Lecture 11:

Using functional proteomics to identify biomarkers and therapeutic targets-II

Welcome to MU course, applications of interactomics, using genomics and proteomics technologies. Dr.joshua La Baer has taught you various, good concepts about, planning for experiments for biomarker discovery. How, a very novel protein microarray platform, like nucleic acid programmable protein array or NAPPA, can be utilized for many applications. today his, giving his last lecture, pair is going to talk to you about a case study ,in which how NAPPA technology, could be utilized for, the functional studies, their various type of modifications ,post translational modification happens, which makes protein functional which gives them different properties and which are very crucial to study ,however in studying Petrium's are not very straightforward ,not very easy, there a variety of modification happens, as you are aware, like phosphorylation, glycosylation, acetylating and there are some newer forms, like addition of a MP, a MP isolation etc. all of those are very crucial, for understanding, a given cellular context , supposedly it will also summarize, all the various studies, which have been covered during his section, especially for the NAPPA technology, as well as, the biomarker, discovery program and other clinical applications, let us listen Dr. Joshua LaBaer, lecture. Okay?, since it's the last lecture of the series, is focus a little bit more on ,sort of functional studies, that we've been doing with the NAPPA, with the fairly heavy n system, system, on our most recent story. But, I just thought it would be useful to wreck, you know, one of the things that I keep saying is that the, the proteins on the array, are active and I think probably one of the best bits of evidence for that, is when we test function, of these proteins. Protein, protein interaction, enzyme substrate activity that sort of thing, we, we usually get it. And so that's kind of how we look at it, so the first, one of the first stories, that we looked at was, ambulation.

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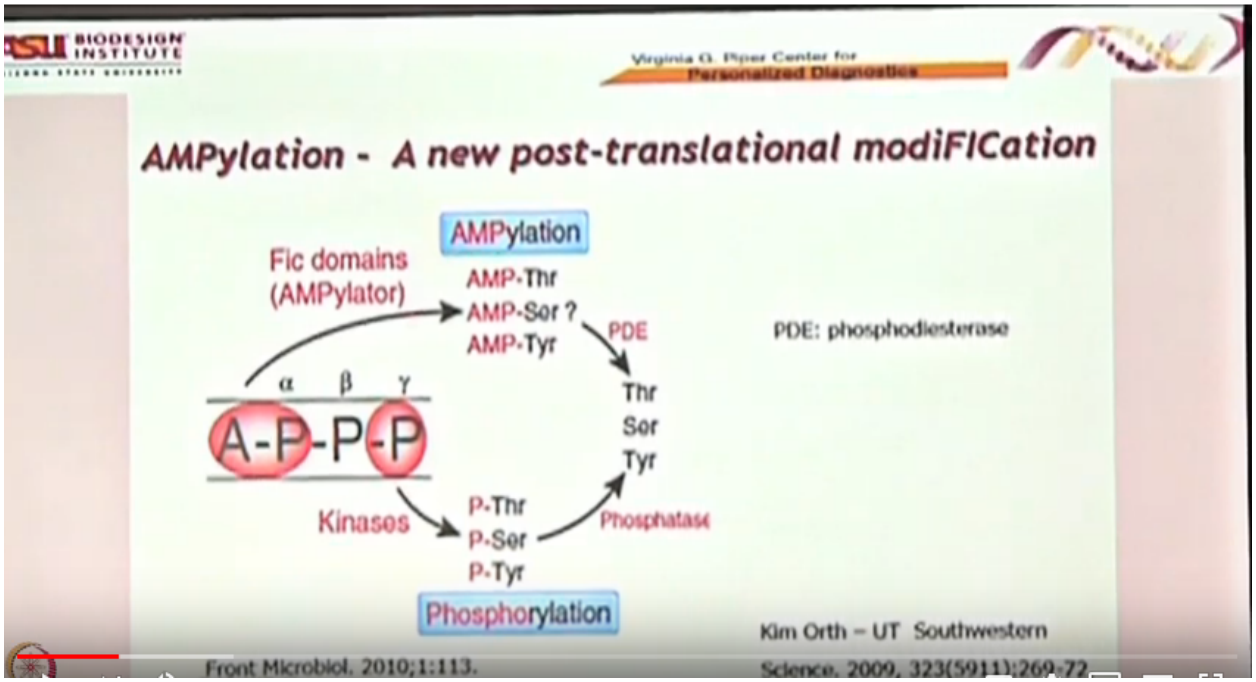

So, you're all familiar with this part of the pathway, where you have ATP and the gamma phosphate on ATP is added to, threonine serine or tyrosine Zhan proteins, in a process that we call ‘phosphorylation’. And that is usually catalyzed by an enzyme called a,’ Kinase’. Right? So, you're all, you've seen that a million times by now. So, it turns out, that in some circumstances, a slightly different but very related reaction occurs, in which the AMP, the adenosine plus the first phosphate get added to a protein. So, you get a mp3, and AMP serine maybe and a AMP tyrosine and that's called, ‘AMPylatio’. So, you're taking the opposite half of the molecule and you're adding it to proteins and it turns out that this process is remarkably well conserved, if you look through evolution. So, if you look at many, many bacteria and even in eukaryotic cells, there are classes of enzymes called, ‘Ample Eaters’ that will that will do this reaction and we don't fully understand, what the biology this interaction is? But, one of the places where we see it the most often is when, bacteria infect an individual and, and then the bacteria use this, to modify host proteins. So, it's possible that pathogens have used this as a way to regulate expression in cells.

So, the, the challenge with AMPylation, is that we don't really know, what the targets are? It you know? For years people have been trying to study, what are the targets that are being modified by these enzymes? And when we began, this work there was probably one bona fide target that we really knew about, there were two or three others that had been proposed but, not verified and the methodologies that people had used they had tried you know? Doing pull down experiments, they had tried doing mass spectrometry experiments; they had done various chemical linkage experiments. But, it was very hard to figure out what the targets were, so we had , we had a very ambitious, postbox in the lab Sabo you and he wanted to, see if he could use the protein array, as a way of discovering what were the targets of these enzymes, so the approach that he took to do this, was ,the idea would be you, you print you imprinted array you express the proteins and you treat it with, with an ample later, that will add the amp group to the proteins. And then you come back later and try to determine, which proteins have the amp group on it. so the initial approach that people thought would work would be, you do this method ,you have the amp group in you come in with an antibody, that recognizes the, the AM P here. But, it turns out that the antibody, didn't work well, it was really not very selective and it didn't, didn't pick up what we wanted and so he came up with, a different strategy that was very creative, so the strategy, he used is based on, click chemistry .Basically you take an alkyne group and an azide group, these are two chemical groups. And they're reactive species but, but they're very selectively reactive species, and in the presence of copper, they will, they will, they will form a chemical reaction, that creates a covalent linkage. And it requires copper for activity but it's very selective. So if you, if you run a, if you have, the azide group on one protein, in a cell lysate and the alkyne group on the other protein, even amongst millions of other proteins. Only those two will linked and nothing else will, so it's very selective.

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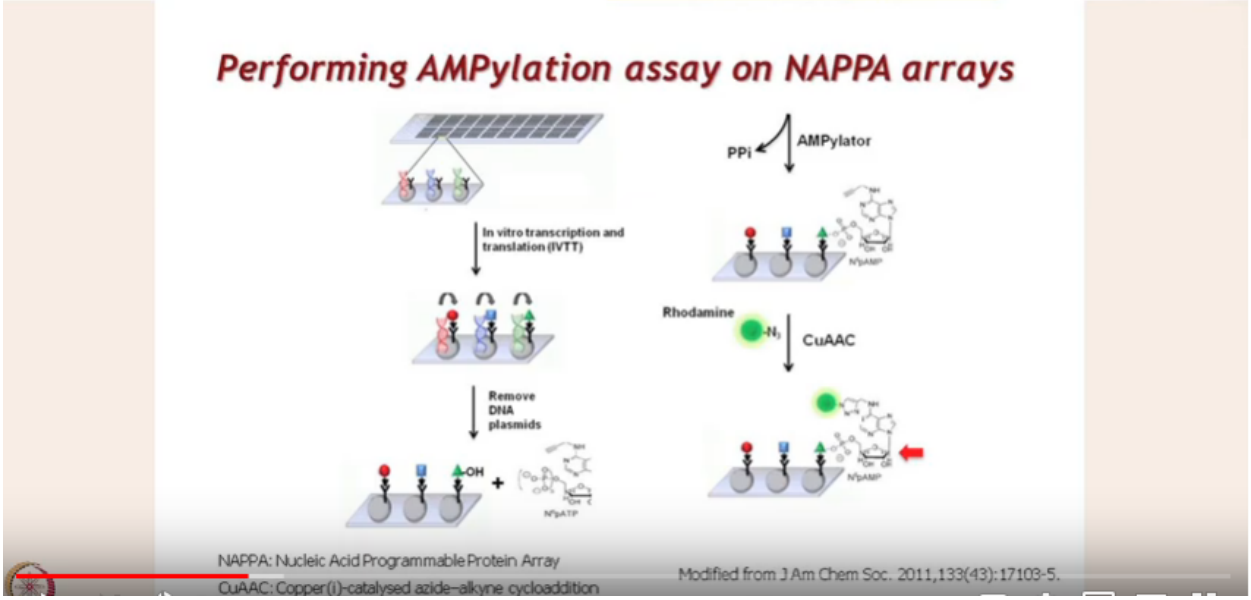

So, so what do you, what he did? Was he worked with a group, Howard Wang in New York? Who had made a, an, a modified version of ATP, in which he'd put this alkyne group right here, that's the alkyne group. On an 8, on an ATP. That was linked to the sugar so it was part of the ANP molecule. Ok? and ,and so ,the idea then would be you take a protein array ,you translate the proteins, you remove it turns out that one of the things that, that Shabbo discovered was that to get this to work, he had to remove all the DNA from the array. so you all realize that we print plasmids on the array to make the proteins, once the proteins are made, you don't really need the DNA anymore .and for a variety of reasons at times if you need to you can digest away the DNA with DNA and you still have your proteins left on the array. so that's what he did, and then he added this, this alkyne modified, ATP, along with an ample after ,that released a pyrophosphate and it added this, this am this, modified AMP, to whatever target proteins were there. and now, it's displaying this open alkyne group, he then came in with an azide linked to, rhodamine which is a fluorescent marker, added that to the array and added copper and that added the this it added it caused the, the covalent linkage and displayed the, the fluorescent tag .so essentially he was marking, the modified proteins with his azide and then he came in with rhodamine, with alkyne and they came with rhodamine linked azide, to find the proteins. so then only proteins, that our targets of the enzyme will light up.

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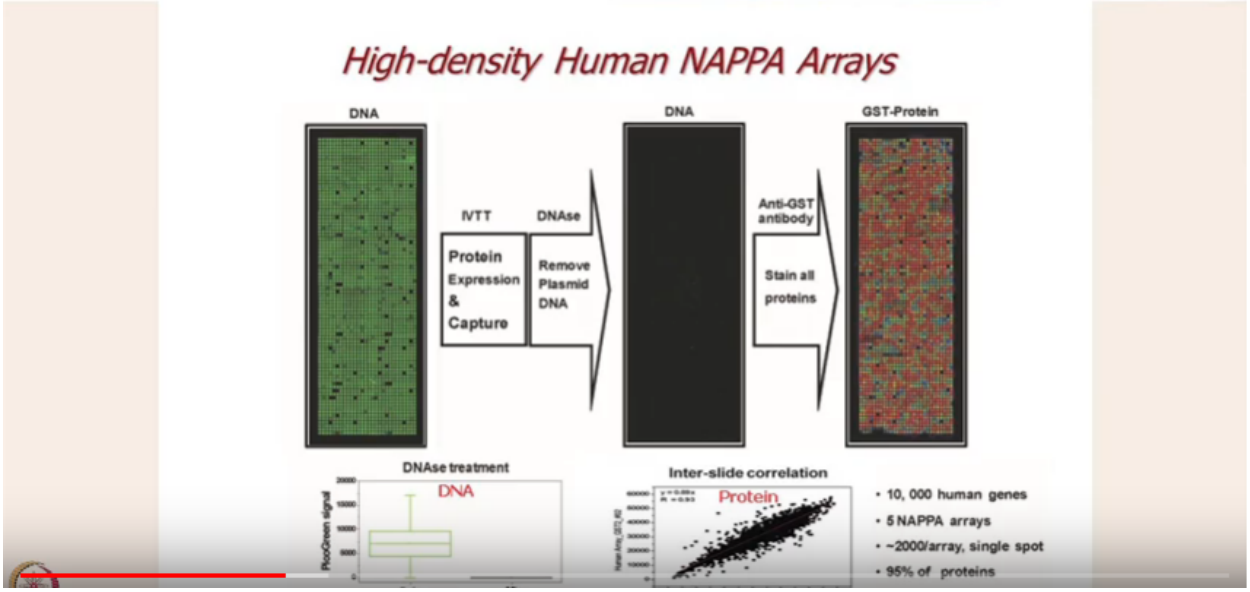

First thing he did ,was to make protein arrays ,here he shows you that he has DNA, remember we stain with a Pico green to look for DNA, that confirms to be a good printing, then he expressed the protein and captured .it and then he digested all the DNA, using, DNA's. And so if he stains her DNA again it's completely gone. Okay?? and then, and this is just showing you, here's the DNA level before and DNA level after treatment, then he tested with anti GST antibody and showed that he still had all the proteins. So this is kind of useful to know, in some circumstances, when you're going to be working with a protein array like NAPPA, if you don't want the DNA around, let's say you're doing a transcription factor study or something like that, you can digest the DNA away and you're still left with the proteins. And it's still a perfectly good protein array, all right. And then this just shows that when he did, two different array studies, he got very reproducible results .Okay? So now, he's got, now he's got this whole protein array, displaying protein, no nucleic acid, no DNA on the chip and he wants to then treat that array with an ample later, plus this, this alkyne modified ATP. Okay?

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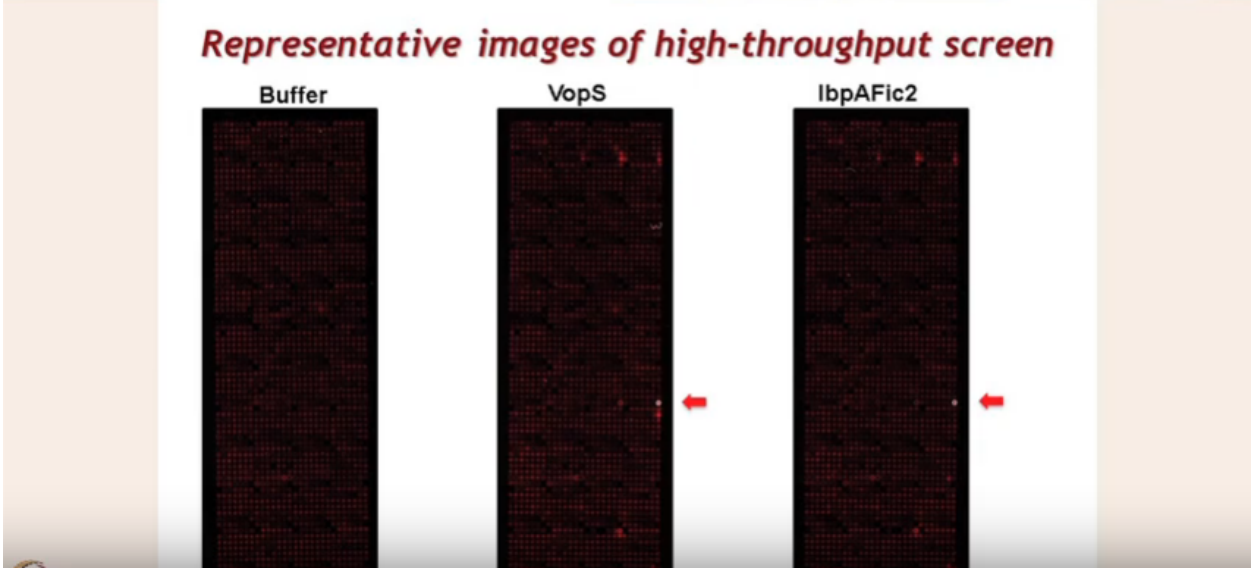

So here is, the array, if he treats it with modified alkyne ATP and buffer alone. So you don't see anything, that's good. Right? you know, if there's no alcohol no AMPA later there, you don't want to see a signal, if you did then that would mean that you had contamination, vopS is a well known ample eater and then I BPA Fick 2, is also a well known ample eater, when he treats with those guys, can you see that, all of a sudden a few, not very many but a few spots, like these guys right here, start lighting up. That's kind of the result that you're hoping for, right. When you're a researcher in the lab, when you're a graduate student the lab and you see only a few spots, light, that's what really gets you excited. Because, if everybody lit up, then you know, if there was a lot of background and it probably didn't mean anything, but if only a few selective ones light up, that's a sign that you really found something.

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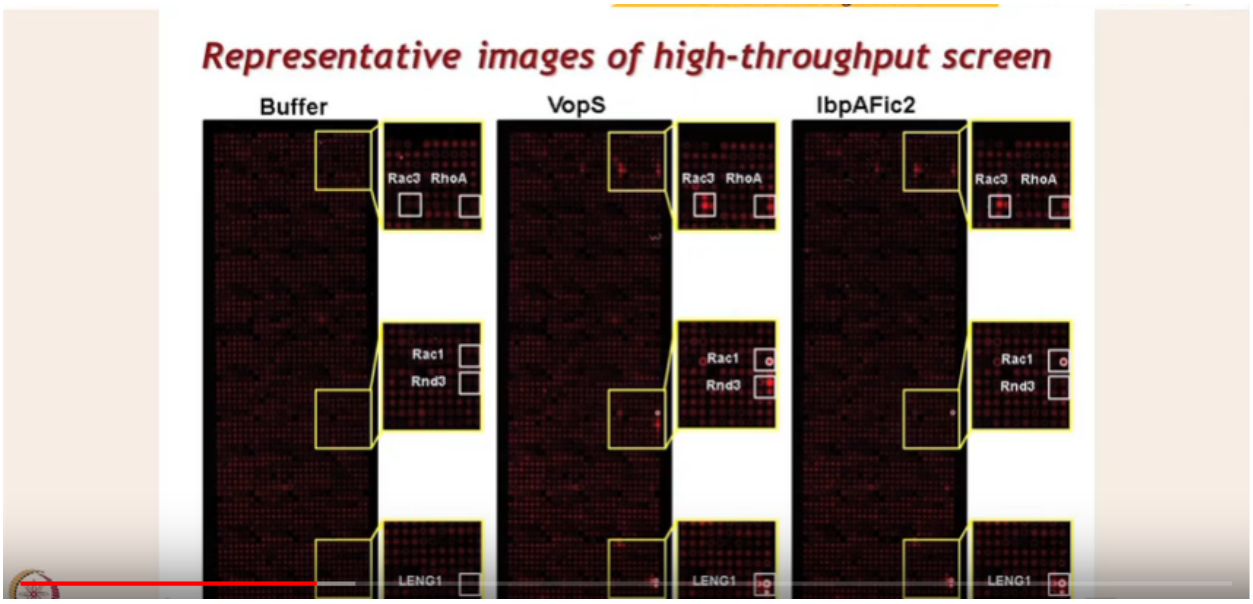

So this is what, with those spots like and of course these are the identities of those spots, you can see that they have very clean signals. Right? And of course you don't see those signals over here, on the control. Right?

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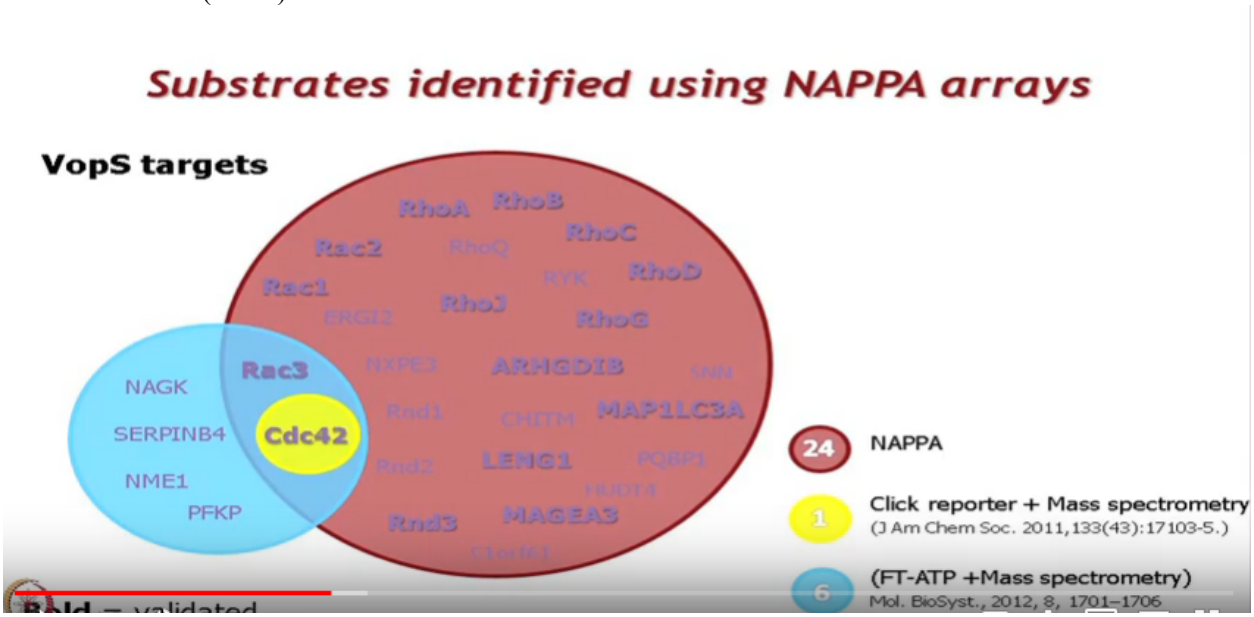

So, by the time he was done with these experiments and I'm not gonna walk, you through all the studies he did of these experiments, he ,he this is what was known before he started, that was the only known target and all of these targets in here, were things that he uncovered ,by screening the arrays. so he found a couple dozen, more new targets for this, when he actually looked at ,the targets and I don't think I have the, the slide to show you, for that work but, he actually found that there was a sequence motif, that was common to all the targets, or at least most of the targets. And so he was able to identify, what it was that the AMPA leaders? Were looking for when they modified proteins. And a lot of the targets of these proteins turned out to be gtp ace proteins. So that's one example, of how you can use the array, to study for enzyme substrate type interactions.

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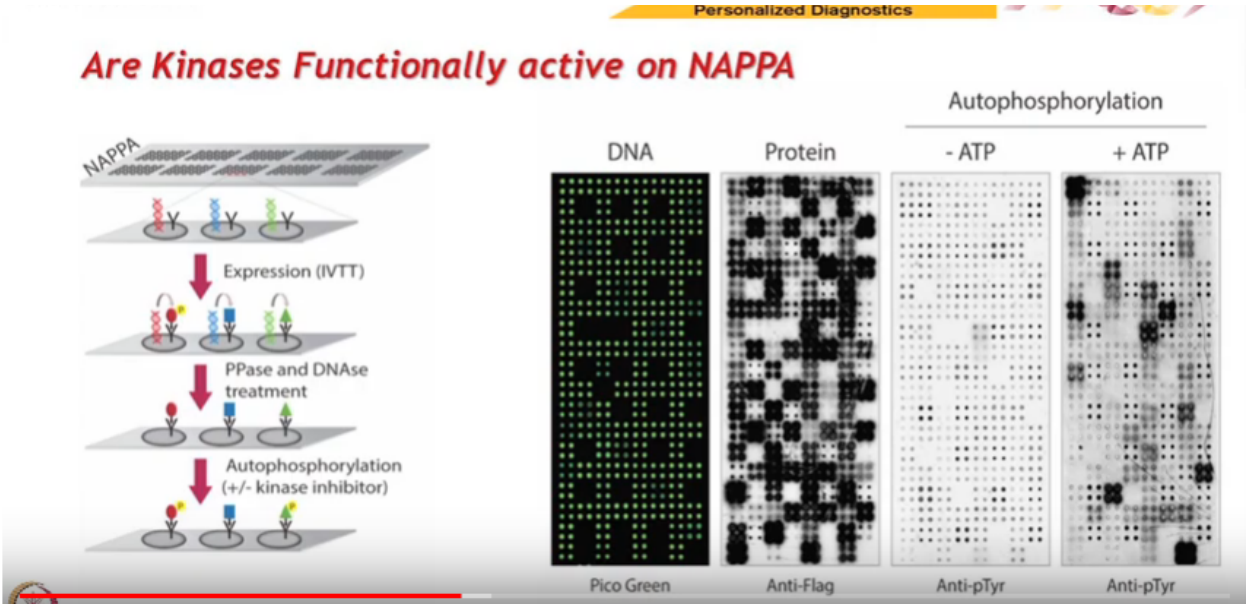

Another, another assay and I'm only going to show, one slide from this. Because, it's still an early study, his work that G Cho in the lab has been doing and again I don't know hopefully you can see the dark spot, there the dark spot, there the dark spot, there. he basically was looking for, proteins on the array that Otto was set alight. and so in this case he was using an a settled group,that ,was labeled and then he was using a settle group that, could be detected by an antibody and then he treated the array with, with acetyl co a right there, incubated the array and just allowed the proteins, to settle ate themselves, washed away all the reactants and then stained with the antibody and identified proteins ,that Ottowa settle a. so this is again a way to look for enzymatic activity, on the array .Okay? so now, I'm gonna switch gears and talk about yet a third application and I'm gonna spend a little bit more time on this one, because I want to kind of walk you, through what I think you, want to do, when you do these sorts of studies. Because, one of them is I see all the time ,as a journal editor, you know and I can't tell you how often, I see this, pretty much every day I'm rejecting at least two papers for this, is people do, one one screen, with a proteomics technology, maybe it's a mass spec screen ,maybe it's a protein or a screen, they get the results and then they write it up, or they get the results they do some informatics and then they write it up. And they don't follow up on any hypothesis, they don't do any subsequent biology, they just simply say here was my screen, here's what I got, you know enjoy it. and you know for me that's not really what scientists should be doing, they should be using this tool to identify a hypothesis and then doing some kind of work to test that hypothesis, you don't have to follow up every lead but, you should follow up at least one or two so that by the end of your story, you've shown something new, that you didn't know before, cuz that's really the goal of science. and so we you know we usually send those papers back to the office and say this is good start, to preliminary, go back and solve a problem and come back just when you have a bigger story. Okay?? So, so this is um, this is a, an app array and what we're going to do here is we're going to express proteins on the array. so first here you see the array stained with pico green, which by now you all know means, it's, it's the amount of DNA and the fact that it's pretty even in its staining means, that we did a pretty good job of printing. and then here, what we've done, is expressed the proteins and then, and then stain them with an anti flag antibody and the reason it's a flag antibody, is in this particular circumstance ,these proteins which are all kinase dash is, happen to have the flag tag and not the GST tag. and it's just a good point to remind you that, we are not wedded to any one tag we've, we've done Napa with Mick tag, with

flag tag, with GST tag ,with halo tag It . It’s a, it's a technology that can be used, a variety of different ways, in this case we happen to have all the human kinase a--’s, in the flag tag, so we use the flag tag. And this gives you a sense, that the proteins are well expressed where they should be, right. Okay?? so now, the question we wanted to ask was, are these proteins phosphorylated .so we, we took this array and if we don't treat it with, if we treat it with, buffer and just but no ATP .and you stain it with an anti phosphotyrosine antibody, none of the proteins light up. so that means, that after, if you strip the kinase ,if you strip the proteins, with phosphates’, to remove the phosphates. and stain with Auto phosphotyrosine antibody’ you won't see any the proteins, won't have phosphates that no surprise ,the question was ,were they where these proteins active and so if we added back ATP to the array and just incubate the array with ATP. Now, some of the proteins are lighting up, all of these proteins are Auto phosphorylation, right. Because, just by adding ATP, to the protein on the array, there, they’re fostering themselves on tyrosine. Okay? So that, that's really good evidence, that all these proteins are enzymatic ally active, on the surface of the trip. Okay? And it was evidence to us, that we had the possibility at least, of exploring now the function of these proteins in the array setting .so one question, that comes to mind is can you inhibit this activity, using drugs.

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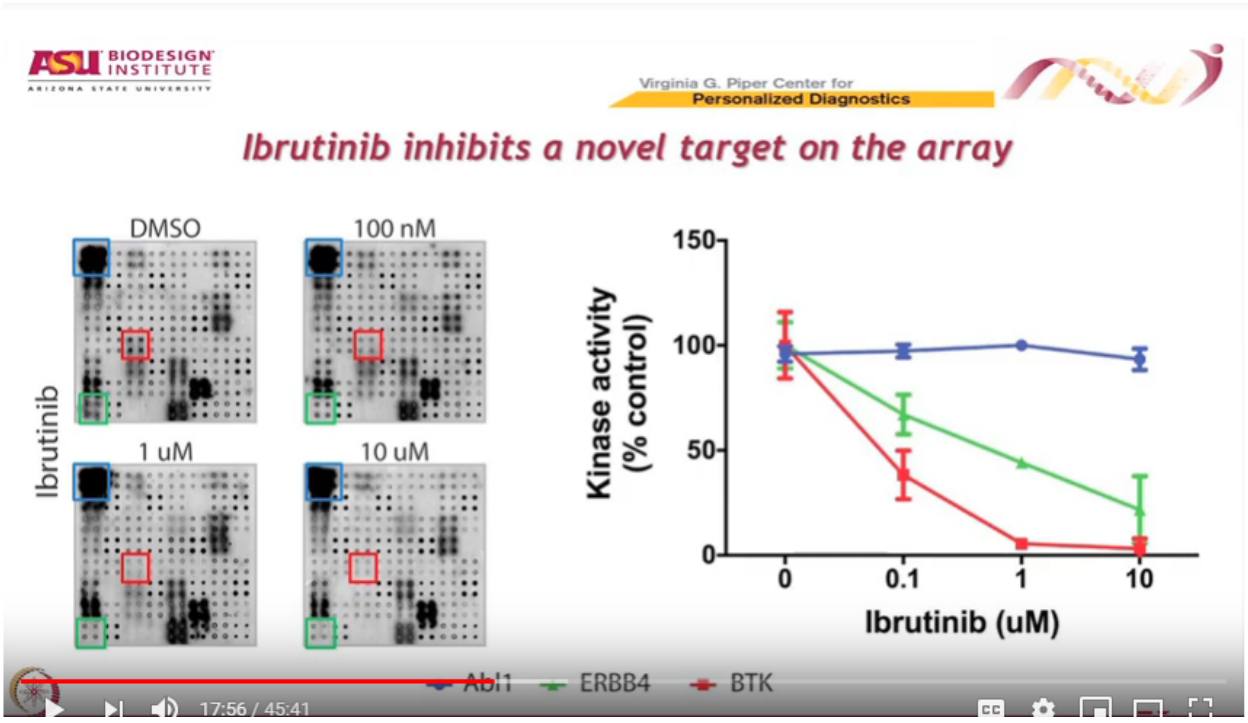

So, the first experiment, that, that Fernanda did, this was my, there was a postbox in the lab at the time. where she took a broad spectrum kinase inhibitor called,’ Stautosporine ’. And Stautosporine inhibits most kindnesses. And she increased the dose of Stautosporine, on the array and so here's no ATP, here's full ATP. And then here's increasing amounts of Stautosporine and as you can see, the kinase activity is headed is, decreasing due to the drug. It’s not completely, wiped out but it's significantly inhibited. So,

that, means that, the enzymes are behaving as we expect them to, a more interesting question is, can you selectively inhibit kinases. So, can you use a kinase inhibitor that knocks out one kinase, but not another kinase and will it also behave on the array right and that's this experiment here. So, many of you are familiar with this drug imatinib, the mat nib is the same thing as Gleevec, Gleevec is the was the first selective drug inhibitor, chemical inhibitor ever used to treat a targeted pathway in cancer. So, I mentioned the other day that Herceptin, was the first targeted pathway that was an antibody, this was the first compound this is Brian Drucker's work, he in you know essentially invented this molecule that selectively knocks out the BCR Abel protein the people, with a type of CML get this trans located enzyme that links the BCR gene, to the, the able kinase and it activates a kinase and it becomes an oncogene, that turns on that creates cancer and using, imatinib you can put people into remission in fact, there are long term survivors, now with that disease who've been treated with imatinib and who have never gotten their cancer back. So, it's, it's a pretty promising compound all right.

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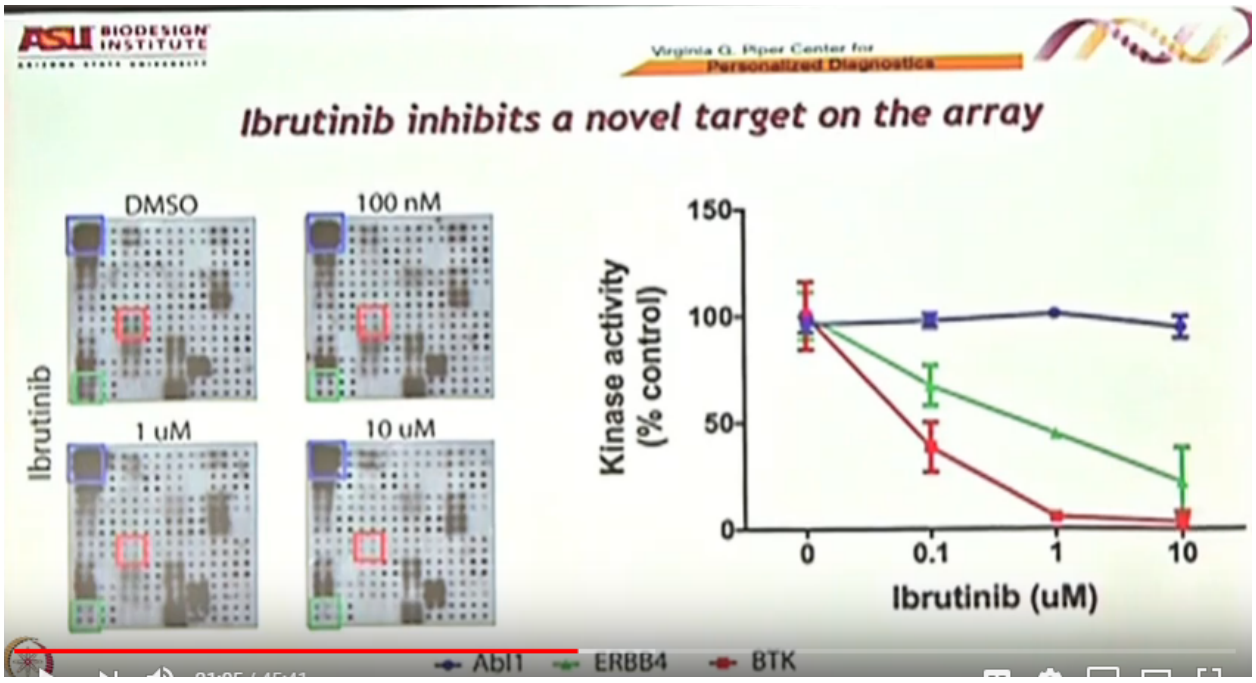

So, what I want you to look at first is this protein in their green circle, I'm Green Square and you can see that, this is the t NK 2 kinase and notice that no matter how much drug we add it's still active. So, the drug is not inhibiting TN K2, but if, you look at BCR Abel here, it is here now it goes down a little bit now it's much down and now it's completely down. So, the drug is selectively knocking down BCR Abel but it's not knocking down t + K2, similarly if you look at Abel which is right down here, you can see that the Abel is also decreased. Okay? So, so on the array these proteins are behaving exactly like you want them to so, we did a number of studies like this to convince ourselves, that the array platform was behaving as we expected and then we then decided, now can we discover something new with that. So,

we started treating the array with other, kinase inhibitors and what we looked, for in particular was were these kind of inhibitor ever hitting a kinase that we didn't expect them to hit. And one of the first ones that fernanda found was this one. So, a Brut nib is a drug that's used to treat, an enzyme called,' BTK' or Burton's tyrosine kinase BTK is an important enzyme kinase, in the B cell pathway and it plays a role in a lot of B cell cancers. So, mantle cell lymphoma for example relies, on BTK and a Bruton, has turned out to be a very useful drug in treating those patients, it inhibits the BTK, it essentially stops the growth, of that tumor and it's well tolerated by patients, not a lot of side effects. So, we asked you know does the Brut inhibit, inhibit anything else. So, the first thing I'm going to point out to you is that and that is able one, able one was the example, in the last slide and you saw that able one was inhibited, by imatinib but able one is not inhibited by, by a Bruton it you can see that the signal is the same in all four spots. Okay? So, then we asked well is it working for BTK which is the one it's supposed to work, for and that's in red and sure enough there's BTK, it's going down it's going down even more, it's going down even more. So, in this case even though it's not affecting this, this kinase it is affecting that kinase and then what Fernanda noticed by carefully reviewing these slides, was this guy down here, strong signal over here weaker signal here, weaker signal there and weak else it weaker still over here. And lo and behold that protein turned out to be herb db4, that was pretty exciting because, if you think about it the herb family right so EGFR receptor or b2 her2/neu those, those are two of the most prominently, known oncogenes in all of cancer, studies right there are very, very successful drugs against both EGFR and an herb eb-2 and now we'd found a drug against Irby b4 and there wasn't a lot of data on Irby b4. And so, that's why we decided to kind of pursue this story a little bit. So, the first question, we wanted to ask was, could, could this drug inhibits cell growth. So, the first thing we had to look for was cells that had early before in it. Okay? Now, the next thing we thought about was what about artifact, what potential confounders, could screw us up. So what else do we know about BTK is a drug, what does it normally inhibit, the Brut nib. What, so it the main target the we the reason it was invented was to target, what kinase? BTK. Right? So, if I put it, if I use it in a selling that has BTK in it. then the reviewers are gonna look at me and say, well how do you know it's due to the Irby before, it's probably due to the or BTK .so our first thought ,was we need a cell line that has a lot of Irby before and no BTK. Right .Okay? so, it takes a little time to, you have this is when you do your experiments, you have to think about them, a little bit and make sure, you're doing them in a logical way.

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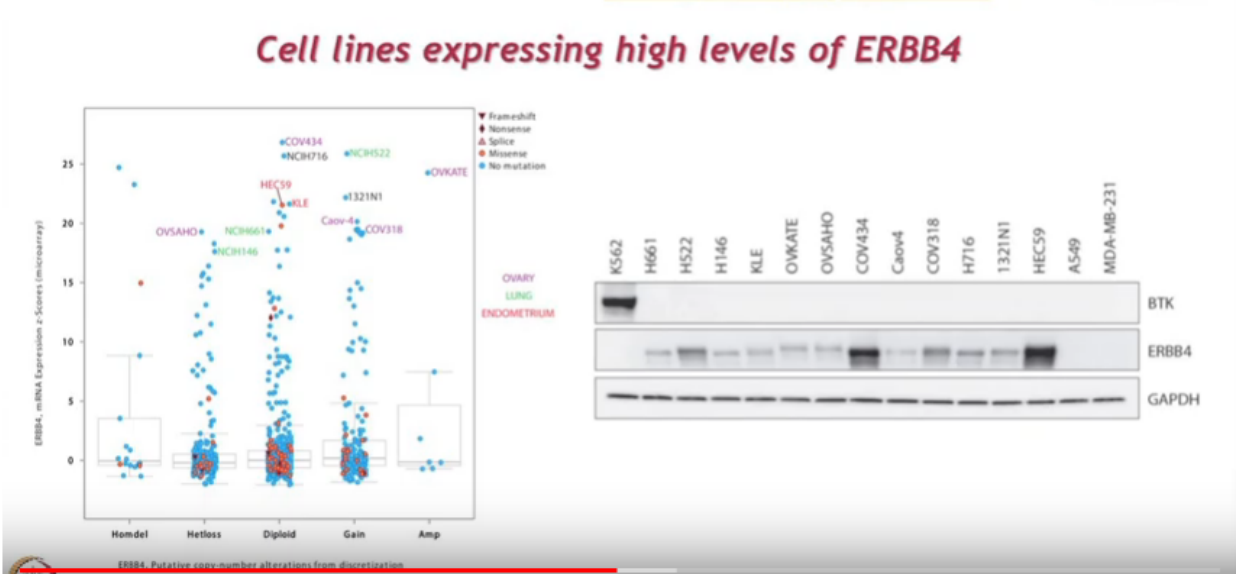

So that's what we did ,we searched to all the ccle, is a ,is a website that has, thousands of cell lines and their gene expression labeled and we ,we scanned all those data, specifically searching for proteins that had, high levels of Irby before and low levels of BTK. And then we ordered a bunch of them. and then the first thing we did was confirm, by Western blot, that these cell lines were as advertised .so here, we show, here's our positive control, that has BTK, all of these cell lines have no BT k. so that, we've taken care of that, that, that's not going to be a confounder. And then all of them have varying levels of RB b4, so the protein is definitely present and then this is just a loading control. Okay? so now, we know, that we have some cell lines that have both. And the first question we want to ask is, will BTK will, will it Bruton have inhibit these cell lines. Because, our hypothesis now, based on our protein array study, is that, a Bruton tip inhibits early before and we think, that that might in some way, inhibit some cell lines, which rely on Irby before for their cell growth, keeping in mind that no one's ever really shown that before.

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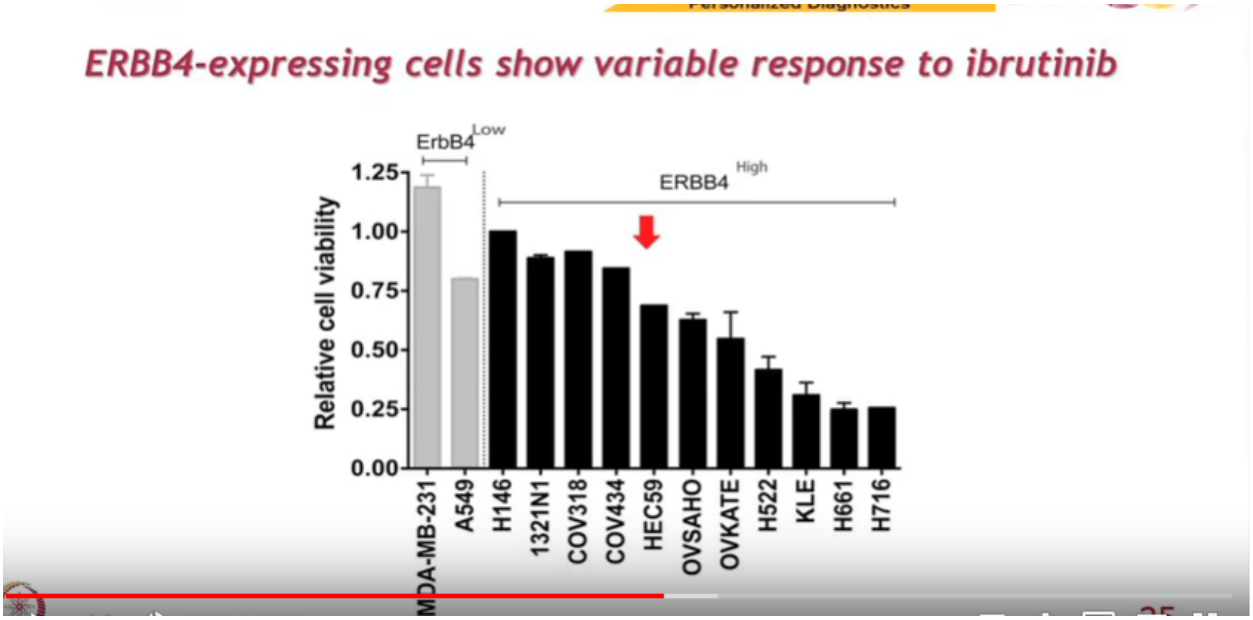

So we treated all these cell lines with, with the, Brut nib. And what we saw, was a range of activity .so some of them, this is relative when I say, relative of cell viability, what that means, is cell growth plus minus drug. So if it's a hundred percent, it means that withdraw gets the same as without drug, if it's, if it's down around 25 percent, that means that with drug gets inhibited by 75 percent. and so you can see that, some of these cell lines out here, were significantly, inhibited by a mat nib, a brute nib and these guys ,not so much, so these guys look like they're resistant to drug, these guys look like they're sensitive to drug. You still with me. Okay? So then, we did a dose response curve, which is the logical thing to do next. And that's what you get.

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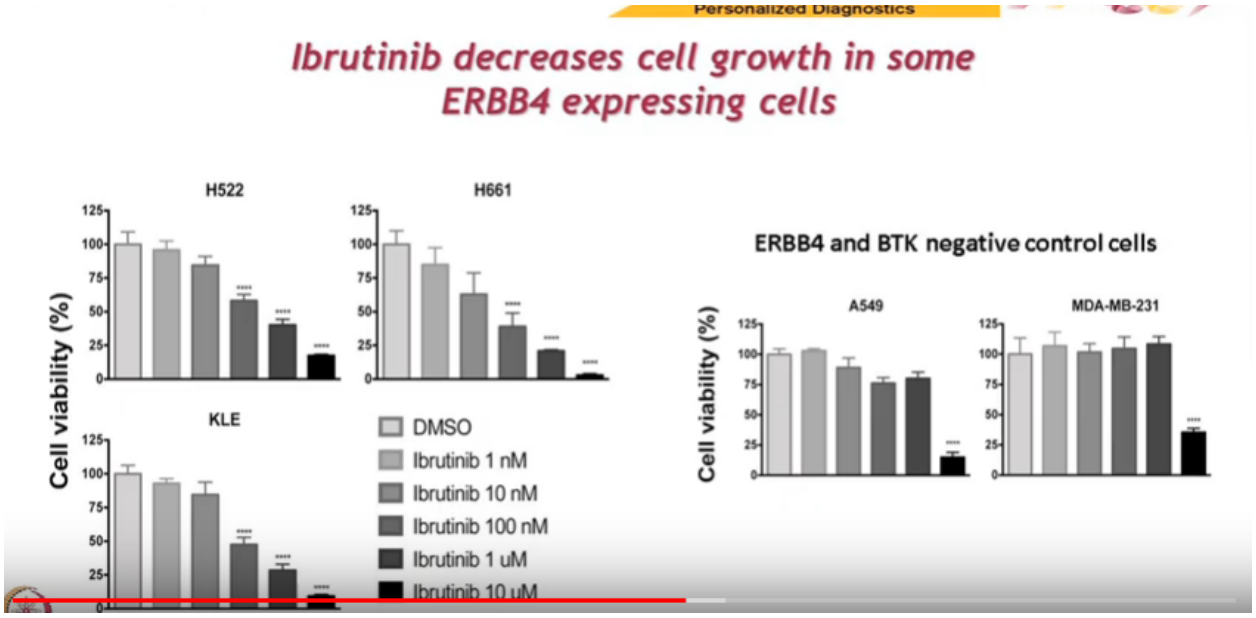

So adding increasing amounts, of drug, you see increasing inhibition of cell growth. So that, looks like, a brute nib is inhibiting a cell line that has, higher B before and no BTK. So you can't, argue that this is due to BTK. Okay? And then and then we did a couple of key controls, we looked at cell lines that were either Irby before negative or BTK negative. And sure enough neither of, these cells were responsive to drug. Okay? so let's, so let's think a little bit about the ER v b4 pathway because, one of the first questions that comes to mind is, Okay, maybe, you've added a drug, that you think you've shown, inhibits early before on your protein array, how do you know, that it's really inhibiting it in these cells, in a way that affects, the cell pathway, the, the, the biochemical pathway, by RB before because, you have to show that right. You can't just say well. Okay, it inhibits, in vitro but I don't know, what happens in vivo. So,

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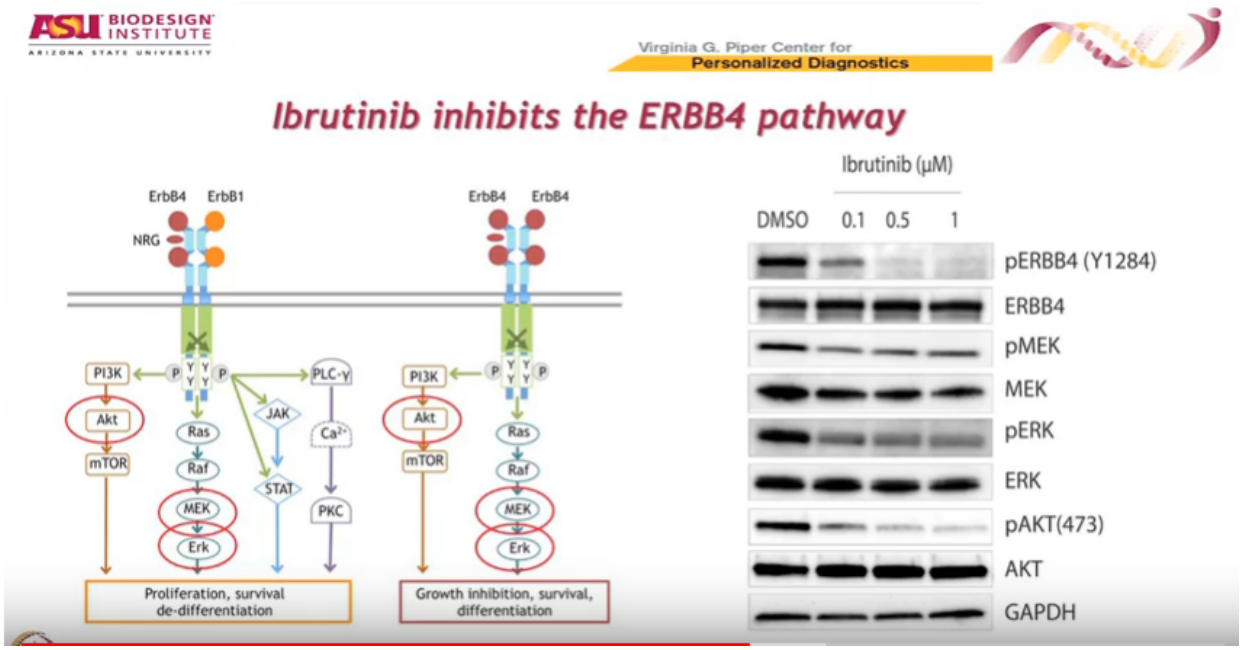

So let's look at the pathway, there are two pathways for Irby before, there's a growth, growth survival pathway over here and there's a proliferation pathway over here. there they're kind of similar, RB before also has an alternate pathway ,depending on the, the splice form that you use .but, you can see that it signals directly is there before like the other EGFR receptors is a dimer, there are, this is an irregular binding here, there are other ligands, that bind to this ,this protein. it sends signals through the rass pathway, the raster pathway. it also sends signals through the pi3 kinase Akt pathway, via mTOR. so these kind of look very similar so, if you were going to think about key, parts of these pathways to test, you might look at these guys, a KT which is a well-known uncle gene, that, that drive cell division and then MEK and ERK which are also, uncle genes and play a role in ,signaling to you the transcription factors like the June Foss and so on. The all three of these proteins, a KT MEK and ERK, ha get phosphorylated when they're active. And we have good antibodies for those phosphor forms, so a good simple test would be, when we inhibit the cells with, a bruit nib, do we see a reduction in the phosphor form, of these proteins which are downstream, of ERBB4.right? and I wouldn't be telling, you this if we didn't do that experiment and that's the result, so here you see that, first of all, ERBB4 itself, is less phosphorylated with drug, so that tells you, right there that it's already,that it itself is being inhibited by drug and then here you see phosphor MEK is going down not dramatically, phosphor work is definitely going down and phosphor a KT is certainly going down. So and keep in mind, that the, the level of a KT is the same. So the protein is still there, it's just not as phosphorylated, the same is true of orc, same is true of MEK, although this is a little bit bigger, and than that I think. But, you get the idea. Right? So, when we treat with them at, when we treat you, with a bruit nib, it inhibits the, the act the downstream pathway, of early before. Okay? So now, what's the next objection, we're going to get from the reviewers, so we've shown that it inhibits the kinase, we've shown that it turns off the biochemical signaling pathway, right. we've shown that it's reliant on Irby before expression, in cells and, and ,and it doesn't matter, the BTK is not there, what else do we have to worry about, what about other, members of the, of the ERBB2 family or herb EB family. Right? So, those proteins are all very similar, there’s a very good chance, that a bruit nib

could also inhibit ERRB2, could inhibit it, could inhibit EGFR and so one of the objections the reviewer might make is well how do you know, that it's specifically, through the ERRB4 pathway and not through these other, members of the of the ERV EB family. Because, after all they're really well known we know, there are cancer proteins. Right? So,

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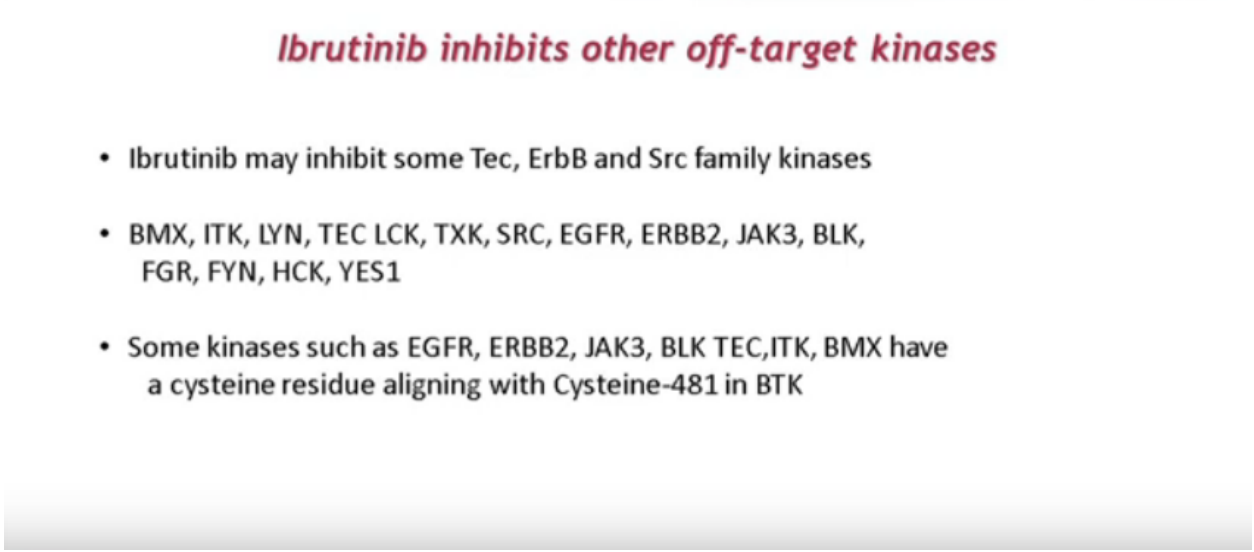

So that was what we have occurred to us, you know, it might, it might, inhibit her, ERBB, Src family members. some of these proteins, in the literature, had been listed by someone, somewhere as being inhibited by her by imatinib, a Bruton him, so I keep confusing those, although the data were not very strong .so and then some of these had, this cysteine residue which is in the binding pocket, where the drug, seems to bind and so it's possible that because, they have that cysteine, like BTK, like ERBB4, they too might be inhibited. so the question, we want to ask was ,if we you know, could EGFR or ERBB2 , be contributing to this improvement response, could it be that this is all due to these guys and not early before. Right? So, so how would you rule that out, knock down? Right? So you would take out, you take these guys out. Right? And still see if you get the effect. Right?

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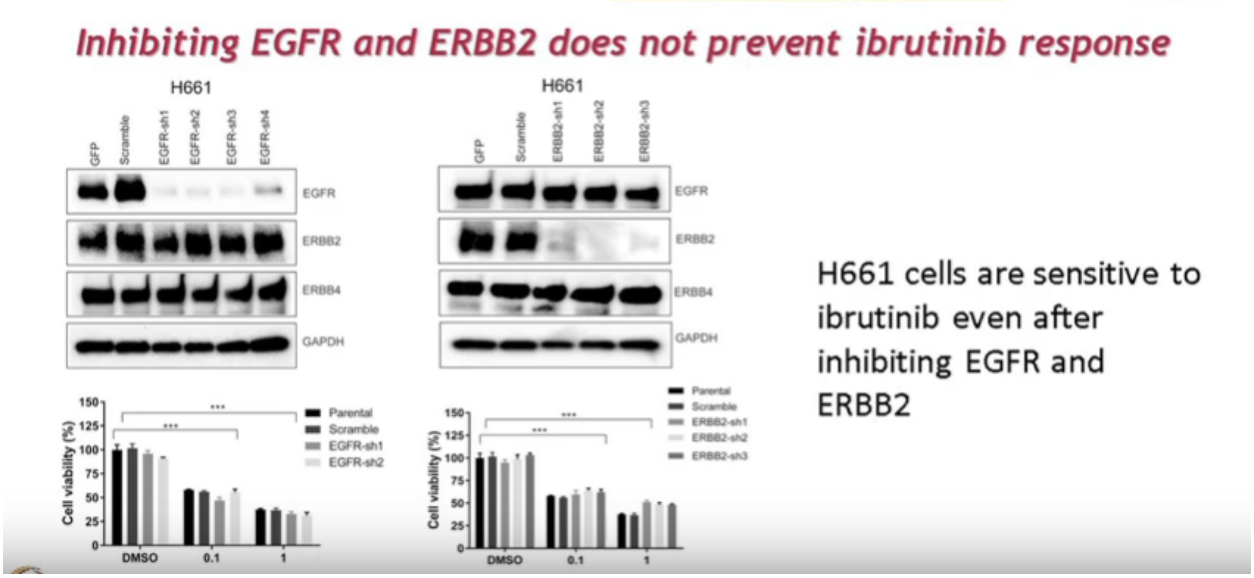

So that's what we did, so we did shrna, here you can see that, this is the level of EGFR, using a during shrna, that knocks down EGFR we could significantly reduce EGFR, notice that these, shrnas do not affect ERBB two or ERBB four. And similarly we had, some ERBB, shrnas that knocked out ERBB2, did not affect EGFR did not affect Irby before and yet, and yet despite that we despite knocking down, EGFR or knocking down or ERBB2 ,we still saw the drug effect. the drugs still inhibited the cells, so now we can say, that ,they're sensitive to Brut nib, even if you inhibit EGFR or even if you inhibit or ERBB2 .Okay? So, so then, then the question was alright. I'm still not convinced, how do you know ,that not can you prove to me that knocking down ERBB4 is really gonna stop cell growth, so we're going to a lot of extreme here because, people have tried to study early before a lot and they haven't the, the people have tested it superficially but they haven't released a lot of time ,on it but, so far the data have not suggested it was Lanka gene, so that's where we wanted to spend some time on it. So, so we wanted to ask the question, can we knock down, ERBB4 without the drug. How would you go about, that shrna. Right? So, that's what we did.

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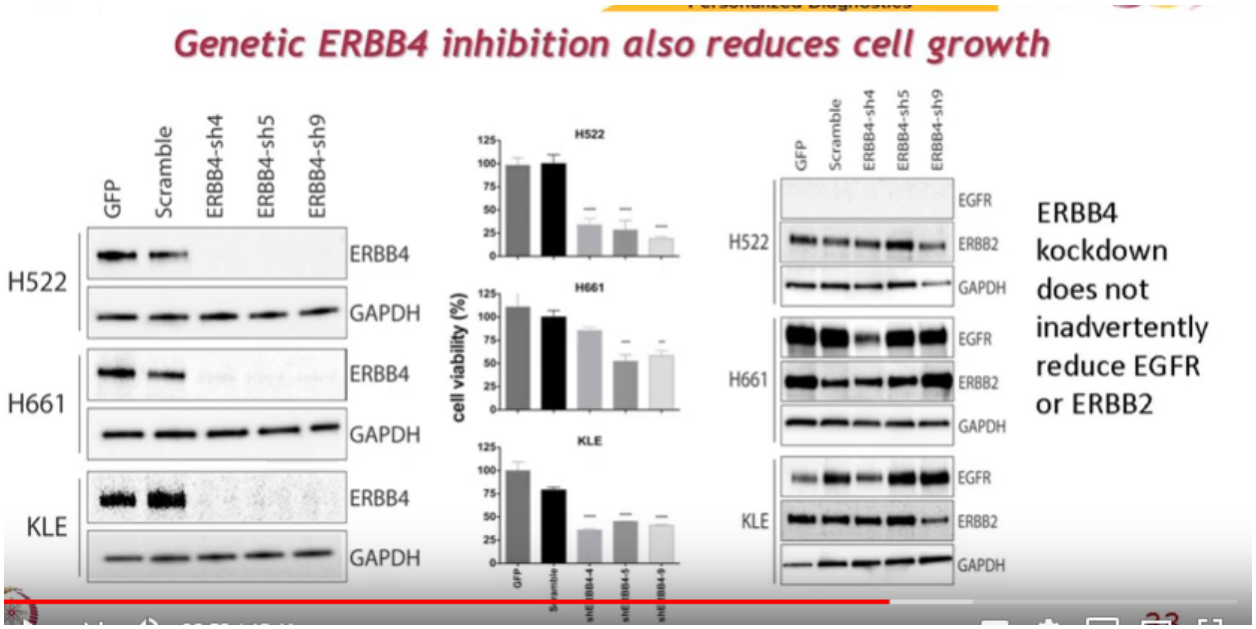

So here, here is in one of our cell lines 5:22, this is one of the sensitive cell lines, here's an herbivore SHRNA clearly knocking down protein, right and stand and if you knock down the protein cell division gets reduced .so just knocking down herbivore is sufficient, to give you cell inhibition. here's, another cell line knock down ERBB4, once again you see reduced cell growth, here's yet another cell line knock it down once again you see reduced cell growth .so knocking down, ERBB4 by itself, using three different SHRNA in each case, is sufficient to knock down cell growth. So, we felt like we had pretty significantly demonstrated that this cell growth inhibition was due to two, herbivore inhibition. And this is just to show you that, when you knock down ERBB4, it doesn't affect EGFR or ERBB2 .so, that's yet another possible criticism as well Okay? you're knocking energy before, maybe you're also affecting EGFR or ERBB2 .but, we can show you that, we actually couldn't detect, your EGFR in these cells, but if you knock down, you don't see any change in ERBB2,no change in ERBB2, no change in EGFR ,no change, no change. so this was really due to EGFR or ERBB4, yeah. there aren't any good drugs ,right now, that we know of I mean, obviously we were, we think, we found one of the first ERBB4, inhibitor you could know you, could knock down, you could block the activity of EGFR or you could have, done that, you could have done that, yeah. That would have been another way; to do we actually just did it genetically, which was easy. But, you we could have gotten the drugs. Right? And then of course, the other way to take out ERBB4 would be to do CRISPR. But, that's a very involved process and we didn't really need, to go that long. Okay? So then, kind of the last piece of this, piece of this puzzle was, asking the question, does this matter at all in biology, right, would it matter in an animal and so we took, we took these cells, created tumors out of these cell, grew tumors out of these cells, in mice. And then, either treated them with a Brut nib or Noah Brut, nib and, and you can see the effect.

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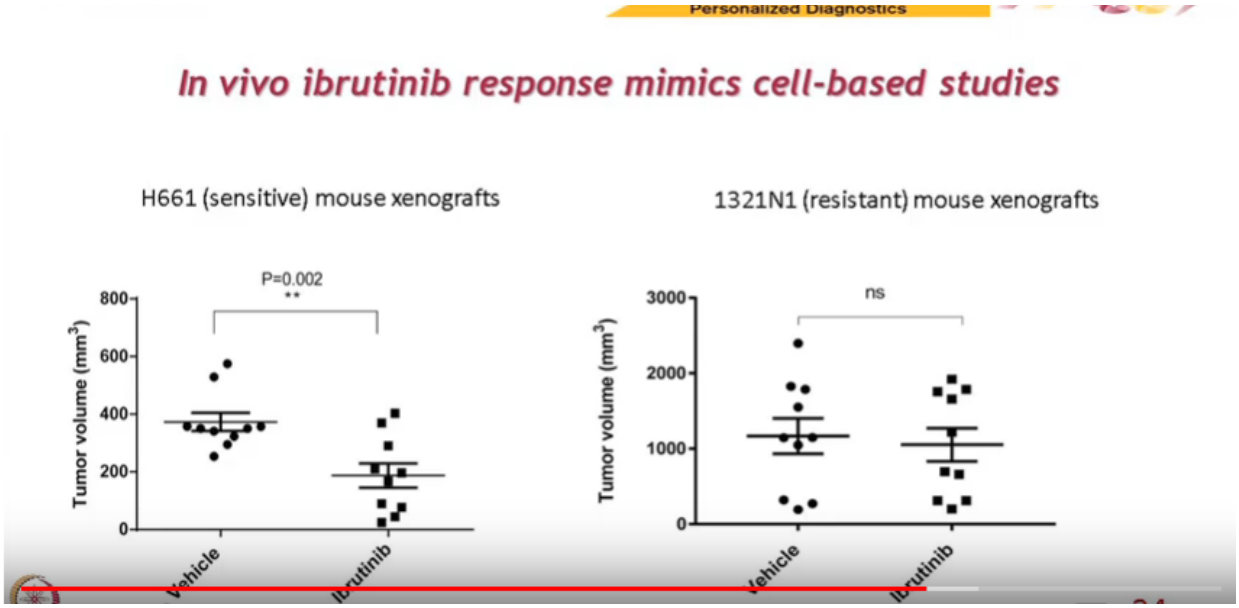

So, this is tumor growth, without a bruit nib and this is tumor growth with a bruit nib. Right? So clearly even in animals, this was working and notice that if you take, if you take, one of the resistance cell lines, that's not a Bruton and responsible, that the use of a Bruton tip doesn't really affect it at all, the difference is really over here. Where, where, where we had a sensitive tumor. Okay? So, now you remember, when I started all this and I showed you a bunch of cell lines, several of the cell lines, were sensitive to the drug. But, quite a few of them were not sensitive to the drug, so the question then becomes, well how come, and why are the non sensitive cell lines resistant. Right? What, what makes them resistant and I think, to me that was the crux of the matter, because historically, ERBB4 inhibition had not been a successful cancer or ERBB4 , had not been an obvious oncogene and I think, part of that reason is because, there's a lot of resistant cells and so when you when you, when people did expect, experiments .they sometimes stumbled on these resistant tumors and they saw no difference, as they decided didn't matter at, all, all right. So, so we looked at the tumors that we had and we looked to see if we could figure out what was different, between the sensitive tumors and the resistant tumors.

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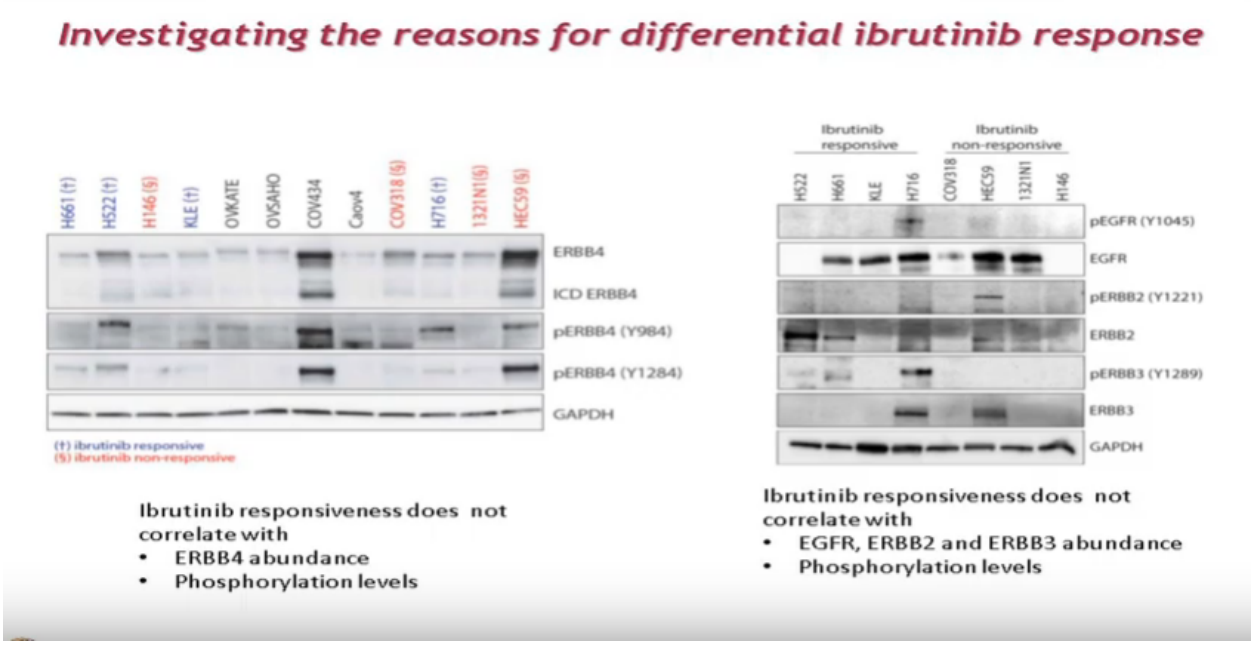

The responsive tumors are in blue. Right? And then, the resistant tumors are in red. and so looking at this at ERBB4 levels, you really, couldn't see anything different from ERBB4, even phosphor ERBB4 ,not really an obvious difference, here's a resistance cell line with very strong phosphor , ERBB4 ,here's a sensitive cell line with really strong PHOSPHO, ERBB4 not, not an obvious correlation, there and we looked at two different Phosphorylation sites, on ERBB4. So it didn't correlate with abundance or Phosphorylation, we also looked to see, if the state of the of the, EGFR or ERBB two or ERBB three, could also have an effect and we looked at their phosphorylation levels and once again, you know, we could spend some time on this I won't bother, comparing responsive to, non responsive there, was no obvious difference. So we were left with this, on you know, unsatisfying situation, of having resistant cells and sensitive cells. But, not really understanding the difference.

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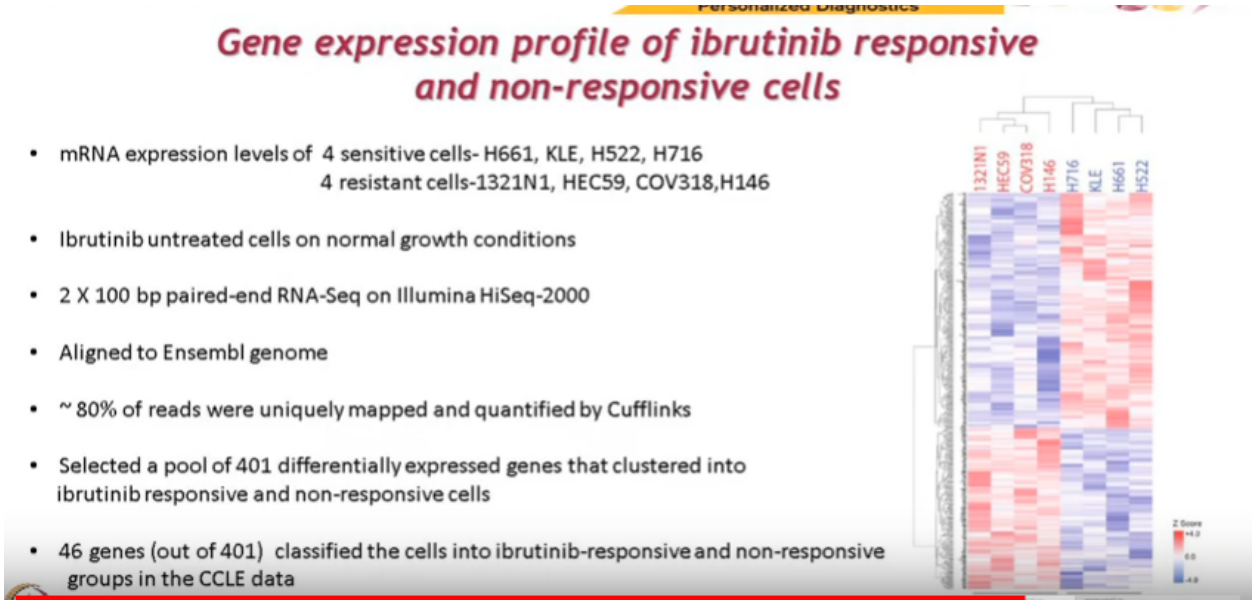

So, we thought, we would do a gene expression profile on these cells. So we looked at, we had these two groups, we had three cells, that were sensitive and, and, and, and four. That were resistant so we put them through gene expression and asked is there anything in their gene expression, that would, correlate, specifically, with responsiveness, could, could we find a difference. Right? so that's ,what you're looking at here, yep, these are the sensitive cells, that I get ,that right ,sensitive cells, resistant cells and right, we ran Tran, that did, did we did them, but we did them, at untreated cells, we did that because, we kind of wanted to know what was at that baseline, we also did the treated ones but that gets more complicated ,we did high seek sequencing, you know, assemble them into genes ,identified what genes responsive and then compared them, compared these results, among this population, to data ,that had where these cell lines have been treated by, but have been looked at in the CC le data ,so we had our own RNA seek data and we also, looked at the CC le data, I'm not going to go through all of the informatics, that we did to kind of sort this out, but in the end, what we did was look at, which pathways, which gene pathways ,were best correlated, with responsiveness.

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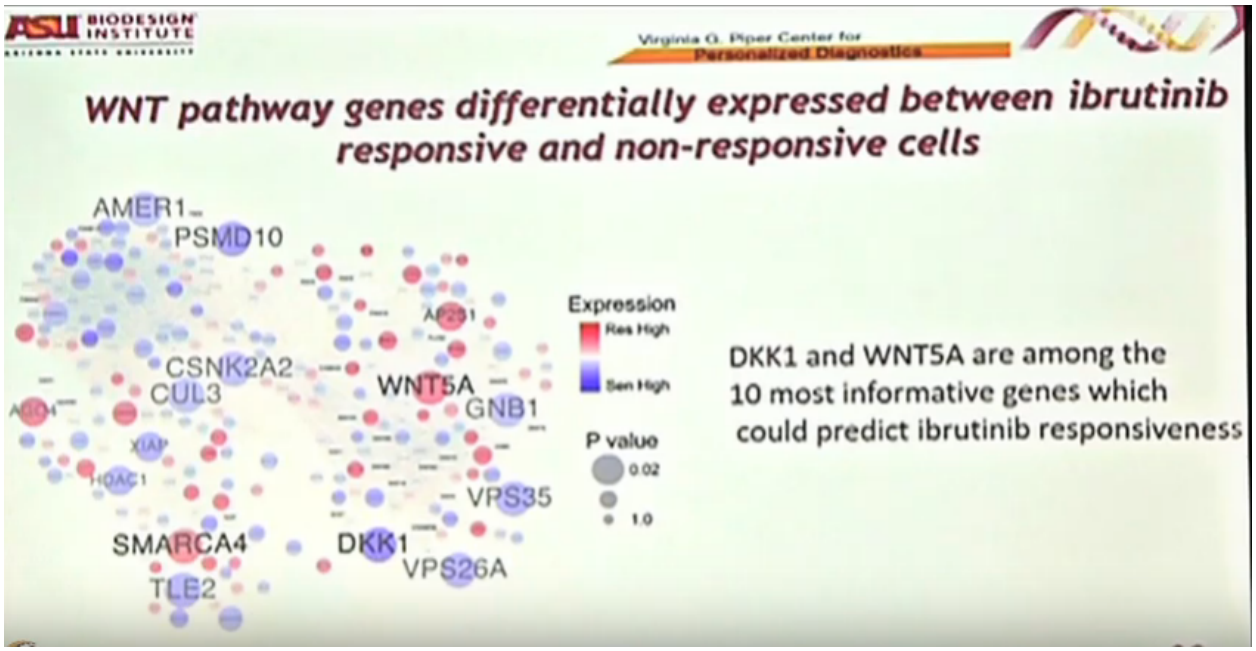

This is a diagram, of gene expression, that shows a difference between, sensitive versus resistant. so red means it drives resistance and blue means it drives sensitivity and the bigger the dot, the more the effect. Okay? Or actually the more the p-value, I should say and so what, you can see is there's so here this mark a for for example, but we were focusing right here, on WNT 5A and DKK one and the reason these two really stood out, for us is these are opposing proteins this guy inhibits that guy, so they are, directly in opposition and they're acting in opposition, this guy drives resistance, this guy drives sensitivity and so, they were among the ten most predictive genes and we decided to follow them, a little bit more closely.

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The one of the question was, can you take a non-responsive, cell and make it sensitive by blocking WNT 5A. So our ball, our model is that when 5a, drives resistance. so the question was, could you, could you change that and so, Famine did this work she took ,she took the cells, she used a win five a, SHRNA ,she tried several of them and she found a couple ,right there, that worked pretty well she created cell lines with knockdown win 5a and showed that in fact, now they are a little bit sensitive to the drug .right? So this is using scramble let's H RNA and then these two are both ,the target attorney, so so, you can make the cells sensitive, by doing that and she did that in two different cell line backgrounds, which is heroic right, so then, then the flip question, is also present so here, we made this cells more sensitive. So the question is, can we take a sensitive cell and make it resistant, by giving it WNT 5A. Right? And so she did, so, yeah.

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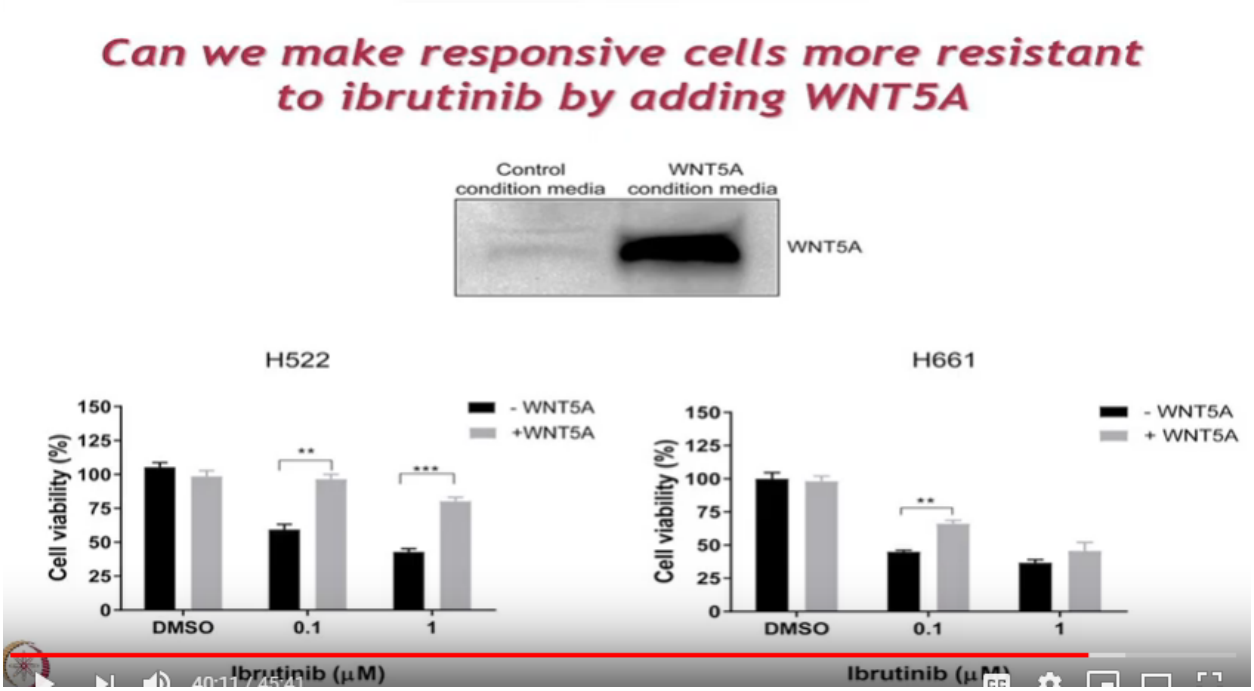

So she did that experiment, it turns out if you take cells that produce a lot of WNT5A. If you take their media, just the cell culture media, you can there's a lot of in the WNT5A media, the proteins right there, and this is the evidence for that and so if she treats the cells, a sensitive cell line, with, with WNT5A. So this is with WNT5A and this is without, you can see that they are much less sensitive to drug, so WNT5A does what we predicted it does. Right? So that's all I have on that story, but it kind, of illustrates for you kind of what, what you hope, that your proteomic studies, will do. Right? they will open up a new idea, a new possibility in this case ,they indicated a new drug that targets a protein that wasn't previously thought to be related to cancer ,allowed us to explore that, that protein as a possible cancer protein and, and kind of pick apart a little bit of a story, that we think reflects back on the biology and the disease, which is what we're really trying to do right, our real goal here is to understand, disease, it's not just us to use a technology, it's to use that technology to study something.

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Points to ponder

- Nucleic Acid Programmable Protein Arrays (NAPPA) is a powerful and innovative technology for the screening of biomarkers and the study of protein-protein interactions
- Functional studies can be performed using NAPPA technology
- Performing AMPylation assay on NAPPA arrays is one such example

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Points to ponder

Other applications of NAPPA technology:

1. Serum screening to identify antibody immune responses for a variety of diseases including cancer, diabetes, autism and infectious diseases

2. Detection of post-translational modifications such as phosphorylation, AMPylation, or citrullination

3. Identification of protein-protein or protein-DNA interactions

4. Non-selective kinase inhibition and identification of drug targets

5. Quantification of protein binding kinetics in high throughput by coupling NAPPA with surface plasmon resonance (SPRi)

So in conclusions, you have learned about ,how to perform, functional studies and especially, a Empress elation assays, using Nappa technology, as I mentioned ,if studying PDMS are not straightforward, you need very sensitive technologies, you need very careful as a design, to really try to capture, how the post-translational modification, happens in cells as ,a result Nappa technology, very elegantly offers you, a very novel platform, to look at, high throughput manner, how the PTAs can be studied .he also studied about, high-throughput screening, on human studies, as well as, the varnish strip, auto acetylating on

NAPPA arrays. Today were also introduced, to the non selective kinase, inhibition, on arrays and how NAPPA technology could be employed for performing such assays very easily. You were exposed to the concept of identification of drug targets, using nappa technology, in them continuation of trying to give you the feel of how protein microarrays and the technology associated with, microarrays, could be utilized for, different applications. In the next few lectures, we are going to talk to you about different type of array platforms and different clinical applications, how this could be realized for, other biologically relevant problems. You will see how to perform a protein microarray experiment, in the laboratory settings. We directly from my proteomics laboratory, some of my senior PhD students, will show you the various are say that the steps performed, in doing microarray based experiments. It will definitely give you, much better idea about this technology, as well as, the intricacies involved, in doing the experiments in the laboratory settings.

In case if you are planning to apply these technologies, in your own research, I think these exposures are very valuable and really needed, to take your understanding, to the actual experiments and try to employ that in your own work. so in the upcoming lectures, we will use different types of microchips, for these experiments, as well as, some demonstrations will be given to try to convey you the protocols involved ,in doing these experiments. also the basic principles and the workflow, almost remains same, whether you use the in vitro transcription translation based, protein arrays like Nappa or you used, purified protein arrays, like you prot which will be also showing or you use reverse fill arrays, variety of these array platforms, the starting materials could be different .but, ideally you will see the workflows remains very similar. But, depending on what the objective Is, you are looking at, a very specific potential in Tractor, you are looking at protein modification, you are looking a at biomarker even available in protein or you are looking at some sort of inhibitor assay accordingly, your experimental design has to be changed and you have to thoughtfully, carefully, think about, what should be my best controls, for giving me answers or the right answers, to address these questions. So you learn about, some of these aspects more, in the upcoming lectures and I hope will be then very confident about, how to use this one of the very promising technologies, for variety of discovery and functional studies, in your own work. Thank you.