TRANSCRIPT

Cellular Metabolism in Hematologic Malignancies:
From Evolving Science to Therapeutic Potential

December 7, 2012

Slide 1. Title Slide

Richard Winneker:

I'd like to introduce Dr. Chi Dang, who will moderate this afternoon's symposium and be its first speaker. Dr. Dang is the John H. Glick, Professor of Medicine and Director of the Abramson Cancer Center at the University of Pennsylvania Perelman School of Medicine in my hometown of Philadelphia, Pennsylvania.

Slide 2. Metabolic targets

Dr. Chi Dang:

Thank you, Richard. First I'd like to also welcome you to this session. And I really want to thank all of our speakers for traveling here today to be with you.

And I would like you to also please kindly take this thing out and silence it, which I'm doing on mine. And also to refer to your workbook and learning objectives and full disclosure information. And then finally, to get your CME credit, to make sure you complete the evaluation form and mail it in within 30 days of receipt to get your certificate.

So I will now begin the session and I'm the first speaker. And what I'm going to try to do in the next 20 minutes is to give you an overview, some background to line up for the other speakers, who'll be presenting their stories. And I'm going to give you an overview of metabolism and then segue a little bit into some of the work we've been doing, to point out whether there might be therapeutic windows and to target metabolism for therapeutic purposes.

Slide 3. Regulation of Cell Growth and Metabolism

First I'm going to address the issue of the regulation of cell growth and metabolism and distinguish that from homeostatic metabolism. And in essence what regulates cell growth and what is required metabolically to fuel the cell for replication.

Slide 4. Energetics of Cell Homeostasis vs Growth

So what you will note is that I try to distinguish here between homeostatic metabolism versus metabolism that's involved in cell growth. So if you look at a cell that's at rest, with the

nucleus shown in the middle here, is that the cell has to maintain membrane potential, for example, turnover of organelles. And this requires continued uptake of energy that's listed here. What we do know is that many adult tissues, not all, will use fatty acids for oxidation as a means of getting the type of energy to maintain the cell, and then the output is the waste products shown at the bottom.

Slide 5. Metabolism of cell growth and proliferation

By contrast, if you look at proliferating cells, they need at least a couple of things. First, nutrients, as listed here, as well as signals for growth, the growth factors that engage the surface of cells. And what we know now from many, many different studies, that many proliferating cells, cancer as well as normal cells, and I will distinguish this later, use what we call aerobic glycolysis, that is, the use of glucose to convert to lactate, even under the presence of oxygen, and glutaminolysis, using glutamine as a major nutrient source. And this gives rise to ATP.

And then using the TCA cycle, these materials, as I will illustrate, also spin up the building blocks for a growing cell. So the resulting reaction here is that one gets two cells as well as waste products. So the big distinction here is that there is a change in metabolic profiles between resting cells and proliferating cells.

And the two major nutrient sources, I already mentioned glucose, shown here, taken up by cells, converted to a 3-carbon molecule pyruvate, which can either enter the TCA cycle, illustrated here in a mitochondrion, or convert to lactate through a process I referred to already as aerobic glycolysis.

What we also know is that glucose also can give rise to the carbon backbones for amino acids, illustrated here. Glycine, for example, once it enters in TCA cycle, it also can spin off other amino acids.

If you look at glutamine, glutamine is also used by the cells to convert to glutamate, and then enters in a TCA cycle as another substrate for the TCA cycle. Glutamine also can rise to proline, for example, and glutathione. And together both of these molecules can give rise to backbones that allow the cells to build lipids. Furthermore, the backbones of these two nutrients give rise to the synthesis of nucleic acids, as shown here. And finally, because of the use of TCA cycle, through high energy electrons, one can then generate a vast amount of ATP, while the glycolytic pathway generates lower amounts of ATP on a per glucose basis, but still contributes to the cell overall energetics.

Slide 6. Nutrients and growth control & signaling

What we know about nutrients and cell growth is the following. If you look at lower organisms, such as the yeast cells, or the baker's yeast, these cells only need to sense nutrients to begin to activate a transcriptional program that allows the cells to begin to build itself in the mass and then divide upon DNA replication.

By contrast, if you look at our cells, particularly adult cells, which are bathed in nutrients, we don't double in size every day because our cells are not programmed to just sense nutrients. Only a subfraction of our cells will proliferate upon stimulation with growth factors. What we do know, from work in unicellular organisms as well as multicellular organisms, is that most of the

cellular mass that increases, increases during the G1 phase of the cell cycle, as illustrated here. When cells wake up with growth factors, they enter through a cell cycle, begin to build up biomass and once it reaches a critical cell mass, the cells will begin to duplicate DNA and divide.

What we do know during this phase of very high cell mass accumulation is that most of it can be accounted for by ribosomal biosynthesis, the main machinery that makes everything else for the cells. And so what we also know over the last several decades is that studies from many different laboratories have revealed what's behind this logic gate that is the integration between growth factors and nutrients. And what we know is that, downstream of growth factors are early response genes, such as Fos and Jun, from many experimental models, including the myc proto-oncogene, an interest of our laboratory over the last several decades. And what I will illustrate very briefly is that, what we know now is that this major switch, metabolic switch, that is a transcription factor, will activate a program that allows cells to take up nutrients, build up biomass and then allows the cells to divide.

Slide 7. Cell growth and proliferation

Now what we know about the myc proto-oncogene is that it's downstream of many different signal transduction pathways, as shown here at the cell surface, represented down here as a cell nucleus. Once engaged, these receptors send signals to the cell nucleus to activate the myc proto-oncogene, which then produces a transcription factor that tethers to DNA with a partner protein, to turn on many genes that are involved in cell proliferation and cell growth.

So what's illustrated on the right, as you'll see, is that once the cell is engaged for the growth factor, activates the program, increases in cell size and divides, and if the growth factors are removed, normal cells will retreat back into a resting state.

However, in those tumors where myc is activated, what we do know from experimental models, if you take normal cells and drive myc expression acutely, what happens is that the cell senses an imbalance and activates a number of checkpoints, including P53. So the resulting fact of this is that, once P53 is activated in a naïve cell, it activates an apoptotic program rather than allowing these cells to continue to grow, unless the P53 tumor suppressors are also mutated, which allows the cells then to continue to proliferate, assay tumorigenic clone.

Slide 8. Cancer & myc target genes

What we do know is that the pathognomonic genetic aberration, that downstream of myc, is found in a lymphoma, Burkitt's lymphoma, it's well known to all of you, resulting in a chromosomal translocation. In fact, we know now from very deep studies that about 40% of these cases in fact have inactivation of P53, unleashing myc to do its dirty work in terms of driving tumorigenesis.

What we know is that the myc gene, which produces transcription factor, activates many, many different genes, as well as represses genes, so we've been very interested. Our laboratory and many other laboratories try to map out the myc transcriptional network, to begin to understand how this master transcription factor works.

So I'm just going to summarize a number of studies, just to highlight how we got to where we are today.

Slide 9. P493: Human Burkitt lymphoma model

So we've used this Burkitt lymphoma model, which is an immortalized human B cell line that's been engineered to be able to activate myc expression very acutely, as illustrated in the western blot shown here. Where the myc protein can come up in a couple of hours, driving the cells from a nonproliferative state to a highly proliferative state, with increasing cell size as shown here by these electron micrographs. And we can also implant these cells in SKIP mice to study them in vivo.

So what have we done with this system? Well, what we've done is to ask the following question: Can we map out where myc is bound on the DNA of the cell line, and then also to map out all the genes that are responsive to myc, and then to begin to understand the biology behind how myc functions.

Slide 10. Expression of Myc-responsive genes

And so what we've done here, just to illustrate for you, over the years we've gone from subtraction cloning to microRNAs, to look at myc-responsive genes. And just simply, I just want to illustrate in this slide at the top here, is what you'll see on each one of those dots, is a single gene. Those dots that are above the diagonal line are genes upregulated in response to myc and the dots below the line are the genes that are repressed. And if you were to drill in into one of these genes, and I'm going to have to go backwards here, if you drill into one of these genes, you'll see that–I'll show you just one of these genes, which is lactate dehydrogenase A. The histograms on the left basically represent the binding of the myc protein on the promoter of this gene, as a function of time that's shown from the bottom. When we turn myc on after one hour, 24 hours, you can see the accumulation of the transcription factor on the transcriptional start site. And then what happens, the gene is activated, the mRNAs illustrated here in the inset, we turn myc off and turn it on, the gene is turned on. So basically what we can do is map out myc-responsive genes, where myc can bind to these genes and turn them on.

So what have we learned to date? What we found is that the vast majority of genes that control the conversion of glucose, the lactate, the so-called aerobic glycolysis, in fact, are directly regulated by myc. So myc in essence can drive aerobic glycolysis.

More recently, however, we want to understand whether myc has a signature that tells us a little bit about its function in tumors as well as normal cells. And to do this we ask the question of whether myc drives a cell type independent set apart of genes. And what we've found, in essence, is by mapping myc target genes in this B cell model, as well as in human embryonic stem cells, and then comparing our target genes to target genes that have been reported in the literature for mouse embryonic stem cells as well as three additional human cancer cell lines. And Hongkai Ji, in the laboratory, derived a 51 gene signature that crossed over many, many different cell types that respond to myc. And it turns out when you subject this list to gene set enrichment analysis, to get a feature out of it, what we found is that many of these genes are involved in ribosomal biogenesis–the same process that allows the cells to build up biomass. And remember, I told you cells coming out of G1 have to build up biomass. In essence, a primordial function of myc is to drive biomass accumulation by driving a ribosomal biogenesis.

And this is not so surprising because studies in *Drosophila* have also made a connection,

genetic connection between myc and ribosomal biogenesis in the following way. This small fly shown at the top here is a mutant fly that has a mutant allele of myc that doesn't function very well, and it leads to small cell size, as well as small body size. And it turns out that this phenotype has been phenocopied by mutations in ribosomal protein genes, thereby linking myc to ribosomal biogenesis.

Now clearly because ribosomes constitute a large part of the cell, that the cell must do additional functions to be able to support these energy-demanding processes. So not surprisingly, over the years we've found that myc also regulates mitochondrial biogenesis. Myc turns on a number of genes that drive the duplication of mitochondria as illustrated here, in the EM; you can see the clustering of mitochondria cytoplasm.

We further documented, over the last number of years, that myc can also change the function of mitochondria because when we purify mitochondria out of these cells in a low myc state or high myc state in these lymphoma-like cells, and run them on high resolution two-dimensional electrophoresis for proteomic purposes, we discover basically that myc can also drive glutamine metabolism because it activates this enzyme, increasing expression of glutaminase when myc is turned on.

So in essence what we've found is that myc can drive glucose to lactate as well as into the TCA cycle, but these observations suggest to us that myc also can drive the uptake of glutamine into cells because it's able to activate an increased expression with this enzyme, right here, that converts glutamine to glutamate.

We further found by interrogating this entire pathway that myc regulates many points of glutamine metabolism, including transporter of glutamine. And in this case we illustrate here on proteomic analysis that the protein levels also increase, but myc drives this gene directly.

We also found that myc through microRNAs actually regulates a translation of glutaminase rather than directly regulating its expression in this B cell model illustrated here.

Slide 11. Myc stimulates mitochondrial biogenesis & glutamine metabolism

Now so on the basis of these observations, I'm going to summarize for you what we know today without getting into further details.

So these are a series of cartoons just to illustrate what we think happens downstream of myc is that here is illustrated a cell that engages a receptor, signals to a MEK ERK to turn on the myc proto-oncogene, which then expresses the myc transcription factor, which in turn induces the transcription of genes–in this case they're related to amino acid transport–to import various amino acids. By the same token, this signal transduction pathway can also turn on AKT, which you'll hear about later. And AKT itself, upon uptake of certain amino acids, can also help activate TOR pathway, which you'll hear about later, and this allows the cells to begin to activate its protein synthetic pathway.

Slide 12. Myc: Biomass accumulation and metabolism

Likewise, myc is able to regulate genes that are involved in aerobic glycolysis and glutaminolysis, as I already referred to. And this allows the cells to take up the nutrients, churn it through the mitochondria, and spin off ATP to support cell growth.

Slide 13. Myc: Biomass accumulation and metabolism

Likewise, by the same means, myc can also activate genes that are involved in nucleotide metabolism. In this case what happens is that the same substrate, glutamine and glucose, can be channeled to make amino acids that then drive it into the synthesis of nucleic acids to support RNA synthesis as well as DNA synthesis.

Slide 14. Myc: Biomass accumulation and metabolism

We've also documented, as well as others, that myc can also drive genes that are involved in fatty acid synthesis, again driving glutamine and glucose, to generate fatty acids; and then, in collaboration with SRABP, to increase cholesterol biosynthesis.

Slide 15. Myc: Biomass accumulation and metabolism

All this bioenergetic support allows the cells to do the following very critical function, which is ribosomal biogenesis.

Slide 16. Mitochondrial & ribosome biogenesis

Myc is the only transcription factor that we know that can activate all three RNA polymerases–one, two and three–allowing the cells to make the components of the ribosomes, so the RNAs are made in the nucleolus of cells. The protein is made in cytoplasm. These are transported back to the nucleolus, and some of the ribosomes then exit the nucleus–and allows the cells to really build up in mass, as illustrated here, as well as the cells undergoing mitochondrial biogenesis.

Slide 17. Cell cycle progression, DNA replication

And then myc kicks off E2F, allowing the cells to undergo DNA replication and upon completion of DNA replication, the cells will replicate into two daughter cells.

Slide 18. Cell cycle progression, DNA replication

Now imagine that when we mapped all this out, we did this in lymphoma-type cells, and so the real question here is whether normal cells also have the same program that I just illustrated for you here, where we used to regulate myc.

Slide 19. Bioenergetics of activated T cells

I just want to highlight a work from Doug Green's laboratory that really addresses this issue. So what Doug's laboratory had done was to isolate primary normal mouse T cells that have been engineered so that you can genetically delete either myc or HIF-1 alpha, the hypoxy-induced transcription factor, to ask whether upon activation of T cells, by using anti-CD3 or with anti-

CD28, to activate the T cells, whether they are dependent on myc or HIF-1 or for cell growth and proliferation.

In essence what they found is that the resting T cells use fatty acid beta oxidation, but upon activation with these antibodies, the cells will wake up, grow in size, and they're completely dependent on myc and not HIF-1 alpha.

So myc in essence drives aerobic glycolysis and glutaminolysis to activation of genes that I just reviewed for you; also in normal T cells. So the question is, if the lymphoma cells have very similar pathways to these normal T cells, could there be any therapeutic window when we begin to exploit these pathways for therapeutic purposes?

Slide 20. Nutrient regulation of ribosome biogenesis, fragility of cancer cells

And I think a lesson may be learned from the yeast studies, as illustrated. And I'm just going to illustrate now one potential perspective of cancer cell fragility, that is, the difference between normal cells and cancer cells. Does this really exist?

And so the lesson learned here is this. Again, this is a yeast cell, sensing nutrients. What we know from Jim Broach's laboratory at Princeton and others, you map out very nicely the signal transduction pathway, how yeast senses either glucose or glutamine, feeding down to ribosomal biogenesis genes. In essence, there are two repressors of ribosomal biogenesis genes that can be inactivated when the cells are exposed to either glucose or glutamine. So when cells see glucose and glutamine, these repressors are shut off, ribosomal biogenesis continues, the yeast cells grow and divide.

Slide 21. Targeting tumor energy pathways

Now what Jim's laboratory has done is to mutate these two transcriptional repressors, rendering these yeast cells constitutive for cell growth. They continue to make ribosome, independent of the presence of glucose and glutamine. And what happens to these cells, they become addicted to glucose and glutamine because when you withdraw glucose and glutamine from these mutants, these yeast cells begin to die. So we reason, on the basis of this, that perhaps cells, cancer cells, are driven by either oncogenes or myc in particular, that drives biomass accumulation in a deregulated fashion, that it might render these cells addicted to glucose and glutamine. In fact, Hyunsuk Shim, in the laboratory in 1998, we did a number of very simple experiments, taking normal cells, at least nontransformed cells, and then to overexpress myc in these cells and we test whether they're sensitive, simply through withdrawal of glucose or whether they would undergo apoptosis.

Slide 22. Cancer cell fragility: myc-induced addiction to glucose or glutamine

What's illustrated in this slide here is on the Y axis is rate of apoptosis and time on the X axis. And what you can see here is that in the parental cells, when you withdraw glucose, these cells retreat back into a resting state in G1, whereas the cells that overexpress myc undergo rapid apoptosis over several days.

Now another group at Cold Spring Harbor a number of years later showed that human

cells, when myc is activated, also can render these cells addicted to glutamine, suggesting that deregulated oncogene expression, if they drive biomass accumulation, that these cells will be sensitive to nutrient deprivation.

Slide 23. Glutamine & tumor tissue metabolism

So we then sought to determine whether we could target enzymes downstream of glucose and glutamine for therapeutic purposes. And what we really want to understand is whether there are differences between nontumorigenic setting and tumorigenic setting, using the same B cell model, and we've used metabolomics to really start to understand how glucose and glutamine are used under aerobic conditions and hypoxic conditions because–at least in lymphoma–because of the neovasculature being imperfect, there are a lot of pockets of hypoxia that might offer a therapeutic window because the genetic program in the hypoxic region would be quite different than what we would find in normal tissues.

So what we imagine is that with oxygen, both glucose and glutamine could be used by aerobic glycolysis as well as oxidation to drive cell growth and proliferation. Those cells that are further away from the blood vessels will be hypoxic. At least we know the anaerobic glycolysis is activated through the hypoxia-induced transcription factor.

What we don't know very well until more recently, through our work and others, is that glutamine metabolism actually remains quite active under hypoxic conditions.

And these are just cartoons to summarize two recent studies that we've published. First is just a summary of the hybrid TCA cycle that we find in nontumorigenic lymphocytes, that resembles lymphoblasts that are EBV immortalized. And these are through metabolomic studies.

What we find is that in these proliferating cells, they use both glucose and glutamine, in this hybrid TCA cycle to support cell growth.

However, when you turn myc on at very high levels, where the cells become tumorigenic, what we found through the work in collaboration with Jamie Young through metabolomic studies, is that while glucose flux increase only about 20%, the glutamine oxidation increased by four-fold, driven by myc, as illustrated here.

Furthermore, we can use the same system to ask what happens when you take the same cells and put them under hypoxic conditions, that is, 1% oxygen.

Slide 24. Oncogenic myc enhances glutamine oxidation

What we learned from a separate study is that when you subject these cells to hypoxia, more glucose gets diverted to lactate and what we found surprisingly is that glutamine backfills to TCA cycle–something that's not really known, at least in the biochemistry textbook. So in essence these two pathways are interdependent.

And so this leads me to the point that clearly because these tumorigenic cells are dependent on very critical pathways to perhaps we could target some of these enzymes. I'm just going to illustrate our work on glutaminase, which is the enzyme that converts glutamine to glutamate, for it to enter the TCA cycle. So it leads me now to ask whether this can be targeted.

Slide 25. Glutaminase & inhibitor BPTES

So we're lucky that there is a compound that is very active in the submicromolar range. It is an allosteric inhibitor of glutaminase. And what's illustrated here is a crystal structure from our collaborator of a tetramer of glutaminase with the inhibitor sitting at the tetramerization interface and locking this molecule into an inactive state.

Slide 26. Glutaminase inhibition diminishes tumorigenesis

So we used this compound as a tool compound for proof-of-concept. And what we found, when we use the lymphoma model, in the 20% oxygen you can see a dose response curve. As we increase the dose, the cells slow down. When we subject these cells to hypoxia they begin to die when they're exposed to this compound. And this work fortunately is supported by the LLS.

Most importantly, however, is that when we took this into a xenograph model, established tumors, what we found is just using as a single agent, its original compound shown up here, we can slow down tumor progression. And through collaboration with a group at Johns Hopkins, to derivatize from this parent compound to more soluble compounds, again work also supported by LLS, we can find that this derivatized compound which is more soluble also has activity in vivo, as illustrated here. Underscoring, at least by proof-of-concept, that interfering with metabolism could have a therapeutic effect.

Slide 27. IDH mutant neo-enzyme and epigenetics

Let me just turn down to a couple of last slides to really turn to a different topic, but related topic to glutamine metabolism.

I just want to turn now to a topic we discuss a little bit more extensively later, which is a discovery of mutations in enzymes that leads to a totally new activity. So this was about a number of years ago, Vogelstein et al was looking at gliomas and through exon sequencing they discovered that there's an enzyme, isocitrate dehydrogenase, which is mutated consistently in the active site at the enzyme.

And I'm just going to only summarize. What we know now is that this mutation leads to a totally new activity of an enzyme that normally does something else. Again, here is illustrated glutamine taken up by cells as a glucose, churned through the TCA cycle, and what you'll see at this step here is that isocitrate can be converted to alpha-ketoglutarate, I've illustrated here, which is mediated by isocitrate dehydrogenases, which comes in different flavors, both the mitochondrial form and cytoplasmic form, IDH1 and IDH2.

Now citrate exiting from the mitochondrion is used both for fatty acid synthesis as well as for this generation of NADPH by these IDHs that are cytoplasmic, illustrated here, by converting isocitrate to alpha-ketoglutarate.

What we believe is that the normal function of these cytoplasmic enzymes is to generate NADPH for biosynthetic purposes, such as fatty acid synthesis, which requires NADPH. However, what was discovered a few years ago is that the mutant enzymes–and now what we also know the connection is quite important–is that alpha-ketoglutarate itself is a substrate for modification of epigenome, and I'll get back to this momentarily and this will also be discussed more extensively later.

So what this cartoon here illustrates is that the normal function of the enzyme converts

isocitrate to alpha-ketoglutarate. What we know is that the mutant enzyme actually takes alpha-ketoglutarate and generates a totally new metabolite to hydroxyglutarate–so a neo-enzyme. In fact, a number of companies now have come up with inhibitors that only inhibit the mutant form of the enzyme and not the wild type form of the enzyme.

Slide 28. Glutaminase inhibition diminishes growth of glioma cells with mutant IDH1

However, what we've done a number of years ago is to capitalize on the observation from metabolomics, that the source of alpha-ketoglutarate for these mutant IDH-containing cells comes from glutamine. Glutamine comes into the cells, gets converted to glutamate, which then gets converted to alpha-ketoglutarate as a substrate for this mutant enzyme to generate this new chemical to hydroxyglutarate.

So simply we reason that if the cells have been rewired they would be addicted, if you will, to a glutamine as well because they require glutamine to feed their alpha-ketoglutarate pores, illustrated here. So we sought to determine whether we could use this inhibitor, the same inhibitor of glutaminase, and see what would happen to cells that we've engineered with mutant or wild type IDH enzymes.

And this is work done by Megan Seltzer, who was at Johns Hopkins, in collaboration with another group. And what we've engineered here is glioma cells that have a μ expression, either wild type or mutant IDH. And here it's just a mass spec showing that the mutant enzyme can generate to hydroxyglutarate.

Slide 29. BPTES inhibits AML cells with mutant IDH1

This next slide is just a dose response. On the Y axis is cell numbers, if you will. And on the X axis, increasing dose of this glutaminase inhibitor. The upper gold line represents the cells that express the wild type enzyme. And the line at the bottom here represents cells that express the mutant form of the enzyme. What you can see is that at all doses of the glutaminase inhibitor, we see that the mutant enzyme containing cells in fact are more sensitive to glutamine inhibition of the glutaminase than the wild type containing cells. And we can partially rescue these cells with 2-hydroxyglutarate, which is downstream of glutamine, as illustrated there.

Now most importantly, particularly for this session, is that we also applied to this concept in acute myelogenous leukemia because these mutations are also found in AMLs. And this is work done by Ashkan Emadi, who's in the audience. And here I just want to illustrate a couple of panels here. On the upper two panels, the dose response of primary AML cells that's been titrated with the glutaminase inhibitor and this was done blindly and we broke the code. Shown here are the more sensitive cells on the two upper panels, in fact, are the ones that contain the mutant allele, and the bottom two panels, those AML cells that have wild type IDH, suggesting that at least the dependence on glutamine could have some potential therapeutic purposes.

Slide 30. Network of cancer genes and metabolism

Let me just end by just reminding you that this whole area is quite complex. All the red dots here represent oncogenes and tumor suppressors that are very intimately tied and connected

to the metabolic pathways, both the mitochondrion and gycologen. So over the next number of years we really need to know how the genetic make-up of tumors is tied to the metabolic network and begin to use these inhibitors in an intelligent way for therapeutic purposes.

And I want to end by just acknowledging people in the laboratory, collaborators and funding agency, particularly The Leukemia & Lymphoma Society.

Slide 31. Dr. Dang - Acknowledgements

Thank you very much for your attention.

So we're going to hold off on–I've been told that we can take a few questions if there are any questions out there.

Audience: Thank you very much for your very illustrated presentation. Do you think the cellular addiction for glucose and the glutamine could explain the new reports about metformin enhancing or sensitizing the cell for this chemotherapy? They claim that this is through autophagic action. Do you think that interference in metabolism could be contributing, especially in metformin potentiation or synergistic effect with chemotherapeutic agents, especially in lymphatic malignancy?

Dr. Chi Dang: I'm sorry, I didn't catch–you're talking about whether myc ...

Audience: Metformin. There are new reports about metformin sensitizing chemotherapeutic or enhancing apoptosis and autophagy, especially in lymphoid malignancy. Do you think this through blocking the addiction of the malignant cells to glucose and glutamine? Thank you.

Dr. Chi Dang: Thank you for the question. So you'll hear more from John Cleveland about this in his talk on autophagy.

So very briefly, I didn't touch on this, but we haven't talked about other means by which cancer cells can use metabolism. One of it is that, once you deprive cells of nutrients they can activate the autophagic pathway, which you'll hear about. And John, I'm sure, will touch a little bit on metformin as well, so I'm not going to pre-answer your question, we just have to wait and hear about that.

And then the other thing I also want to highlight for the audience is, what we learned over the last year or so is that cells can also take fatty acids from the outside world and also some new work that suggests certain types of cancer, mainly not hematologic cancers, that actually can eat nutrients from the outside, literally taking in albumin, digest these substrates and then to feed themselves. So there are many ways by which cancer cells can feed themselves beyond using glucose and glutamine.

Audience: Now IDH mutation occurs very likely during tumor progression. How do you expect that this therapy might work?

Dr. Chi Dang: So Dr. Croce points out that IDH mutations, just to summarize that, leads to 2-hydroxyglutarate, which is now known to interfere with enzymes that are involved in epigenetic

modulations. So what we don't know at the moment is whether that's an early event, that is, early driving event that the cell is no longer dependent on this driver; that we do not know at the moment. I think the new experiment is going to be coming out with new models that are available, will really teach us whether it's a hit and run or the cells continue to be dependent on this. In our very kind of cursory look at dependence of these cells on glutamine, it appears that there's still some dependence on glutamine to drive the amount of alpha glutarate for homeostasis. And if you tip the scale there it might be enough for those cells to tip over into cell death. But we don't know that at this moment. So I think it's a very important question.

Great. So we'll move on to the next speaker. Delighted to introduce our next speaker, who's Dr. Jeff Rathmell, who's an Associate Professor in the Department of Pharmacology and Cancer Biology and Immunology at the Nutrition and Metabolism Center at Duke University. And he's going to present metabolism and apoptotic pathways in leukemia. Thank you very much, Jeff.

Slide 32. Metabolism and apoptotic pathways in leukemia

Dr. Jeffrey Rathmell:

Thanks. It is a pleasure to be here. So I will go ahead and get started, talking about mainly more focusing on some of the apoptotic pathways in leukemia, but also just how this relates to normal immune response.

Slide 33. Metabolism and apoptotic pathways in leukemia

So our interest has really been in thinking about how metabolism is regulated in hematopoietic cells with largely a focus on T lymphocytes, although we've seen basically the same thing in B cells as well. So I'll talk about T cells, but everything relates to B cells.

And the basic premise is that a resting lymphocyte doesn't have to do very much. They're just on an immune surveillance sort of mode, so they need a lot of ATP for migration, but they don't need to do a whole lot else. So the cell in the middle there is a cell that is just sort of sitting and they are largely performing an oxidative metabolism. So a resting lymphocyte expresses nutrient transporters, such as the glucose transporter GLUT1, as well as transporters for some amino acids and they take up lipids. And ultimately all the nutrients feed into the mitochondria, where they get oxidized for maximal ATP generation.

When the cells are activated or if there's a transformation event in a case of leukemia or lymphoma, the cells switch and all of a sudden they need to be able to grow and divide. And of course, an activated lymphocyte will be dividing every four to six hours, so very remarkable cell division. So the metabolic demand is completely switched. And just as Dr. Dang discussed, the cells now need to have a premium on growth metabolism.

So what the cells do, both for an activated lymphocyte and leukemic case, is they upregulate glycolysis, and upregulate glucose expression of the glucose transporter, GLUT1 in particular. Glycolysis is then used, glucose flows into the cell and lactate is secreted. And essentially the cells are doing the same kind of metabolism.

Now at the other end of the spectrum, at the end of a response, or say if you wanted to eliminate a cancer cell, what ultimately happens if the nutrients become limiting, the cells will

internalize their nutrient transporters, if the signals are gone, then transporters such as GLUT1 get degraded in lysosome, so the cells can't even take up the glucose that's surrounding them. And then the cells turn on a process called autophagy, which allows them to digest their intracellular components for a short while, but then eventually the cells undergo apoptosis.

So our interest has been to look at the sort of spectrum of metabolism that happens in– primarily glucose metabolism has been our focus–but the spectrum that happens from the activation events on one hand, down to how do the cells die if the nutrients are not sufficient or how blocking metabolism actually kills cells.

Slide 34. Metabolic reprogramming in lymphocytes

So the big metabolic reprogramming that happens in lymphocyte activation and in transformation is really something again that was summarized quite nicely by Dr. Dang. But really you go from a case of a naïve resting lymphocyte, or a nontransformed cell, and really they're burning a number of different fuels. They are burning things like glucose through glycolysis, they're using glutaminolysis and they're also burning lipids. Again this is all oxidized in the mitochondria for maximum ATP.

Upon activation everything switches and the cells now increase their glycolysis, that's what we think is predominantly the most dramatic change, they also increase their glutaminolysis, again summarized nicely in the previous talk. They no longer oxidize lipids to the same extent; they sort of start to conserve their lipids so they can use those to grow. And ultimately everything is geared towards the growth of the cell. So that's sort of the metabolic transition.

Slide 35. Metabolic reprogramming towards Warburg

So in normal lymphocytes, what's the data that this actually happens? So this is data that we generated from peripheral CD4 T cells from patients. It just shows some metabolic measurements here. We look at a naïve T cell on the left versus the activated cells on the right. After stimulation there's an increase in glucose uptake, an increase in the acidification of the media, so this is basically lactate, this is a measurement of glycolysis. There's also an increase in the oxygen consumption rate of the cells. So the cells are more active, very much generally. Glycolysis is up as well as mitochondrial oxidation. Everything gets turned up.

But on the balance, if you look at sort of the overall shifts in what happens, the ratio of oxygen consumption to media acidification or glycolysis, the ratio actually decreases. So the balance of the cells switches more towards a glycolytic phenotype. So they're much more active in general, but on the whole more glycolytic.

And one of the things that we think contributes to that and helps drive that is, cells also induce the expression of this transporter, GLUT1, from the resting cell there to the blue line, where the cells are stimulated. So they upregulate GLUT1 and start to become more glycolytic.

Slide 36. Glut1 is required for T cell activation and proliferation

So is that important for the cells? Well, we can test this a couple of ways, one very basic way is if we use an siRNA to eliminate the expression of the glucose transporter GLUT1. So

again we're not getting rid of it, we're just preventing the upregulation in this case. And then if you measure the cell proliferation, in this case using a dye called CFSE, as the cells divide, they dilute the stain of the dye, so the staining gets lower. So in a normal case, using a control siRNA, when the cells are stimulated the dye gets lower as the cells proliferate. If we knock down expression of GLUT1, again not eliminating it, just reducing it, the cells can't proliferate any more. And actually, it's not shown here, but there's a reasonable amount of cell death that happens in the culture as well. So they have to be able to turn on this program to go and divide.

Slide 37. Leukemic T cells are highly glycolytic

Alright, so that's a normal T cell. What about in leukemias? Again, leukemias are basically the same. So this is an image, on the left here, that I suspect you might run across again in the other presentations, this is Otto Warburg, he was the initial person in the 1920s who described this glycolytic phenotype for cancer cells. And leukemic cells do exactly the same thing.

So here looking at them, this is actually measuring glycolysis in resting versus stimulated T cells. You can see there's quite a big shift up in the glycolytic rate of a stimulated T cell. It goes up 40- or 50-fold. Actually, in this particular experiment it's more like 100-fold, and it went up in those two days.

But at a higher rate of glycolysis, we get a nice stimulation. Actually pretty much exactly matches the glycolytic rate that you'll measure if you take T cell leukemias. So the T cell leukemias in the activated cells are along the exact same route; very comparable metabolically.

Slide 38. Human T cell acute lymphoblastic leukemia cell express increased Glut1

And this is not just that the glycolysis goes up. I mentioned that GLUT1 expression is important for normal T cells. We see the exact same thing in leukemias. So this is looking at the expression of GLUT1 in some T cell acute lymphoblastic leukemia samples. On the upper left, this is actually message expression from a panel of samples compared to normal resting CD4 T cells. Western blot. And some cell lines shows that it's a little heterogeneous, but generally GLUT1 is more highly expressed. And if we do flow cytometry from primary T-ALL samples from patients, we see that pretty much uniformly they have high GLUT1 expression. So GLUT1 is overexpressed in these cancers.

Slide 39. Murine T-ALL undergo metabolic reprogramming

So we've modeled T-ALL in animal models to try to decide, try to investigate this a little more deeply in terms of the regulation and the role of GLUT1 and glycolysis and their fate. And the system that we've generally used has been one where you can infect hematopoietic stem cells with an activated form of the notch receptor and get activation of the notch signaling pathway. This is known to be disregulated in a large percentage of T-ALL.

So if we do this we generate these sort of characteristic CD4, CD8 double positive cells and the animals get sick with the pretty uniform transmission here.

So these cells do have the same metabolic transitions that we would maybe predict based

on the human samples, in that they have upregulated the transporter GLUT1. And actually normal bone marrow you detect a fair–normal hematopoietic stem cells–you detect a fair amount of the transporter GLUT3. And there's a transition away from that, so GLUT3 is downregulated and GLUT1 sort of replaces it as the cells start to divide rather rapidly. And that's not the only change. There are also changes in other metabolic enzymes such as hexokinase, there's a shift from hexokinase 1 to hexokinase 2 as well. So the cells are turning on this metabolic phenotype.

And the most important thing is, if you take these cells and then try to block glycolysis, it's a real problem for them. So this is where we're just measuring cell viability, cells cultured in normal media or with the addition of the glycolytic inhibitor 2-deoxyglucose. And we've used a number of other inhibitors as well, but this just illustrates the point. And basically the 2-deoxyglucose wipes out the cells. So they have high rates of glycolysis and they're very, very dependent on that glucose to keep the cells alive.

Slide 40. Human primary T cell acute lymphoblastic leukemia cells require glucose metabolism

So that's the animal model. We can do this with patient samples as well and I'll show data from just a couple of patients. We've now done this with quite a number more.

But if we take the peripheral blood of a T-ALL patient and we can use the CD4, CD8 markers to sort of identify what we consider the T-ALL cells themselves that are CD8 positive and little bit CD4 in this particular case. And we can compare them to the CD4 cells that are in that exact same patient–so from the same patient within the same population. And then we can treat the cells with 2-deoxyglucose and again we've used other inhibitors as well, but this is the most clear example.

The normal CD4 positive cells, they're not very glycolytic, as I mentioned to begin with. They're using a lot of oxidative metabolism; they use a lot of lipids and amino acids. You block glycolysis, they don't particularly care, doesn't have any effect on them whatsoever. But the T-ALL samples, these cells all of a sudden have a pretty rapid cell death. And you can see this is showing two different patients here, the control, the T-ALL cells die; the 2-deoxyglucose, the control T cells do not.

So there's a little bit of a selectivity in that these cells have increased levels of metabolic dependence. Again, exactly this metabolic fragility that Dr. Dang discussed.

Slide 41. Human primary T cell acute lymphoblastic leukemia cells require glucose metabolism

So then the question that I want to talk about for the rest of it is, if we have this idea that cancer cells are very glycolytic and they have high levels of expression of transporters such as GLUT1, and if we target these pathways and block this glycolysis through whatever means, such as the inhibitors that Dr. Dang was mentioning, ultimately we want to be able to kill the cell, we want to be able to induce apoptosis, on the other end here.

So what's that stress pathway, how do you actually end up at apoptosis? So we take these cells and if they're metabolically starved, where do they end up? How does this cell death happen? As I mentioned, there's sort of this characteristic response. The first thing that'll happen

is, if the nutrients aren't coming in, the cell will try to digest itself. So it'll activate autophagy. And we're going to hear a lot more about autophagy from Dr. Cleveland. I just have a couple of slides on it. But then ultimately focus on apoptosis.

Slide 42. Growth factor or metabolic stress induce autophagy in leukemic cells

So autophagy again, it's a process of self-digestion. Cells aren't getting enough nutrients from the outside. They start to eat themselves. And of course, this is a process described from yeast on up and every cell will do it. And this just shows some data from our lab with some leukemic cells to show essentially that if they're starved, either from growth factor or from glucose, they do activate autophagy. There's a couple of markers with some EM to show this very characteristic membrane structure as well as this aggregation of this LC3 marker and you can see the processing of LC3 in these two T-ALL cell lines down here.

Slide 43. Autophagy provides lipids for survival in metabolic stress

So leukemic cells will activate this. But what does it really do? Well, one of the things that we've done to try to get at the role of autophagy and how it affects cell fate is, we made a model where we can now acutely delete one of the autophagy-essential genes. In this case it's ATG3. So we've taken animals that have conditional floxed version of ATG3, crossed them to a conditionally active cre-recombinase. We can immortalize these cells by expressing TLX 1, a T cell leukemia-associated oncogene, it's a HOX oncogene. And basically prevents these cells from differentiating. They're immortal and just proliferate. If we treat with hydroxy-tamoxifen and delete ATG3, so now we have an autophagy-incompetent cell line that we can compare to this isogenic control. And this just shows that we can delete it and if we do so, all these markers of autophagy show that these cells can no longer induce autophagy.

So what does autophagy do? Like I said, the data would really argue that it's a source of nutrients and that's pretty much exactly what we see. So if we take these cells and we subject them to some metabolomic sort of measurements, where we can measure the intracellular metabolites, in this case measuring the mitochondrial metabolites, we see when cells, in the upper right, the control cells or the tamoxifen-treated cells, if they're starved, removed from normal growth factors, ordinarily they'll induce levels of these lipid intermediates, these acylcarnitines here, C16 and 18, the oleate and palmitate mixture. But that's dependent on autophagy. So if the cells can't do autophagy they can't get those lipids into the mitochondria. So autophagy is a source of lipids for nutrient-starved cells.

And if you take cells and then block glycolysis, ordinarily they're going to try to switch to autophagy, but if you block autophagy as well, then you get a tremendous amount of cell death. So blocking glycolysis on its own not necessarily enough to kill very strongly, autophagy same deal, you put the two together, it can be quite toxic. So autophagy is a survival mechanism. So that can keep cells alive.

Slide 44. Role of Bcl-2 family when aerobic glycolysis is disrupted

But again we'd like to talk more about apoptosis. Actually want to kill the cells in the end.

So if the process is, there's induction of autophagy, then apoptosis is the next event and ultimately the cells would die necrotically. What you really want to do is try to see how is apoptosis regulated. And one of the really key findings came from data from Craig Thompson's lab, showed that BAK and BAX deficient cells, which are sort of the effectors of the BCL-2 family, if you don't have BAK and BAX cells can live for a very, very long time in the complete absence of glucose. And what those cells will do is they will turn on autophagy as a nutrient source. So you can live without these supposedly necessary nutrients for quite a long time, and autophagy will keep the cells alive. So really the limiting factor, so the constriction point in killing a cell is, how can you activate BAK and BAX.

So we've looked at this and sort of focused on the upstream mediators of the BCL-2 family, the anti-apoptotic proteins like BCL-2, BCL-X and MCL-1. And then there's a series of regulators called BH3-only proteins, the pro-apoptotic proteins. I'm going to focus on one called PUMA here and another called BIM.

So to try to get at how these proteins are ultimately going to regulate BAK and BAX, the first thing we've done is taken cells, glucose starve them, which is a pretty blunt tool really, but it lets you see what's going to happen if you block metabolism, and then try to see what are the changes in the BCL-2 family proteins based on expression at least.

Slide 45. Glucose deprivation leads to changes in expression of select pro-apaptotic Bcl-2 family members

So these are western blots for PUMA, BIM, BAD, NOXA–all pro-apoptotic BH3 proteins, as well as BAK and BAX. And BAK and BAX don't change at all when the cells are nutrient-starved. Neither do BAD or NOXA. But we do see a nice induction of both PUMA and BIM when cells are metabolically starved.

So that is really important. And actually if there's going to be one take-home, this would be the take-home slide that you should all remember, and that's if you don't have these guys, the cells don't die. You can block metabolism all you want, but cells just won't die.

Slide 46. A Bcl-2 family regulated metabolic checkpoint is essential for rapid cell death

So in this case what we've done to show this is, we've knocked down BIM and PUMA and taken the cells and completely withdrawn glucose from these cells. Ordinarily in the control cells, BAX gets activated and this leads to a rapid cell death. If you don't have BIM and PUMA, the cells no longer can activate BAX and again they can live a very, very long time without inducing apoptosis. And again, at this point we believe the cells are surviving on autophagy rather than being able to use any outside nutrients. Okay, so you have to be able to activate BIM and PUMA to die. So how do you do that?

Slide 47. How PUMA is regluated

Well, PUMA itself is an interesting molecule. It was cloned as P53 responsive gene. That's what the P stands for in PUMA. So you can see if you glucose starve cells, you actually get an activation of this tumor suppressor P53, there's a phosphorylation event there. That's

coincident with the upregulation of PUMA. So that starts to fit, maybe P53 is involved. There's a lot of data now, that it's sort of a general cell stress responder.

Slide 48. p53 contributes but is not necessary for PUMA transcriptional induction

So you can go then and use P53 NOL cells and try to see how much does this actually impact the NOL8 PUMA generation. And so if you use P53 NOL T cells, activate them and then do a glucose starve, they will still induce PUMA. So you don't actually need P53 for this. But there's a pretty significant drop-off in the amount of PUMA. It's about a 50% drop-off in the PUMA induction, both at the protein level as well as at the mRNA level. So you really don't need P53, but it does contribute. You can do sort of the opposite experiment. Start with the P53 NOL cell, like the Jurkat T cell leukemias, and they will induce PUMA when they're metabolically starved. If you put P53 back in, they'll induce quite a bit more. So it's kind of a complicated process. We don't really at this point know what else is regulating PUMA. Something that we're very interested in, though.

Slide 49. Pyruvate can replace glucose to rescue PUMA and cell survival

But we do know that it's not just glucose that's important. It's not just having glucose is the key thing. Because you can give downstream metabolites and give the same effect and you can rescue the effect. So in this case we're providing methyl pyruvate. So this is the end product of glycolysis. And methyl pyruvate just allows it to be cell-permeant.

But if you give methyl pyruvate to glucose starved cells, you have no effect whatsoever on the BIM induction, but you can at least partially suppress the PUMA induction, and methyl pyruvate then can keep cells alive in the absence of glucose. And this is just on the far right, just shows this by DNA content.

Slide 50. Alternative fuels also rescue puma and cell viability

So again, not just methyl pyruvate, though, it's just sort of nutrients that we think are important. So if you can add lipids or an alternate sugar, so giving oleate and palmitate or giving an alternate sugar here, fructose, and in both cases you can at least partially rescue the cell survival problem, when cells are glucose starved, and you can suppress the induction of PUMA. Again, showing that we get an effect on BAX activation as well as the cell survival and DNA content assay to repeat the cell death.

So it's not just that you need something glucose, it's just fuel, really. PUMA just responds to fuel.

Slide 51. Mcl-1 is dependent on continued metabolism

So that's the pro-apoptotics. And to say a few words about the anti-apoptotics, and I really want to focus in on MCL1 here. If you starve cells from glucose, BCL2 and BCLX don't change at all. They're just sort of rocks. MCL1 starts to decrease. It doesn't go away, though. You may be at most a 50% loss of MCL1. That may not seem like very much, but actually if you do the

experiments and knock down MCL1 by about half, that actually is significant enough to really sensitize the cells. So MCL1 seems to be a protein that's sort of kept on the knife's edge of how much do you have to really be a rheostat for the sensitivity to apoptosis.

Slide 52. Mcl-1 is dependent on continued metabolism

So this is not–in this particular case, where glucose starved cells, if you treat with glycolitic inhibitor oxyglucose, MCL1 goes down. You can do this in activated T cells, glucose starve them, MCL1 goes down. Not an effect of the cells starting to die or undergo apoptosis, because if you overexpress BCLX, this keeps the cells alive, even though they're glucose starved, so there's no death in the culture, MCL1 levels still go down. So this is something that happens prior to the commitment of the cells to death.

So what is it? So MCL1 is thought to be regulated largely through protein degradation. It's a very short-lived protein. But when we look at the turnover of MCL1 and metabolic stress, we don't see that there's any difference in the protein stability. In this case, treating with cyclohexamide to block new protein synthesis, and you basically look for the loss of MCL1 and the half-life is about the same, so it's not really different.

Slide 53. Mcl-1 expression decreases due to reduced

What is different, though, is the translation rate of MCL1. So MCL1 translation has to be pretty rapid to make up for this short-lived nature of the protein. And when cells are metabolically stressed, there's a decrease in MCL1 translation and this is actually mediated through TOR. So it's a TOR dependent translation that has to happen. Again we're going to hear a lot about TOR in the talks coming up. So you need to have TOR to maintain MCL1.

Slide 54. Metabolic stress and apoptosis are closely linked

So overall model then for how metabolic stress actually kills the cell, so if you have nutrients coming in and just showing glucose here, although glutamine and others can be important as well, glucose comes in and fuels pathways such as the hexosamine and glycosylation pathways and we actually think that BIM responds more to an ER stress, a metabolic disruption. We haven't proven this, but we think this is the case.

Whereas glucose via pyruvate and potentially other fuels like lipids fuel the mitochondria and PUMA is really responding to some mitochondrial stress, some just energetic signal. Because again, there's nothing special with pyruvate. Lipids will do the same thing. P53 is involved here, although it's not the only component. MCL1 as an anti-apoptotic protein is dependent on being able to maintain activity of the TOR pathway, the mTORC1 pathway. Which of course we'll hear about TOR. It's also downstream of the NPK kinase, which will inhibit the mTOR pathway, so that all kind of links together to maintain MCL1 translation.

Slide 55. Metabolic stress and apoptosis are closely linked

Now cells that are glucose starved, you lose input of the hexosamine pathway, this causes

ER stress, we think that in both BIM. You have limited input into the mitochondria. P53 can play a role in inducing autophagy and lipids can help relieve this a bit. But at the same time you're inducing PUMA, TOR pathway is turning down, so MCL1 is going away. So at the end you have this position where you're inducing proteins like BIM and PUMA, and NOXA is already there, it's pretty toxic, and all these guys are really reliant on MCL1 to suppress the pro-apoptotic function. So MCL1 is kind of a linchpin to prevent these other proteins from killing the cells. So as it decreases, these pro-apoptotic proteins can then have their function to actually kill the cells.

So we think MCL1, actually this change, that loss of TOR-dependent translation, might be sort of the centerpiece.

Slide 56. Lymphoma cells develop resistance to ABT-737 by Mcl-1 and/or A1 upregulation

So how can you then sort of test this, start to look at what this really may be doing in vivo. And one sort of interesting application of this is in regards to BCL2 inhibitor compound called ABT-737. There are other versions of this that are in clinical trials. But this is a drug that blocks BCL2 and BCLX and apoptotic function, but it doesn't touch MCL1. And MCL1 in fact has been shown to be a resistance marker for this drug. So if you take diffuse large B cell lymphoma cell lines here, using a couple of different ones here, and you treat with this drug, they generally are sensitive to the compound. But they do develop resistant clones of these that will not die or take very high concentrations of the drug to die.

Slide 57. Metabolic interference of Mcl-1 synergizes with ABT-737

And what happens for the resistant clones is again they largely have induced expression of–upregulate expression of MCL1. In some cases they express other anti-apoptotic proteins like DHL4 R2 here, as expressed, this other protein, called A1. And when they've done that they are now relatively resistant to the drug. But again, MCL1 is metabolically sensitive. So if you take these drugs, take these cells, and then culture them with 2-deoxyglucose at a sublethal dose, this is a rather low dose, sort of barely even tickles the self proliferation of the cells, and then you couple that with the drug, the ABT-737 drug, again, cells are resistant to that drug on their own, but you put the two together, this LY1 R10 cell line is very sensitive to this, the DHL4 R2 line is intermediately responsive. And this is actually an interesting control because the mechanism resistance for this R2 line is partially induction of MCL1, but there's also an induction of another protein, this A1, which is not affected metabolically nor is it a target of the ABT drug. So this is sort of a nice specificity control really.

Now the MCL1 responds itself, to maintain MCL1, that's a TOR-dependent translation, so you get the exact same data if you inhibit the mTOR signaling pathway as well, using this TOR kinase inhibitor or rapamycin, although the TOR kinase inhibitor is a little more dramatic.

Slide 58. Metabolic interference of Mcl-1 synergizes with ABT-737

So our overall model, and I'll end with this, is that normal lymphocytes when they get activated, they turn on this Warburg metabolism, this glycolytic metabolism, leukemic cells and lymphoma cells again doing the exact same thing. Expressing lots of GLUT1, lots of glycolytic

flux here, and this makes the cells very dependent on glucose for survival. This I think is the therapeutic window that Dr. Dang was referring to. Those are now reliant on this pathway, or at least certainly the leukemic cells are reliant on this pathway.

When metabolism is decreased, ultimately the cells will have a stress and they induce autophagy and then there's a regulation of BCL2 family proteins, MCL1, BIM and PUMA here that can lead towards apoptosis.

Now there's an alternate arm here that I have added to the slide and this is in regards to regulatory T cells, which again for time's sake I didn't include. But regulatory T cells have a little bit different function than your activated T cell in that they shut down an immune response. And interestingly enough, they actually don't use this Warburg metabolism and instead use an oxidated metabolism. So as you block glycolytic phenotypes, you block leukemic cells and you block effector lymphocytes, but you don't block the regulatory or suppressor cells.

So we think that this sort of shift in metabolism is going to be important for treating leukemias and lymphomas and going to provide some new targets, as well as in normal immune responses, and gives you a way to target these specifically as effector T cells and leave the regulatory T cells intact.

Slide 59. Dr. Rathmell - Acknowledgements

And with that I will close. So this is the group in my lab who've done the work. And I want to highlight that one of my students is here and she has a poster coming up, Tingyu Liu. As well as a number of other collaborations and support from The Leukemia & Lymphoma Society.

Audience: Do you check if metabolic–you inhibit MCL1, you use the ABT-737, that has a good effect in CLL? Where both oncogenes are overexpressed considerably.

Dr. Jeffrey Rathmell: Yes, with CLL, do have higher–again this is on the poster from my student–so CLL tend to have high level of BCL2 and they also can have MCL1. So in our hands this ABT-737 drug is quite toxic to the CLL cells. So presumably that MCL1 is not sufficient, but once you knock the BCL2 out, then the cells are quite sensitive. Okay, thank you very much.

Dr. Chi Dang: Thank you, Jeff. Our next presenter is Dr. Mariusz Wasik, who is Professor in the Department of Pathology at the University of Pennsylvania, Perelman School of Medicine. And he's an attending physician in hematology/oncology as well as has been trained at DFCI, at Harvard Medical School, and he'll tell us about mTOR links cell signaling and metabolism.

Slide 60. mTOR links cell signaling and metabolism

Dr. Mariusz Wasik:

So let me start with a few words of introduction, though this subject probably doesn't require much of an introduction for this audience.

Slide 61. mTOR

So mTOR, which translates until recently as mammalian target of rapamycin, and since we had vincristine as mechanistic target of rapamycin, probably to acknowledge the fact that TOR, regardless of derivation, acts very similarly, in other words, TOR in humans behaves in a very similar way as TOR in yeast.

So it's a C103 kinase, which is expressed ubiquitously through human tissue. And it can form two complexes, which have a number of proteins in them. Some are shared, but some are complex-specific, so for example, raptor is characteristic and functionally very important for a complex called mTORC1, and rictor is important and unique for the complex 2, mTORC2.

So, as the name indicates, this kinase is inhibited by rapamycin and actually rapamycin was found a long time before this kinase and its target have been identified, hence the nomenclature.

Slide 62. Inhibitors of mTOR kinase activity – type I

So there are two types of inhibitors, which was alluded to already in the previous talks. One is the group of rapamycin and its derivatives, it's colloquially called rapalogs. And what's important is that they are not acting as classic kinase inhibitors, they inhibit protein-protein interactions which among other things inhibits function or binding of raptor to the complex and hence inhibits the activity. They also very specific and they are very potent.

Slide 63. Inhibitors of mTOR kinase activity – type II

As far as the type 2 inhibitors are concerned, they roughly can be divided into two classes. One of which is fairly specific for mTOR kinase and by definition they inhibit both mTORC1 and mTORC2. Some of them, however, will interact also with PI3 kinase AKT. In general, since they are analogous to, let's say, Gleevec® and other ATP binding competitors, they are not totally specific and they inhibit a number of kinases. So that's a striking difference between type 1 inhibitors, rapalogs, which are specific for mTORC1 and actually inhibit only partially its function, versus the type 2 inhibitors, which are preferentially inhibiting mTOR, but inhibit other kinases as well.

Slide 64. mTOR complex 1: activated signaling pathways and biological functions

So as far as the signaling is concerned, and this is a prevailing model, but as you'll see in a moment, fairly incomplete, is that as far as mTOR is concerned, there are two branches below, S6 kinase and S6RP and also 4E-BP. And both of them are involved in a number of functions in cells, most of them are acting through protein synthesis, but not only. And many of the functions actually recapitulate what c-myc does on the gene level, so there is plenty of cooperation in terms of regulation of cell functions between c-myc regulating genes on the gene level, whereas mTOR regulating it on protein level.

The functions are cell growth and that means physically that the cells become larger, and to become big and to divide they depend on mTOR, but also androgenesis and many other functions.

Slide 65. mTORC1 signaling and its inhibition in transformed B cells

So activity of mTOR can be tested using antibodies and the antibodies are uniquely specific, not only for the targets of mTOR, but actually for the phosphorylation sites. And as far as we know they're completely unique and specific, so they are great to analyze signaling pathways of this or any other kind.

What's important from a practical perspective is that many of these antibodies can be adapted to be used in immunohistochemistry. So they will act–detect in tissues, not only the presence of the specific proteins, but more importantly the status of activation.

Slide 66. Cells

So here I'm showing our data done with post-transplant lymphoproliferative disorder, which happened to abstain by virus or either one positive, and as you can see the cells are very, very positive for both phosphor S6RP and phosphor 4E-BP, indicating that mTORC1 pathway is highly activated in such cells.

Slide 67. mTORC1 activation in lymphomas

Over the years we have been working on a number of lymphomas and actually our first study from the year 2000 was the first to link mTOR activation to human malignancy, where we worked on PTLD, coming from the idea that since mTOR is immunosuppressive, perhaps it can prevent development of PTLDs in such patients when used, let's say, instead of cyclosporine. And as you can see there is a whole group of lymphomas in which mTOR is activated.

Slide 68. Mechanism of mTORC1 activation

As far as the signaling pathway is concerned, even a decade ago or so the system was very straightforward and resembling other pathways, at least they are presented now, namely the system was fairly linear, in a sense that the first signaling, which was identified in the setting, was insulin growth factor receptor, which acted through P13 kinase AKT, which inhibited the tuberous sclerosis complex, which in turn inhibited Rheb, which if released, activated mTOR and both branches below the kinase.

Slide 69. Mechanism of mTORC1 activation

Some time ago, though, another very interesting finding was made, mainly that this kinase activity depends very much on so-called second signal, which was provided by nutrients, such as glucose and certain amino acids. Both signals are necessary for the kinase to be activated. So in other words, it's not enough to obtain the signal from IGF and it's not enough to obtain the signal from nutrients at this point. Both have to be present for the kinase to be activated. Hence, if you look at it from this perspective, mTOR is the coordinator of the cell activation.

Slide 70. Mechanism of mTORC1 activation

Relatively recently the MEK ERK kinase pathway has been identified as being upstream of mTOR, though at that time it wasn't clear what's upstream of it. So reasoning that EGFR cannot be down in the pathway which activates mTOR, given its ubiquitous activation and I showed you many of the lymphomas do express it, we used a couple of lymphoma models to look at what may be the activation, what the activation was caused by.

Slide 71. Anaplastic large cell lymphoma expressing ALK

So one of the models we work with is anaplastic lymphoma kinase model. And a great aspect of it is it's a highly oncogenic kinase, hence a lot of oncogenicity depends on expression of this kinase by the lymphoma. In its native form, ALK is expressed only in the development of neuronal cells, so the presence in lymphoma certainly represents a great therapeutic opportunity and actually there is a very good response to ALK inhibitor in such tumors. The reason why ALK is expressed in lymphomas and other malignancy is typically through chromosomal translocation.

Slide 72. Translocation t in anaplastic large T-cell lymphoma

The first one was identified in lymphoma, hence the name, anaplastic lymphoma kinase, and this one involves NPM and ALK. Both of these fusion proteins activate a number of pathways identified by us and other laboratories,

Slide 73. Mechanism of NPM-ALK-mediated cell transformation

which the pathways, as you can see, include both PI3 kinase and AKT pathway, as well as MEK and ERK, suggesting that perhaps mTOR may be downstream of it.

Slide 74. Mechanism of NPM-ALK-induced mTORC1 activation

Since this work has been done some time ago, and for the sake of time, I'm just summarizing the results. So yes indeed, NPM and ALK does activate mTOR and what was surprising to us at that time, it was at both pathways at the same time. So in other words, it's not only PI3 kinase AKT, but it uses MEK ERK and in repeated experiments MEK ERK pathway was much more important and provided a stronger signal. So I think the pathway should not be linear any more. This is really a network and there is a contribution of various pathways, feeding into activation of mTOR. The cells were very nutrient-dependent. Removal of both glucose and amino acids completely shut down the pathway in the cells. And cells were very sensitive to rapamycin.

Slide 75. Cutaneous T-cell lymphoma

Another model which we worked with was cutaneous T cell lymphoma and which we have evidence that this lymphoma depends on the gamma change signaling cytokine such as interleukin-2.

Slide 76. Stage-dependent mTORC1 activation in CTCL

And as many of you know, this disease is also interesting in a sense that one can easily follow the stages of the disease and very often early on the disease presents as a patch, then as a plaque, and then develops tumors. There is a separate subform of the disease, and there is still an ongoing discussion of how these two forms relate to each other, is the so-called generalized erythroderma, which very often has circulating cells called Sezary syndrome.

So when we look at the activation of the pathway in this disease, in a stage-dependent method, something interesting came out. Namely it turns out that the pathway is activated, but you can see activation only in the large atypical cells. Building on the talks which we've just heard, this indicates that mTOR is present or activated only in the activated cells, not in the resting cells. So this is the stage in which the cooperation with the oncogenes discussed will occur. And this is very strikingly indicated here, that in the, let's say, the patch stage, only a few and atypical cells will stain for the phosphor proteins downstream ___ mTOR is activated. Whereas when the cells undergo lots of transformation, as you can see, every single cell very strongly expresses this phosphor protein, indicating a high level of activation of mTOR.

The same data we have obtained with normal lymph nodes. Mantle zone, it's completely negative. In germinal centers, only the large transformed cells are positive, small cells are negative.

Slide 77. Mechanism of mTORC1 activation in CTCL cells

So as far as the activation is concerned, the picture is very similar with some differences to anaplastic large cell lymphoma. Again both PI3 kinase and MEK ERK pathways are engaged. This time PI3 kinase and AKT is dominant. And we did side by side experiments, indicating that this is the case. Again, the cells are totally signal mTOR nutrient-dependent. Again, building on statements made by Dr. Dang, that this may represent a very attractive therapeutic target in the future. And the cells are very sensitive to rapamycin.

Slide. 78. MEK-ERK and PI3K-AKT pathways

This interaction or interdependence, not only on PI3 kinase ATK, but MEK ERK has been also noted by others. And as summarized here in the review from John Blandis' lab, one can see there is a lot of interactions between ATK and MEK ERK pathways, as well as ERK is able to inhibit mTOR or interact with mTOR on multiple levels. One is an indirect one, by inhibiting tuberous sclerosis complex, but also direct one on mTOR cells or indirectly RSK kinase.

Slide 79. mTORC1 generates negative feedback to suppress cytokine/growth factor signal

So again, a lot of complexities, lot of interdependence, but certainly indicated, at least both these pathways, and certainly this is not the end of the story. There are indications that other pathways may be involved and are upstream and have emerged.

Slide 80. mTORC1-generated negative feedback: impact of mTORC1 inhibition

Another important aspect is that inhibition of mTOR has its consequences. And if one used the inhibitors of the type of rapamycin, in other words, rapalogs would partially inhibit complex one, then this effect would disappear.

Slide 81. mTORC1 generated negative feedback: impact of direct mTOR inhibition

So one of the feedback mechanisms which is relatively well known, is on the left hand side, in which mTOR TORC1 through S6 kinase inhibits IRS. And the second described this year, it goes through the GRB10 adaptor proteins which affect receptors themselves. So at least two different mechanisms through which this happens.

So when you think now about the effect of mTOR inhibition through rapalogs, these negative feedback mechanisms are gone, which means that there is a hyperactivation now of PI3 kinase AKT, and also mTORC2 complex at this point.

The mechanism, by the way, is probably not to totally shut off the pathway, but rather fine tune it, presumably to match it with the second signal, which is the metabolic signal. So in other words, this needs to be very well orchestrated in a sense of how much signal comes from growth factors and how much from cytokines and how much comes from the second signal. And this is the part of the interaction. So this is not specific for tumor cells. This occurs in the normal cells, though obviously tumor cells use this mechanism to their advantage.

If we think about using direct mTOR inhibitors, and I've mentioned, some of them are quote-unquote mTOR specific, whereas the others also hit PI3 kinase, in principle this situation will be different. That if you use now at this point the direct mTOR inhibitors, you will inhibit not only mTORC1, but also mTORC2. So you have at least part of this feedback mechanism taken care of, so to speak. But again activation of AKT still remains in the system.

So when you use the inhibitors which inhibit now both mTOR, meaning mTOR complex 1 and 2, but also inhibit PI3 kinase, at this point you will have inhibition of the entire pathway. So in principle, this obviously provides a great opportunity. In practice obviously there is a question of the concern of toxicities. But if you are so radical and attribute everything, so to speak, in the cells, or at least many basic mechanisms and certainly PI3 kinase, AKT and mTORC1 and 2 belong to such mechanisms, the question would be, what would be the effect, though the preliminary data in patients who are treated with both types of inhibitor seem to show that they tolerate this type of treatment fairly well.

Slide 82. mTORC1 activation by diverse metabolic signals

So the numerous studies, and this is reviewed by Sabatini, recently indicate that the activation is more complex, so it's not only signal 1 and as I indicated, the signal 1 is very diverse. I believe that any cytokine or growth factor which activates cells will engage TOR. And there are data indicating that many of them do. But also in terms of what we call metabolic signals, they are also diverse. It's not only nutrients such as amino acids and glucose, which have already been discussed, but also oxygen, which also makes perfect sense, if the cell needs to be activated, it does need oxygen, though it can rely on the DEPTOR phenomenon for only so much. So it can adapt to a certain degree and it does, but again lack of oxygen at least slows down the cells, including malignant cells, and making them to turn on some adaptive mechanism for the

production of HIF-1 alpha. Distress will also do it and also the energy level.

As far as the effects are concerned, they are multiple at this point. Some of which we have already discussed or heard about, such as autophagy and production of protein, which seems to be a main critical function of this particular kinase.

Slide 83. Amino acids-mediated mTORC1 activation and translocation

So the pathways which activate mTOR, the so-called second signal, are being worked out pretty extensively at this point. The main breakthrough came very recently regarding amino acids. So it turns out that amino acids would activate the complex, containing the RAG protein and the newly discovered complex called, very cutely, ragulator. And you have a composition on the right hand side of multiple proteins. The idea is that when you have this regulator, bringing together this GTP, in this case A and C, they activate phosphorylation, which in turn activates mTOR, also causing these translocations from the lysosomal lumen to surface and induce its action.

Slide 84. mTORC1 activation increases nutrient uptake

Similar to first signal, also a second signal is activated by mTOR and that's again parallel with what we've heard about c-myc on the genetic level. So through induction of a number of proteins, mTOR will also secure delivery of the substrates by inducing expression of transporters of both amino acid and glucose. So again, myc on the genetic level, mTOR on the protein level.

Slide 85. mTORC-mediated regulation of key cell functions

As far as the functions are concerned, and again for the sake of time I will just mention some, in a general statement, as you can see, the proteomics in other studies and phosphor proteomics show that the pathways become more and more complex. So as far as the protein synthesis is concerned, it's not only S6 kinase as an S6 protein, but you see also a number of depicted additional targets. Lipids beside the proteins are also affected. And the idea behind it is probably that if the cell is to proliferate, it will have not only to recapitulate its protein mass, but also its membranes, and that's why mTOR is involved in the lipid synthesis.

Energy, again, I've mentioned already HIF-1 alpha. This probably is not the only factor because HIF-1 alpha is barely present in normal normoxic conditions. And also there is some indication of TORC2 being involved in cell metabolism, acting primarily by inducing translocation of FOXA protein from the nucleus, to cytoplasm binding to scaffolding protein and inducing the intracytoplasma function.

Slide 86. Rapamycin-induced inhibition of hexokinase II expression

So mTOR also acts on the RNA level. And this is our study in lymphocytes. As you can see, many of the elements of the glycolytic pathway are affected. The one which is affected by far the most is hexokinase 2, which as some of you may know, is the critical gatekeeper for the entire pathway. So really its level and activity regulates the presence or the absence of activation of glycolytic pathway because it sits at the surface of mitochondrion and actually absorbs glucose

from there.

We confirmed this dependence of mTOR, of hexokinase 2, also in the protein level, with the kinetics of 24, 48 hours. So the effect is not immediate. It needs to wait until the protein wears off.

Slide 87. Rapamycin-induced inhibition of several metabolic pathways

A study by the other group, David Manning from Harvard, was much more comprehensive as far as the pathways are concerned. And they made the finding that it's not only glycolysis enzymes which are involved, but also enzymes which are involved in the pentose phosphate pathway and also the lipid synthesis pathway. So really, involvement of the pathway in various types of cells. This was done in epithelial cells and yet, as you can see, hexokinase is among the–hexokinase 2 is among the activated genes, showing that the process is universal.

Slide 88. Rapamycin-induced inhibition of mTORC1 signaling, cell proliferation and glycolysis

If you think about it, this may have an important practical implication, which actually with the technologies which have been used in diagnosis of lymphoma for a number of years, but I think based on this data, one has to really think about the philosophy, how they are used and interpreted. And what I'm talking about is using the glycolysis system to identify activation of cells or growth of the cells.

At this point the PET technologies, and certainly they are solidly based on the glycolytic pathway because after all this is a labile glucose and it's binding to hexokinase 2, is what is being measured, using PET scans at this point. In my opinion are still used more as diagnostic tools to measure volumetric effects. In other words, the drugs are given and then PET is done and one looks at–has the tumor shrunken and this usually done much later in the treatment of the patient. If one wanted to use it for detection of inhibition of signal transduction or metabolism, and as I've indicated, mTOR is downstream of many other factors, so if you inhibit ALK, if you inhibit BCR-ABL and so on and so forth, you will inhibit mTOR, therefore you can use this type of an assay to check whether the Gleevec has worked, whether ALK inhibitors have worked, and so on and so forth at this point. So probably the two aspects need to be taken into consideration. One, I think this assay should be done soon and I will say 48 to 72 hours would be the optimal period. And the question should be asked, is the pathway activated, rather than whether you have an antitherapeutic effect, because the other is–the latter is much more complex and depends on the other factors. So I see a great application of this particular technology to be used now, to study the effect of targeted therapies, again coming from the premise that many of them will affect metabolism through mTOR.

Slide 89. Rapamycin-induced inhibition of tumor growth and glucose metabolism in vivo

So this can be done in vivo and here we are showing this in xenotransplant mouse models. And as you can see on the left hand side, we have a tumor in which mTOR induced stasis and you can have some increase in the lactate expression at this point, or concentration within the cells.

The cells would respond with a decrease in the tumor volume. This effect is much more pronounced. And timewise they are identical. So really looking at this, we can see what is happening with the tumor, again, providing the proof-of-principle that this technology should be very powerful when applied in a similar manner in patients.

Slide 90. Effect of mTORC1 inhibitors as single agents in cancer

Now we move to the therapy and as many of you know, mTOR inhibitors, primarily mTORC1 inhibitor, rapalogs, are being tried very widely in a number of tumors. There are over 70 clinical trials happening at this point. They are very effective in lymphomas, as shown primarily through the work of the Mayo Clinic, but also leukemias, as shown by the Children's Hospital of Pennsylvania, primarily when it comes to the lymphoid leukemias.

A number of other tumors are responsive to the treatment and as many of you know, the FDA has approved it for five different applications, with the most recent being breast carcinoma in menopausal women, with the estrogen and progesterone positive HER2 negative malignancies. None of these tumors, mind you, are of hematopoietic origin at this point so far. Though there are active trials in both mantle cell lymphoma and diffuse large cell lymphoma.

Slide 91. mTOR inhibition may enhance the antitumor effects of other therapies

What the studies show, though, is that the responses are usually partial and primarily there is stability of the disease. Furthermore, they are transient, six to nine months later the patients progress. Not anything unique, as you know, when you use a single targeted therapy. This is a fairly universal finding. And as far as the inhibition of mTOR, for that matter, any other single anti-oncogene, it can be argued that the future is in the combined therapies.

What the combinations are going to be, one can pick up his or her favorite at this point. In essence, one can think about combining them with the standard therapies like chemotherapy radiation. But one can think about new therapies and combining them with the new targeted therapies, to optimize the effect and limit the toxicities.

Slide 92. Biological effects of mTORC1 inhibition in CTCL cells

So to address this question, we came back to our cutaneous T cell lymphoma model, and did a very simple experiment of exposing the cells to various amounts of mTOR inhibitor. And although this inhibitor is exquisitely–and that's rapamycin itself actually–is very active in a sense that nanomolar doses inhibit growth of the cells, but once we reach the threshold, which you can see correctly here, it's about one nanomole for this particular cell line, it reaches a certain plateau. So in other words, you cannot go any further. It can do whatever it can do, but it cannot inhibit completely the growth of the cells. And this applied to both cell lines and the primary tumors.

Slide 93. Rapalogs efficiently inhibit S6K but not 4E-BP1 phosphorylation

So the question is why. And one of the answers is something which I've alluded to, that rapamycins or rapalogs are so specific that they inhibit only part of the pathway. In other words,

they are extremely efficient to inhibitor S6RP, but they are not very active and it's very much tumor-dependent as far as the 4E-BP arm is concerned. In some tumors it's no inhibition. In some it's partial. Others, it's almost complete. But in general it's much less efficient than when it comes to this other arm.

Slide 94. MNK kinase phosphorylates eIF-4E

So what can be done about it? It turns out that actually a number of years ago another kinase, which is called MNK, which did not get much attention in the literature, is able to phosphorylate and activate EIF-4E which is downstream of 4E-BP1. So obviously the question would be what happens when you do inhibit now mTOR and do the combination with inhibition of MNK kinase.

Slide 95. Combined inhibition of mTORC1 and MNK results in marked suppression of growth

And these are the data. So as you look at that, there's a tremendous inhibition of the cell growth at this point, almost disappearance as far as this particular line is concerned. But what I found exciting at this point is that now we have an induction of cell apoptosis. So mTOR inhibitors or rapalogs by and large inhibit cell growth, but do not induce apoptosis. To the contrary, they induce autophagy. Whereas here we see that combination leads to inhibition of the cell, cell viability at this point, suggesting that perhaps, perhaps when you target, when you use targeted therapies and you use a smart combination, based on the biology of the disease, the effects may be quite profound.

Slide 96. Summary – part 1

So to summarize my presentation, I have two slides. What I talked about today is that mTOR pathway, and for that matter mTORC2 as well, are ubiquitously activated in lymphoma and many other types of malignancies. And it mostly relates to the size of the cells, since most of the malignant cells are anaplastic or atypical, therefore they have these high requirements for metabolism and activity of mTOR. Many of the cytokines and growth factors, and as I said, I expressed my opinion, all of them probably which are able to activate cells, do activate mTORC1 and this is the so-called first signal. Importantly, mTOR is not only a passive receiver of the signals, but also actively modulates this type of the signals by affecting in a feedback, negative feedback, the factor receptors as well as downstream elements such as IRS1.

mTORC is highly sensitive to nutrients, both amino acids, primarily leucine and arginine rather than glutamine, and also glucose and other metabolites. But also regulates actively the pathway and uptake of the nutrients.

Slide 97. Summary – part 2

When it comes to the clinical or therapeutic applications at this point, mTOR activation can be monitored in vitro and in vivo by the functional cell metabolism, glucose metabolism in

this case, and lactate production assays. Inhibition of mTORC alone universally leads to inhibition of cell proliferation, but typically does not induce substantial apoptosis, however, the combination with MNK kinase led to apoptotic cell death, at least in this cutaneous T cell lymphoma model.

Slide 98. Contributors - 1

I have two slides of acknowledgments. Original work was done with Novartis group, which provided us with RAD41, the first rapalog.

Slide 99. Contributors - 2

And these are the other critical investigators listed in these types of projects. And also with the follow-up project with the imaging study done in collaboration with the imaging experts, led by Dr. Jerry Glickson.

Slide 100. Genetic and metabolic alterations in myeloproliferative neoplasms and acute myeloid leukemia

Dr. Chi Dang:

We'll move on to our next speaker, on the genetic and metabolic alterations in myeloproliferative neoplasms and AML, presented by Dr. Ayalew Tefferi from the Mayo Clinic in Rochester, Minnesota.

Dr. Ayalew Tefferi:

My presentation is going to be mostly more of a perspective from the standpoint of the clinician investigator. As you know, I am primarily a caregiver/clinical investigator, and when you listen to all this fantastic science, you need to actually put it into perspective as to how it somehow plays out in the broad spectrum of cancer biology and treatment. And that's what I'm going to try to do today.

Slide 101. Myeloid malignancies

Now at this point the dogma is that all myeloid malignancies start with the genetic transformation of the hematopoietic stem cell. It could be the true ancestral stem cell or it could be a level or two below, then that subsequently gets the stem cell-like property. That's beside the point. It is a stem cell disease, they're all stem cell diseases. And at this point, believe it or not, more than 95% of the cases, we really don't know what that disease initiating disease allele is. In other words, we don't know exactly what sets off the disease. There's a lot of talk about predisposition of alleles, but we don't even have a good understanding of what the actual disease allele is.

And because of that, we don't have a very nice accurate molecular classification system, which would be therapeutically relevant. But instead we are classifying it based on morphology of what we are seeing and for all practical purposes, you can think of all myeloid malignancies as

three big groups. The acute myeloid leukemias, as you can see, where the programming of the mutation or mutations ended up in completely abrogating the differentiation capacity, all you see is leukemic blasts. Or some sort of differentiation is retained and then you have the chronic myeloid malignancy and there we have the myeloid dysplastic syndrome, where dysplasia is mostly the morphology. And the myeloproliferative neoplasms, where actually there's an accumulation of mature-appearing cells. That's pretty much it as far as classification is concerned.

Now if you look at these three groups separately and you want to look at the genetic makeup of this and obviously the metabolic consequences come from the mutations, I think the metabolic consequences become important in treatment because you don't always have to go after the mutant protein, you could potentially go after the altered metabolism, as very well outlined by Chi in his first talk.

Slide 102. Acute myeloid leukemia

When you look at acute leukemia, what is hidden in those ugly looking cells is a spectrum of cytogenetic abnormalities, as you can see.

Slide 103. Cytogenetic abnormalities in AML

And like chronic myeloid leukemia, there is no one specific oncoprotein. There are many, or the Philadelphia translocation.

Slide 104. Cytogenetic abnormalities in AML

What you have is a bunch of cytogenetic abnormalities and then there are normal ones, but normals are not exactly normal because if you take those normal cells

Slide 105. Dyserythropoiesis

and do a much more submicroscopic exploration of the genetic alterations, you could see tons of somatic mutations and alterations that are listed at primarily NPM1, CEBP1, FLT3, and what have you. In other words, there's a complex multitude of both cytogenetic abnormalities and submicroscopic molecular changes.

Slide 106. MDS cytogenetic abnormalities

Now the story is very similar in myelodysplastic syndromes, where it's not just the proliferation of these immature leukemic cells, but primarily there is some differentiation, but the cells are more follicularly dysplastic.

Slide 107. Gene mutations and karyotype in MDS

If you take those, again, the same story, cytogenetic abnormalities, tons, spectrum, and molecular abnormalities as you can see, most of them very similar to what you see in acute

leukemia, but again, there is nothing normal here. In fact, if my memory serves me right, there are actually 18 genes that have been affected with one or more mutations. And most of these mutations are not mutually exclusive, interestingly.

Slide 108. Manifestations of classic MPN

Now you go to myeloproliferative neoplasms where the phenotype is primarily excess, big spleen, high white cell count, and so forth, so in other words, the differentiation is fine, it's just accumulation of mature appearing cells and what have you.

Slide 109. Primary myelofibrosis

And if you go there, again the story is very similar. We've looked at–now we have about 2,000 patients in our myelofibrosis database–and as you can see, there's a spectrum of cytogenetic abnormalities, there are less cytogenetic abnormalities compared to MDS and acute leukemia. There are about 30%. And if you go to essential thrombocytopenia, that's even less than 5%. But when you look at it molecularly, again tons of mutations. Some of them more frequent than the others.

Slide 110. Mutations in myeloproliferative neoplasms

Everybody here has heard about the JAK2 mutation that occurs in almost everybody with polycythemia vera, but then there are many other mutations, whose mutational frequency is actually often below 10%, sometimes between 10 to 20%. In other words, you're dealing with many, many mutations and therefore many, many subclones of the ancestral oncogenic clone.

Slide 111. Mutations in frequency and distribution

Now beyond JAK2 and myc though, and in fact I'm happy to share with you some of the data we're going to present on Monday or Tuesday, one of those days, and we actually looked at epigenetically relevant mutations and other mutations, trying to look at prognostic impact and trying to understand the distribution and the pattern. And as you can see, we've looked at specifically four genes because they have been individually reported to be prognostically relevant, ASXL1, EZH2, SRSF2 and IDH. And as you can see, 30% of the patients had an ASXL1 mutation. And in fact, if you look at them stage by stage, the high risk group, almost 60% of patients have these mutations. So these mutations are not infrequent and as you can see, the other mutations follow. The interesting thing is there appears to be come clustering of some of the mutations, even though they're not mutually exclusive, there appears to be, between IDH and SRSF2, there appears to be a significant clustering of sorts. And some patients actually have almost all of the genes in them. So this is a complexity. The question is, what are you going to target?

Now so how does one navigate through this? I showed you these three diseases, all spectrum of cytogenetic abnormalities, molecular abnormalities, what do you do with that? Where do you begin to put some order here?

Slide 112. AML Classification

Now obviously that has started. For example, in AML, some of the cytogenetic abnormalities have been associated with some phenotype and therefore based on that, the WHO has incorporated these as recurrent cytogenetic abnormalities, which suggests that there is a subset of those acute leukemias that are driven by a particular set of mutations. And therefore, if you have something that targets something, it's not only always a subclone, it could be a subset of a different disease that you can then identify and use a particular targeted therapy.

So as you can see in AML, there is the 15;17 translocation that everybody talks about, acute promyelative leukemia. There's going to be a plenary session at ASH this year that you can actually treat that disease without chemotherapy just using arsenic trioxide and ATRA. And then there are the core-binding factor acute leukemias, 8;21 and so forth, which again needed to be identified separately because A, they respond beautifully to high dose ara-C, but then also if you have targeted therapy you have to distinguish those from the others, lest you do not succeed or do not show activity of that drug because you are using it in the wrong patients.

Slide 113. Dysmegakaryopoiesis

And it's not only acute leukemia, but also in myelodysplastic syndrome, where for example, patients with 5q minus syndrome, which have the 5q minus in the cytogenetic abnormality, appear to have a phenotypic manifestation of small mega karyocytes, so-called micromega karyocytes. So the question is, is there phenotype patterning here.

Slide 114. Spliceosome mutations in myeloid malignancies

And then came the spliceosome mutations. Now we were talking about all those mutations, how frequent they are, and all of a sudden somebody told us about a completely different set of mutations, which in fact appear to be even more frequent than the mutations we knew.

Slide 115. Ringed sideroblasts

And here, the main message here is, it appears that some phenotypes such as ring sideroblasts, that you can see in some patients with myelodysplastic syndrome, but also in other myeloid malignancies, appear to be almost consistently associated with a particular spliceosome mutation, which is SF3B1. So in other words, if that mutation and phenotype pattern a mutation.

Now this is not trivial. Because let's say that you have the best anti-SF3A1 small molecule. So you give that to the patient. So what are you going to achieve? Get rid of the ring sideroblasts? What did they do to you? They're not the disease-initiating mutation. You can get rid of them, but you don't necessarily get rid of the disease. So it is very important to have these associations and to know what you're targeting and what the endpoint might be after you target these targets.

Slide 116. Clonal evolution and mutations in myeloproliferative neoplasms

Now in myeloproliferative neoplasm, we got lucky. Most of the good things happen by luck. And as you know, the BCR-ABL1 appears to be the disease-initiating mutations and as a result, a good drug was able to put the disease into sleep forever. Doesn't cure it. I think one of the stories we've learned from the imatinib CML luck is you don't really need to cure cancer in order for it not to kill the patient. You can put it to sleep forever, which is as good as cure.

Now unfortunately that's the exception rather than the rule. If you look at the other myeloproliferative neoplasms, we do not have a BCR-ABL1 equivalent. What we have is a series of mutations that I have listed there, including some which are very frequent, like JAK2, some that are not very frequent, like IDH, and the question is, what are they doing? They're not being traced back to the ancestral core, so they're probably not disease-initiating. If not, what are they if they're not disease-initiating? Are they phenotype patterning? Some appear to be. In other words, if you have a myeloproliferative phenotype, in order to have erythrocytosis as part of the component, you need a JAK2 mutation. No JAK2 mutation, no erythrocytosis. You can still have myeloproliferative neoplasm.

The subsequent exercise from that kind of thinking is, if you have a fantastic JAK inhibitor, get rid of the erythrocytosis, but not necessarily the disease.

The same thing with the SF3B1 as I showed you, ringed sideroblasts. IDH, sometimes there is a phenotype or so-called couplet nuclei. In other words, there appears to be some phenotypic patterning. And then the main question, if I have ET, or myelofibrosis, and if God says alright, I'm sorry I gave it to you, but you know what, I'm not going to make that go into acute leukemia. Then I won't die. I'll live with my ET forever. I can't cure diabetes, I can't cure arthritis, I can live with it forever, as long as it doesn't transform into something that is fatal. Therefore, it is as important to find those mutations or alterations that actually transform the disease because then we can target those and let the patient live with the ET or myelofibrosis forever and ever.

So the question is, do any of these mutations contribute to this leukemic transformation and, therefore, can they be targeted. And there have been some of these mutations. For example, SRSF2, IDH, ASXL1, EZH2, these four mutations have actually been associated with poor survival, but some of them like IDH1 and SRSF2 with vulnerability to leukemic transformation. Especially IDH1. IDH1 seems to be the bad one. Acute leukemia, myelodysplastic syndrome, myeloproliferative neoplasms. And yet the metabolic consequences appear to be similar. So what gives?

Slide 117. AML: Prognosis of cytogenetic markers

And so, we have all these. Now as clinicians and taking everything into a patient, how do we use this, all these things? Well, first is the easy part. Just use these markers as biomarkers, prognostic biomarkers.

Slide 118. Outcomes according to cytogenetic risk group

For example, in AML, you can distinguish between favorable, unfavorable, and intermediate risk, and have a survival code which is beautiful and you can tell patients you've got this mutation, so you're going to do good. This mutation, it's important, so as a prognostic

biomarker very important.

Slide 119. Molecular studies in AML

The same with the molecular mutations in AML. Some are good, some are bad. And again based on that, for example, everybody under the sun knows that you have an NPM1 mutation without the FLT3, you do very well. If you have FLT3, doesn't matter whether you have NPM1 or not, you don't do very well.

Slide 120. CEBPa and NPM1 mutations in AML

And now we are understanding more and more CEBP1, good; IDH1, bad; IDH2, don't know. There are so many things.

Slide 121. Prognostic relevance of gene mutations in MDS

So prognostically we can do this exercise in MDS as well. And as you can see, this was published, pretty much the same bad mutations for every disease.

Slide 122. Survival and prognosis in primary myelofibrosis

In myelofibrosis, despite having a very robust cytogenetic inclusive prognostic model, if you add molecules here, and here is for example, this is a study that we're going to present at ASH on Monday, here we've looked at these three extremely important prognostical biomarkers, ASXL2 mutation, SRSF2 and IDH1.

Slide 123. Three-tiered DIPSS-plus stratified survival data in 297 patients

If you take these and then you molecularly classify these patients, you can actually distinguish in very low risk patients who is going to act as high risk and therefore therapeutically is very important.

So having these mutations is prognostically important. And it's not very difficult, as long as you have a very good database in DNA to achieve this.

The problem comes, and the challenge, I wouldn't say it's a problem, the challenge comes as to, what do you do with it in terms of treatment. Could you use any of these things as drug targets?

Slide 124. Potential drug targets in myeloid neoplasms

Now most of you here know that you can acutely classify mutations into those that are class 1 or whatever they are. Driving mutations, perhaps. Mostly kinases, FLT3, JAK2, C-KIT. These are usually kinases. And then we say, there are the class 2, the transcription factors, RUNX1, AML1-ETO, and so forth. And then there are others. And now we have spliceosome mutation. The epigenetically relevant mutations. And they're all intertwined. All of you know

that, for example, IDH inhibits TET2. So if you have an IDH mutation, it's like having a TET2 mutation. And so forth.

And the question then becomes, what do you target in this intertwined mechanism? Do you target the metabolite, for example, 2-ketoglutarate? Do you target the IDH mutant? Do you just deprive the cells of important nutrients? And the question then becomes, most important question in all this, despite how nice it looks, is okay, let's sit back here and think. These are not disease-initiating mutations. If you measure them at any one time the amount, if you quantify them, some of them are very small subclones. In other words, the majority of the clone, 99% of the disease does not have, for example, IDH, does not have, for example, SRSF2. So if you have a fantastic target or whatever approach you go, and you get rid of that subclone, you still have not gotten rid of the major clone. Could you identify a disease that is primarily driven by that? And there was a question about some patients go into acute leukemia and that's when they acquire IDH1. Well, can you prophylactically use treatment so that they don't? Or once they go in there, then can you just kind of get them back into the chronic phase by getting rid of the IDH acquiring cell clone. It becomes pathogenetically very complex.

Slide 125. JAK

Now I'm not just talking here. There is proof here. Now here are the JAK inhibitors. Everybody has heard about JAK inhibitors. I have listed them for you. There are a lot of them. Some of them are looking better and better. Ruxolitinib was the first one, but now we're getting better, more potent EC4 and there are tons of them.

Here is a pattern. They're all good at shrinking your spleen. They don't make it disappear, like imatinib does in CML. 20%, 30%, 50% sometimes. They all make you feel very good. And by the way, feeling very good means getting rid of pruritus, night sweats and so forth. And guess who else can do that as good? Everolimus. Which is a rapalog. An mTOR inhibitor. Right there at the end, which tells me that perhaps at least those aspects are mediated by mTORC1.

So in other words, we have this symptom relief, not only from the JAK inhibitors, but appears to be mTOR-mediated. We have them, this decondition of the spleen, but that's it. That's it. The fibrosis is still there, the cytogenetic abnormality is still there. And the JAK2 mutation is still there. The JAK2 mutation is still there. So what did we do?

We don't have a good antineoplastic drug here. What we have is an anti-paraneoplastic drug here. That's what we have.

Now the second problem is that they also have side effects. And this is very important, especially when you're thinking of combination therapy in the future.

Slide 126. Two pathogenetic faces of myelofibrosis

Now what we have learned, however, from the JAK inhibitor trials, is that in myelofibrosis, the phenotype, the patient's getting very sick, losing weight, cachexia, and not being able to eat or sleep, having night sweats, is not only the consequence of the clonal proliferation, but it also is the accompanying inflammatory state that cancer has. There's a lot of aberrant cytokines that we have reported on and we have shown that the use of JAK inhibitors profoundly affects the cytokines. So in this cytokine story, this cytokine store, the host's reaction

to the disease contributes to the phenotype of the disease. Whether it's a big spleen, whether it's anemia, whether it's night sweats and what have you. So in other words, you make the patient feel better, but you don't necessarily have a good antineoplastic drug.

Slide 127. Long-term outcome of treatment with ruxolitinib

And at least in our hands, and we have had the longest study, we have not seen any improvement in survival. Others claim that they have. But okay, even if they did, the survival curve differences are like this. It's not exciting, it's not exciting.

Slide 128. Concluding remarks

So what am I saying here? What I'm saying here is myeloid malignancies pathogenetically are extremely complex. It's not an imatinib CML-like paradigm here. So we have to be prepared for that and our paradigms have to be different. Yes, the science has to be done, but within the context of what we are trying to achieve.

Now the other thing that we have learned from the JAK inhibitor trial is that JAK study is important in the accompanying inflammatory state of cancer. In fact, these drugs can be used for any disease. They're not myelofibrosis drugs, they're cancer drugs. They're anticachexia drugs. You could use them for any cancer, I bet you the patients will feel better. It's all inflammatory state.

And if really we want to change cancer radically rather than just incrementally, add this and that, we have to be bold in how we're thinking. And what I have heard has enlightened me, and I just felt good that people are actually serious about looking at more than just mutations, metabolism and so forth. And the amount of work and the quality of work is wonderful. But it has to be within the context. Context means that the beast that we are facing is not a simple beast. It's got many heads. So we probably have to attack it in a multiprong fashion. But the most important thing, when you do that, everybody says oh, combination therapy, and this and that. But combination therapy has to be scientifically driven. You have to remember when you combine something, not only could you possibly increase the efficacy, but you can often increase the toxicity. That's common knowledge.

But most importantly, what really upsets me, every time I turn around I see these combination drugs. Not based on science that it makes sense to combine different–but depends on what the industry, the drug company that has a JAK2, what else that drug company has and will combine it with that, no? Get two for our money.

It is done this way at this point. But I hope, I mean I think the outlook is better than fixing our budget deficit. So I am not really that pessimistic. I think it will be resolved. But I think the scientists are going to drive this, not the clinicians because there's so much corruption and so forth. But I think this kind of science and coming in with new drugs and so forth and doing in the laboratory what makes sense to combine or what have you, is I think what's going to help us. Thank you very much.

Audience: With JAK2 inhibition and the same things that you mentioned about the mutation and transformation to acute leukemia, do you think that these anti-paraneoplastic drugs, they

eventually increase the clonal evolution of other mutation and increase the leukemic transformation, acute leukemic transformation in otherwise people that I don't think that live ever and ever, but we are not expediting their longevity with like probably shorter life with maybe better quality of life.

Dr. Ayalew Terrefi: You know, that's the profound question. And it's very important because people are thinking oh, why don't we treat them earlier with these new drugs, make them anemic. Because they're not anemic, they're doing fine. But more importantly, what you say is, what do you know about the long term side effects of these drugs. I mean, you're changing things here. You're suppressing tumor necrosis factor, you're suppressing IL6, for a long period of time. How is that going to affect the clone? Or actually the emergence of new cancers and what have you. So it's very, very important, that your point is very, very important, for whoever wants to treat patients with early stage. The drug companies want to treat the early stage because it expands the market. And so we have to be very, very careful. Now alternatively, they might prolong life not because they address the cancer, but they address things that will kill you. Other things. Consequences of cachexia and what have you. So most points are there, but we have to err on the safe side and make sure that patients who are otherwise going to live a long time, don't die earlier because we get too greedy, for something that's not very far from imatinib. We don't have an imatinib-like drug right now. So I agree with you 100%.

Audience: I want to ask, should patients' diet be considered, because we are talking about nutrients and all those things.

Dr. Ayalew Tefferi: You know, today I got enlightened. It's unusual for me to get enlightened because it's the same old, same old. But today I really got enlightened because it's a different–and it really, especially the glutamine deprivation, I mean that is very, very interesting. And it's real. He's shown it in leukemia cells and so forth and so forth. So that is so easy, isn't it? Diet is so easy. But the question is, you have to have very strong experimental proof for advising the population about this and that. Now I think that's all we can say. I don't think anybody can say more, but at least this, what is presented today, suggests to you that diet is not an innocent bystander in this whole scenario of cancer and so forth.

Audience: So Dr. Tefferi, I have a question regarding the myeloproliferative neoplasm. For a long while we believed JAK2 pathways really plays a key role in the pathogenesis. Not only do we discover JAK2 mutation, also the MAPO mutation. Now your clinical trials suggest there's something else. So beside JAK pathway, and what do you believe with such a wealth of information about mutations, do we have a central theme and what are the most important pathways to go forward from here and try to target them?

Dr. Ayalew Tefferi: I hear you. I mean it's clearly an advance. It's an advance to understand that JAK studies have been activated. It's an advance to know that cytokines are important and so forth. So it is a drug. But we know for sure that it is not an antineoplastic drug. The question then comes is, we have more work and we have more targets to consider and we need to think about combination therapy. So what's alternative to combination therapy? I always think of an

alternative to combination therapy. Can we have a common denominator of cancer, especially leukemia stem cells? There's so much work on leukemic stem cells. Can we come up with something that is common to all mutations, that we don't have to go after the mutation, but after that common denominator. Example, telomers. Everybody needs telomers to survive. And you inhibit telomerase, maybe they die. Maybe if you know how to use a telomer inhibitor, maybe you can knock out a few things here and there. That's just an example. Or something metabolically important for leukemia stem cells, but not just IDH-mutated. But any kind of mutation. I think that's probably going to result in a more radical fashion. So what are we doing? We're doing a lot of studies, we're doing telomer inhibitor studies as we speak. We're doing anti-WNT pathway drugs in order to hit the stem cell level, to have a more common denominator target, rather than subclone target. It's easier said than done. But we just continue to work.

Dr. Chi Dang:

Our final presenter is Dr. John Cleveland, who will address targeting autophagy in hematologic malignancies. And he's Professor and Chair of the Department of Cancer Biology at The Scripps Research Institute in Jupiter, Florida.

Slide 129. Regulation and role of the autophagy-lysosomal pathway in myc-driven lymphomagenesis

Dr. John Cleveland:

You don't have a lot of pages in your book for my talk because most of it's unpublished. This is actually the first time I'm going to roll it out. And I think it's nice from the perspective that the topic that we're talking about today is kind of the endgame from the topic that Chi told you about in the beginning. So cancer cells are highly anabolic.

Slide 130. Eμ-myc

The primordial function of a gene like myc, as Chi has shown quite nicely, is to make ribosomes and ribosomes can make lots of proteins and cells can grow.

And the autophagal lysosomal pathway is the principle recycling center of the cell. So consider it the major catabolic pathway. And so what I'd like to leave with you today, hopefully to convince you at the end of the talk, is that this a coordinated response. Myc is not only regulating anabolism, for example, by increasing mitochondrial biogenesis, that Chi showed some eight or nine years ago, but it's also coordinately downregulating the autophagy pathway and lysosomal biogenesis. And this is quite counterintuitive. And this is a classic case where you let the data show you the way. Because going into this we had preconceived notions that myc would upregulate the autophagy pathway and that would be important for tumor progression and tumor resistance and et cetera. And I'll tell you it's exactly the opposite.

So for a long time our group and others in the audience, like Clemens Schmitt, have used a transgenic model of Burkitt lymphoma called the Eμ mouse model which was created by Alan Harris in Jerry Adams's lab back in 1985. This myc is a powerful genetic tool because one can actually isolate B cells. So in this case myc is driven by the Eμ heavy chain enhancer, so it phenocopies the translocation of what occurs in Burkitt lymphoma in human beings, where myc is

driven by translocations that juxtapose immunoglobulin gene enhancers to myc. So myc is overexpressed. They have a protracted premalignant phase. Then they have a neoplastic conversion of that. And we and others like Clemenz in the audience have shown that several checkpoints are important, that have to be bypassed for tumors to then arise and for the mice to get fulminant lymphoma and die. This usually happens within 100 days, so this is a very nice in vivo test tube.

So our studies have shown, so has Clemenz, that the Arf P53 tumor suppressor pathway is a very important checkpoint and so that indicated that the apoptotic program had to be bypassed during tumorigenesis. And these studies were actually those that showed that this is a common feature of all human malignancies.

The second inroads really came from work by Ulrich Keller in the audience, which showed that the hyperproliferative response driven by myc is due to overexpression of an allosteric regulator in the SCF complex that controls P27 degradation.

And the third checkpoint listed here is DNA damage response, which Clemenz had discovered along with Bruno Emadi as an important checkpoint. And the Bartek and Lucas labs basically showed DNA damage is probably an initiating event in all human lymphomas.

So we've been using this model for a long period of time and more recently over the past four years, have been asking questions about, really, Chi's seminal work, what are the changes in metabolism that occurred during the premalignant phase in neoplastic conversion. And how can we exploit this.

Slide 131. Chloroquine abolishes the pre-cancerous response

So one of the curious findings that we had was that an antimalarial drug called chloroquine, which you take if you're going to Africa, as a big pill, was actually a very effective chemoprevention agent in this model. And so the model manifests with increased white blood cells in the premalignant phase, you get extramedullary hematopoiesis in the spleen, so the spleens get huge, and so you can measure a spleen right here. And you could see that animals that were treated with chloroquine IP at doses of about five-fold less than you would take if you take the drug, effectively abolished this premalignant state. So that was curious.

And then we noted that the premalignant state was basically disabled in these B cells from any myc mice. What you're looking at here is a western blot. And the key checkpoints that are activated are P53 and PUMA and Arf goes up when P53 goes up, so Arf is induced by myc and P53 comes up because Arf inhibits MDM2 and then P53 transcriptionally activates PUMA. And all of these have been shown to be important in disease progression in this model and in human malignancies. So this looked really quite interesting.

Slide 132. Antimalarial drug chloroquine blocks the autophagy pathway

And one of the curiosities was that well, then how does it actually work? And so it was actually known that chloroquine was a weak base and as a weak base, it accumulates some lysosomes, which are in acidic compartment. And if you take untreated cells and look at them under EM, this is the typical picture that you see. You see a nucleus in the middle there, you see lysosomes that are empty, that's because they degrade in everything they get as fast as they get it.

And if you take cells and treat them with chloroquine, one sees very rapidly, this is within four hours, a disruption of this process. And if you're looking at these organelles under EM you see that the lysosomes and autophagosomes that deliver their cargo to the lysosomes for destruction are packed full of cargo, you're not destroying anything. In effect, you've blasted the heck out of the recycling center, the garbage disposal no longer works, you're filling up with garbage and then you die.

So that's been shown to happen in many tumor cell types and chloroquine is actually being used clinically. I believe, last check, there were 16 different clinical trials on NCIclinicaltrials.gov, of investigators trying chloroquine in certain malignancies. And certain malignancies, such as melanoma, and it appeared perhaps pancreatic adenocarcinoma, are exquisitely dependent upon and attack autophagy pathway and therefore chloroquine is being tried in those malignancies.

Slide 133. Chloroquine prevents myc-driven lymphoma development

It also works in blocking lymphoma development in this model of Burkitt lymphoma. So the black line is the Kaplan-Meier survival curve of mice that were treated with vehicle and the red line is the mice that were treated with chloroquine. So it had a profound effect on lymphoma development.

So we thought at the time that this was all due to the inhibition of the autophagy pathway. But there were actually genetic tools that were developed at that time, to actually allow us to critically address this issue. So the question is, is chloroquine really disrupting lymphoma genesis, preventing the premalignant state, delaying cancer progression because it's really hitting the autophagy pathway, or is it really doing something else?

Slide 134. Macroautophagy

Okay, so let me give you a little bit of primer on the macro-autophagy pathway, which is what I'm talking about, and which most of academic investigators and the pharma industry are actually targeting.

Autophagosomes are formed by isolation membranes called phagophores. After autophagosomes form, they're double-membraned garbage bags that then fuse with the lysosome and deliver their components to the lysosome where they've been degraded, to allow cells to recoup essential building blocks like amino acids and energy. This process is regulated by, strictly requires a protein called LC3. You've seen a couple of blots of that. That undergoes a very unique modification of phosphatidylethanolamine residue, is hooked to a terminal glycine. That is initiated by an E1 enzyme called ATG7. ATG7 is somehow turned off and on by a kinase called ULK1. There are actually four of these kinases in our genome, ULK1 through 4. At least a couple of them appear to be functionally redundant. It's not known how ULK1 turns on this pathway really.

And also another key component is a cysteine-dependent protease called ATG4, which clips off the last five C-terminal amino acids of LC3, which is made as a proform. That exposes a glycine. That's the glycine that gets modified by phosphatidylethanolamine.

And so there are a lot of investigators that are very interested in targeting the kinase,

myself included. We got a grant on that. We're getting there. Also the cysteine-dependent protease is a very interesting target. There are actually four of these in the genome as well. But this turns out to be a targetable entity, this is quite a unique proteolytic reaction. And then if you want to get real brave, you could try to target an E1 enzyme like ATG7.

So there's a lot of activity in the pharma industry and in academia, going after these targets and others in the pathway.

So once LC3 becomes modified by phosphatidylethanolamine, it decorates these autophagosomes and along with other cargo that these garbage bags swallow, they're then delivered to the lysosome where it gets degraded. So that's how the pathway works.

Okay, so we reasoned that we should be able to really exquisitely disable this pathway by targeting ATG7. And so the title kind of tells you the disappointing results of the experiment. So we took Eμ myc mice that have a CD19 cre-driver, so cre-recombinase is expressed in stages B through FB cells, where the disease arises normally in these mice. The cross to a conditional knockout allele of ATG7 flox to generate ATG7 flox-flox Eμ myc CD19 cre-mice. These lymphomas lack ATG7, so we've confirmed that by western blots and by southern blots. The gene's gone, the protein's gone. And so what happens to disease? Absolutely nothing. So the post-doc that was working on this went to Eli Lilly. Chuck, are you out there? No.

So that was disappointing. But we haven't stopped there. And so because I'm trying to figure out why the heck isn't the autophagy doing something. So the conclusion from these experiments is that autophagy pathway is at least dispensable for myc-driven lymphomagenesis. But no worry, because the autophagy pathway is supposed to be important after tumors develop, when they're in their restrictive environment, when they're exposed to chemotherapy, etc.

So the next experiment we tried is, does ATG7 loss have an effect on the tumorigenic potential of myc-driven lymphoma? And the way we did this is, we got lymphoma out of an ATG7 flox-flox Eμ myc CD19 cre-mouse. So the lymphomas coming out of here lack ATG7. We harvested lymphoma and then we transduced those lymphoma cells with retroviruses that expressed either GFP or ATG7 and GFP. And then we transplanted them into recipient mice. And so the thought is that those that lack ATG7 will surely show less tumorigenic potential than those where we've restored the pathway.

And so we've harvested the lymphomas coming out of these mice and this is the result. So in the red are those tumors that lack ATG7. So this is done in immune-competent mice. This is done in cynegetic mice, ___ where these mice were derived. And so the tumors, lymphomas arise much faster than those that we've reconstituted ATG7. And the same is true in immune-deficient mice. Didn't really matter if you had a functional immune system or not. These tumors that lack the autophagy pathway actually have more robust tumorigenic potential in vivo. They also grow faster ex vivo. So you're getting rid of a major catabolic pathway in the cell and you're actually ramping up tumor cell metabolism. So that's one of the zingers for the talk I think.

Slide 135. Myc suppresses expression of components in the autophagy pathway in vivo

Okay, so why does this all happen? So it turns out that myc effectively suppresses the expression of components of the autophagy pathway. So what you're looking at here are real time PCR analyses of wild type versus Eμ myc B cells. The full change is the full change in Eμ myc versus wild type B cells, so these are cells before they become a tumor. As a control I showed you

that Arf protein was up before. Myc activates the transcription of Arf and Arf transcripts are up, we've shown previously that BCL2 and BCLX are repressed by myc. Those transcripts are down, so that's kind of a control.

Then here are the experimentals. This is kind of another zinger. Well, myc actually is shutting down the expression of components of this pathway, so here are the cast of about 15 components and enzymes in the pathway, almost all of them are suppressed in wild type–sorry, in Eμ myc B cells versus wild type B cells.

Okay. The autophagy pathway is intimately linked to lysosomal biogenesis. And this has come out from some recent science papers, indicating these two are cordantly regulated.

And so then we next asked well, if myc is suppressed in the autophagy pathway, are you also suppressing the pathway to the organelle that they're delivering their cargo, lysosomes? So there's a cast of about, I think, around 80 or so genes that are involved in lysosomal biogenesis.

And to get at this initially we used the same model that Chi was talking about at the beginning of the session, these P4936B lymphoma cell lines, where one has an inducible myc transgene that's TET repressible, one removes TET and looks at myc on the western blot, one sees robust levels of myc protein being produced.

And you can actually measure the numbers of functional lysosomes in cells, simply by FAP analysis, with a dye called LysoTracker®. And if one looks at conditions where myc is off, off versus myc is on, one can see that when myc is on, there's a shift to the left, so there's less lysosomes, okay? And this is after 24 hours.

And then if you look at the genes that myc regulates in these cells, and these are from existing databases on these cells, one sees that in no myc many of the genes are up, so yellow indicates up, blue indicates repressed, and as myc is going up most of these lysosomal biogenesis genes, this cast of 80 or so, are actually going down. So myc is suppressing the autophagy pathway. It also appears to be suppressing the lysosomal pathway.

These systems, one could argue, certainly reviewers will, are rather contrived. And so, is this really actually going on in vivo? So we harvested RNA from premalignant cells, this is from B220 cells assorted out of bone marrow, from Eμ myc mice and wild type mice, and looked at genes involved in lysosomal biogenesis. And again, I think I can quite easily convince you that genes involved in lysosomal biogenesis are expressed in wild type B cells and they're repressed when you're overexpressing myc in these B cells.

And it also happens in human beings with Burkitt lymphoma. So this is an array, it was done by the German group, I believe, and what you're looking at here, so Burkitt versus non-Burkitt B cell lymphoma. Burkitt obviously has myc involvement. Almost all of the genes involved in lysosomal biogenesis are off. You can see this other class of lymphomas is very much different. It's very high.

So wherever we've looked, this is the case, ___ of myc involvement have this kind of a signature.

Okay, can we actually show functionally? I've just shown you expression of genes that there is actually an effect in vivo. This is not an easy experiment because there's not a lot of great cargo that have been specifically shown to be associated with the autophagy pathway. But one of them is P62 Sequestrin. It's gobbled up by phagophores and it's delivered to lysosomes. And so if this pathway is inactive in Eμ myc B cells and in tumors that arise in these mice, you should see an accumulation of this protein. And that is in fact the case. So this is bone marrow B cells from

Eμ myc mice and wild type B cells, these are litter mates. You can see the P62 is up. And almost all the lymphomas have elevated P62 levels. So this indicates that the pathway actually is functionally inactive as well.

Okay, so how does this make sense? I think this makes sense because in this system and probably every other system, you're cordantly upregulating anabolic pathways and down-regulating catabolic pathways.

Just to give you a flavor of what the other side of the coin is, mitochondrial biogenesis. That's what this looks like. So mitochondrial biogenesis is, in general, low in wild type B cells and is, in general, high in pre-malignant Eμ myc B cells. And the opposite is true of Burkitt lymphoma. I mean the same is true in Burkitt lymphoma. So again, Burkitt, whereas high levels of myc show massive upregulation of genes involved in mitochondrial biogenesis, and it's rather more sporadic in other lymphoma subtypes, without clean or direct myc involvement.

And you can also measure this in vivo in cells from these mice by staining them with MitoTracker®. That's what this experiment shows. So this is wild type B cells and these are pre-malignant Eμ myc B cells and one can see a massive increase in MitoTracker® or CMXRos staining.

So again functionally and by gene expression arrays, I think what we've really got going on is a symphony, where myc is cordantly upregulating anabolic pathways and downregulating catabolic pathways, the autophagy and lysosomal pathways.

So one of the questions comes up, they're actually two linked, so is myc's ability to suppress lysosomal biogenesis linked to its ability to suppress the autophagy pathway? We actually have tools we can address this. So we have paired wild type and ATG7 null NEF and B cells, doesn't matter where you do this experiment. And we can transduce those cells with retroviruses that just express GFP or myc and GFP. And when one sees the blue line here, is when you have myc. One sees downregulation of LysoTracker® staining, in either scenario. So it's not absolutely dependent upon that, you can separate that. So how is myc actually affecting lysosomal biogenesis.

Slide 136. mTOR-TFEB circuit controls lysosome biogenesis

And so this is the next question. What is the mechanism by which this happens? So it actually has been very well worked out over the last few years by several groups with some very prominent papers, how this actually works.

mTOR actually phosphorylates a transcription factor called TFEB. And that phosphorylation is actually necessary to keep TFEB out of the nucleus. So in a nutrient-replete state, when mTOR is active, indicated by the green color, TFEB is phosphorylated and it's not getting into the nucleus where it can activate its lysosomal and autophagic gene transcription targets. When cells are starved, mTOR is off, red color, TFEB gets dephosphorylated, relocalizes to the nucleus and then activates these genes. Well, it's this question of putting two and two together. What is TFEB?

TFEB is a B HLH-Zip transcription factor. In fact, it's a member of the same super family. It also regulates target genes by binding to E-boxes CA*NN*TG. And so the lysosomal genes that it activates have these E-boxes. So the question then becomes, does myc actually antagonize TFEB and is this important for lymphomagenesis.

Okay, so first question, this could happen–if myc is going to antagonize TFEB it could happen at several levels. Maybe TFEB is a myc transcription target that's repressed by myc. And so this is the same kind of datasets I showed you before. There's Burkitt lymphoma, this is expression of myc, this is non-Burkitt, there's TFEB, it's off in these tumors. Okay? Not so much off in these other tumors, where you're seeing these lysosomal biogenesis genes. So TFEB strictly correlates in Burkitt lymphoma at least, with expression of these genes, and it's repressed where myc is on. So one arena that we're following up is, is myc actually directly or indirectly blocking TFEB transcription. And to our mind it's indirect. So you can activate myc in this P4936 system that both I and Chi talked about, TFEB transcripts go down, but it takes quite a while. Doesn't look like a direct transcription target. But nonetheless, TFEB transcription can be shut off and yes, it's probably relevant for human malignancies.

What about competition? I told you that the two transcription factors bind to E-boxes. And this is data from the Encore database and on the left we slapped on some expression analysis of TFEB gene targets from our Eμ myc system. Wild type B cells, Eμ myc B cells and lymphomas that arise in mice. This is one target, it goes down a little bit in this system. Myc actually chips quite well to this promoter regulatory gene–promoter regulatory region that has this E-box for this gene. Here's another one. This was more impressive, I think. It's repressed in myc-expressing B cells. Myc actually chips dead on, where TFEB would chip, at these E-boxes. And here's yet other one, GNS, also robustly suppressed. The chip data aren't quite as convincing, but you can see that myc is there at the E-box where the promoter is.

So we've done a little bit more of this. So here are two other targets that are activated by TFEB. They're definitely repressed Eμ myc B cells and even more so in Eμ myc lymphomas. That's the amplification that one talks about when one talks about myc regulating gene expression I think.

And if you do chip analyses, and on the same cell system that Chi and I have been talking about, and when myc is on, myc actually chips to several of these targets, that it represses.

So myc actually can compete with TFEB to block transcription of some lysosomal gene targets. So that's mechanism number two.

How else can this happen? It also can happen by TOR. So in the same cell system that both Chi and I have been talking about, if you activate myc and you look for hallmarks of TOR activation, you see them. So there's phosphorylation of P serines, 2448 mTOR, P70S6K, P85S6K, get phosphorylated when you activate myc. This is actually TOR driven because this is one of those specific inhibitors was talked about, torem, that blocks these effects, and one also sees phosphorylation of 4ABP.

The next data are not so good, but the antibodies against TFEB aren't so good either. So one idea is that I told you before that TOR regulates TFEB nuclear localization, cytoplasmic nuclear localization. So the idea would be that if you overexpress myc, most of TFEB should actually be in the cytosol, not in the nucleus. And that is the case. So lane two, when you're activating myc and you separate cytoplasmic and nuclear fraction as monitored by KREB and tubulin, one can see that most of the TFEB that's there in myc-expressing cells is in the cytoplasm, it's not in the nucleus. So that's the third mechanism by which this can happen.

Okay, who really cares? Is there a functional consequence of this, right? So is suppression of TFEB function or expression or is competing with TFEB important at all? And so we tested this doing lymphoma transplant studies. And the first thing we did is, we took these wild type

lymphomas and infected them with the GFP virus and you let them grow. With time there's really no selection for loss of growth. All the cells are GFP positive. In the red you're looking at cells that were analyzed after two days in culture after infection with the TFEB GFP virus, so most of them are still GFP positive, but a lot of them are not. Much more so than the GFP control. By day six, you can see there's a huge shift. This is an antiproliferative effect, it's not an apoptotic effect. You're shutting down these cells and you're selecting against cells that are expressing TFEB.

So TFEB selects against the growth of Eμ myc lymphoma cells ex vivo. What about in vivo? So we did the same kind of experiment. We took lymphoma from an Eμ myc mouse, we harvested the lymphoma, we transduced that with a virus that just expresses GFP or TFEB and GFP. And we took these unsorted cells, so they have a mixture of uninfected, so GFP negative and GFP positive cells. And then we transplant them into mice. And then we analyze the percent GFP, the percent GFP positive tumors in the peripheral blood, the spleen and lymphoma. So if it's an advantage, you'll have more GFP. If it's a disadvantage it'll be selected against.

So you can see when we analyzed peripheral blood, there was seen a reduction in the percent GFP and the TFEB GFP cohort. So GFP is selected against those cells in peripheral blood, in the spleen and in the tumors.

Okay, so the bottom line is that TFEB actually has tumor-suppressive functions in the context of myc-driven lymphoma. But it's not really simple tumor suppressive functions. It's probably just mucking up the metabolic state of the cell. The cell is highly anabolic, now you've restored a catabolic pathway, and this equals metabolic catastrophe. This is a hypothesis at this point. I don't have any solid data to say yes, I'm right. But I thought it's compelling enough and it certainly dramatically fits with this session.

So we would argue that a myc TFEB circuit is pretty important for controlling lymphomagenesis. We think myc can actually, may affect TFEB transcription of TFEB. And then that blocks lysosomal biogenesis, is repressing it. We think that myc can actually also compete with TFEB, so for some of its targets, certainly not all of them, but some of them. And there's also the mysterious pathway, myc is activating TOR somehow. I don't think anybody in this audience really knows how that happens. And this also affects lysosomal biogenesis. And I've shown you that that's probably pretty important for lymphomagenesis.

So I think the end message is that myc-driven cancer is certainly a symphony. It's coordinating anabolic pathways and catabolic pathways in opposite fashions to really ramp up the anabolic state of the cell. Is this also going to be the case in other malignancies driven by other oncogenes? I doubt it. Because I don't think most other oncogenes have effects on the anabolic state that myc does. So I don't think those kind of catabolic-anabolic conversions would really happen. In fact, the data indicate that a lot of Ras-driven malignancies, this is Eileen White's data, are actually quite dependent upon the autophagy pathway.

Slide 137. Dr. Cleveland - Acknowledgements

So the work I've shown today, most of it was by Franz Schaub with help from Stepanie Demetriou, a talented MD/PhD student, and a terrific technician, Weimin Li. The autophagy work was initiated by Frank Dorsey and Meredith Steevos, gone on to greener pastures in some sort at Eli Lilly. And we got help from Stephanie Prater. And we've all got help from Chunying Yang.

Audience: So with your autophagy inhibitor, hydroxy chloroquine for example, if you want to envision to combine it with conventional cytotoxic chemotherapy, where do you think that would be the best, up front, combining it, or as maintenance therapy?

Dr. John Cleveland: I wish I knew the answer to that. I honestly don't know. With results like this, I think all bets are off. Let's say we have a real specific kinase inhibitor for ULK1, which everybody's after. Will we really get the therapeutic response that we suspect? I think what you're going to have to do is, you're going to have to tailor it to the kind of malignancy where you've shown a clear dependence. And in this kind of a tumor it would be a bad idea. Because actually what you're driving is the metabolic state of the cell, it's going to make the patient worse, you're going to end up in court. So you don't want to do it for that. But in terms of what kind of other regimens that you could envision, I would start at the old standard ones. Do it intermittent with radiation, something like that. I'm not really convinced that–one would think that mTOR, PI3K inhibitors in combination with a ULK1 inhibitor would be the perfect storm. We've actually treated these mice with torem and with BEZ235 and both cohorts, they actually behaved the same. So it's working through other things and torem has other targets. So I don't really know. I think it's going to take a lot of careful work and when we roll these things out we'd better be darn sure that we're tailoring it, so they don't just die on the vine. I think they're going to be very important for some kinds of malignancies, but we'd better be careful.

Audience: That was really very provocative. Just wondering where you place the necroptosis pathway in the overall scheme of autophagy ...

Dr. John Cleveland: So we've done the experiments. I'm not sure if you've done this. So we crossed cyclophylen D knockout, which is supposed to be a key regulator of necrosis. At least that level. I mean it's really important in cardiac ischemia. And we're not seeing anything interesting whatsoever. So that's where I place that. But I have to say that if we didn't force the envelope, we wouldn't have made this interesting either. So just takes more experiments, that's all.

Dr. Chi Dang: John, perhaps you can address the question that I didn't address about metformin in terms of what's thought to be its mechanism and–because you have some experimental data to suggest ...

Dr. John Cleveland: Yeah, I didn't give that talk. The metformin, I guess it was, so the metformin synergism is because it's going to force cells towards–down a glycolytic path. And so if you feed cancer cells, you know, inhibitors that specifically hit the glycolytic pathway, for example, they're going to be acutely sensitized to metformin. So we've shown this with some in-house agents that block MCT, inhibit, block MCT1, a function that is a lactate transport inhibitor. But conceptually there are many points of intervention in the glycolytic pathway that metformin should show a benefit when added together. Including inhibitors you've got.

Audience: Actually I have two questions, not one. Knowing that hydroxy chloroquine, metformin and with some __ clarithromycin as well, helps inhibition of autophagy. Can we tailor the drug to be given in combination with chemotherapy according to the comorbidity the patient has? For

instance, for diabetics we might push in the direction of adding metformin for others who are having a comorbidity preventing one of these three or others, we might know later on. This might urge us to tailor or it will dictate what to use for this particular patient. This is my first question. And my second question is a sort of mental exercise. We know that the function of autophagy with malignancy is a bell-shaped. Why do we focus on inhibition of autophagy? Why we don't push more hardly to the other side of the pendulum, enhancing very much autophagy, pushing the cell hardly for self suicide.

Dr. John Cleveland: I'm going to handle the second one first because I'm not sure if I got the first one. The second one is, so let's just think about that. So let's push the autophagy pathway and give the cancer cell more building blocks to do its nasty job. So that's one way you could answer that, right? Because the pathway is there as a recycling center. So if I throw in an antagonist to the pathway and I activate it, in the face of a tumor cell that is able to get its nutrients, I think this is not a good idea. And so I'm not sure–I don't really buy into that, I really don't. It'd have to be a perfect storm. And speaking from personal experience, cancer cells find a way. They will quickly capture that one I think.

Audience: Because would it be a recycling process, it will be a suicide process later to really eat itself, I mean the malignant cell. It's just a mental exercise.

Dr. John Cleveland: Okay. And your first question was again what? Sorry.

Audience: To tailor or to choose according to the comorbidity, which agent to give.
Dr. John Cleveland: Yeah, I agree with you, but that's for the docs in the audience, right? I'm just a PhD, just discovering stuff and saying hey, we should look at this. Like discovering that chloroquine was actually doing something, it's exciting to see that in the clinic and I hope it all goes well, but I don't think we really know how chloroquine works. I know there are better derivatives out there and I know depending upon how you modify the structure of this molecule, you can find activators of the autophagy pathway and inhibitors of the autophagy pathway. So there's a lot of work to be done.

Audience: Do you think it could be, rather than comorbidity, disease-specific, for instance?

Dr. John Cleveland: Yeah, it should be, for sure.

Dr. Chi Dang: Maybe I can reflect on that very briefly. So we, on a totally separate effort to try to look at metabolic therapies for pancreatic cancer, John referred to the fact that autophagy is a big deal there. So we thought that perhaps by using metformin, which will stimulate autophagy and we can combine it with hydroxy chloroquine, which then could have a synergistic effect. And so we embarked on very large xenograph studies and found that about 30% of animals treated with a combination actually died from the therapy. And we have done this now back in the host animals without tumors. So I think that when we combine these things, depending on the host, that actually even though single agents may not be toxic, the combination could be quite toxic on normal tissues, so I think that's just a cautionary note. Although there are anecdotal reports

because we were doing this, that there are physicians actually giving some patients this combination and some are doing well.

Dr. John Cleveland: Yeah, you can get it from the pharmacy, right? Plaquenil and metformin, hello. It's everywhere.

Audience: I have a question. So Burkitt's lymphoma is known for the high apoptotic rate. Do you think that's due to the inhibition of the autophagy?

Dr. John Cleveland: Possibly, yeah. I don't think so, but it's possible, sure.

Audience: So when you messed around with the autophagy, did you ever see the apoptosis?

Dr. John Cleveland: Nope, nope. No change. No change in proliferation, well, the proliferative rate goes up, right. It's just the opposite of what we were hoping for. They grow faster.

Audience: Yeah, related to that question, since you see the delay in the onset of the lymphoma after you knock off ABT7, I wonder whether you have checked any difference in the frequence of transformation under the given transduction of your ABT7 knockout is there? Or you get higher proliferative rate of the given __? Do you see any difference in them?

Dr. John Cleveland: I don't have the answer for that. We haven't done it in that kind of a detail. So you're asking how many tumors are arising per mouse?

Audience: [Inaudible] ... susceptibility to the leukemia genesis by giving rise to the higher frequency of transformation.

Dr. John Cleveland: Oh, no, we haven't done that experiment.

Dr. Chi Dang: So John, I actually have a question to follow up on that. So we think of autophagy as the general macro-autophagy, but certainly the same mechanism is also used for mitophagy.

Dr. John Cleveland: Sure.

Dr. Chi Dang: So have you looked at mitochondrial content when you manipulate the autophagy pathway?

Dr. John Cleveland: No, we haven't done it yet.

END