylated and dragged to the proteasome, whereas caspases attack and degrade the proteasome. J. Wells proposed an attractive model whereby a low level of caspase activity can be shut off by the proteasome, leading to non-apoptotic effects, whereas prolonged caspase activation overwhelms the proteasome and leads to apoptosis. Chris Overall (Vancouver, British Columbia, Canada) presented an array of elegant proteomics methods that his group developed to identify protease cleavage sites. For instance, by using an approach to delete amino termini, they were able to specifically enrich for neo-ami termini generated by proteases and to analyze them by mass spectrometry. With over 500 putative proteases encoded in the human genome, there will be no shortage of work for researchers in peptidomics.

**FUTURE CHALLENGES**

Our understanding of the ubiquitin and ubiquitin-like system has tremendously progressed in the past several years. It has also become clear that, with over 1000 human genes involved in ubiquitylation and Ubl conjugation, the system is extremely complex. We are facing a daunting task to assemble all the pieces of the puzzle. One challenge is to identify the sites and the functions of the different ubiquitin and Ubl modifications. Specific enrichment and analysis methods for phosphorylated peptides have led to a burst of new data in recent years,
permitting the identification of several thousands of phosphorylation sites. For comparison, only a few hundreds of ubiquitylation sites have been identified so far. Researchers should continue to develop new proteomics approaches to promote further discoveries in the ubiquitin field. With unquestionable evidence for multiple different ubiquitin chain topologies in vivo as well as the possibility of forked chains, the roles of these different architectures need to be addressed. New and effective strategies to identify substrates modified with different chains are thus required to enable detailed biological analysis. In addition, comprehensive strategies to uncover which substrates are targeted by the different E3 ligases need to be improved. Kay Hofmann (Bergisch Gladbach, Germany) presented some elegant sequence and phylogenetic analyses to help with several of the challenges in the field. Systematic proteomics analysis of E3 ligases, as performed by the Harper group, will likely provide a plethora of new results. A major enterprise in the field will be to adequately store and share the information, and researchers should properly deposit new results in current or new databases. One great outcome of the Proteomics of Protein Degradation and Ubiquitin Pathway conference was to bring together major key players in the proteomics and ubiquitin field to discuss these different issues. We are looking forward to continuing this dialogue in the future.

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