

CASE STUDIES

OPENING DOORS TO A WHOLE NEW VIEW OF BIOLOGY WITH MASS SPECTROMETRY

Going beyond snapshots to monitor real protein dynamics at work with multiplexing strategies using tandem mass tag (TMT) solutions

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Introduction

Multiplying the power of proteomics

Researchers working in proteomics can be forgiven for feeling envious of geneticists. Sequencing entire genomes has become easy, and changes in the expression of thousands of genes can now be simultaneously quantified with remarkable accuracy. In contrast, similar experiments in the protein world have generally been confounded by limited sensitivity, poor throughput, and difficulties in identification and quantitation.

Fortunately, things are changing. The advent and rapid maturation of isobaric labeling reagents, such as tandem mass tag (TMT) reagents, are opening up exciting new horizons for sophisticated proteomic analyses in diverse research settings.

The combination of liquid chromatography and mass spectrometry (LC-MS) is highly effective for separating and identifying proteins from a complex biological sample. However, comparative analysis of complex samples via LC-MS is undermined by the tendency of sequential injections of a sample to yield variable results that can be easily misinterpreted. This variability greatly reduces the usefulness of LC-MS for some clinical research applications, where subtle alterations in protein levels can be critical in understanding the etiology and progression of disease. “This run-to-run variance can give you a lot of false positives,” says Gerold Schmitt-Ulms, who studies prion biology at the University of Toronto.

The development of stable isotope labeling in culture (SILAC) in 2002 by Matthias Mann and colleagues at the University of Southern Denmark offered a critical step forward for such experiments because it enabled direct comparison of the protein content from two different samples in a single experiment.¹ For SILAC, living cells are fed with isotopically labeled amino acids so the cells synthesize “heavy” proteins that incorporate the label. These labeled proteins can then be compared against cells that were not fed the label, and therefore express unmodified, “light” proteins. The resulting pairs of peaks that appear during MS analysis make it possible to determine relative differences in protein levels between the two samples. However, SILAC is limited to specific cell lines, cannot be applied to tissue samples, and is not suitable for higher levels of multiplexing.

An alternative approach based on isotope-coded affinity tags (ICAT)—first described in 1999 by Ruedi Aebersold, then at the University of Washington, and colleagues—overcomes the limitations of metabolic labeling with a procedure that introduces chemical modifications onto target proteins after a cell-lysis step.² However, both ICAT and SILAC are confounded by separation issues arising from the differences in mass associated with isotopic labeling, undermining the accuracy of MS analysis.

The development of TMT technology in 2003 by Christian Hamon and colleagues at Xzillion and Proteome Sciences addressed many of these issues.³ Like ICAT, these are isotopically modified tags

that are conjugated onto target peptides after cell or tissue lysis, each with its own distinct mass. However, each TMT label is also linked to a complementary “mass normalization” domain that ensures that each sample is modified with an identical final mass. The linker joining these two domains can then be broken during fragmentation in tandem MS (MS/MS). Analysis begins with the isolation of peptides of interest from a mixture of TMT-labeled lysates during the first stage of MS; at this point, all the differentially labeled peptides are still equal in mass. The labeled peptides subsequently undergo fragmentation, which breaks the linker and releases the variable-mass TMT reporters. By comparing the peaks generated by each TMT reporter, one can determine the relative levels of peptides and in turn proteins of interest.

TMT labels are also highly amenable to multiplexing. “In the case of ICAT, it was only binary,” says University of Cambridge researcher Kathryn Lilley, who uses MS analysis to study protein localization in different cellular compartments. She notes that the further commercial development of TMT reagents made it



“By comparing the peaks generated by each TMT reporter, one can determine the relative levels of the protein of interest in each sample.”

possible to do six-plex and even 10-plex labeling experiments. “That meant it was possible for us to use more fractions per experiment and leave in every subcellular niche, more or less,” Lilley says.

Most current implementations of TMT technology use a three-stage tandem MS strategy (MS³), developed in 2014 by Stephen Gygi’s group at Harvard Medical School.⁴ Early experiments with TMT were potentially vulnerable to interference from random ions present in the sample, which would cloud the signals generated by the TMT reporter ions. The MS³ approach eliminates this interference and thereby produces stronger and clearer reporter signals for peptide quantitation. Gygi’s method achieves better performance by identifying fragments indicative of the

identity of the peptide in the second stage of MS. A selected number of these fragments are then collected and further broken down, resulting in the release of TMT reporters that are then detected in the final stage of MS³. “With this approach, you obtain a cleaner, low-mass spectrum containing the masses of interest—the signature ions—and you can determine their relative intensities,” Schmitt-Ulms says.

The case studies that follow illustrate some of the advantages of the combination of TMT and MS³. This approach is called synchronous precursor selection (SPS)-based MS³. For example, the potential to analyze six, eight, or 10 samples in parallel allows unprecedented multiplexing in a proteomic experiment, as demonstrated by the Lilley group’s atlas of the mouse pluripotent stem cell proteome. This multiplexing is also valuable for ensuring the accuracy and reproducibility of data obtained from relatively limited biological samples, as Chris Overall of the University of British Columbia demonstrated when he used TMT to reassure the diagnosis of a rare genetic disorder based on proteomics. Finally, the ability to look at many samples in parallel means researchers have the ability to detect aggregate signals from relatively rare peptides that might go missing in a single sample. “If you have limited amounts of material but have nine or 10 samples, they add together,” says Gregg Morin, head of proteomics at the Genome Sciences Centre of Canada’s British Columbia Cancer Agency, who is using the technology to identify novel protein biomarkers from tumor tissue samples. “So you’re able to get detection, which also means you get higher density of coverage.”

The commercial release of TMT 11-plex reagents in May 2017 enables even higher throughput protein analysis at very low levels. Collectively, these studies highlight exciting new opportunities for exploring the richness of the proteome and reveal important progress toward unlocking the potential of the proteome as a resource for both biological and clinical research.

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Postdoc Claire Mulvey (left) and PhD student Aikaterini Geladaki (right) from the Lilley laboratory. Image provided by Kathryn Lilley.

CASE STUDY

1



Kathryn S. Lilley
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Mapping cell-wide distribution of the proteome

Scientists have long been interested in cataloging the protein content of various organelles, such as the mitochondria, endoplasmic reticulum, and nucleus. But this level of cataloging requires a precise cellular dissection difficult to achieve, as Kathryn Lilley had learned. She recalls her early attempts to study the protein-modifying machinery of the Golgi apparatus. “We naively thought that we could isolate and purify the Golgi apparatus,” says Lilley, who is director of the Cambridge Centre of Proteomics. “It turned out we couldn’t because the physical properties of the Golgi are too similar to other parts of the secretory pathway.”

Research At A Glance

- **Application:** Proteome mapping
- **Description:** After cell lysis, the Lilley group isolated subcellular fractions containing different organelles via density centrifugation. These fractions were subjected to TMT 10-plex labeling and subjected to SPS-MS³ with an Orbitrap Fusion system. The resulting data allowed the Lilley team to assign different peptides to specific cellular compartments.
- **Sample Type:** Mouse pluripotent embryonic stem cells

Lilley’s group has made strides in mapping the cellular proteome since those early days, with a technique based on mass spectrometry known as hyperLOPIT (localization of organelle proteins by isotope tagging). The technique relies heavily on the multiplexing capabilities of tandem mass tags.¹ Lilley and her team begin by gently lysing cells to leave the subcellular organelles largely intact and then applying the cellular contents to a density gradient, a centrifugation-based approach that can efficiently separate organelles based on their mass and volume. Fractions that best represent each cellular compartment are then subjected to isobaric tagging with TMT, which allows each cellular compartment to be quantitatively measured and compared with other organelles in subsequent tandem MS analysis.

Initial iterations of LOPIT were hindered by limited multiplexing capabilities, with only a few isobaric tags available, and tended to suffer from poor resolution. With the combination of 10-plex TMT reagent sets, a more robust analytical method, and more sensitive MS instrumentation, however, Lilley’s group was able to achieve a breakthrough analysis of proteome localization in mouse pluripotent stem cells. “We could get a pretty decent overall cell map in a single experiment,” Lilley says.

This initial demonstration of hyperLOPIT tracked distribution of proteins in numerous compartments—including those residing in organelles such as the mitochondria, lysosomes, and nucleus, as well as proteins selectively associated with macromolecular structures, like ribosomes or chromatin. All of this data now reside in a searchable database. Lilley notes that she has already worked with many colleagues to help track down where their favorite proteins reside. “Many standard proteomics experiments give you information about the total amount of a protein, but they’re not giving you spatial information,” she says.

One of the most striking findings from this study was the fact that many proteins maintain multiple addresses in the cell. Lilley notes that these findings have subsequently been borne out in other hyperLOPIT-based analyses, including a newly published extensive survey of the human proteome that her group conducted in collaboration with the Stockholm-based Human Protein Atlas consortium, which employed large-scale antibody-based immunofluorescence microscopy.² These results lead her to believe that this multi-site localization is not a mere technical artifact but rather a core feature of the proteome. “It’s quite intriguing to imagine that proteins moonlight more than we imagined they did, and have different functions in different places,” Lilley says.



“One of the most striking findings from this study was the fact that many proteins maintain multiple addresses in the cell.”

To date, hyperLOPIT mapping has generally been applied at the level of groups of proteins, without attempting to distinguish closely related variants. However, Lilley’s team has shown that it is feasible to focus on peptides that make it possible to, for example, distinguish multiple isoforms of a given protein. This data could fill in critical blanks about how protein localization is regulated and ultimately, lay the foundation for a truly comprehensive map of the proteome.

References

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A principal component analysis (PCA) plot of the Lilley group’s hyperLOPIT data shows where in the cell each mouse protein can be found. Image reprinted with permission from *Nature Communications*.

A researcher in Huang's laboratory at UC Irvine. Image provided by Lan Huang.

CASE STUDY

2



Lan Huang
Professor of Physiology & Biophysics
University of California, Irvine, School of Medicine

Reconstructing proteomic interactions

Rather than having a constant structure, most proteins undergo routine conformational changes as they go about their biological business. Furthermore, proteins seldom work alone—they engage in complex webs of interaction that enable proteins to chemically modify one another or to assemble into larger complexes with specialized functions. Identifying these interactions within and between proteins is a critical task in deciphering their roles in the cell. Many researchers study these interactions with cross-linking strategies that chemically shackle proteins to one another. Lan Huang's group at the University of California, Irvine has now taken this approach a step forward with an approach based on mass spectrometry that makes it possible to precisely and reliably characterize protein-protein binding in a highly multiplexed fashion.¹

Research At A Glance

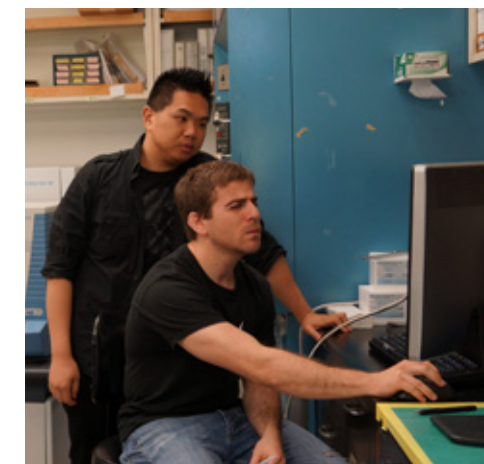
- **Application:** Identifying intraprotein and protein-protein interactions
- **Description:** Huang and colleagues first treated cytochrome c with their cross-linking reagent, disuccinimidyl sulfoxide, to tether adjacent segments of the protein. The cross-linked protein was then divided in two aliquots and labeled with TMT duplex tags. After performing ID-MS³ and SPS-MS³ analysis on the samples with a Thermo Scientific™ Orbitrap Fusion™ Lumos™ system, the researchers were able to identify interacting peptide fragments that had been cross-linked in the protein.
- **Sample:** Bovine heart cytochrome c

Huang's team has developed special sulfoxide-based MS-cleavable cross-linking reagents, which tether interacting polypeptide sequences together with a bond that can subsequently be broken during the MS analysis process. "Our strategy is to use multistage tandem MS to identify the cross-linked peptides," she says. In initial iterations of this approach, Huang's team used a binary, deuterium-based labeling strategy that allowed them to compare protein-protein interactions across two different samples. More recently, however, she has shown that the use of tandem mass tag (TMT) in conjunction with MS³ analysis allows for much more detailed and quantitative analysis of these interactions.

In this quantitation of multiplexed, isobaric-labeled cross-linked peptides (QMIX) approach, cross-linked peptides are detected at the MS¹ level. The cross-links are uncoupled from each other during fragmentation at the MS² level, and the joined polypeptides are then precisely defined and quantified during the third and final stage of MS analysis. This strategy combines two parallel approaches. One approach is called ID-MS³, which is optimized for specifying the proteins contained in a given complex. The other approach is based on a technique called "synchronous precursor selection," which improves the accuracy of detection and quantitation of the identifying peptide ions. TMT reagents are an important means for ensuring accuracy. "They allow you to do repeated experiments and have biological replicates in one sample," Huang says. "You increase your throughput and also enhance the signal, and you don't increase the complexity when you have multiple samples to compare."

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Graduate students Clinton Yu (left) and Craig Gutierrez (right) performing an LC-MS analysis in the Huang laboratory. Image provided by Lan Huang.

QMIX is well suited for understanding the interactions between individual proteins and can also offer insights into physical rearrangements in a protein's structure. For the initial proof of concept, Huang and her colleagues performed a relatively simple demonstration in which they used TMT 2-plex to characterize the internal interactions that take place in the protein cytochrome c. Since then, however, she has made greater use of this system's multiplexing capabilities. "We have applied 6-plex quantitation to multisubunit protein complexes to study their composition-dependent structure changes and to understand their action mechanisms," Huang says.

She adds that QMIX is especially well suited for monitoring proteomic reorganization resulting from specific perturbations, such as the addition of a drug or change in cell culture conditions. The Huang group's sulfoxide-based cross-linking agent is now commercially available, and she is hopeful that other labs in the proteomics field will find exciting applications for QMIX. "These techniques we developed can easily be applied if people have experience with TMT labeling," Huang says. "It shouldn't be a problem."

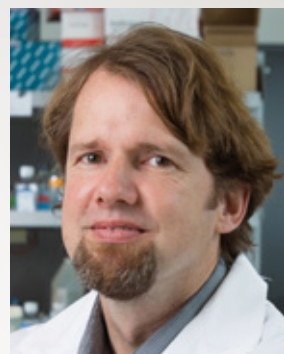
Reference

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Declan Williams, a postdoctoral fellow in the Schmitt-Ulms laboratory at the University of Toronto. Image provided by Gerold Schmitt-Ulms.

CASE STUDY

3



Gerold Schmitt-Ulms
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Picking apart the prion puzzle

P rion protein (PrP) can essentially act as an infectious protein because the misfolded PrP molecules have the capacity to trigger misfolding of otherwise-normal PrP molecules within a host. The resulting misfolded protein assemblies are responsible for diseases such as Creutzfeldt-Jakob disease and bovine spongiform encephalopathy (also known as mad cow disease).

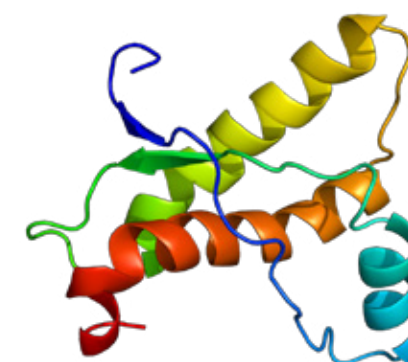
Research At A Glance

- **Application:** Functional proteomics of the prion protein
- **Description:** Schmitt-Ulms and colleagues used CRISPR/Cas9 to determine how the absence of the prion protein affects the proteomic content of various cell lines. After labeling with six-plex TMT, the researchers used an Orbitrap Fusion Tribrid system to perform comparative SPS MS³ analysis on wild-type cells, prion protein knockouts, and cells with a partial “knockdown” phenotype. In a preliminary analysis of one cell line, NMuMG, the researchers identified roughly 120 proteins whose expression levels are meaningfully affected by the presence or absence of prion protein.
- **Sample Type:** Mouse cell lines

Gerold Schmitt-Ulms of the University of Toronto trained with Stanley Prusiner, the scientist who discovered prions and won the Nobel Prize in Physiology or Medicine for his discovery in 1997. But even after studying PrP for almost two decades, Schmitt-Ulms is still struggling to understand exactly why we produce PrP in the first place. “PrP has been known for more than 35 years, but its function has been elusive for the longest time—and it still is controversial,” he says. In order to get to the bottom of this mystery, Schmitt-Ulms has used mass spectrometry and tandem mass tags to determine how the absence of PrP affects cellular proteomes.¹

Earlier studies in fish have suggested that PrP regulates signals that cause epithelial cells to convert to a more undifferentiated cell type known as mesenchymal cells—a process seen in some cancers. NMuMG is a widely used mouse cell model for this epithelial-to-mesenchymal transition (EMT). Schmitt-Ulms and colleagues used the genome-editing tool CRISPR/Cas9 to eliminate the gene encoding PrP from NMuMG cells. They then compared the proteomic contents of these PrP-deleted cells relative to unmodified NMuMG cells.

Isobaric labeling with TMT or iTRAQ reagents offered a valuable tool for facilitating this comparison. “There is a lot of run-to-run variance in MS—you might get as much as 30% difference when you analyze the same complex protein sample twice,” says Schmitt-Ulms. “If you have two different samples that you want to compare, it can be a major challenge to figure out which differences are real and which are run-to-run variance.” By analyzing their samples with multiplex isobaric labeling, Schmitt-Ulms and his team were able to study many replicates in parallel, building their confidence that any findings were real. Because each protein was represented by several peptides that



A structural model of the human prion protein. Image from Wikimedia Commons.

were being independently quantified, it became possible to distinguish even subtle changes from background noise. “We could see changes in abundance that were no more than 0.3-fold,” says Schmitt-Ulms, noting that older methods for comparative proteomic analysis had the ability to accurately reveal changes only greater than 1.5- or twofold.



“We could see changes in abundance that were no more than 0.3-fold”

This first study served as an initial survey of how the absence of PrP alters the NMuMG proteome. “We identified around 200 proteins whose levels were changed,” says Schmitt-Ulms, noting that this was the largest number of PrP-associated protein expression changes identified to date. However, this work also laid the foundation for the subsequent identification of a protein with an apparently close relationship to PrP, known as NCAM1. Schmitt-Ulms’s team discovered that PrP is responsible for promoting the addition of a particular chemical modification to NCAM1.² This not only promotes onset of EMT but also appears to drive numerous other critical biological processes. “This modification has key roles in cellular reprogramming that leads to cell motility and is important for neurogenesis, circadian rhythms, and more—biological processes with which the normal PrP had previously been associated,” Schmitt-Ulms says.

These findings about the PrP-NCAM1 relationship open the door to finally establishing the natural function of this protein, but Schmitt-Ulms has been unable to connect this interaction to the pathology of prion diseases. He is therefore delving back into the proteome in search of proteins whose behavior is specifically altered when the misfolded form of PrP is predominant.

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Shane Colborne, a research assistant in the Morin laboratory. Image provided by Gregg Morin.

CASE STUDY

4



Gregg B. Morin
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Proteomic discovery of tumor biomarkers

These days, researchers regularly interrogate the genomes and transcriptomes of tumor samples in search of abnormalities that might prove clinically informative. These interrogations are more challenging for proteins, which is problematic, given that gene sequences and RNA expression levels do not necessarily predict how much of a cancer-related protein will ultimately be produced.

Research At A Glance

- **Application:** Tumor proteomics
- **Description:** The Morin team performed multiple rounds of TMT 10-plex labeling with various collections of tumor specimens representing three different subtypes of ovarian cancer. To maximize the sensitivity of their analysis, the researchers used a sample preparation system developed in their lab called SP3-CTP, which boosts the efficiency of protein isolation. After performing SPS MS³ analysis with a Thermo Scientific™ Orbitrap Fusion™ mass spectrometry system, they were able to identify at least one protein that appears to be a potentially clinically relevant biomarker for certain ovarian cancers.
- **Sample Type:** Formalin-fixed paraffin-embedded (FFPE) human ovarian cancer specimens

As a result, cancer researchers have been missing out on critical data that could inform important treatment decisions. “You just weren’t able to get enough accurate information that was potentially useful, or get enough coverage of the proteome to be able to get good signatures for a tumor,” says Gregg Morin, head of proteomics at the Genome Sciences Centre of Canada’s British Columbia Cancer Agency. This is especially challenging because most banked tumor samples come in the form of tiny slices of formalin-fixed paraffin-embedded (FFPE) tissue, which requires exquisite sensitivity and accuracy to obtain robust results.

But Morin and his colleagues are starting to overcome these challenges by making use of isobaric tandem mass tags for mass spectrometry analysis of tumor samples. The first phases of TMT analysis are additive, measuring peptide levels in all the samples at once, which allowed the researchers to home in on proteins that would be too scarce to detect in a single specimen. In an initial investigation of FFPE specimens, Morin’s team used a 10-plex TMT reagent kit to simultaneously analyze multiple samples that represented three different subtypes of ovarian cancer.¹ Ovarian cancer generally is an aggressive and often fatal disease; classifications of the cancer subtypes are highly informative in terms of making a prognosis and selecting the appropriate treatment. To properly classify the disease, it would be immensely helpful for researchers and clinicians to have molecular markers that could aid in early, accurate diagnosis and perhaps even reveal new avenues of subtype-specific treatment.

TMT multiplexing allowed Morin and his colleagues to accurately measure and compare the quantities of a broad range of proteins from multiple tumors in parallel. Morin’s team also benefited

from the use of a bead-based sample preparation technique known as SP3, which was developed by lead author Christopher Hughes. “It has very high efficiency, so we lost very little material,” Morin says. Using this combination of SP3 and TMT, they were able to quantify thousands of proteins from ovarian high-grade serous carcinoma, clear cell carcinoma, and endometrioid carcinoma. According to Morin, the use of TMT analysis allowed them to quantify between two and three times as many proteins as previous-generation techniques.

This study also yielded a promising biomarker, a protein called cystathionine gamma-lyase, which was notably overexpressed in clear cell carcinoma relative to the other two ovarian cancer subtypes. “It has really opened up the field in terms of identifying candidate ‘cells of origin’ for clear cell carcinoma,” Morin says. “We’ve already submitted three additional grants and a manuscript based on that.”



“They were able to quantify thousands of proteins from ovarian high-grade serous carcinoma, clear cell carcinoma, and endometrioid carcinoma.”

Morin and his colleagues are based at a major cancer center, so they have access to a vast array of tumor specimens. The team has already begun to apply their MS strategy for detailed proteomic analysis for a variety of other cancers, including breast, bladder, and endometrial.

Most importantly, this rich stockpile of tumor-derived MS data is now forming the backbone of a publicly accessible database. This database is the kind of resource that genomic and transcriptomic researchers take for granted but which has been largely absent for proteomics. “Instead of waiting around for somebody else to do this, we’re going to do it ourselves,” Morin says. “We’re now doing this Canada-wide.”

Reference

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Histological analysis by Morin’s team showed heavy staining for cystathionine gamma-lyase in a subset of ovarian tumors (lower right), suggesting this protein might indeed offer a useful cancer biomarker. Image reprinted from *Scientific Reports*.

Theo Klein, a postdoctoral fellow in the Overall lab and one of the lead authors on the MALT1 study. Image provided by Christopher Overall.

CASE STUDY

5



Christopher M. Overall
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Explaining a rare disease with the help of proteomics

Chris Overall's group at the University of British Columbia is primarily a basic research laboratory. So it may seem strange that Stuart Turvey, a pediatric immunologist at the same institution, turned to Overall for reassurance on a diagnosis he made for one of his patients. It turned out to be the right call because it led to a detailed diagnosis as well as new insights into immune system function.¹

Research At A Glance

- **Application:** Characterizing an ultra-rare genetic disease
- **Description:** The Overall group used TMT analysis to identify proteins processed by MALT1 in order to understand the cause of the severe immune symptoms in a young female donor lacking this protease. They used a technique called TAILS for the targeted enrichment of cleaved proteins from B-cells from the donor, who was homozygous for MALT1 deficiency, and her healthy, heterozygous sibling and mother. Samples from unstimulated B-cells as well as B-cells that had been activated with ionomycin or PMA were subjected to TMT 10-plex labeling and analyzed on an Orbitrap Fusion Tribrid system; this revealed a differentially-processed protein with a prominent role in regulating immune cell activation.
- **Sample Type:** B-cells collected from the donor and her heterozygote brother and mother.

The patient was a 14-year-old girl with debilitating autoimmune disease. “She had small stature, weighed 22 kg, and had fragile bones, with unrelenting inflammation in her gut and on her skin,” Overall says. “She had a 100% chance of dying in the next couple years without a bone marrow transplant.” Turvey contacted Overall because of a mutation discovered during genome sequencing. “She was found to have a mutation in a protease called MALT1. She was the only living MALT1-deficient donor in the world, and she had just enough MALT1 to keep her alive,” Overall says. His group specializes in these sorts of protein-cleaving enzymes. They had developed a mass spectrometry approach called TAILS (terminal amine isotopic labeling of substrates), which makes it possible to accurately identify specific protein cleavage sites targeted by individual proteases.² TAILS relies on specific labeling of N-terminal protein ends—both naturally occurring ends as well as those resulting from the snipping action of proteases. By comparing B-cell samples from the patient to those collected from her healthy brother and mother, Overall's team sought to detect protease targets that were no longer being cut when MALT1 was defective.

Tandem mass tags played a critical role in this study; the Overall group worked closely with scientists from Novartis and Thermo Fisher Scientific to perform their analysis. “Every sample had to work the first time,” Overall says. “You can't keep doing 50-mL blood draws from a 22-kg patient.” By using 10-plex TMT for the TAILS labeling procedure, the researchers were able to achieve highly multiplexed analysis of multiple samples from all three family members. This multiplexing was particularly important: MALT1 is specifically activated in response to immune cell signaling, so the researchers wanted to monitor changes at various points post-activation. This experimental approach allowed them to confidently

focus on a single target that was no longer being cleaved in the B-cells of the donor—a protein called HOIL1.



“We think this is the first disease to be phenotyped and mechanistically explained by proteomics”

Previous studies have strongly suggested that MALT1 switches on a protein called NF-κB in response to T- and B-cell activation, which in turn stimulates immune cell proliferation and an increased inflammatory response. As they dug deeper into the relationship between MALT1 and HOIL1, however, Overall's team uncovered a more complicated picture. The lack of functional MALT1 results in an overactive NF-κB rather than preventing activation of NF-κB. This excess stimulation occurs because cleavage of HOIL1 normally sets into motion a cellular process that inhibits further immune activation by NF-κB. “Most people think about activation, but inactivation is just as important,” Overall says. “Although MALT1 initially is involved in stimulating NF-κB, it then actually applies a brake on the process in T-cell and B-cell stimulation.”

These findings explained why the young donor's immune system was raging out of control. “We think this is the first disease to be phenotyped and mechanistically explained by proteomics,” Overall says. “This didn't guide her treatment, but it was reassuring to her doctors.” Fortunately, bone marrow transplantation profoundly improved the girl's health and growth, and she is again attending school and leading a normal life.

Overall sees this study as a victory for collaboration, as the three lead authors—Theo Klein, Shan-Yu Fung and Florian Renner—hail from both academia and industry, and from both basic and clinical research. He also hopes to achieve similar wins in the future, with additional TMT/TAIIS-based clinical research studies now under way in his and Turvey's laboratories.

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In healthy B-cells (left), there is extensive colocalization (yellow) of MALT1 (red) and HOIL1 (green) after immune activation (bottom row). This did not happen in B-cells from the MALT1-deficient patient. Image reprinted with permission from *Nature Communications*.

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