Proteomics Meets Cellular Signaling: Exploring Post-translational Modifications by Mass Spectrometry Webinar 1 May 2013

[0:00:10]

Slide 2

Sean Sanders: Hello and welcome to this *Science*/AAAS audio webinar. I'm Sean Sanders, editor for custom publishing at *Science*.

The focus of today's webinar is the intersection between post-translational modifications and mass spectrometry. Mass spectrometry can be a powerful tool when applied to the identification of individual modifications, particularly when coupled with antibody-based peptide enrichment.

In this webinar we will examine how antibody-based proteomics approaches can be used to identify, study, and characterize known and novel post-translational modifications and elucidate their role in the regulation of large signaling networks as well as be applied in target identification, validation and biomarker discovery.

It gives me great pleasure to introduce our speakers today. They are Dr. Chunaram Choudhary from the University of Copenhagen in Denmark, Dr. Vipin Suri from GlaxoSmithKline in Cambridge, Massachusetts, and Dr. Cloud Paweletz from the Dana-Farber Cancer Institute in Boston, Massachusetts. It's a pleasure to welcome all of you to the webinar today.

Before we get started, I have some information that our audience might find useful. Note that you can resize or hide any of the windows in your viewing console. The widgets at the bottom of the console control what you see. Click on these to see the speaker bios, additional information about technologies related to today's discussion, or to download a PDF of the slides.

Each of our guests will give a short presentation followed by a Q&A session, during which our panelists will address the questions submitted by our live online viewers. So if you are joining us live, start thinking about some questions now and submit them at any time by typing them into the box on the bottom left of your viewing console and clicking the submit button. If you can't see this box, click the red Q&A widget at the bottom of the screen.

Please remember to keep your questions short and concise as that will give them the best chance of being put to our panel. You can also log in to your Facebook, Twitter or LinkedIn account during the webinar to post updates or send tweets about the event. Just click the relevant widgets at the bottom of the screen. For tweets you can add the hash tag #sciencewebinar.

Finally, thank you to Cell Signaling Technology for their sponsorship of today's webinar.

Slide 3

Now I'd like to introduce our first speaker for today, Dr. Chunaram Choudhary. Dr. Choudhary is currently an associate professor and group leader at the NNF Center for Protein Research at the University of Copenhagen in Denmark. His laboratory is interested in investigating the dynamics of protein post-translational modifications in cell signaling networks using quantitative mass spectrometry-based approaches. A very warm welcome to Dr. Choudhary.

Dr. Chunaram Choudhary: Thank you, Dr. Sanders, for your kind introduction. In today's talk I will talk about mass spectrometry-based global analysis of lysine acetylation and ubiquitylation.

Slide 4

Before going to this, I will give a brief overview about post-translational modifications and mass spectrometry-based quantitative proteomics, and then I will talk about lysine acetylation and ubiquitylation, how we apply this technology to investigate these two PTMs. And in the following talk, you will hear more from Dr. Vipin Suri about acetylation and enzymes that regulate this PTM.

So as you can see on this slide, there are diverse types of PTMs that can occur in eukaryotic cells. In fact, it is estimated that almost all proteins in eukaryotic cells are modified with the different PTMs at some point in the life, and several hundred different PTMs are reported in the later term. But only a few of these are studied in detail including phosphorylation, ubiquitylation and acetylation.

It is estimated that all together, post-translational modifications can increase the complexity of human proteome by at list an order of magnitude. PTMs can critically regulate protein functions. For example, phosphorylation plays an important role in signaling downstream of cell surface receptors. Acetylation and methylation can regulate gene expression via modification of histones, and ubiquitylation regulate proteins via the proteome system as illustrated in this cartoon.

The regulatory PTMs function in a highly controlled fashion and have to precisely control their cell fate. Thus, understanding the complexity and the dynamics of PTMs is highly valuable in understanding the mechanisms of biological processes.

[0:04:55]

Slide 5

In human cells, cells put really enormous resources to control these PTMs. For example, phosphorylation is controlled by about 750 kinases and phosphatases together and about 350 or 400 proteins that recognize these modifications, then release the signals.

Similarly, ubiquitylation is controlled by over 1000 proteins that regulate these modifications very dynamically.

Acetylation is controlled by nearly 100 proteins, and for each of these modifications, thousands to tens of thousands of sites have been identified in human cells. This implicate that these modifications have very broad regulatory scope in cells; and therefore, it is important to get a systems view of these modifications in cellular networks.

Slide 6

To do this, we use the so-called shotgun-based proteomics method, and what we do is we take cells or tissues, extract proteins and

proteolyse them with enzymes such as trypsin and enrich the modified peptides using affinity-based reagents such as antibodies or different mutases that bind to modified proteins which results in enrichment of modified peptides.

And this is important because eukaryotic proteins are highly complex and many PTMs occur at low levels in cells. And the identification is challenging and enrichment has to detect these low-abundance PTMs which may play important regulatory functions.

Slide 7

And there are different types of enrichment reagents which can be used for enriching different types of PTMs. Since post-translational modifications are different in their chemical and structural properties, these reagents can be selected for one or more types of PTMs. For example, phosphorylation can be enriched using metal or metal oxide-based chromatography. Also, tyrosine phosphorylation and lysine acetylation can be enriched typically and it's using modification-specific antibodies. Glycosylated peptides can be enriched using lectin-based chromatography.

Slide 8

And here's a workflow for a shotgun proteomics. After enrichment of PTM-containing peptides, samples are analyzed using mass spectrometry. We use liquid chromatography coupled with electrospray ionization to separate peptides based on their biophysical properties and ionize them before transferring them to a mass spectrometer. Their mass spectrometry is recorded and subsequently individual peptides are isolated, fragmented, and fragmented spectra are recorded.

Raw mass spectrometry data are then searched using a computerized algorithm to identify proteins and peptides. This can be used for downstream bioinformatic analysis.

If combined with quantitative approaches such as SILAC or other quantitative methods, relative and absolute evidences of modified peptides can be quantified.

A key strength of this technology is that it allows identification and quantification of thousands of PTM sites in a relatively short period

of time in an unbiased fashion, and this technology is also highly versatile and can be applied to investigate the dynamics of PTMs in response to different cellular perturbations and different model systems.

Slide 9

We use this technology to investigate the regulatory scope of lysine acetylation in human cells. As you can see, we use three different cell lines to monitor or measure acetylation, and together in these cell lines we identified over 1700 acetylated proteins that contain about 3600 acetylation sites. These results provided a first view of lysine acetylation in human cells and indicated that lysine acetylation plays broad regulatory roles. And before this study, of course, acetylation was known to play important roles but mostly its role was known in context of histones and some nucleoproteins. And our data suggested that acetylation may have regulatory functions outside the nucleus.

Slide 10

In subsequent studies, we investigated lysine acetylation in Drosophila melanogaster, in yeast, Saccharomyces cerevisiae, and mice in their tissues.

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And here is an example in which we compared acetylation frequencies with well-known modification phosphorylation and checked how the frequency of these two modifications is in different organisms.

As an interesting example, you see in the left-hand side of the slide, we note that in prokaryotes, phosphorylation is relatively not that abundant, typically about 75 in each of the four bacteria that have been analyzed so far.

In contrast, acetylation appears to be more frequently occurring in two of the bacteria that have been analyzed so far. When we look in eukaryotes; that's in the right-hand side of your slide; you see that phosphorylation is by far much more abundant modification and much more frequently occurs in these organisms compared to acetylation. This gives a contrasting picture that the frequency of

acetylation and phosphorylation differs between bacteria and eukaryotic cells.

Slide 11

When we look at the frequency of these two modifications in the mitochondria which are believed of prokaryotic origin inside the eukaryotic cell, it's interesting to note that the frequencies of these two modifications are similarly to bacteria. Their phosphorylation is more often observed outside the mitochondria, and acetylation is more frequently observed in the mitochondria. In this picture we saw frequency of acetylation in cancer cells; and in human cells, about 15% to 18% of modified acetylation sites occurs on the mitochondrial protein.

And more dramatically, we have data and also published data such as that this trend is even more dramatic in tissues where nearly half or more of the sites can occur on mitochondrial proteins, suggesting that the phosphorylation and acetylation targets very different types of proteins. Although their functions are significant, particularly in context of bacterial acetylation and mitochondrial acetylation, it remains to be fully investigated.

Slide 12

Next, I will talk about proteomic investigation of lysine ubiquitylation. Ubiquitin is a small molecular protein that can be conjugated to lysines via a fifth aminoglycine. And after conjugating ubiquitin to proteins, trypsin proteolysis of proteins, these proteins generate so-called di-Gly motif. In this case, the last two amino acids of ubiquitin remain conjugated to the lysine, and these then generate so-called di-Gly modified peptides which are branched peptides and they can be analyzed using an antibody that was used by Xu, et. al. in 2010. And we applied this method combined with the mass spec analysis to analyze ubiquitylation in human cells.

I should mention that the di-Gly remnant is not a unique segment of ubiquitin. It can also be generated by two other ubiquitin-like molecules, NEDD8 and ISG15.

Slide 13

Using this technology, we identified over 11,000 ubiquitylation sites in human cells which vastly expanded our knowledge about this

modification in human cells. And when we compared this modification with previously known acetylation sites, it's interesting to note that about one-third of all acetylation sites can also be modified by ubiquitin, suggesting that there might be a potential cluster between these two modifications, although it remains unclear that the ubiquitylation and acetylation occurs on different pools of proteins or they may directly compete for the target site.

Slide 14

Furthermore, we used guideline or this approach in mouse tissues to investigate ubiquitylation in five different tissues -- liver, kidney, brain, heart and muscle -- to demonstrate that this approach can be used in much larger and complex organisms for getting a systems view of this modification. And this approach now provides a way to look at ubiquitylation on a global scale in a relatively short time and should provide an important way to look at this modification and its regulatory properties.

Slide 15

We also investigated ubiquitylation by di-Gly profiling in human cells that were treated with a proteasome inhibitor MG-132.

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Proteasome regulates protein stability and it is known that inhibitional proteasomes should increase ubiquitylation, that this modification is best known to play a very important functional role. Then we treat human cells with the proteasome inhibitor and look at changes in ubiquitylation. As you see on the right-hand side of this chart, about half of ubiquitylation sites showed a dramatic increase.

More interestingly was that a substantial fraction indicated in blue also showed decrease in ubiquitylation within four hours after proteasome inhibition. And then we look at these proteins, these proteins are nucleoproteins like histones and many other proteins that have chromatin-associated functions, suggesting that inhibition of proteasome can decrease ubiquitylation on proteins that are located in the nucleus, and these ubiquitylation may have non-proteasome regulatory functions.

Slide 16

Finally, we applied this technology to investigate ubiquitylation dynamics in cells treated with ultraviolet or UV radiation, and we identified several hundred ubiquitylation sites that were increased after UV treatment, and this natural diagram shows some of the proteins that were dynamically regulated by UV radiation and this includes some of the less known proteins like PCNA, FANCI, RPA2 and RPA1. These were previously known to be important for damage repair after UV and regulated by ubiquitylation. Now we map sites on these proteins and also implicate many other proteins which are regulated by ubiquitylation in this context.

Slide 17

To summarize, mass spectrometry-based proteomics is ideally suited for global analysis of ubiquitylation and acetylation networks in cells and tissues.

Lysine acetylation frequently occurs on highly conserved mitochondrial metabolic enzymes.

Ubiquitylation and acetylation show a substantial overlap at a site level.

Quantitative ubiquitylation analysis identity sites regulated by proteasome inhibitors and UV-induced DNA damage.

Slide 18

With that, I would like to thank the members of my laboratory whose work I have presented here and also funding agencies who have generously supported our research.

Thank you.

Sean Sanders: Thank you so much, Dr. Choudhary.

Slide 19

We're going to move right on to our second speaker for today, and that is Dr. Vipin Suri. Dr. Suri is Director and Head of Pharmacology at Sirtris, a division of GlaxoSmithKline in Cambridge, Massachusetts. His research there is focused on the sirtuin family of protein deacetylases, specifically understanding the regulation of

protein acetylation and the role of sirtuins in metabolic, inflammatory, and neurodegenerative diseases as well as the discovery of novel sirtuin modulators as therapeutics for diseases.

Welcome and thanks for being with us, Dr. Suri.

Slide 20

Dr. Vipin Suri: Thank you, Sean, for inviting me on your panel.

Slide 21

Chuna gave an excellent introduction and overview on acetylomics so I won't belabor it except to just give a little bit of an introduction to the enzymes that regulate acetylation.

So the acetyl groups in proteins come from acetyl-CoA through the action of acetyltransferases and deacetylases removed from the acetyl groups.

Slide 22

And this is the family of histone deacetylases.

Slide 23

The enzymes that we are specifically focused on at Sirtris are the sirtuins, and the sirtuins differ from other histone deacetylases in that they use NAD as a substrate. So the sirtuin reaction is acetylated protein plus NAD gives you deacetylated protein nicotinamide and the acetyl group is transferred to O-acetyl-ADP-ribose.

Slide 24

The questions that we're interested in are can we use SIRT1-dependent acetylome to (A) provide biomarkers for sirtuin modulators, and (B) to generate testable pharmacological hypothesis? So what we're trying to do is try to connect a pharmacological phenotype of a SIRT1 modulator with the substrates that are involved in generating those phenotypes, at the same time discover pharmacodynamic markers that we can use in preclinical studies and clinical studies.

Slide 25

And so the workflow is to identify the acetylated substrates of SIRT1, to validate those substrates, and then to develop assays based on those substrates.

[0:20:09]

Slide 26

And so because one of our goals is to understand the potential of this substrate as biomarkers, we work in many different systems. But before I go to that, let me just point through a few studies in literature that were published in the last year or so that are applied to identify the SIRT1-dependent acetylome, and these are useful to review because the methodologies are slightly distinct.

The key problem with these acetylome approaches is how do you enrich for the acetylated proteins? The better the enrichment, the better your DNA quality.

So Anderson and colleagues published this really interesting strategy about a year and a half ago, and they were interested in looking at the sirtuin substrates in Xenopus cytosol. This was an in vitro strategy. In this case, what they did was they made extract from the cells, Xenopus cytosol, and blocked all the free lysines with the NHS esters, treated the blocked lysines with SIRT1 in vitro with NAD as a co-factor, and SIRT is going to deacetylate a certain number of lysines and the deacetylated lysines were then modified by an NHS ester with a biotin tag on with it. And then you can use biotin to enrich the substrate. So this is a good strategy to do mass spec to identify the substrates.

So this is a good strategy for an in vitro work. One potential issue to keep in mind is that the specificity of these enzymes in vitro and in the cell or in the tissue could be different. So you could pick a superset of substrates this way. But in this case, it was a pretty successful strategy.

Slide 27

The other methodology was from Peng and colleagues where they were interested in looking at the differences in the acetylome in SIRT1 knockout murine embryonic fibroblasts versus wild type and

they did SILAC quantification in this case. But the interesting method was so they enabled the wild type cells and the knockout cells and mixed them, and then they did the IP prior to the trypsin digest. And then they analyzed the IP on an SDS-PAGE and then mass spec.

So in this case, the fine acetylomic mapping wasn't done, but it was an acetyl lysine IP followed by proteomics. So this is also a useful strategy. It gives you good information. The information it lacks is on fine-mapping of the acetylome site.

Slide 28

And the most comprehensive study was from Yingming Zhao's lab about a year ago where again he was interested in looking at the differences in the acetylome in wild type murine embryonic fibroblasts and SIRT1 knockout fibroblasts, and he did an extensive fractionation strategy so he SILAC labeled the cells, mixed the lyases, trypsinized the lyases, then fractionated and then did the acetyl lysine IP and got excellent information on several thousand acetyl sites.

Slide 29

There's a lot of very good quality data that was provided by Yingming. And the observations were that many of the SIRT1 substrates are nuclear. They are in specific complexes that are tied to transcription, that are tied to DNA repair, spliceosome and other pathways.

And one of the other interesting observations was that some of the substrates for SIRT1 were actually acetyl transferases so P300 and MYST family were also identified.

Slide 30

For us, as I mentioned, the important goal is to understand pharmacology of SIRT1 modulators and identify biomarkers. So we have looked at the SIRT1 acetylome many different ways, in many different situations, and the reason for doing that is because we want a very robust set of markers that we can reliably use preclinically and clinically.

So we have looked at animals. We have looked at mouse liver from conditional knockouts, developmental knockouts, catalytic domain, deleted construct, full deletion, pharmacological inhibitors. And then in the cells we have looked at murine embryonic fibroblasts as well as U2OS cells where we have knocked out SIRT1 with Zinc Finger Nuclease technology. So we have a very closely matched pair of cells, one of which expresses SIRT1 and the other one lacks SIRT1 protein and activity.

[0:25:05]

Slide 31

This is data from mouse liver. This is a volcano plot. The Y-axis here is the false discovery rate and the X-axis is $\log_2$ fold change in knockout versus wild type.

And in the mouse liver, so there are three sets of dots here. There are the blue dots, so these are sites that are not changing between the knockout and the wild type; the red dots that meet our false discovery rate criteria which is in this case 10%, but the fold change is less than twofold; and the green dots which are the robust deregulated sites.

And you can look at the green dots to see that you get -- on some of these sites there are that dramatic changes, 50-fold, 30-fold, in the absence of SIRT1 which again (A) overlaps very well with Yingming's data, and secondly, tells you that SIRT1 robustly regulates specific sites and is presumably the sole deacetylase for these sites.

Slide 32

And then if you compare the pathways that are affected in our studies versus what was seen by Yingming, there is a fairly good correlation. So again, we get a lot of nuclear pathways, RNA splicing, DNA damage, and then the bottom graph here shows the correlation amongst the z-scores in the two studies, which again points to similar pathways being affected.

One observation we did make was that the proteins are very often the same even if sometimes the site that we saw was a different site from what was seen in the fibroblast. The complexes were very often identical in the studies that we have done with what's been published.

Slide 33

And then the next question we asked was can we replicate the effects of the genetic knockout with a pharmacological agent? And so we used EX527 inhibitor of SIRT1. This is about a 100 nanomolar SIRT1 inhibitor in vitro, about a micromolar in cells. We didn't know the selectivity of the compound in cells.

But here's the data from the study. So the left panel here, the Y-axis is $\log_2$ fold change, and the X-axis is the different sites. There are two sets of dots again. The blue dots are not specifically significant change sites. The red dots are specifically significant change sites. The left graph here is the effect of EX527 in the wild type cells where you see a number of sites change fairly robustly, and then the right panel is the effect of the same compound, the same concentration in knockout cells where you see very few sites change.

Slide 34

So this tells us a couple of things. One is that the inhibitor replicates the effects of the knockouts, so that was quite useful. And secondly, the inhibitor is fairly specific in the absence of SIRT1 so there are very few sites that are changed by the inhibitor.

And then the bottom panel shows the correlation between knockout versus wild type on the Y-axis and the inhibitor versus vehicle on the X-axis. And again, there is a fair correlation between the two, which was very useful for us as we developed these modulators.

Slide 35

The next set of experiments for us was how do we validate the sites that we have identified. So I'm going to focus just on one site here which is the nucleoporin 50 as an example of the sort of methods we're using for validation.

That protein reproducibly showed up across many studies, and the specific site that shows up is the lysine-8. So as we validate the site, we wanted to get away from the acetyl lysine IP and we wanted methods that were free from any bias that the antibody could bring in. So what we did was transfect tags. In this case it was a FLAG tag,

Nup50 cDNAs into wild type and knockout cells, doing immunoprecipitation and then observe on the Western blot the acetyl lysine antibody, the acetylation status, and you can see that in wild type cells that Nup50 K8 acetylation is barely detectable while in the knockout there is a robust signal.

Slide 36

Before that we wanted to quantify the acetylation and we used SILAC quantification in this case. So we transfected the DNA into wild type and knockout cells, going under heavy and light media, lysed it and then trypsin-digested it into the mass spec.

Slide 37

And we could identify a number of sites on Nup50.

Slide 38

Out of which only one site was robustly regulated which was the lysine-8 which changed about 30-fold in knockout and it's greater than 30-fold because we couldn't see it in the light media.

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And there's another further site that we could easily see, but the acetylation was not changed in the knockout cells. So this is the point I was making earlier is that the selectivity of these enzymes are very, very good in cells and in tissues and sometimes you lose that in the in vitro studies.

Slide 39

The other strategy we've used for validating the sites as well as assay development is targeted proteomics. So this would be a multiple reaction monitoring. And these assays are great because they are very sensitive, they're very reproducible, and you can multiplex them.

So again, as we think about assay development for these sites, for example, Nup50, the ideal assay would detect total protein, acetylated site that is SIRT1-dependent, so SIRT1-dependent acetylation, and a site that doesn't show a SIRT1-dependent acetylation all in the same sample.

And in addition, because we've been able to identify 10 to 15 robustly regulated shared sites across multiple systems, we would like to assay all of them at the same time if possible and MRM is a method that lets you do that.

Slide 40

And so this is Nup50 MRM assay development. In this case, we monitored transition pairs in the mass spec, and again, the information we get is very similar to what we see with SILAC-based approaches where they raise a dramatic change in Nup50 K8 acetylation in the SIRT1 knockout samples as well as inhibitor-treated samples.

Slide 41

And then the third approach which we're working on is to use antibodies to develop acetyl-specific antibodies and use these for quantitative multiplex assays. The challenge here is developing good antibodies because it's not trivial and we've had only limited success.

Slide 42

The last thing I wanted to say was that much of the work we have done and much of the work in literature is looking at acetylome in the static state, so it's a sampling at a given point of time on what's happening to the acetylome.

But very recently, we have started looking at dynamic acetylome and how the acetyl sites change on a protein over time, and this is an experiment that we did in collaboration with folks at KineMed, Inc. in which we infused mouse with 13C-acetate for different times, two and eight hours in this case, and then took the liver out and then did acetylomics with antibody enrichment.

And the rates of acetylation can be very different at different sites. Even in the same protein they could be very different. In this case we have four different proteins where there's a hundredfold fold difference in the rates of acetylation. So we think that the dynamics of acetylation can really give very unique insight especially when we try to understand compound action and try to correlate it with

other downstream pharmacological endpoints. And this is likely to be very useful in the future.

Slide 43

I just wanted to conclude with our operations, and that we have been able to identify a conserved set of SIRT1 substrates in mouse and human cells and tissues.

Generally, those substrates appear to be involved in nuclear processes and then the rates of acetylation can vary dramatically at acetyl sites and its rate information can be very useful as well.

Slide 44

And I just want to thank our colleagues at Sirtris and GSK as well as Cell Signaling, Biognosys, Kinaxo, KineMed, Yingming Zhao and the Buck Institute.

Sean Sanders: Wonderful. Thank you so much, Dr. Suri.

Slide 45

Our final speaker for this webinar is going to be Dr. Cloud Paweletz.

Slide 46

Dr. Paweletz is Head of the Translational Research Laboratory of the Belfer Institute for Applied Cancer Science in Cambridge, Massachusetts. He was recently part of a team that is helping to drive the development of mass spectrometric-based diagnosis and protein microarray-based disease detection.

Welcome, Dr. Paweletz.

Dr. Cloud Paweletz: Thank you. Can you advance the slides for me, please, Sean?

Sean Sanders: Sure. No problem.

Dr. Cloud Paweletz: Thank you. Second slide, please.

Slide 47

That was a very nice introduction, so I want to take the whole idea of post-translational modification and using proteomics to apply them to scientific problems initially toward some sort of a more actual problem.

And the way I look at proteomics, the way I actually look at any kind of dynamic changes in the body and apply them to drug discovery is to really think about it either in a compound-centric way or patient-centric way. And the idea obviously is if you look at the compound, if you look at the phosphorylation changes in a compound-centric way such as in the phase one, we think about pharmacodynamic, we think about target engagement, pathway modulation. What does the drug actually do to your body? What are the pathway changes?

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A patient-centric way is really thinking about patient stratification, what is the second line treatment, what is the regression.

And the reason I look at phosphoproteomics specifically is to really… When we look at cancer, if you really have to look where we are in molecular targeted therapies, we really can say that yes, we have seen some dramatic responses in some selected subsets of patients, very small percentages. The responses are usually very, very short. They correlate to some sort of molecular phenotype. But we really still have idiosyncrasies despite genotyping.

Slide 48

And if you really think about strategies here, if you have a kinase inhibitor that actually acts on a drug with the target mutations, we have some sort of tumor response, we have regression of the tumor. But sadly, what we actually see is that we have resistance occurring. If that happens, usually we see resistance after about six months.

And these can actually happen for pretty much three reasons. One of them is the second mutation in your kinase target that's actually when does your kinase struck inactive. You can have bypass mechanisms or you can have activating downstream effectors. And ultimately, those really end up in the pathway reactivation.

So the strategy and the way we actually use phosphoproteomics for this problem is that when you think about you can take a patient, you can have a diagnostic biopsy, you can do a cell protein network analysis, you can come up with inclusion, exclusion, pharmacodynamic biomarkers and really monitor the treatment.

Once you actually have the treatment, we have resistance, we can have take a post-therapy biopsy, do the same sort of analysis and then keep and change the therapy.

Slide 49

What we need to do and the goal really is really understand how can we monitor simultaneously critical phosphorylation events across many, many different pathways in response to compound treatment? Or another way, what are the phosphorylation changes that actually happen upon compound treatment?

Slide 50

So I'm going to give one example. I'm going to just -- again, it's very similar, PhosphoScan Direct. The example that I'm going to be using to look at the PI3-kinase pathway in the context of cancer. And the strategy that we use is that if you look -- we know quite a lot about pathways and we have exquisite compounds of incredible sensitivity and specificity to different nodal points of those pathways and we can really dissect the pathway.

So in the case that I will be explaining, if you actually look at the PI3-kinase pathway in the pathway-dependent context, we use the three different compounds. You look at the general PTEN, PI3-kinase inhibitor, we look at the PDK-1 inhibitor and we look at the AKT inhibitor.

Slide 51

So the way we went about doing it is we decided to use a PTMScan method approach. There's really no reason to go to the right side anymore. That's what I described.

Again, just to the right side is you have the PhosphoScan. We can think about actually -- we heard a little bit about ubiquitylation. You heard a little bit about acetylation, methylation. And I'm going to talk about the PhosphoScan and the PhosphoScan Direct.

Slide 52

So the idea here, as I was saying, is to look at the PI3-kinase pathway. We looked at three independent inhibitions at different nodal points, either at the PI3-kinase pathway in itself or PDK-1 or MK2206. The way you can think about is that we actually dissect the PI3-kinase-dependent arm of the pathway and the PI3-kinase-independent arm of it.

Again, the strategy that we use too is to look actually at motif antibodies where we actually immunoprecipitated known motif antibodies to those inhibitors. So for example, we could look at phosphotyrosine which we did. We can also look at the AKT substrate motif antibodies and actually say, okay, what are the substrates that actually are modulated by AKT inhibitor, as well as we can actually look at a PDK-1 inhibitor, FxxFsDFxxR.

What we get in the end, I tried to schematically show to it in the bottom right corner, is that you will actually get a list of phosphoproteins that shared a motif that we expect to trace, so that's one. We see changes that happen upon AKT inhibition, we see changes that happen upon PDK-1 inhibition, and then obviously if you use a PI3-kinase PTEN inhibitor which happened with our PI-103 inhibitor.

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You will see when we actually do this analysis, you can see, okay, phosphorylation changes actually happen pathway-centric, pathway-independent and are compound-specific. So that way we can really distinguish what phosphorylation changes are compound-dependent and what are pathway-dependent.

Slide 53

As described previously, we actually decided to use a stable isotope labeling for quantitation. The one point that I would like to make on this slide is in the middle, is that for each study that we do, we actually do not just one labeling. We actually also do the reverse labeling.

So in the case you can use C12-arginine/lysine, vehicle treatment and then use compound treatment. But then we reworked and we

actually looked for certain changes with the compound to actually use it as a filter to say those are robust changes.

The experiment that we did was actually quite a big experiment involving 52 different immunoprecipitations. In this case, we used a PC3 cell lines, PTEN deficient, plus/minus treatment, six different immunoprecipitations. And then for the reverse labeling, we do all quantitation first and we then actually only identify the changes, the proteins and peptides that are changing.

Slide 54

So then we summarized them. These are the results. So the top left is very similar to the schematic that I've shown before. We identified 375 fossil peptides that shared the motif, and of those 375, we identified 19 phosphopeptides that was shared and they changed with AKT inhibition as well as PDK inhibition, and then you can break it out differently. And again, as I described, you can use the Venn diagram to see which phospho changes are dependent on the PI3-kinase, which one are the PI3-kinase-independent changes.

All the way to the right you see the identification of those 19 proteins that change upon AKT and PDK-1 inhibition. And non-surprisingly, not only did we identify the translational arm of it, but we also identified arms of the PI3-kinase pathway that actually are changed upon modulation.

Slide 55

When we look at the next slide, we then actually decided to move forward to go a little bit deeper into the pathway, and then we decided to use what we call a PTMScan Direct mode. And that is rather than using antibodies against particular motifs, we actually used an antibody—and this was done in collaboration with...

Slide 56

…Cell Signaling Technology—against 130 serine/threonine kinases. There's about 300 different sites. So rather than just looking at the RXXS or the PSSP motif, we actually had an antibody cocktail against pretty much everything in the CSP catalog.

Slide 57

Just as I've told you for the AGC arm of it, what proteins you would get out of it. For example, you would identify the serine-241 site for PDK-1. You would identify AKT threonine-308 site, et cetera, et cetera, et cetera.

Slide 58

When we combined now both experiments, the substrate motif antibody experiment as well as the PTMScan Direct experiment, this is the result that we get. So in this network, demonstrate -- it's not a linear pathway network, but it's actually a GO annotation network that links the function of your protein with proteins that are linked and enriched in the dataset.

So what we identify, obviously, we identify -- this is just one particularly slide in the AKT pathway. We identify targets that are phosphorylated by AKT in the cytosol**.** We identify the mTOR mediated signaling end. We identify the FGFR arm. But what's actually really interesting in this example is that cyclin-dependent kinase 1 was actually not believed to be involved in AKT signaling. And by using a very well-defined pathway system, a very, very well-defined signaling system that we know which pathway of ubiquitylation we have as well as very, very well-defined compounds, we are able to actually link PDK-1 exclusively to AKT.

We actually tested this by antibodies. The proteins that we identified to be changing by mass spectrometry we actually identified by Western blot as well. And this is just an example thereof.

[0:45:08]

Slide 60

I just want to acknowledge a lot of people. Most of this work was done actually at the Merck Laboratory and its published in *Science Translational Medicine*. Obviously it's a large, large effort for a lot of people. Thank you.

Slide 61

Sean Sanders: Great. Thank you, so much, Dr. Paweletz.

I wanted to thank all our speakers for the excellent presentations, but we're going to move right on to the questions submitted by our online viewers. Reminder to those watching us live that you can still submit questions by typing them into the question box and clicking the submit button. If you don't see the box on your screen, just click the red Q&A icon and it should appear.

So the first question I'm going to put to you, Dr. Choudhary. It's about reproducibility, and this viewer asks if you can comment on the qualitative and quantitative reproducibility observed when you are using motif antibody enrichment. And I'll come to you Dr. Suri and Dr. Paweletz afterwards. But Dr. Choudhary?

Dr. Chunaram Choudhary: With motif-specific antibodies we had variable success. For acetylation, so far we have used polyclonal antibodies and then we observed batch-to-batch variabilities even from the same vendor and much greater variability from different vendors.

And using the same batch, we get about 60% to 80% overlap between independent biological replicates. So with the same batch we get relatively good reproducibility. However, in one case, we tested two different methods and reproducibility was only about 40%.

So I think in this case it depends on really from antibody batches. And with the monoclonal antibodies, for example, in this case of di-Gly modified peptide, we had much greater success both from Cell Signaling and also from the other antibody that was published from Xu, et. al. So it's a good idea to really think that before you perform large experiments.

Sean Sanders: Great. Dr. Suri?

Dr. Vipin Suri: Yeah, I would agree with Chuna. There's very significant variability with acetyl antibodies. So across vendors, you can get very different results with different antibodies because the different vendors make them differently. But even if you stick with one antibody, there is lot-to-lot variation and you have to optimize each time. So if you're planning to do a number of acetylome studies, you should really load up on one lot of antibody if you can from a vendor.

Sean Sanders: Excellent. Dr. Paweletz, anything to add?

Dr. Cloud Paweletz: Yeah. Actually, I would only add that the way we try to actually account for this is the fact that we're using the reverse labeling approach. So yes, there's variability in your antibodies, but by actually focusing on the experimental design, you can actually control a lot of those variables.

Sean Sanders: Great. Let me stay with you, Dr. Paweletz and ask you a related question, not on reproducibility but on specificity. So what kind of specificity can you obtain in immunoaffinity purification of PTM peptides?

Dr. Cloud Paweletz: Well, obviously, the specificity is guaranteed by the mass spectrometry. So the whole idea, it obviously comes from the topic of discussion of phosphoproteomics. So the strategy that we use is to use very well-defined pathway effects and use compounds thereof.

So for example, for AKT, we decided to use an AKT-specific motif antibody. Now, will there be nonspecific peptides hold out? Yes. But we will use the power of the mass spectrometer to really divide and really dive into that information and use that as a filter.

So the specificity, I'm not that concerned because I used the power of the mass spectrometer to actually really divide and understand the pathway effects.

Sean Sanders: Excellent. Any other comments from the other panelists?

Okay. I'm going to move on to a question for you, Dr. Choudhary. Are there any changes in the phosphorylation or acetylation ratio during development of eukaryotic organizations that you have observed such as Drosophila?

Dr. Chunaram Choudhary: Yes. That's a really, really interesting question. And what we observed and also started noticing is that the number of phosphorylation sites increase with evolution from prokaryotes to eukaryotes, and the relationship seems not to hold true for acetylation. In fact, if there's anything, it's contrary. We observed far more acetylation in bacteria and in mitochondria that phosphorylation is much less frequent.

Sean Sanders: Excellent. A question I'm going to put to you, Dr. Suri. Can antibody-based enrichment be utilized to understand and explain phenotypic differences? Do you believe?

Dr. Vipin Suri: Phenotypic differences, I mean that's what I would be trying to do is to connect the acetylome data, in our case, with phenotypes.

[0:50:10]

It depends on how you set up the question. You can use it. It just depends on how you set up the question.

Sean Sanders: Great. Dr. Paweletz, I thought you might have some comments on this as well. Can antibody-based enrichment be utilized to understand and explain phenotypic differences?

Dr. Cloud Paweletz: Yes, I think it can be. It depends. It really comes down to the fact do you understand some of the biology that is linked to the phenotypic differences? So if you understand some parts of the biology, it will help us to expand to that knowledge.

Sean Sanders: Excellent. Dr. Choudhary, back to you, is there any crosstalk between the different modifications and analyses that you've done?

Dr. Chunaram Choudhary: So what we say is usually overlap between the modifications. That means that we detect different modifications occurring on the same residue. And I showed you about overlap for ubiquitylation and acetylation. That doesn't really directly tell that there is a crosstalk between these two modifications. So what we say is that these modifications can occur on the same amino acid and they can potentially have a crosstalk, but we really don't have a direct evidence to say indeed they have a crosstalk and they functionally impact each other. This is actually important to understand in the future studies.

Sean Sanders: Excellent. Dr. Suri, this is a question for you and I think Dr. Paweletz as well. What are some of the data analysis challenges when comparing reverse SILAC labeling versus normal SILAC labeling?

Dr. Vipin Suri: Reverse SILAC labeling which is normal?

Sean Sanders: Yes.

Dr. Vipin Suri: I'd like Cloud to take that question. For us, I think the bigger challenge is that when we do the label-independent quantification, but I'd like Cloud to take that question.

Sean Sanders: Sure. Dr. Paweletz?

Dr. Cloud Paweletz: We actually designed and developed a software, a custom software tool to do so. The biggest challenge is actually identification of the peptides and actually labeling and aligning the peptides pair.

So if you think about -- let's think about an example. So if you have a mass difference of three and you have two items, you actually have to really a high-accuracy mass spectrometry to really identify and quantify the differences. So it's one too many differences that you have to account for.

Dr. Chunaram Choudhary: I have a comment here actually. For SILAC it's important that between the isotope labels, there should be typically about four differences to really accurately quantify and use labeling in case if you have a contaminant or something because they always are like-labeled. So reversing SILAC labeling then has to really distinguish from contaminants to real candidate proteins.

Sean Sanders: Great. Thank you, Dr. Choudhary.

I'm going to put a question to you, Dr. Paweletz, now. This viewer says that they're familiar with the two primary means of enriching phosphoproteins prior to mass spec which is immunoprecipitation with phospho-specific antibodies and IMAC which is immobilized metal affinity chelate phospho-enrichment kits. Can you comment on the pros and cons or the strengths and weaknesses of these two methods?

Dr. Cloud Paweletz: I think it depends on the question you want to answer. So IMAC is actually very, very simple in most of the phosphoproteins. It's kind of taken a very, very big position to really identify what.

If you look at pathway-dependent phosphorylation change, it's very, very difficult to align all of the phospho changes that you see where the pan-phospho-enrichment approach such as IMAC into the pathway-dependent change. What I mean by that is that just because of phosphorylation changes doesn't mean it's linked to the pathway.

By using pathway-dependent immunoprecipitation, it makes my bioinformatics analysis a little bit easier, and that can actually look - for example, again, the example that I showed was by looking at phosphorylation changes in a patient-dependent context for AKT

inhibition. By looking at an AKT substrate motif antibody I was able to actually link the phosphorylation changes to it, to that particular pathway. So actually, I see them actually complementary. It depends what you want to answer.

[0:54:55]

Dr. Chunaram Choudhary: And if I may, one more thing to add here, actually, this IMAC or similar method are really good for getting a totally unbiased rework modification. If you are interested in going to certain pathways, then as Dr. Paweletz says, it's good to get motif-specific antibodies and so on.

The second thing that often we and others have observed is that IMAC and titanium oxide enrichment are usually much more efficient in terms of getting percent off modified peptides. So thus, AKT antibody is very cheap compared to antibody-based engagement. But, of course, once you go to pathway, then antibodies are more helpful.

Sean Sanders: Fantastic. Dr. Suri, what are the possible clinical and diagnostic indications of this research? It seems like this might have been in your area with your work currently.

Dr. Vipin Suri: Yeah. I mean Cloud can comment on that too. So for us, we're looking for biomarkers, for target engagement in our trials. So we are hoping that with targeted proteomics, now that we know where to look, the substrates to look at, we should be able to design assays, either fluid biomarkers or tissue biomarkers, to measure target engagement in the clinic.

Beyond that, again, depending on the situation, it could be useful for diagnostics as well as there's more and more information into these conditions. People start to generate more and more data and information.

So again, for our application, it's mostly biomarkers, but I can see that in a few years. There would be diagnostic implications as well.

Sean Sanders: Dr. Paweletz, anything to add to that?

Dr. Cloud Paweletz: I think that's very accurate. So again, some of it is biomarker focused. Some of them is understanding the basic biology to drive the next generation of inhibitors for example. You can think about

basic biology. You can think carbon CFPs. Right now it is again in the field of molecularly targeted CFPs it's mostly used in the biomarker space and then you can think about using this sort of enrichment strategies and linking those into MRM analysis to actually develop a very, very robust assay.

Sean Sanders: Excellent. Dr. Choudhary, I'm going to come back to you with this question. The frequency of acetylation and phosphorylation could be dynamic and may be changing constantly. So is there a possibility of technological limitation that might bias analysis and how would you handle this?

Dr. Chunaram Choudhary: It is possible and it is quite true that frequency and dynamics of modifications change. However, looking at all across the organism from yeast, Drosophila, bacteria, mouse tissues and human cells and comparing that to phosphorylation and acetylation, generally speaking, this trend holds true. So it is possible that some of these differences can be accounted due to their changes and what about technical differences, but it's unlikely that that can explain these great differences in bacteria and eukaryotic cells. So we believe that this is generally true that acetylation and phosphorylation have different frequencies in prokaryotes and eukaryotes.

Sean Sanders: Excellent. Any other comments from Dr. Suri or Dr. Paweletz on this?

Dr. Vipin Suri: Yeah, I agree with Chuna in general. I think the information we're getting from these studies is static but still very useful, and probably the conclusions are correct. But I do think that dynamics is where the next big findings are going to be, which is why we're investing in acetylation dynamics.

Sean Sanders: Excellent. Dr. Paweletz? No additional comments. Great. Okay.

We're at the end of our hour so I'm just going to put one final question to the panel and that's looking at where we are right now and where you would like to be with your work, can you talk a little bit about what technologies you would like to see that would help you drive your research and where you see the research possibly being in the next two to five years? I'll start with Dr. Choudhary.

Dr. Chunaram Choudhary: I think one of the real questions that we would like to know and many people in the field would like to know is occupancy of modifications, how much they occur, to what degree they occur in

proteins and how they are dynamically regulated. And, of course, improving the reproducibility and getting good reagents that we can analyze the modifications better, that would be really very helpful.

And also, I should mention there are many other PTMs that we had no specific and good working reagents for enriching those types of PTMs and developing those kinds of reagents would be really very important in understanding different PTMs and relating them to each other.

Sean Sanders: Great. Dr. Suri?

Dr. Vipin Suri: Yeah, I completely agree with Chuna. I think occupancy is an important outstanding question. And only recently I think there are a few studies where that's measured. And this is especially important with acetylation which is not a high-stoichiometry modification.

The enrichment reagents, I think improvement in enrichment reagents is always good. It gives us better quality data.

[1:00:00]

The novel PTMs, again, there are very interesting modifications that are functionally relevant. This paper came out recently on palmitoylation of TNAP. And I think reagents to identify those would be important.

Targeted proteomics I think is going to find more and more applications. Technology is getting there and needs to improve a little bit more. And dynamics is the other interesting frontier.

Sean Sanders: Fantastic. Dr. Paweletz, I'm going to give you the last word. Just where you'd like to see the technology moving in the next two to five years and where you see the field being in that time span.

Dr. Cloud Paweletz: So I think where I would like to see the technologies moving is to really see it applied in the clinics. So we target proteomic approaches certainly moving in the right direction. So I think that is on the application front and the diagnostic field.

Where I would like to see the technology as far as enrichment and mass spectrometry and pathway-based proteomics moving is

actually a way to use top-down proteomics. So there are certainly some inherent limitations of bottom-up as well as middle-up sort of approaches, and I really think that the idea of top-down proteomics is really kind of the ocean that we have not really seen yet.

Sean Sanders: Excellent. Well, unfortunately we are out of time for this webinar, but I wanted to thank our speakers very much for providing such fascinating talks and very interesting discussion. Dr. Chunaram Choudhary from the University of Copenhagen, Dr. Vipin Suri from GlaxoSmithKline, and Dr. Cloud Paweletz from Dana-Farber Cancer Institute.

Many thanks to our online audience for the fantastic questions you submitted to the panel. I'm just sorry that we didn't have a chance to get to all of them.

Slide 62

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Again, thank you to our panel and to Cell Signaling Technology for their kind sponsorship of today's educational seminar.

Goodbye.

[1:03:00] **End of Audio**