Lecture 31: Use of SPR in unravelling domain motif interactions of proteasomal assembly chaperones

Welcome to MOOC codes on applications of Interatomics, using genomics and proteomics technologies. Today's lecture will be conducted, by Dr. Prasanna Venkatraman, who is principal investigator and scientist at act track, Mumbai Dr. Prasanna lab is interested, in general understanding of, mechanism of cellular homeostasis, both in health and disease, her group is trying to develop, a model system, to navigate through, the various steps, involved in protozoa rumen degradation. She is also interested, to understand, how the communication, between substrates and the proteasome then translates into

downstream events, like unwinding, of the polypeptide chain and its subsequent degradation, included lecture Dr. Prasanna will further explain, the surface Plasmon resonance SPR technology, to know why and when it is applied, to study the protein, protein interactions, select we welcome Dr. Prasanna for today lecture. Hi, very good morning, this is the, title use, use of, SPR in unravelling domain mode of interaction it's of producible assembly chaperones. But before directly going into SPR I wanted to give the philosophy of, what this domain mode of interaction is maybe, not all of you are familiar with it and especially in the context of a non-core protein, where instead of one on one protein interactions, the protein interaction exists as a network, which changes from the normal, to the cancer cells you need to understand, how would one look at, from a network, network perspective and even if you looked at the network perspective, at the end of the day, you want to inhibit these protein, protein interactions, with small molecules. So, how do you get to the details of it, from a global perspective and you narrow down it to single protein, protein interactions or destabilize the network in turn of interactions, because you have a common principle that drives this interaction and that is basically, the domain mode of interaction concept, that can be extrapolated in the context of a protein interaction Network. I'll show you one example, I don't have time to go for two both and then in the end, I will tell you how having done different techniques, I'll ask you a few questions of how SPR is going to help us, in taking it to the next step.

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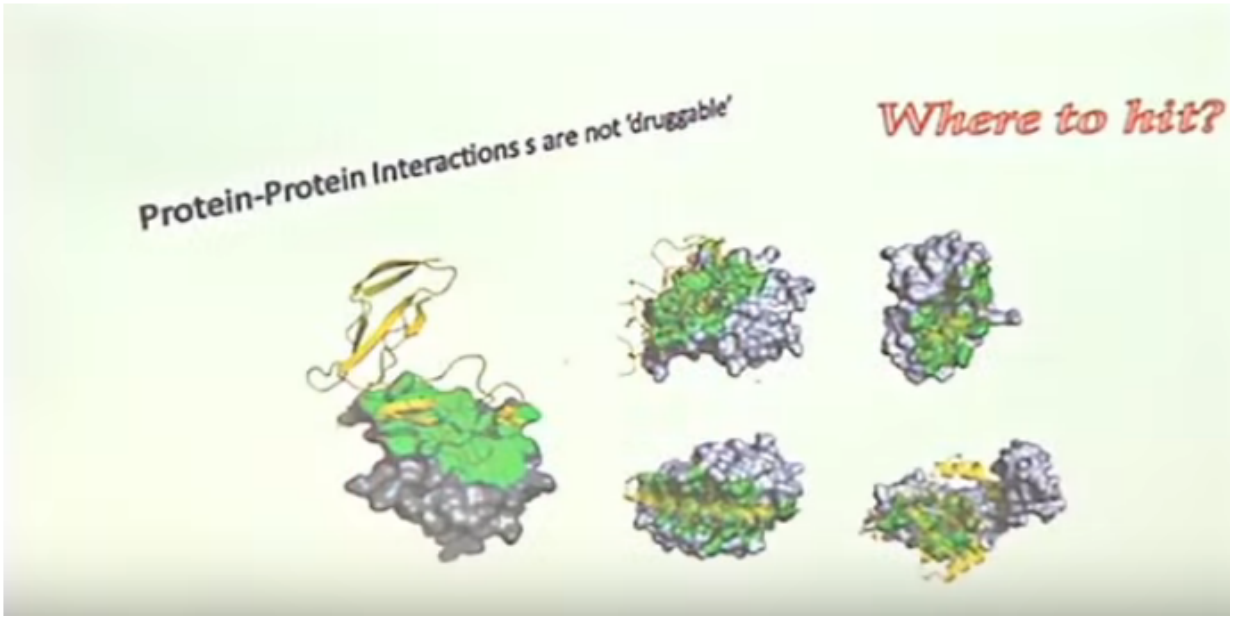

So, these are some of the structures, of protein, protein two proteins interacting it doesn't matter what they are and those of you were very familiar, with small molecule protein interactions, in terms of a substrate enzyme interactions, you know that they will occupy a very small site, active site is very small and the

molecules that bind to the active sites are very small. So, you burry only a small surface region, like a 600 Armstrong square, region when the small molecule binds, with a protein, whereas if another protein interacts, with a protein then they barely large surface areas, which can go up to 4,500 Armstrong Square, of buried surface region that's what contributes to a high affinity, of interaction. And because you have a large surface area, that covers the interface, you can't find a principle that will inhibit this interaction. Right? So, the simp so complex and they are enveloped against, each other over a very large, surface area, for you to pinpoint that there is a specific region that I would like to target with a small molecule, becomes difficult and that is why people say, these are undruggable. Now if they are undruggable are they really, so and can you convert, them into druggable targets, using principles of biophysics and biochemistry. Now long ago,

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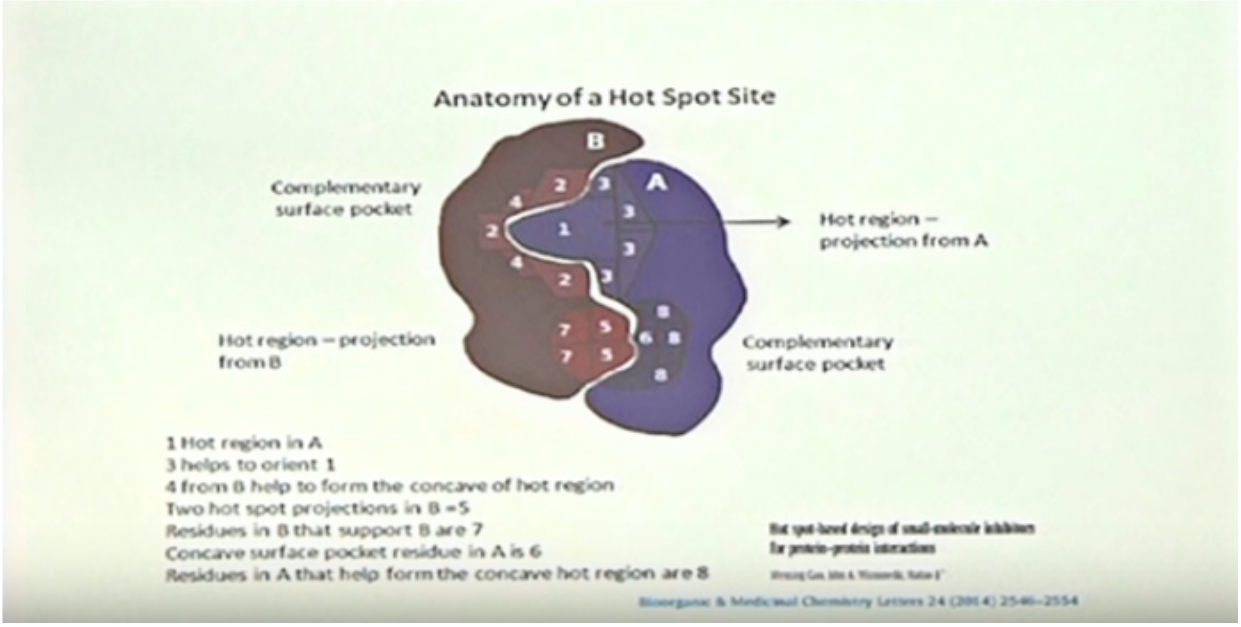

people worked on these, protein, protein interaction dissected them. And this is your reductionist picture of a protein, protein interaction, even though they envelope over really, lot surface area, you can see this protrusion that is a knob that is sitting into this hole. Right? And if you mutate, any of these residues within this region, which is normally one or two, this will fall apart. So, despite a large surface area that, dictates the interaction, the key interactions are driven by very few residues at, the interface and these are called as,' Hotspots', in many cases it can be a single amino acid that is dictating this bulk of the binding energy. And if you make the mutation and this residue, you will weaken this interaction that they will not, be able to associate with each other. So, this is the concept of a hotspot interaction and this has been increasingly shown, to be true in a number of interactions, where you are able to identify, such a key residue that is responsible for interaction, it seems easy, it does not reveal, but it is being increasingly observed, that this can be true for many, many protein interactions.

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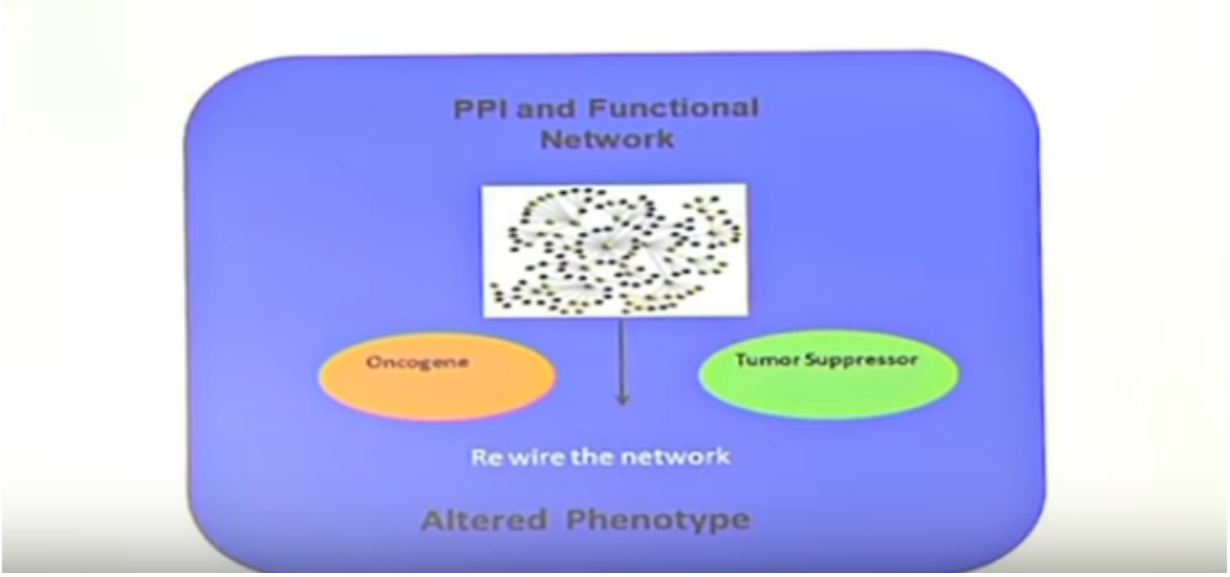

So, I said that no longer can we think of single protein, protein interaction, we love to view protein interactions, as a network, where there are different hubs, with their own islands of interactions, there are unique, edges and these edges are connecting different hubs as well. So, this is the kind of interaction that exists, in a normal cell and if you had a oncogene or a tum or suppressor, because of their levels, mutations they can what they can do is that they can rewire, the network, because protein interactions are primarily, determined by affinity. So, if you have over expression or a mutation that can I think this binding you will deregulate this network, some of these interactions, may get strengthened they just may be stronger, some of these interactions get weaker. And that is how you rewire the network and that is the difference between a normal and a cancer cell. Now how do you view this? How do you view this? In the concept of protein, protein interaction. How do you study it, using biophysical techniques such as SPR?

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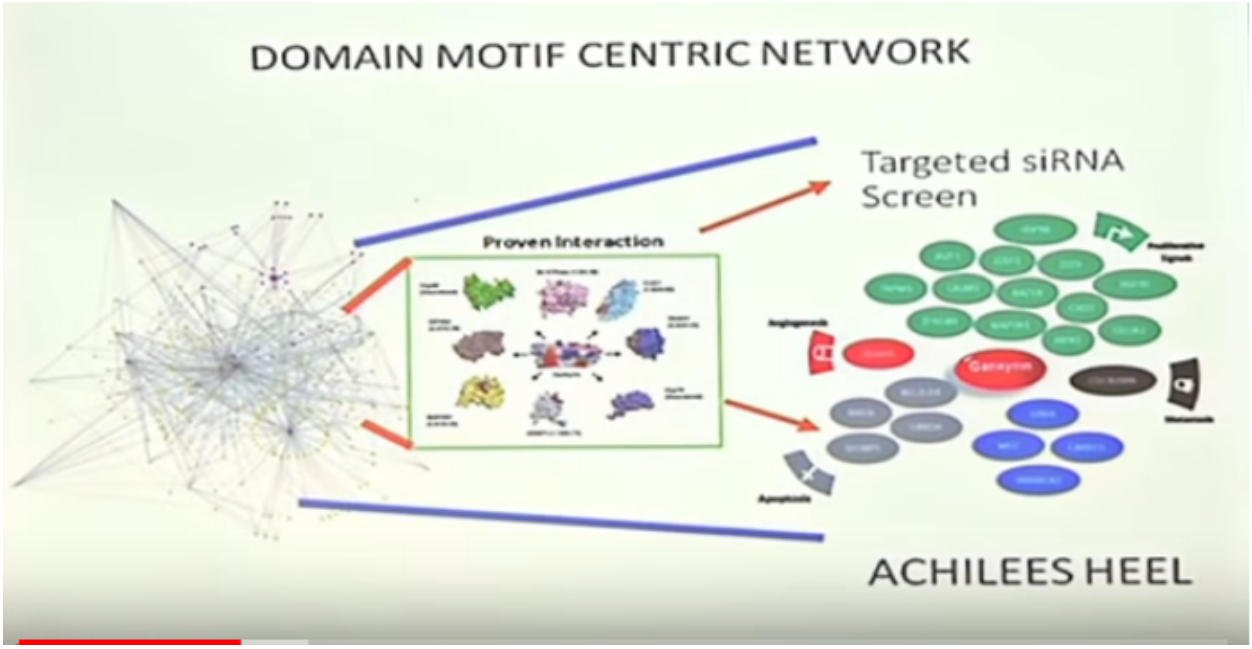

So, as I said this is the network, this is made of multiple interactions. So, what you're trying to do is that, can I find a sub Network within this. That I can describe in terms of loan structural and by physical principles. So, if you consider that there is a domain and a motive and these are interacting and this interaction, is a conserved interface, what most of the time happens, is that helps generally borrow, this common interface, for example, I have residue something like EVD, which will come in the context later, there are four residues that are at the interface, in this complex, the hub will borrow this interaction, interface use it, site to interact with multiple proteins, which carry the same motive. Now this proteins need not be homologous, they need not have carry any sequence similarity, but they happen to carry the short sequence within them, what are called as,' Short Linear Sequence Motive '. And if you have this conserved across multiple proteins, these would interact with this hub. So, now you have a hub centric network, dictated by a single motive and say for example, I understood what is the residue that is contributing, to this interaction among the EVD or maybe just these three residues are really, really important for interaction. I mutated, I destabilize. So. I destabilize, not just a single interaction, I is destabilized multiple interactions, got the concept. So, this is how you go from principles of domain mode of interaction, to the network. And then you come to the reductionist, approach of destabilizing the network, using the very same principles, that's what we had employed in our study, to look at gang Karen it's a producible chaperone, assembly should have chaperone as well as a non coprotin then what you could do is that, to know which one of them may be functionally relevant, you can couple, it to a genomic, approach where you assess siRNA to knock out each of these genes. And see pick up, really the functionally relevant ones or which one actually dictates cell fate, malignancy, metastasis and then you could go for that particular interaction, in the cancer type that you have. So, this is the global picture of what our concept, is our philosophy is and we call this a trying to find out, the Achilles heel in cancer.

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So, now let's go to the directly to the example. So, this is PSM de 10 Morgan Caron it is a protease normal assembly chaperone, don't worry too much about, it tourism is like huge machinery, for it to work it needs to be assembled from different, protein subunits and these chaperones help in the assembly. And what happens is that Gankyrin also turned out, to be an oncoprotein; over expressed in multiple cancers and it is as you can see in this it, is involved in a plethora of cancers and plethora of hallmark, cancer properties.

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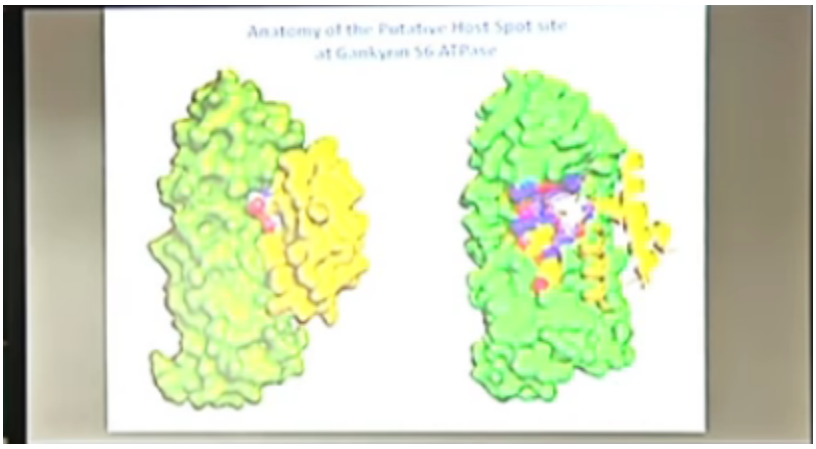

What was available to us, was a crystal structure of Gankyrin and one of the assemble, assembly component, which is a ATPS of the proteasome and you see that there is an interaction, between Gankyrin and six ATPS through similar to the knob and the protrusion that I showed you earlier. So, we recognize this as a hotspot, the crystal structure is available, but we recognize this as a potential, hot spot. We looked at residues that are there and we found that e EVD. Okay?

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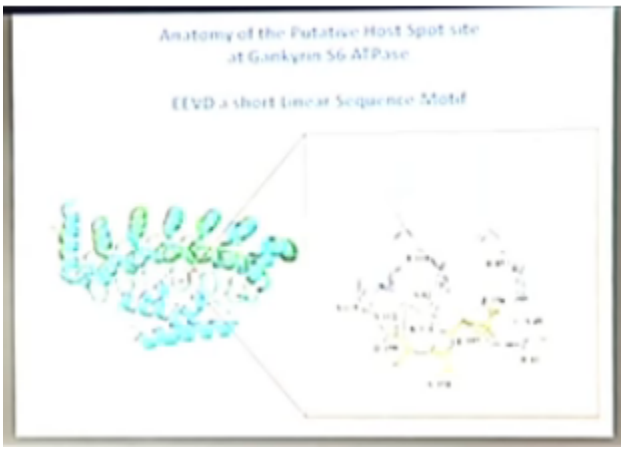

From the s6 ATPS protrudes into the Gankyrin, surface and if there are proteins, which have EVD on the surface, of Onyx surface, they are likely to interact, with Gankyrin that is the prediction, a bioinformatics prediction.

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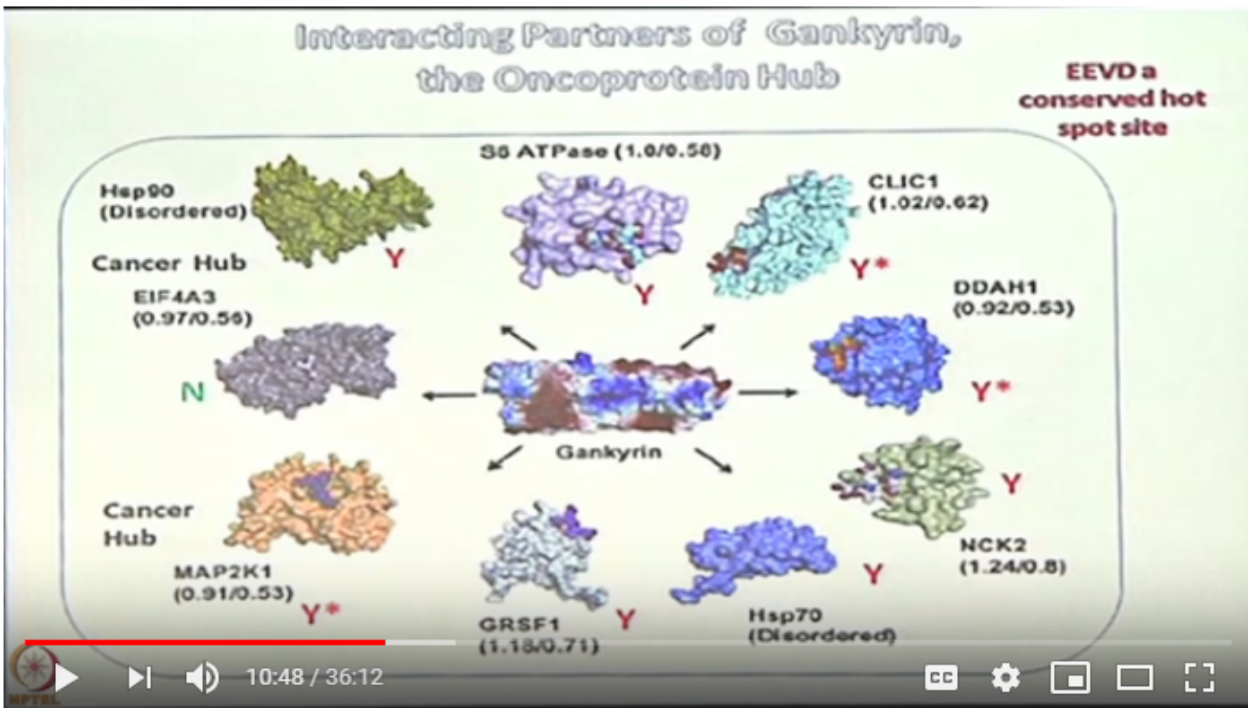

And we had some 32 proteins, for which the crystal structures were available. So, we know that these are in the accessible regions of the protein. And we asked tested eight of them, as to how many of them would interact and then except for this one protein we found all of these that we predicted, interact with Gankyrin in experimentally, if you mutate the EVD, here you lose interaction, this is already published and as you can see that seven of them, drives this interaction through EVD. So, this is a sub network within the Gankyrin EVD interaction.

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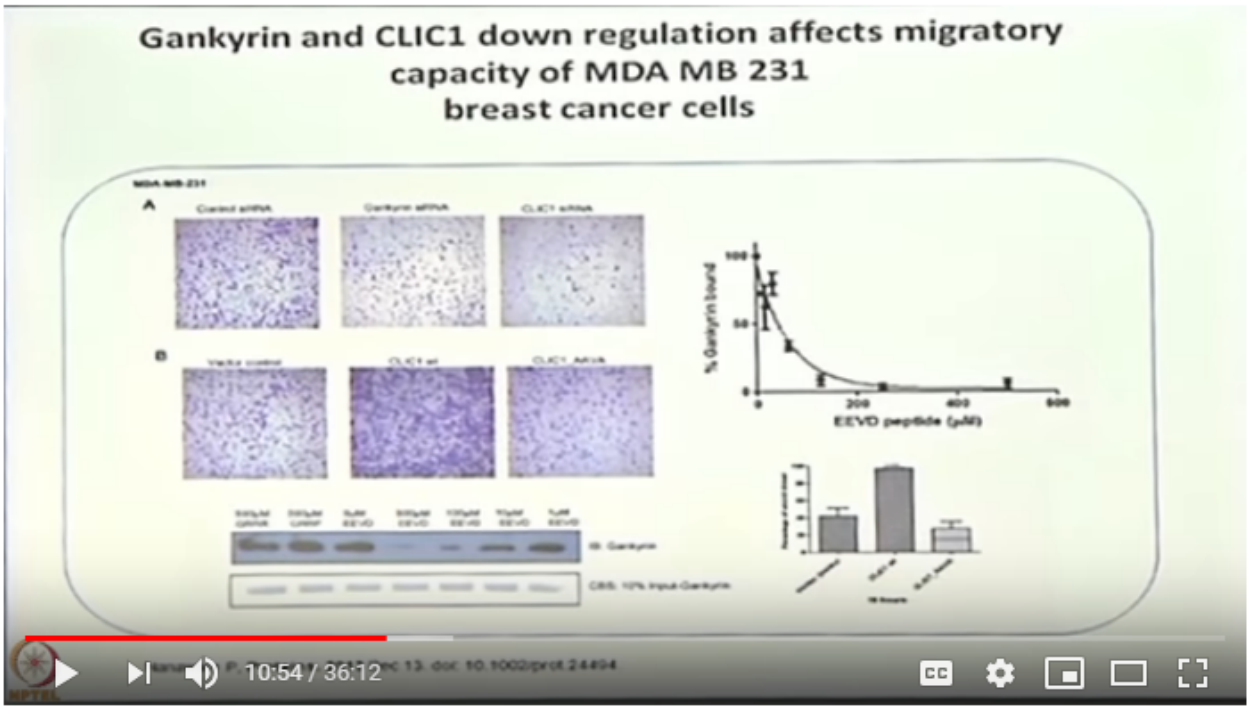

Now is it functionally relevant. So, you have to demonstrate that this physical interaction makes sense. We did a series of experiments, where you knock out the, one of the interacting partner and then you over express a wild type or the mutant. The wild type is able to rescue the phenotype, the mutant is unable to rescue the phenotype the peptide alone a short EVD peptide, is able to inhibit this interaction. And that can be quantitated and you can get a nice 50 of about 50 micro molar. So, that is important. Right? When you're saying this peptide is responsible mutation is one way of doing it. And throwing in the peptide to inhibit the interaction is another way, of establishing that.

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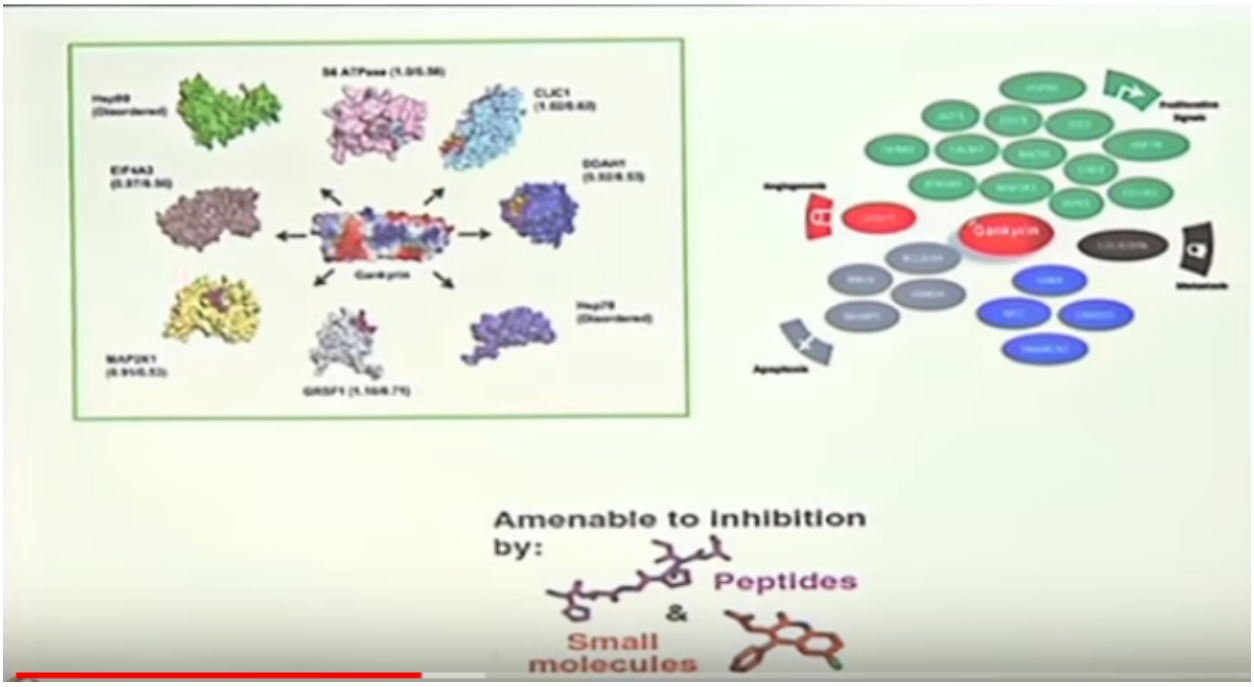

So, this we did and we know that we can go this route, to find the Achilles heel and you can see that many of these proteins are involved in, many cellular activities, like angiogenesis apoptosis proliferation and metastasis. So, we are looking at the functional network of Gankyrin through this domain mode of interaction. And a conserved hotspot site surface, why is it important because, now you have reduced, the surface like a small molecule, EVD is now like a small molecule, you can perturb it by peptide, derivatives or small molecules, you are converting a large surface area. Now into something that will mimic a small molecule protein interaction. So, what was non druggable earlier, is now, now becomes druggable, because you are looking, at the key interactions, that stabilize the complex and therefore you can put up them, with small molecules and peptide derivatives.

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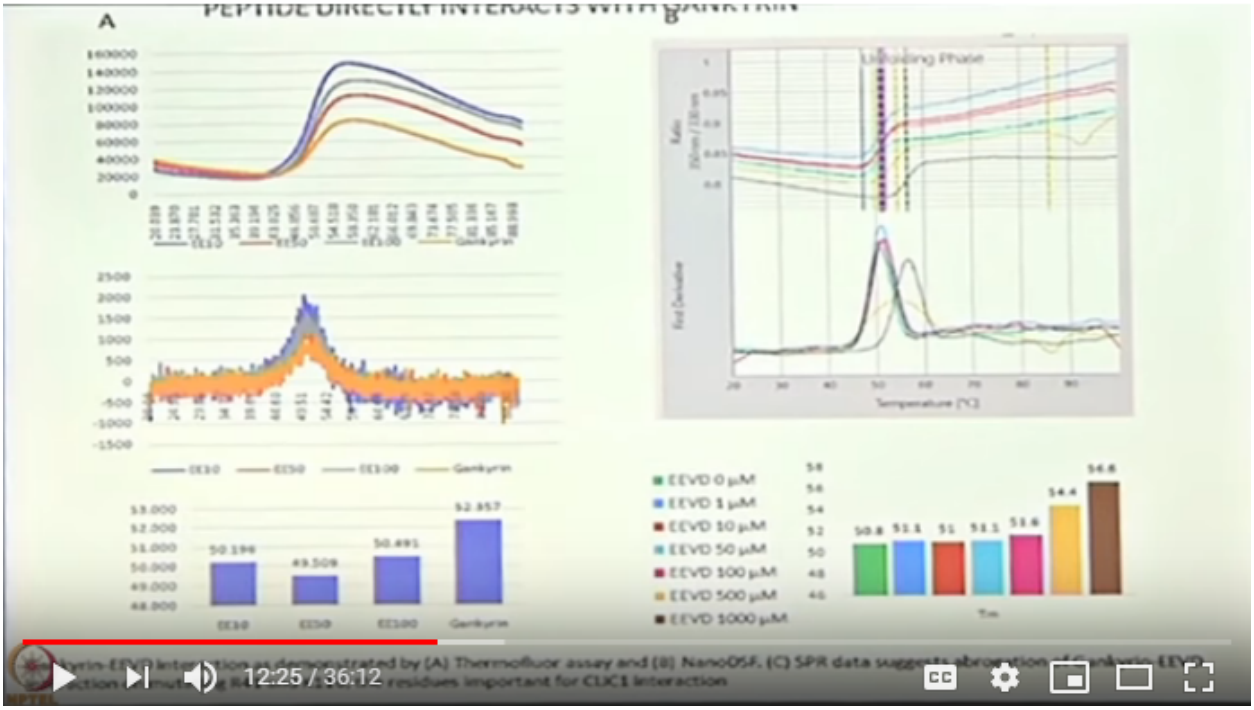

So, now that's the peptide directly interact with the protein, you need to do a series of experiments, to establish that the peptide directly interacts of course it interacts because, it inhibits the complex, but does it directly interact. We did a series of experiments where there was a trauma floor assay and there was also a danno DSF I think you are familiar with, it. Now that the courses have been conducted. So, you can look at these and then establish that there is a direct interaction.

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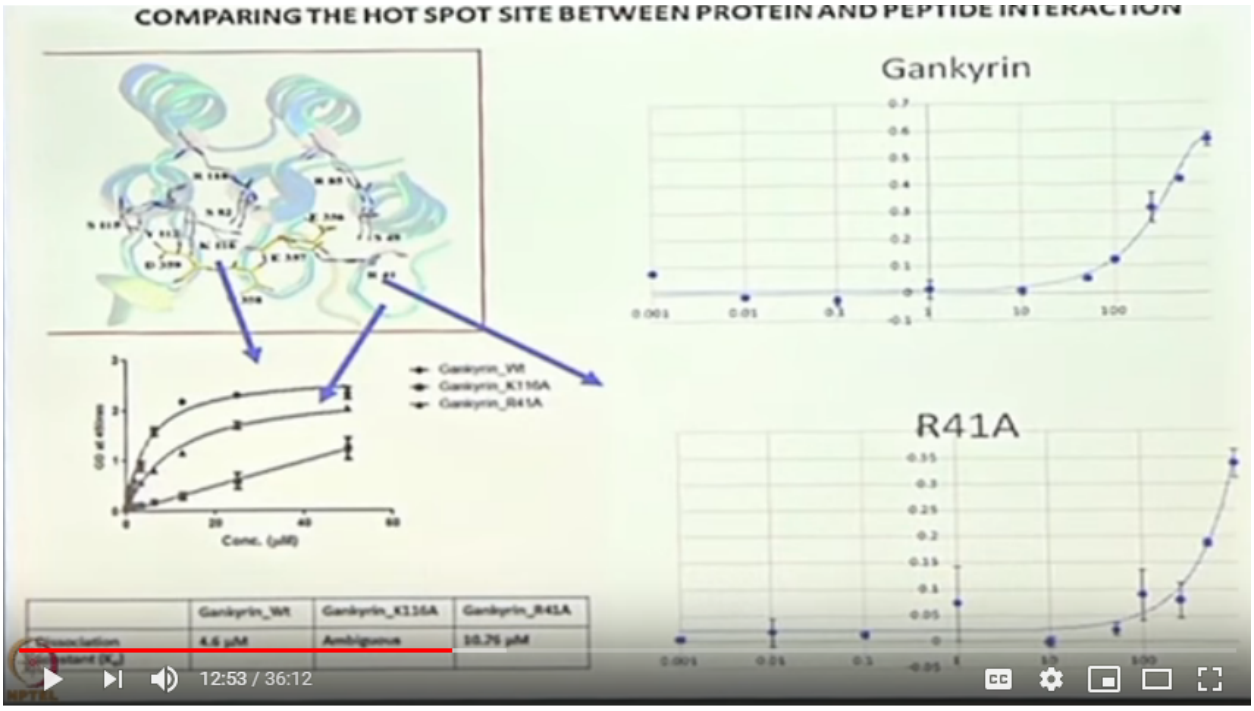

We went back to our old technique of Eliza, where you can look at peptide protein interaction, only that the peptide is now labelled with a biotin. So, that you can pick it up with a streptavidin alkaline phosphates, you can see this is the wild-type peptide interaction, it hasn't gone to the saturation this is still in process, we probably have to get new peptide preparation, this is hot from the oven and then you can mutate, the same residue that we think is important for interaction from the Gankyrin interface, because you say saw that the s6 ATPS comes, with EBD other proteins are coming with EBD in their sequence, Gankyrin, uses lysine and lysine, hundred and sixteen and our 41 to interact with the 680 PS. We believe that we'll use the same residues to interact with other proteins, as well as with the peptide are we. Right? Here is an interaction, with click one, one of the interacting partners, which we suspected would interact with EVD here, you can see when we make a lysine or an arginine mutation, you abrogate interaction, you do that similarly for the peptide and I show direct binding of the peptide to the protein from the other essays.

So, we have now narrowed, down to the peptide protein interaction, using this shot motive. Now that we have all these answers, we know the peptide binds a peptide binds at the intended place; it is able to compete with the protein. So, why should we do SPR? That's a question to you why do we need SPR? We also have a crystal structure of the protein by the way, it's published but we can solve the structure we are soaking it with a peptide to determine that structure that is different. So, if we can go to that, extent why do I need SPR kinetics, very good what this kinetics? How to kinetics going to help me? Nice kinetics but, why how's it going to help me why should it help me why should I bother to study kinetics, I should compare the on and off rate, very nice but why should I compare on and off rain why am I not status white with, dissociation constant equilibrium dissociation constant KD, What is the relationship between KD and on and off rate KD is equal to, K off by K on these are rate constants. Right? They are equated to the equilibrium, rate constant, but I equilibrium dissociation constant. But why? So what? So, once you have the ratio, what does it mean? The ratio will determine the equilibrium dissociation, what does that mean then? So, if I have the same KD, what do you think will happen to the on and off rate? I have so,

four molecules I have the same KD for them, very good that's a reason to do SPR. Okay? But still, even if I knew that the on and off rates are different, how does it help me in the next step, how can I use this, how can I apply the SPR derived information, which can be given by no other technique. Right? Real time kinetics of interaction is there any other technique that can truly give you, the label freeze SPR is the one that will give you. So, why should I do? It what am I going to get out of knowing what is the K on and K off. Okay? Okay? Correct? So, how do I engineer it now? If suppose I want to make it more, I want to dissociate it slowly or I want to have a rapid Association. So, most likely you want to slow the dissociation of a drug. Right? And you don't it to slow, because you know exactly. So, what would I do when I look at the structure, can I can it help me in any way, can structure help me in any way. So, independently, K on and K off I have and I want to improvise, this in producing a drug that will compete, with this peptide or it will compete with the protein, protein interaction, I want a drug. And I want to get the drug, small molecule inhibitor which will go, into a drug, with all the properties that are necessary.

So, simple on and off rate will tell me or what watched I do to? So, how do I improvise on the drug, how do I engineer the drug? Now you alter the Pharma course of these. Right? You look at the interactions, with the protein and then you find out what are the molecules? What are the residues that are interacting? Whether it is a hydrogen bond, whether it is a salt bridge whether is a hydrophobic interaction and knowing the residues that are interacting, can I now put up these and can I engineer a better drug or if I engineer a better drug I'm thinking I'm engineering, a better drug and I come and look at the SPR, maybe, I did not achieve the goal, I actually made it into a poor a it had a poor outcome as compared to what I expected. Right? So, you couple the structure, guided drug design, look by with the SPR, on and off rate, you can actually, begin to look at, how to get you the best drug that is possible engineer it, with the properties that are favourable, for a drug protein interaction that will displace, the protein. Okay? So, this is an example, where you had the same KD.

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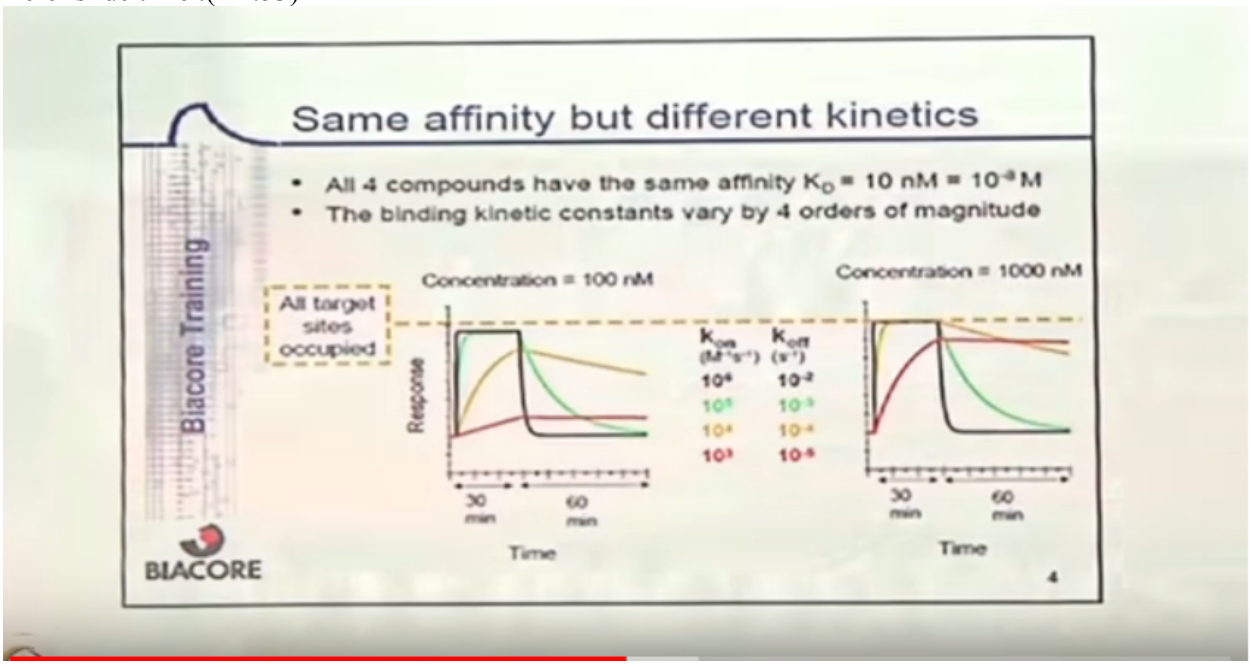

But different on and off and as you guys rightly pointed out. So, the ratio is going to change, you can have the difference, you can find out the molecules that are different.

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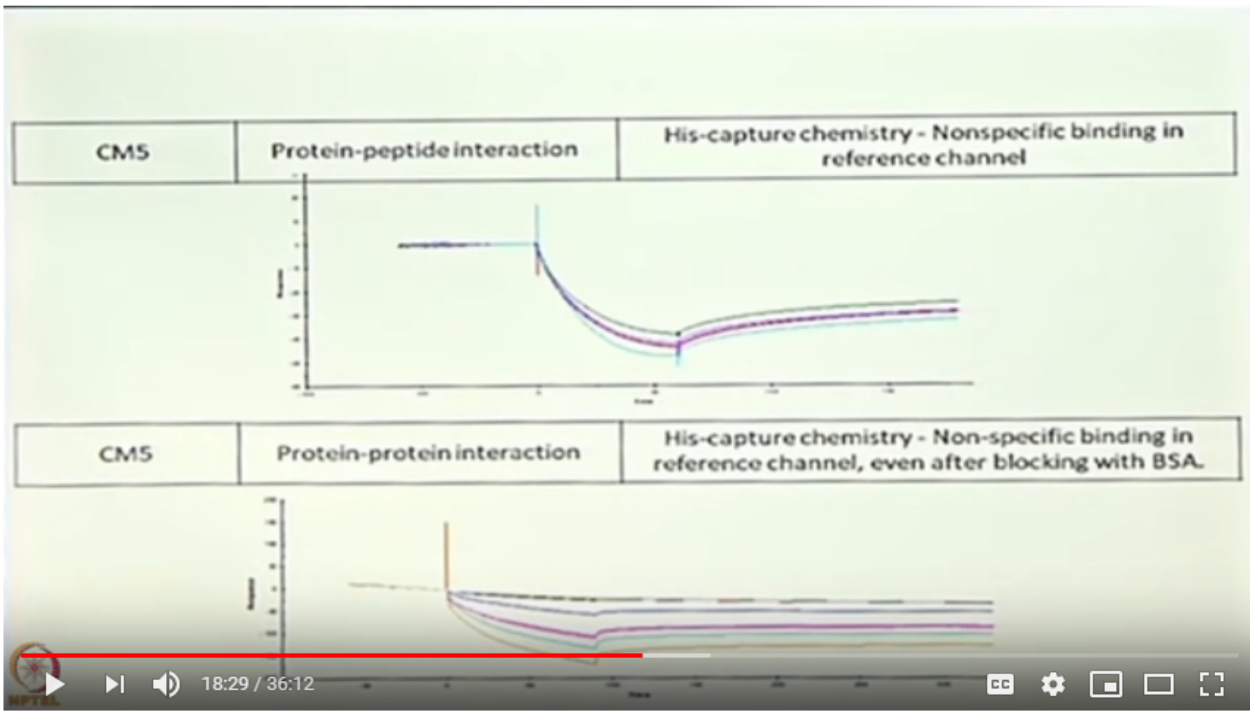

So, you should look at the bioroid bulletin and the GE bulletin to, understand these things yeah. So, we started off trying. Now we had a fairly good idea of how the peptide is interacting with the protein. And then in the crystals that we soaked with the peptide we do, get the crystals, but all the crystals that we have diffracted still now have a vacant binding site we haven't formed the peptide there. But the process is on-going. So, here you can see that we tried the have you do you know about the SCM fight tips, cm3 chips, yeah. So, these are chips, with the Dexter molecules, to which the protein binds, you can immobilize them, yeah, so Okay? So, we tried the CM 5 and chips here, which is normally used for these kind of studies protein peptide interaction. And we also tried that he's captu, his beam capture chemistry by having the nickel nta there on this in. All the cases what we found is that there is? A nonspecific binding to the reference cell. So, once you get subtract, you don't get any interaction and then nonspecific, but hey again in the reference, cell even after blocking with the BSA and we have not been able to successfully get the CM 5 working then we went to CM 3 one of the suggestions because, what is the difference between a CM 5and the CM 3 dextran chips exactly. So, they do not that so, they have a lesser surface to bind and therefore you can control your binding, your occupancy, better and therefore you are going to avoid this nonspecific binding that's the logic. We did some improvement.

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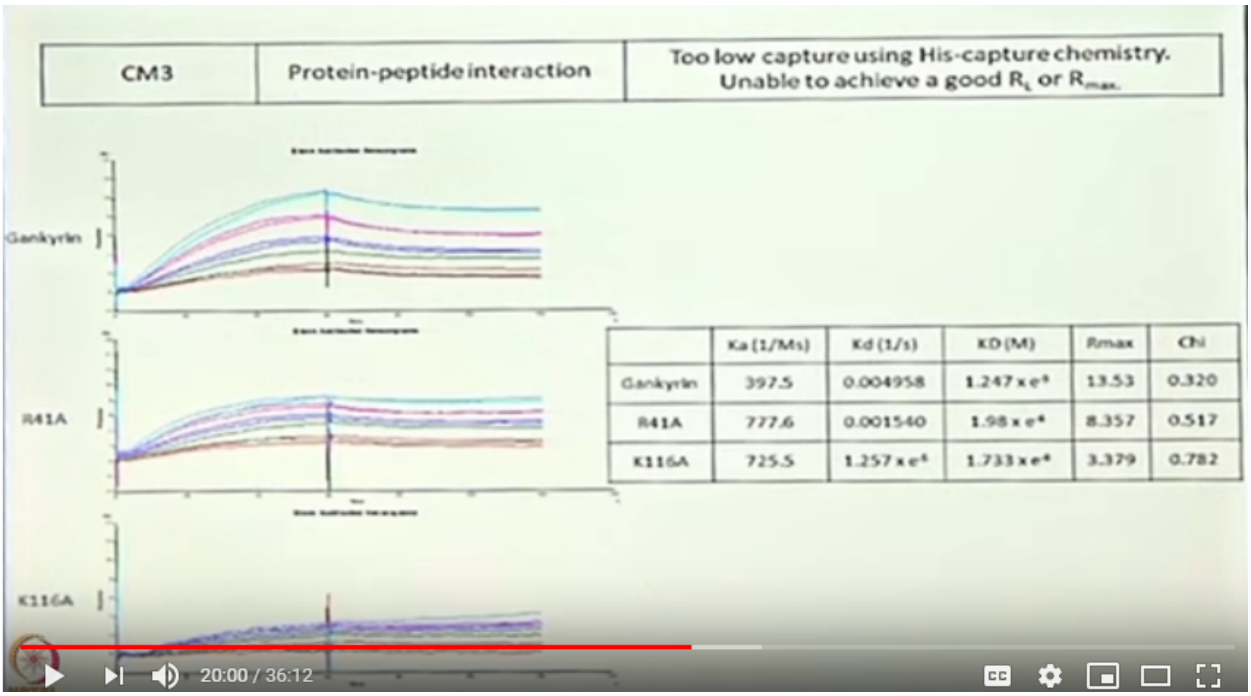

To this you can see this is the wild type protein and the peptide interaction. And in each case you see that we have now done a duplicate run with a peptide. And then you have the r 41 and k1 16 a mutants because, we know that interferes with the interaction, you can see they do interfere with the interactions. So, what is your take on these SPR sensor grams do you like it? Do you not like it? Is it ideal is it interpretable, what are the features that you see? What are the features you should see? And what are the features that you see? So, do you know how to interpret a sensor grams? What is the x axis? Time what is in the y axis? Are you units. Okay? And then what is the sensor grams telling us, there is an association phase and the dissociation phase what and in between the Association and the dissociation phase. So, when do you start associating, how do you determine for what time that I need to run the analyte, when do I stop and start the Association, very critical, can I say arbitrarily, I will run it for 10 seconds I know I just wash it off no. So, what determines it do?

We call it a saturation, saturation by definition is what? Saturation by definition is all the sites are occupied; will that happen when you have lower than cade lower, concentration and KD it won't. So, what do we call that as equilibrium, yeah? So, you need to achieve equilibrium, have you achieved equilibrium here? Maybe, maybe not maybe, but we should try and extend the time for equilibrium, this you learn by trial and error you fix some time for the different concentrations and as the time goes you improvise on that but this is not too bad. Okay? And what's happening with the dissociation? Is dissociation critical, I already got this why do I worry about that I just flushed, get all the analyte out of it should I wait for the dissociation, to happen, why is this association important very nice. So, what does it determine? What does dissociation determine here? Exactly the off rate. Right? That is so, what is so, in something important about the afraid, why should I, if I didn't have off rate in SPR, I will convert this into a normal equilibrium study where I get the KD values. Okay? It makes no fun, unless I get the K off. Okay? And that comes from the dissociation phase and how should the dissociation be, are you happy with this dissociation, criticized you are happy with the decision, why is that very nice, it is very slow. Right? So, if then if I want to keep do you think I'll ever achieve, complete dissociation in this case, why not? So, I will keep it for three days no. Okay? Yeah? But didn't imagine over the days it, will come like this. Okay?

Here it'll definitely come, but that's not the point. So, how do I now so, what should I do now? I won't get a good off rate from this. So, what should I do? And without afraid my kinetic estimations are not going to be. Right? Why is it why is it K off so, important, why can't I look at K if it seems very good and if I achieve the equilibrium I should get that K, what is so, unique about K off K on as diffusion control, if it is it, it is diffusional control and there is also it is controlled by another parameter. What is the difference between K on and K off? Excellent, the first one is concentration dependent K off is independent of the concentration, because now I am looking at a complex A B, it is there it does not determine by concentration.

So, the inherent property, of the binding, comes from this independent, variable that is K of independent constant, which is chaos and if I can determine that I my all my kinetic estimates are going to be more or less accurate. Right? And imagine I have the. Right? K of I have KD, which is an equilibrium constant I did determine by, n number of methods. Right? What can I get? I know K off; I know the equilibrium dissociation I can get K on. Right? I can get a rate constant for Association, independent of the instrument and can I go back and verify whether I got the same K 1 or not yes. Right? And do you think the KD that I determine by SPR that is a equilibrium dissociation constant, is going to be very different from the ones that I determined elsewhere, say for example analyser it shouldn't. Right? And how do I get an equilibrium constant from the SPR data, steady state measurement, how do I do a steady-state measurement? Different concentration and what do I plot? How does it look like? Very nice. So, you guys are experts here huh. So, then there do I need equilibrium or do I need saturation, do I need saturation very nice. So, equilibrium whether it is a kinetic measurement or equilibrium measurement you need, equilibrium and you need saturation, which is determined by what in SPR? What is a unit? Saturation.

The first and foremost thing that you do, before you start the SPR, there you go ARMA X. Right? So, why, should we, determine on ARMA X, what is the unique? Property of our max that allows you to, interpret your SPR data faithfully. So, suppose I said I want 400, RU as the ARMA X Right? I want 400 RU as the ARMA X value, I know the molecular weight of the ligand, I'm on know the molecular weight of the analyte and what is another parameter that is in the equation? Okay? Now, tell me, why should I go back and calculate what the ARMA X? Exactly. So, if I got something weird, then what I expected, there are two things that happen. Right? One, there is some weird nonspecific binding going on or two, the estimated stoichiometry is not correct, the second you come, you interpret later, the first thing that you should worry when you get a more, ARMA X than what you expected, because you know, what you immobilized? Right? And if it is 1 is to 1, no matter what you do? Those mathematical equations have to be satisfied. So, whether you understand the kinetics, whether you fit it into a one is to 1, binding or more complex binding isotones, the first, level of checkpoints, have to be done. Okay? So, when you're doing SPR you be careful about all these things, your calculations, you first do a predetermined calculations, you go back and look at your sensor grams, and see whether you, have got all these values correct, you estimate the equilibrium dissociation constant by even, if you can't get the kinetics, estimate the equilibrium dissociation find that your, binding interactions are very similar, to what you are obtained, by other studies? It is better to do SPR, after doing some, kind of measurements that tell you the equilibrium constant, is there any other technique that allows you to look at equilibrium constant, any by physical technique? So, you do ITC, you will get the stoichiometry there, yeah! It's very, good for Stoichiometric measurements and then you get the KD there as well and then you combine it with K on and K off, you get a delta s, Delta G, Delta H, the stoichiometry, KD on and off you are done. Right? So, that's a way, to prove the protein, protein interactions in depth and if you have a select example: that you started with the hypothesis and it has ended up, giving you the expected results, then you try and do all these parameters and see, whether either by screening, molecules to find the inhibitor or you start with the structure guided

design by docking or you start with already known peptide: that it binds and then you begin modifying the peptide, using chemists, help and then designing their molecules, so that you can get, better and better inhibitor. This is the reason, why we are doing SPR and so far it seems, you guys rightly, pointed out, what are the problems but, what is the, what is the positive aspect of this data? What's the positive aspect of the data? Hey, very good, you'll see some binding and then, what happens with the mutants? The mutants behave like they are expected to behave they do not bind very well. Right? Now, you see here, it's not so bad, we still fitted these binding kinetics and why do I say it is not so bad? Chi-square and why, why is chi-square important?

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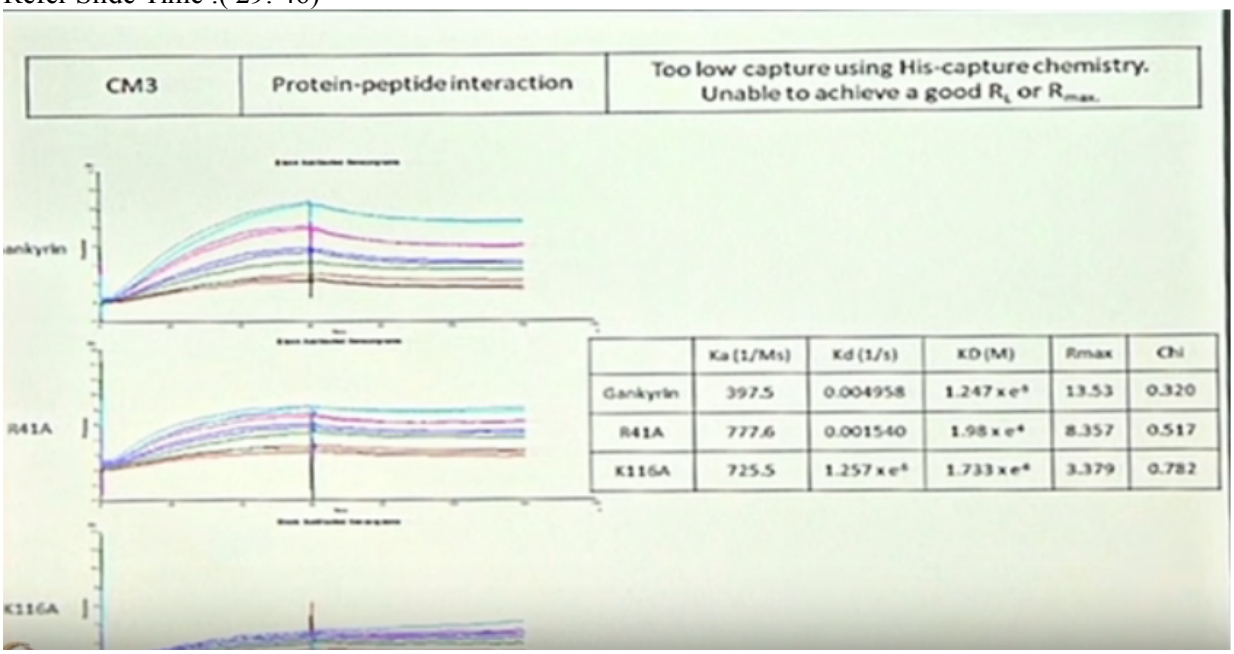

What is it that you look for? When you look at the chi-square, expected and observed and what should it look like? Residuals. Right? So, it is the deviation, from the residual that gives you the chi-square value, this is pretty type, so you can believe all these. Right? So, you look at the gam Karen and we get, KD is around, 12 micro molar, is it right? This is something that we expect around 12 to 50 micro molar is what we expect and the KA and KD and you look at the mutants. Okay? The mew it seems to suggest: that the mutants have better on rate. Right? And very, similar or a slightly, faster of, store afraid than concur in, the wild-type and you look at all these KD measurements and look at the ARMA X, despite poor binding, they seem to be behaving pretty well, in terms of the kinetic constant, we do not understand this, I'm showing you what the graph values are, but, we do not understand this yeah! We are trying to interpret this as to what may be, the problem and definitely we are not happy because, if you look at it is not very, clear to you from here, the it is and we are achieving up to 15 our use, on the binding and which is not very, good and we are not able to achieve ARMA X, because the chip whatever we have used, whether it is his tag, nth chips, or we have tried direct immobilization, we are having problems and nonspecific binding to the flow cell there seen and some of the times many times, some of the flow cells do not work and what we have, come to conclude, by looking at many, many interactions of this kind, changing the chemistry, you definitely need to change the chemistry, either it is a tile or it is about instructor or direct

immobilization or a capture, you need to do multiple things to confirm that these are behaving the way you should, so, what we have understood now is that, the IFC that the fluid Excel, itself is a problem and that reads a replacement every six months, costs about six lakhs. But, we are also trying alternative chemistry, as well on this and trying to reverse, so we took the streptavidin ship and try to bind the biotin, peptide and then come up with the protein, what is the advantage that I have in that case? The analyte is bound know: that is a biotin lifted peptide to the streptavidin ship and I am passing the ligand, instead of having the ligand immobilized and I pass, the analyte, which is a short peptide, which is better. Bigger molecule on the tip, how is the, how is RU generated? Why is it reverse, you finish your thought, exactly? So, when you design, experiments that you want to see, so here is where the sensitivity, question of sensitivity, comes in and if you are looking at very small molecules, it is better to have, the ligand and then you bind the analyte, but, G bosh of T 200, to be able to capable of detecting small molecules, but once probably we, get these things going straight and especially, the fluidic cell, we should be able to see these things happening. But yes: that's the main point

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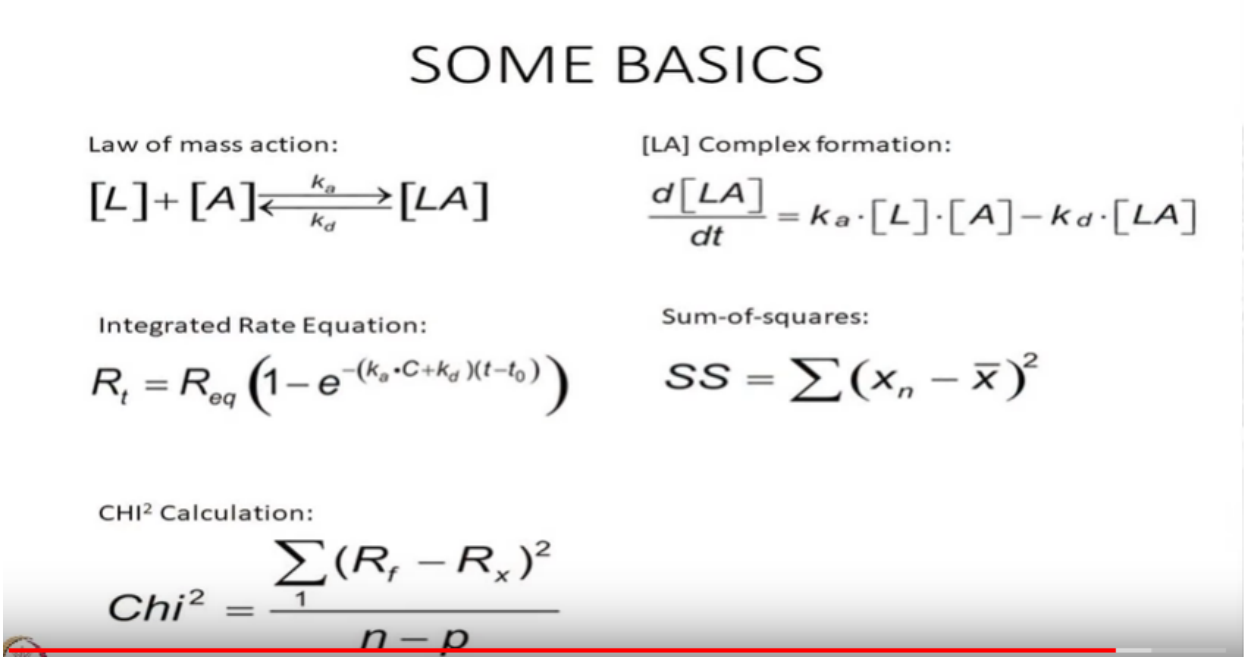

and these are before, you start an SPR experiment just, keep these things in mind, you calculate, you find the expected values, go back interpret your sensor gram, don't try to fit the data, before understanding the sensor gram, you can fit it to any, equation that you want, you will get a value, you will get a chi-square, never ever do that, most often, it is the simple one as to one binding: that is happening, just because your sense of you did not do the experiment right, a second-order fit or a third-order fit might give you a very bad, very good fit, because now you are increasing the parameters to fit, so the your sensor grams will look nicely fitted, but that may be your wrong interpretation, most often it is, the first one is too binding that you should try, if there are after doing many, many experiments you think: that things are not explained, by one as to one binding, then you begin to use, other equations on this. So, these are some

things I definitely have to look at, you have to look at equilibrium, try to look at and how do I, how do i enhance dissociation, can I, enhance dissociation, i can change the PH, can i tell you anything else? Flow rate, very nice, anything else, why should I change PH? What happens if I try to PH? Why should the interaction get weak, I change the proto nations. Right? So, what is the other way of doing it, dissociating two proteins? Salt. Okay? Then I'm speaking to experts, there was my last site.

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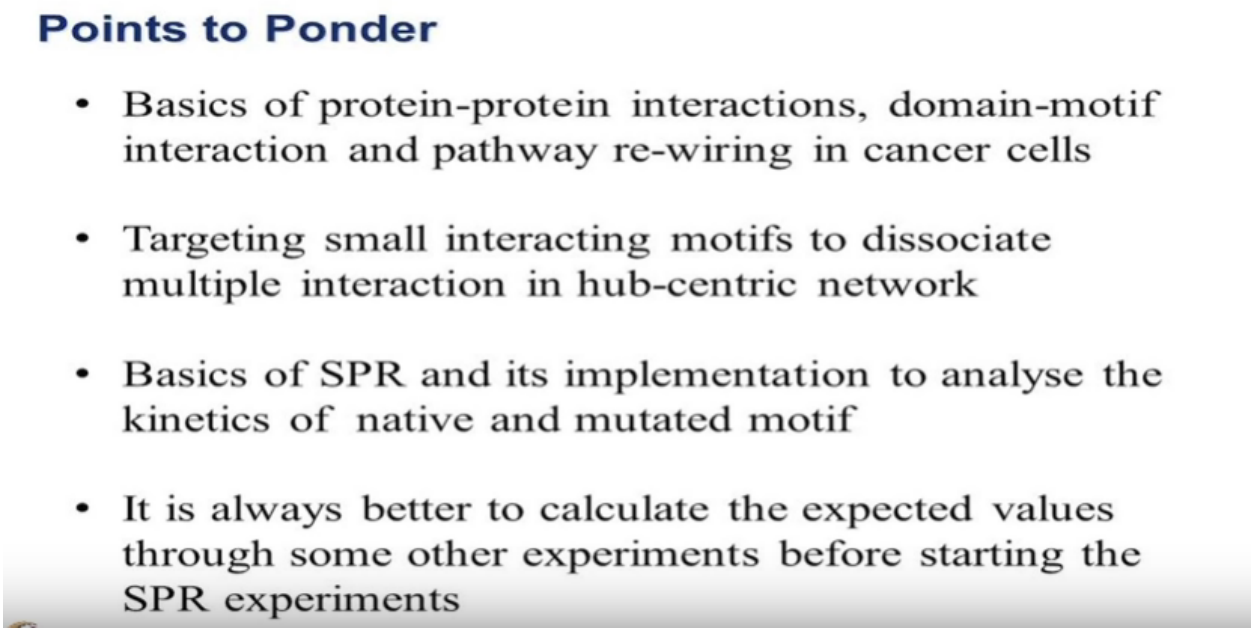

Thank you very much. So, I hope, now you're convinced: that SPR based systems, is a very powerful platform, to generate high quality data, for bio molecular interactions, especially to obtain, the dissociation constant the KD values, on rate, of freight the kinetic data, which could provide you, very, quantitative valuable information, we'll discuss about, some other technology platforms, in the next lectures. I hope till then, you will study the SPR and associated, label free bio sensor technologies, in more detail. Thank you.