ELIZABETH NOLAN: We're going to move on with GroEL/GroES and a few more comments about where we closed yesterday and then talk about experiments that were done to determine what polypeptides are folded by this machinery.

So I'm just curious. Has anyone stuck trigger factor or GroEL into PubMed to see how many hits you get? Yeah, so Rebecca's question yesterday, or on Monday, about trigger factor and active versus passive folding motivated me to take a look. So just to give you some scope, if you put trigger factor in PubMed, as of last night, there's 11,810 hits there. GroEL is closer to 2,000 to 3,000-- in that range.

If you put trigger factor active folding, you end up with 34 hits. Most of those are about using trigger factor in protein overexpression. So if you also express trigger factor, does that help? And it looked like there was one paper in those 34 that suggests an active folding role for one of the domains. But that is just looking at an abstract.

And so, the point there is there are many, many studies that consider these chaperones and a huge literature to search. So what we're able to cover here is really just the tip of the iceberg for that.

There's also a new review out on GroEL/GroES, which is not required reading, but we're posting it on Stellar. So it just came out last month, and I really enjoyed reading this review. I thought they did a very good job of talking about current questions that are unanswered yet in terms of models and presenting different models for how this folding chamber works-- so passive versus active, for instance. And they also give a summary of the substrate scope-- so the experiment we'll talk about today.

So where we left off last time, we went over the structure of this folding chamber and here's just another depiction of the overview. So effectively, we have to back-to-back heptamer rings as shown here. Some polypeptide in its non-native state can bind. It initially binds up at the top by these apical domains, and there are some hydrophobic interactions.

OK, ATP also binds, and we have all seven ATPs found within one ring, the ring that has the polypeptide.

OK, we see the lid come on, and then this polypeptide has some time, a residency time, in this chamber to fold. And then after the residency time, which is generally quoted on the order of 6 to 10 seconds, the lid comes off, and it gets ejected. And during that time, the ATPs are hydrolyzed. So somehow, this ATP hydrolysis gives conformational changes that drive this cycle. OK, and then we see, again, we flip to having function in the other ring.

So one point to make involved cooperativity, so I hope you've all seen cooperativity before, probably in the context of hemoglobin. We have examples here of positive cooperativity and negative cooperativity. So within one heptamer ring, ATP binds to all seven subunits. So that's positive cooperativity.

And then we can think about negative cooperativity between the two rings, where we only have ATPs bound to one ring. So the other heptamer ring will not have ATP bound here.

So what is happening inside this chamber? The polypeptide enters the chamber, and it's given this protected environment to fold. And we saw that when the GroES lid comes in that the hydrophilic nature, hydrophobic nature of the interior changes. And it becomes more hydrophilic.

So I just want to point out-- and this also builds upon Rebecca's question from last time-- is this passive folding in the chamber so effectively in Anfinsen's cage, where the primary sequence dictates the trajectory? Or does the actual chamber itself play a role? So that would be active folding. And effectively, is there forced unfolding or refolding by GroEL itself?

So perhaps the apical domains can force unfolding before polypeptide is released into the chamber. And the cartoon that was just up indicated that to some degree. Maybe the cavity walls are involved.

And what I would say is that the pendulum on this has swayed quite a bit over the years in terms of whether or not GroEL is a passive folding cage or actively involved in folding. And some of the debates in the literature have resulted from experimental set-up that may bias results to indicate one thing or the other. And that's something the community is striving to work out these days. And I'll talk about that a bit more on the next slide. But I'll just note--

these questions are still there, and the recent review I just noted discusses these questions.

There was a study just a few years ago that was performed with very dilute polypeptide substrate-- so below one nanomolar. And what they conclude from this study is that GroEL is involved in active folding of a maltose-binding protein mutant. One question I'll just spring up with this is, maltose-binding protein is a nice model polypeptide, but what happens for a native GroEL substrate? And is there utility in studying those?

So why have I emphasized this dilute protein sample point here? So what happened in some early work, in terms of studies that were done to try to differentiate active or passive folding, is that there were some complexities in in vitro studies.

So, here, I just have a cartoon of folding in the chamber. And if we think about only one polypeptide within the GroEL chamber, it's folding in isolation. So there's no possibility for it to form an aggregate or a ligamer with other polypeptides. It's all alone here. So this folding in the chamber avoids the complications of the folding landscape we talked about in the introductory lecture to this module.

So what happens in aqueous solution, right? There's the possibility that, depending on your conditions, maybe there's some sort of aggregate that forms. And if this aggregate forms, what does that mean in terms of what you see?

And so, in earlier work, there were some in vitro kinetic studies that indicated GroEL accelerates folding relative to folding in dilute aqueous solution. But some of these comparisons weren't appropriate, because as it turns out, oligomerization might compete with what you're watching for. And so, if there's some oligomerization happening, it might indicate that the rate is slower than you think.

So there's ways to monitor for this. And it's just a point in terms of what control studies do you need to do to make sure your experimental setup is appropriate there. I think it'll be exciting to see what's to come in future years about this question and what kinds of biophysical techniques are applied, including single-molecule studies here.

So where we're going to go, moving on, is to think about what actually are the substrates for GroEL. So what polypeptides get folded in this chamber? And how do we begin to address that question from the standpoint of what's happening in the cell? OK, so first, we're just going to consider some observations. And then we're going to go into the experiments here.

So here are some observations. So the first one is that polypeptides, up to 60 kilodaltons, can fold in this chamber. So that's quite big-- 60 kilodaltons.

Some proteins or polypeptides need to enter the GroEL multiple times to be folded. So that means the chaperone has the ability to bind and release and re-bind the polypeptide here.

So when studies are done in vitro, what's found is that almost all polypeptides interact with GroEL. So you just saw even an example of that in terms of this non-native maltose-binding protein. So many polypeptides will interact.

And this really contrasts what's observed in the cell, where, in vivo GroEL is involved in only folding about 10% of E. coli proteins here.

OK, so what observations three and four suggest is that GroEL has some preference for particular endogenous polypeptides. And what we want to answer is, what are these polypeptides, and what are their properties here?

OK, so Hartl's group did some nice studies to look at this, what needs to be done. First of all, there needs to be a way to isolate the polypeptides that are interacting with GroEL in the cell. And then, once these polypeptides are isolated, they need to be analyzed in order to learn about their identity and properties.

OK, so we're going to look at experiments that were done to address this. And they involve pulse-chase labeling of newly synthesized proteins, amino precipitation, and analysis here. So in terms of addressing what are these substrates, we're going to begin with pulse-chase labeling.

OK, so basically, the goal of this experiment and why we're starting here is we want to determine which proteins interact with GroEL. And, in addition to which proteins, we want to determine how long they interact.

OK, so what is the experiment? These experiments are going to be done with like E. coli cells. So we want to know what's happening in the cell.

So imagine we have an E. coli. And so these bacteria are grown in some culture medium. And the trick here is that they're going to be grown in medium that's depleted in methionine. So incubate, or grow, in medium with no methionine. OK, so effectively, we're depleting them of that amino acid.

OK, so then after some period of growth, what are we going to do? We're going to spike the culture with radiolabeled methionine. And this is the pulse.

So we're going to add 35S methionine. And we're then going to incubate for 15 seconds. OK, and so that's the pulse with a radiolabeled amino acid.

Then what are we going to do? And after we go through the steps, we'll go through why. After this stage, we're going to add excess unlabeled methionine. And we're going to then continue this culture for 10 minutes. OK, this is the chase here.

And during this chase period, basically, samples will be taken at varying time points. OK, and then, at some point, we're just going to stop this. OK, so just say, stop culture and experiment.

So what's happening in each of these steps? And why are we doing this?

So what we want to do is think about newly translated polypeptides. OK, so we have a living E. coli. It has ribosomes. And these ribosomes are going to be synthesizing polypeptides over the course of this experiment.

So during the pulse period, all proteins, or all polypeptides, synthesized are radiolabeled. Right, because the methionine has been depleted from the culture medium. And so effectively, the methionine that these organisms are seeing are the S35-labeled methionine. And all polypeptides have an informal methionine from the initiator tRNA and what other methionines are in the sequence.

So, if we think about doing this for 15 seconds, and we think about the translation rate, which I gave as 6 to 20 amino acids per second when we were discussing the ribosome, we want to think about how long are these polypeptides going to be? So we have a translation rate of 6 to 20 amino acids per second. OK, so, if we think about 15 seconds of a pulse, we're getting polypeptides on the order of 90 to 300 amino acids synthesized during that time.

So newly synthesized polypeptides in these 15 seconds are radiolabeled. What happens next?

OK, we have this chase period where we flood the system with unlabeled methionine here. Why are we doing this? So certainly, there are some polypeptides that are longer than 300 amino acids that still need time to be synthesized. And if there's new peptides being synthesized that start in this stage, we won't see them, because this unlabeled methionine is in

vast access over the radiolabeled methionine that was added early.

So here, we have, the synthesis of larger polypeptides can be completed. And we have, no longer producing radiolabeled new peptides. OK, so this allows us to only see the peptides that were radiolabeled during this pulse period here.

So what are we going to do in terms of the sampling at various time points? So let's say we want to sample at one minute, five minutes, ten minutes. What do we need to do? So can we just aliquot some of these E. coli and put them on our bench?

We could, but that's not going to be very helpful to us, because what we want to do is stop the translation machinery and all of the cellular machinery here.

AUDIENCE: You need some kind of clench?

ELIZABETH NOLAN: Yeah, we need a clench. And not only do we need a clench, we're dealing with a living organism too, right? So we need to break open the E. coli in whatever this condition is to stop the reaction.

OK, so we're going to take aliquots at varying time points. And basically, we care about time, so you have to immediately lyse, or break open, the cells. And this was done in the presence of EDTA.

So what is EDTA?

AUDIENCE: Ethylenediaminetetraacetic acid.

ELIZABETH NOLAN: Yeah, ethylenediaminetetraacetic acid. So it's the chelator. And why might this lysis be done in the presence of this metal chelator?

AUDIENCE: [INAUDIBLE] processes like-- [INAUDIBLE] magnesium, which would help [INAUDIBLE]

AUDIENCE: Are the proteases that are not binding?

ELIZABETH NOLAN: There certainly are zinc proteases. So that that's one class of protease. So EDTA will chelate many, many different metals. The main point here is we want to stop stop translation, shut down processes here.

OK, so we have these samples. What do we need to do next? We need to detect these newly synthesized proteins that interact with GroEL. And we want to do this at each time point.

So how are we going to do this? We have a very complex mixture that has all of the cellular components.

So the next step in this will be immunoprecipitation. And so, what will happen in immunoprecipitation in these experiments is that the researchers had an antibody that binds to GroEL. And this antibody was put on a bead and used to fish out GroEL from this complex mixture. And we need to talk about these antibodies a little more.

But just in starting, I imagine there's a bead. And we think about antibodies as being Y-shaped biomolecules. So here, we have a GroEL. And imagine that, in this mixture, we have GroEL that has some polypeptide bound. That's one of its endogenous substrates.

So, if these are mixed together, then the antibody binds GroEL with the polypeptide attached. OK, here, we can imagine "capture" of this species here and using the bead to separate, say, by centrifugation.

So let's think about this a little bit and a little background to have everyone up to speed. If you need to learn more about antibodies, please see a basic biology textbook for further details. But these are Y-shaped molecules that are produced by a type of immune cell called B cells. And they're used by the immune system to detect foreign biomolecules and help to neutralize them.

And so, in these, the tip of the Y contains the paratope that ideally binds specifically to a particular epitope-- in this case, GroEL here. And so, we often think about a lock-and-key model with antibody and think about the antibody binding its target with precision here.

So for these experiments that were done, just realize the researchers had to come up with an antibody to GroEL. How is that done? They may have immunized, say, a rabbit or given a rabbit GroEL and allowed that rabbit to produce antibodies. And then they isolate the antibodies here. So something we want you to take home from this course is, yes, the antibodies should bind the target with precision. But there's huge problems in terms of use of antibodies in research.

This is just the start of an article that was published last year around this time. And it's focused on pharma and clinical trials. But this is much more broad. And often, antibodies aren't as specific as indicated by the label on the container from the supplier here. And it's pretty dismal

what they quote in this terms of how difficult it is to reproduce data here.

So if you're going to use an antibody, you always need to test it to see whether it is selective or not for the species of interest that you want to detect there and have that information on hand so you don't misinterpret your data here for that.

So what are the steps for this immunoprecipitation? Basically, as shown on the board, beads will be functionalized with the antibody and then just added to the cell lysate. And the antibody can recognize GroEL. And the goal and hope are that whatever polypeptides are associated with GroEL are pulled down together.

So that's something a bit incredible here that these polypeptides remain bound to GroEL during the steps of this process. You can imagine, if there's a low-affinity binder, it could be lost.

So the sample can be centrifuged. And then, you can isolate these beads here.

So, in cartoon form, a complex cell lysate in your microcentrifuge tube. You can add the antibody, centrifuge. And see, down here, we've pelleted the beads with GroEL attached. And then some sort of workup needs to be done to dissociate the protein, or polypeptide, substrates here. And then they can be analyzed.

AUDIENCE: How long do they do that for? Do you know how many--

ELIZABETH NOLAN: How long do they centrifuge for?

AUDIENCE: No, for the immunoprecipitation. Is it 30 minutes? Is it--

ELIZABETH NOLAN: I don't know how long the incubation time is. Need to go back to the experimental, but that's getting right back to this question as to how do they stay bound.

AUDIENCE: How do they stay bound?

ELIZABETH NOLAN: Yeah. So, see the point here. If you have a high-affinity complex, that's one thing. If you have low-affinity association between GroEL and the polypeptide, you can imagine it might get lost during this workup. And how much do we know about those affinities there?

AUDIENCE: You said that they would just give rabbits GroEL, and hopefully antibodies would just happen.

But if a rabbit's immune system encountered GroEL, would it actually see it as an antigen that it had to develop antibodies against?

ELIZABETH NOLAN: So, yeah. So here's the point-- would it? So, if it's E. coli GroEL, would the rabbit recognize this, yes or no? And if no, then what can you do to provoke an antibody response?

And so, what can be done is, say, you could take a GroEL subunit and attach that to something immunogenic. So there are carrier proteins that will mount an immune response.

So one of the subunits of cholera toxin is an example that can be used. And then the idea is you're mounting an immune response against that carrier protein. But you'll also get antibodies to whatever is attached. So that's another strategy for doing it if direct injection doesn't work.

And too, not going off on a big tangent, but there are some decisions that need to be made. So would they use the full-length GroEL? Or maybe they would just use a polypeptide region, like some shorter polypeptide that's a portion of GroEL. So there's a lot of possibilities there in terms of what you use to generate the antibody for that there.

And it's something that a lot of companies do these days. You can send them your protein or your polypeptide fragment. And they'll conjugate it to one of these carriers and treat the rabbits or whatever animal and then isolate those antibodies. And then they need to be characterized there for that.

OK, so how are these samples going to be analyzed? That's the next step. So, for the analysis, effectively, we're going to have some mixture. And, at the onset, we don't really know how complicated this mixture will be.

I told you initially that about 10% of E. coli polypeptides are thought to be substrates for GroEL, which is quite a large number if we think about the total number of proteins in E. coli. And the other point is we have this radiolabel, which we're going to use for detection there.

OK, so, for analysis-- OK, there's two things. We need to separate these various polypeptides in each sample. And then we need to determine what their identities are here.

So-- that were bound to GroEL from one another. OK, and then, we need to determine identities. And once we know the identities, we can think about their properties. And this needs to be done in every sample that was collected along this time course, which is also going to

give some temporal information.

So what are the methods that have been used? So, in order to separate the proteins in this complex sample, the method is a 2-D gel-- so 2-D gel electrophoresis.

OK, and in terms of determining the identities, what's done, once these polypeptides are separated, is to do a protease digest and then mass spectrometry.

Has anyone here ever run a 2-D gel or seen the equipment? One person. Has anyone heard of 2-D gels? Fair number.

OK, so, we'll go over this briefly in terms of 2-D gel.

So, in terms of 2-D gel electrophoresis, we talk about running these gels in two dimensions. And, in each dimension, we separate based on a different property.

So, in the first dimension, the separation is based on charge. And effectively, we can talk about the pI of a protein. So the pI is the isoelectric point. And it's the pH where the net charge on the protein is zero.

And so, the type of gel we use here is called isoelectric focusing, or IEF. And effectively, what's done is that the gel electrophoresis is done through a continuous and stable pH gradient. And, in this gel, the protein will migrate to a position where the pH corresponds to the pI. Then the anode is low pH and the cathode high pH. So that's quite different than SDS, where, in an SDS-PAGE gel, we're coating the protein with negative charge.

So then, the second dimension is something most of us are familiar with, is SDS-PAGE. And so, what happens in SDS-PAGE? We have separation based on size here-- on molecular weight.

So has anyone not run an SDS-PAGE gel? And this is totally fine. I never ran one till I was a postdoc. So it's not something to be ashamed about if you haven't. OK, so everyone has.

So what's the ratio of SDS molecules to amino acids? So if you take your protein sample and you put it in your loading buffer and run your SDS-PAGE, what is the ratio of binding?

What is SDS?

AUDIENCE: Sodium dodecyl sulfate.

ELIZABETH NOLAN: And what does it do? What happens to your protein in SDS?

AUDIENCE: Denatures it.

ELIZABETH NOLAN: OK, what else? So it's a denaturant. So it denatures the protein. So why does SDS-PAGE let you separate based on molecular weight, more or less?

AUDIENCE: It coats the protein, more or less, uniformly with negative charge.

ELIZABETH NOLAN: Yeah.

AUDIENCE: Do we know the exact ratio of binding?

ELIZABETH NOLAN: Yeah, so what's the ratio of binding that can be done in terms of grams of SDS per grams of protein or number of SDS molecules per amino acid. What is it? And there'll be some error, but there's approximates. But it's something to think about, right? You're putting your sample into this.

So it's about 1.4 grams of SDS per gram of protein. That's the ratio there. And as said, the idea is that SDS is giving the protein a large net negative charge. So it's going to override whatever the intrinsic charge is of the protein. And so, it gives all proteins a similar mass-to-charge ratio here.

With that said, sometimes, there are proteins that migrate in the gel in a manner that's not reflective of their molecular weight. That's just something to keep an eye out on. So within the slides that will be posted on Stellar, there'll be some background information about both of these methods-- the IEF gel and SDS-PAGE, which I encourage you to take a look there.

OK, so back to the 2-D gel-- how is this actually going to be run? So it's one gel. First, it needs to run the IEF gel. And you need a special apparatus for. This it's called a cylinder, or tube, gel-- so not flat like what you're all accustomed to for SDS-PAGE.

Then, this gel needs to be equilibrated in the SDS-PAGE buffer. And then, you run the SDS-PAGE separation. And, in this step, just to note, the gel is rotated 90 degrees.

OK, so what you get-- you get a gel where we have molecular weight here. We have pI here. And if it's a cell lysate, there's going to be many, many spots. These should all be spots unless

you did a poor job running the gel.

So this 2-D gel is being used, because it's going to provide better separation than a standard 1-D gel. Imagine trying to separate peptides out of some cell lysate using just a 1-D gel. Even after this immunoprecipitation, we'll see that these samples are very complicated here for that.

So what we need is some way to detect the spots that indicate different polypeptides. So what are methods? Maybe Coomassie stain for total protein. We can use the radiolabel-- autoradiography, for instance, which is what's done here. We're looking at the S35 radiolabel-- or maybe Western blot here.

So how are we going to get from this gel to knowing the identity of each of these spots?

AUDIENCE: You have to identify your spot, excise it, extract the protein from the gel, adjust it, and then run NS and line it up with known protein for evidence.

ELIZABETH NOLAN: Exactly. So what will be done is that each spot of interest will be cut out of the gel. So you need a way to mark them. You'll see they're numbered in the data that we'll look at. The protein needs to be extracted out of the gel.

Then the protein will be incubated with a protease that will give some number of fragments. Trypsin was used in this work. And then that digest can be analyzed by mass spec. And so, for each sample, you get all of the m over z values for the different polypeptides that resulted from the digest. And then, effectively, you can compare that to some database of E. coli protein sequences.

So further details are provided throughout here. So what are the major questions? And what are we going to look for answers for in the data here?

So first, how many proteins interact with GroEL? We can imagine getting an answer to this by counting the number of spots. What are the identities and structural features and properties of the proteins that interact with GroEL? We're going to get that from the mass spec analysis and then literature studies.

And then another question we can get at is asking, how long do proteins interact with GroEL? Because we're calling the pulse-chase samples were taken at various time points over that 10-minute period. So, at two minutes, do we see the same polypeptides associated as we see at 10 minutes? Or if we monitor one given polypeptide, when does it show up and potentially

disappear from the gels?

So all of these samples can be addressed with these methods. And where we'll begin on Friday is going through the data in some detail. But just as a prelude to that in the last minute, here's the data from the paper for these gels.

So this is looking at the 2-D gels for, on the top, total soluble cytoplasmic proteins at zero minutes and then total cytoplasmic proteins at 10 minutes. So this is without the immunoprecipitation. And then, at the bottom here, what we're looking at are the polypeptides that we're isolated from the immunoprecipitation with the anti-GroEL antibody at zero minutes and 10 minutes.

And so, before we meet next time, what I encourage you to do is take a close look at these gels and see what information can you pull out just from a qualitative look. So simple questions, like, we see a lot of proteins here. And please don't go and try and count all the spots. I'll give you the numbers next time.

How do these gels here from the immunoprecipitation differ from these up top? And it's not just the total number of proteins. There's some additional subtleties in these data.

OK, so next time we'll begin examining these data, looking at what polypeptides were pulled down. And then we'll move into looking at the chaperone DnaK, DnaKJ system there.