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**JOANNE STUBBE:** The last time, we were finishing the first part of the reactive oxygen species module, focused on how we as humans fight bacterial or viral infections using neutrophils, the white blood cells. And I introduced you to this cartoon. So here's our neutrophil, all of this blue stuff. It has an unusual-looking nucleus. Neutrophils have weird nuclei. They somehow sense the bacteria you saw, the bacteria getting chased by the neutrophil.

And then somebody asked me this question. Somebody asked me this question last time about where the NOX2 proteins end up. And let me reiterate again that they can be found in multiple membranes. The predominant membrane in neutrophils is in these little vesicles within the cell, OK, but the bacteria out here, so they need to somehow engulf the bacteria. So they can also be found in the plasma membrane. And they need to engulf the bacteria to form phagosomes.

And so here you see them again. So this is the phagosome with the NOX2 protein, with this predisposition where the NADPH is in the cytosol. OK? So you see that the location here, the NADPH is also in the cytosol. And so you need to think about where you're located. And we'll see in a few minutes that there are lots of different kinds of NOX proteins, and they all have different locations and all have different factors that control the regulation of all of these things.

OK, so what I want to do today is start out by looking, talking about the NOX complex, what the chemistry is, because it's not only involved in killing bacteria, but we'll see-- and you've already seen in last week's recitation-- in the signaling process. It's the same protein. And so once the bacteria are engulfed, you now have a phagosome within the neutrophil. And here's the NOX complex. Remember, it's composed of two proteins, the 91-kilodalton glycoprotein and a 22-kilodalton protein, both membrane-bound. I drew those on the board last time. And the NADPH/NADP is in the cytosol. But all the chemistry is going to happen in the lumen of the phagosome.

OK, so somehow the reducing equivalents from the NADPH need to be transferred into the

lumen, where oxygen is going to be reduced to superoxide. So I want to talk a little bit about how that happens first, given what we know about the cofactors in NOX2 that are essential for this process. And then what we'll do is look a little bit at how the superoxide that's generated in the phagosome gets converted to hydrogen peroxide, and ultimately with another protein we're briefly going to discuss, a heme-dependent protein, myeloperoxidase, which uses chloride, can form hypochlorous acid. OK? So that's where we're going.

This is a cartoon I drew on the board the last time, where you can see that we have a flavin domain, a flavin domain that's located in the cytosol. And then you have two hemes. And the unusual part about this system is that these hemes-- we've seen heme before with reversible binding of oxygen in hemoglobin-- they're both hexacoordinate. So there's no binding site for oxygen. So the chemistry is not happening by reversible binding of oxygen to the heme. In fact, you know, how close this is located to the surface, we don't have any structure of this, but somehow this reduction has to occur by an electron transfer process that probably occurs through the edge of the perforin system.

So what you have here-- and I think this is an important teaching point, because I think there are only two ways inside the cell that you control all the redox balance. The redox balance within NADPH and flavins really play a central role in everything, so you really need to understand how these cofactors work, and how they're controlled. And so if you have a flavin-- and this is also written-- you don't have to write this down. This is written on the next handout, so you don't have to draw all this stuff out.

OK, so I'm going to draw the business end of the flavin. OK, so this can be flavin adenine dinucleotide. So this is the oxidized state. And the two important places where redox chemistry, but look at flavin. If you don't know anything about heterocyclic chemistry, it's confusing. But in fact, we know a huge amount about flavin chemistry from studying model reactions in organic chemistry. Decades ago, Tom Bruce did that.

And so the two places where the chemistry in general happen are either the inside-- so this is 1, 2, 3, 4. This is the N5-- or the C4A position, and this is the C4A position here. And I'm not going to go through this in detail but this is the oxidized form, and what we have is the reaction of this oxidized form with the reduced form of NADPH. And I'm not going to draw out the whole structure here either. But hopefully you all know now that NADPH and general works by hydride transfer, so it's almost always a two electron transfer. The one electron chemistry is really outside the realm where it would normally happen inside the cell.

So basically the chemistry involves transfer of a hydride, a hydrogen with a pair of electrons to the N5 position. And so you go from the oxidized state to the reduced state. And so again I'm not going to draw out the whole flavin. The rest of the-- I guess I don't have a picture there but-- well, let me just show you where we're going.

So the key is, which is interesting about this, we need to get across a membrane. So how do you get across a membrane? So the flavin domain is way over here, and we need to have two hemes. Remember you can do electron transfer over 10 to 15 Å with very fast rate constants. Somehow these reducing equivalents from NADPH need to be transferred to the flavin. And the major function of the flavin inside the cell is to mediate two electron one electron chemistry.

And here's an example that. The two-electron chemistry is being provided by the NADPH as a hydride. But what we have to do is the hemes in this system are in the plus 3 oxidation state, so what we need to do is be able to convert-- ultimately we want to reduce oxygen to super oxides, so we need an electron. So that electron is coming from NADPH.

So we need to have an electron transfer-- a single electron transfer to the heme because it only can-- iron can only be reduced by one electron. So we end up then with this system. So this is the reduced state of the flavin.

And you can draw a resonance structure. This is deep-- you can draw all kinds of resonance structures with flavins. That's why I can they can do one-electron chemistry. So one-electron chemistry, you can make the one electron oxidized a reduced state depending on which state you're starting in and the electrons are delocalized. They turn out to be blue, or they turn out to be red depending on the protonation states.

So what you can then do is-- let me just write that over here. So I'm just drawing a resonance structure of that so you can see-- let me not. And again let me just show you that that's there so you don't need to write that down. You don't need to write this down. It's all there. So just pay attention to me.

So you're going to do an electron transfer to reduce an iron 3 to an iron 2. Then you've got another iron 3 because we got to get through this membrane. So that heme-- and again, this is where the redox potentials become critical-- can transfer an electron because now in the reduced state to the other heme so that becomes in the iron 2 state. So the one out here is in

the iron 2 state. Now it can transfer electron from oxygen to form super oxide.

But at the same time during this process, we're transferring the two electrons that the Flavin received from the NADPH one at a time, so then we can repeat that process. And that's why you get the stoichiometry of the overall reaction. You get two super oxides produced.

So you have a resonance form of this. Let me see. I think I need to write over here. So anyhow you have a resonance form of this where you--

And now we're ready to do-- so this is the same as-- so this is a resonance form. These are the same structures.

In the flavin, this is attached either to adenine or to a ribose biphosphate FMN versus FAD. And now what you're ready to do is you have the heme. And so the heme again is embedded in the membrane. And so now what you're doing is electron transfer.

So you do an electron transfer reaction and you get this structure. So make a big dot for the radical.

And now we've reduced one of the hemes to iron 2. And if you look at the redox potentials-- I haven't ever read these original papers-- but they're close to being matched in terms of redox potentials. I haven't read how they measured these kinds of things, but the system needs to be set up so that you can do transfer the electrons across the membrane and ultimately reduce the super oxide over here.

And so now what happens so you've gotten to this stage. So now you have a semi quinone form of the flavin. this guy can then be re-oxidized by the next heme, generating the iron 2 form and regenerating the iron 3 form. So this guy then-- so let's-- to distinguish between them-- again I don't think this-- hopefully most of you were seeing this, but you have another one, so it donates an electron to covert the iron 3 form so we have an iron 3 form to the iron to form. And it itself becomes re-oxidized.

And so now what's happening is you're set up to do another electron transfer where this is going to go to the iron 3, transfer it again, and in the end this guy is now in the lumen-- adjacent to the lumen. I don't know where it's located. We don't have a structure but this guy is probably through the heme going to convert oxygen into super oxide.

So is everybody following that? The main point here is you all hear about the flavin being the

major mediator between one-electron chemistry and two-electron chemistry. Most of the time people don't draw out the details of this, but these things can all be observed spectroscopically because they're colored. Yeah. The second iron reduction happens from the semi [INAUDIBLE]?

**JOANNE STUBBE:** The second iron-- yeah. Happens from the semi--

**AUDIENCE:** So you generate your process.

**JOANNE STUBBE:** Right. Right. So you regenerate the oxidized form, so in the end over here, you go all the way through this. And again I haven't looked at the kinetics in the paper very carefully, but it very efficiently does this and shuttles the electron across.

So you're doing the same thing. You're reorganizing the reduced form of the flavin, but you're doing it one electron at a time. So here's the key take home message. So all of this then happens in the phagosome.

And so what you're generating then is superoxide, OK? Now what happens to superoxide? So superoxide could potentially do chemistry, but we talked about, last time, what are the properties of superoxide? It's not all that reactive, and frankly having read a lot of papers, I think we don't really understand all the details of how the bacteria die when they're engulfed by the phagosome-- but a key player in all in this overall process.

And it's certainly not the only player, because you can actually wipe out my myeloperoxidase, and you can still kill bacteria. So it's much more complicated than what I'm telling you, but a key player in when most people describe this is myeloperoxidase, which is a heme protein. I'll show you that in a minute. But it turns out that these myeloperoxidases exist in little granules. Just like you saw the little vesicle with the NOX2 it, that was predominantly sitting inside the neutrophil, you also have little vesicles. And the vesicles are stuffed with myeloperoxidase. And somehow there's a signal, and the myeloperoxidase then fuses with the phagosome. So this is a phagosome, and you have a huge amount of protein in there. It gets dumped into the phagosome, so you have a heme protein.

And that one's dumped in here. Inside the cell, you generated a gradient, so there's some complicated independent reactions. You need to sort of neutralize the pH, which happens. But once you get inside the cell of the myeloperoxidase, the protonation state is such that you can rapidly protonate superoxide to hydrogen peroxide, and we'll see that hydrogen peroxide

reacts with myeloperoxidase, which then reacts with chloride which is also present. In the hypochlorous acid, we'll see is a key player, and how can you tell that? Because if you isolate the proteins that come out of the phagosome, they're all chlorinated. So you generate-- if you go back, and you look at the little sheet I showed you about reactivity-- hypochlorous acid is really reactive. It's reactive, very reactive kinetically and also thermodynamically.

OK, so the myeloperoxidase-- so once we get-- so we've gotten our superoxide, so now we're in the phagosome. And now we want to look at myeloperoxidase, and so that catalyzes the reaction of hydrogen peroxide and chloride to form hypochlorous acid. And this is myeloperoxidase, and it's a heme dependent protein. And so the question is how does this work? And so what do we know about myeloperoxidase? People have been studying this for decades, and you're going to see the chemistry is actually quite complicated. It's very important, so people are always trying to figure out the details of the chemistry. But the devil is in the kinetics in the environment of the phagosome, so it's not so easy to sort all this out.

But if you look at the structure, number one, you see this is-- this is from an X-ray structure. It's bent, so it has an unusual structure. It has an axial ligand that's a histidine, and there's no second axial ligand. And it's covalently bound in two places. There are parts in the heme that are hanging off the protoporphyrin IX, where it's covalently bound to the protein, and the covalent attachment's distinct from most of the heme. So that's all you need to know, in terms of what we're going to be talking about.

So you have a heme protein, and most of the time I don't talk about the heme systems. But I think the heme systems-- you guys ought to know something about hemes. We've talked about hemes with reversible oxygen binding. You've seen hemes in cholesterol biosynthesis. In many of the natural products, biosynthetic pathways, you have hemes that do hydroxylation reactions or epoxidation reactions. Hemes play a central role in many reactions inside the cell.

And this one, the general reaction, I think is pretty straightforward to understand. So what you have-- and so again this straight line is protoporphyrin IX, so I'll just write protoporphyrin IX. And again, it's ligated to histidine, so this is part of the protein. And there's no second axial ligand. You take the superoxide which rapidly disproportionates, so this can be rapidly disproportionated in the presence of protons to form hydrogen peroxide and oxygen gas. And we've already gone through that reaction.

And so now what happens is the peroxide is going to bind to the heme, and so this is the key

to the reaction. So you lose a proton, and the oxygen binds to the heme. And you generate that species. Now this species-- and again, this is dependent-- this is where it becomes distinct when trying to think about all the chemistry that hemes can do. You need to look at the two axial ligands, and what the environment is around the ligand. So that's another thing. I've tried to stress how important these ligands are-- and the second coordination sphere around the system. So what happens now, in this system, is in some way the enzyme catalyzes heterolytic cleavage of the oxygen oxygen bond, and forms what is formerly an iron IV species. But it's not an iron five species, so we've lost a molecule water.

Somehow this leaves as water, so we have some groups in the active site that can facilitate that cleavage. And this is formerly an iron V species, so we're using electrons from the iron porphyrin system to facilitate cleavage of that bond. Now how do we know this? We know this because we know a lot about the spectroscopy of hemes and of the iron in the hemes, and we can actually look at all of these intermediates. And so what does this mean here?

What happens is-- remember your porphyrin. Well, you can see the porphyrin, but you have this. You have all these pyrroles. And so iron V is a hot oxygen. Nobody's ever seen the iron V in any of these systems, so what you see-- spectroscopically, you see an iron IV and one electron oxidized porphyrin ring. OK, so that's what this is. This is a one electron oxidized porphyrin ring. So now what do you want to do?

In the normal reaction, what you want to do-- the one that forms hypochlorous acid is a two-electron reaction, and the chloride can come in-- and you're going to form hypochlorous acid. So the chloride comes in and attacks, and one of the electrons goes back to the iron. And the other goes back to the porphyrin. So these one-headed arrows means you're doing one-electron transfers, and so what you've generated then is hypochlorous acid. The pKa of this is 7.4. And then you've generated back your iron III porphyrin, so you're ready to start again. So you're doing a two-electron process, and we know that the driving force for this reaction is large. It's 1.16 volts, so this is a very favorable reaction.

Now if any of you have thought about heme-dependent systems-- before, if you have an iron oxo, this is a hot oxidant. It's dying to be reduced. It can be two-electron reduced, but it can also be one-electron reduced, depending on what small molecules are around here. And myeloperoxidase does both kinds of chemistry. The predominant chemistry-- so this is what you need to look at the rate constants for the reaction. The predominant chemistry is thought to be this, but I will show you a slide where we know it can catalyze a lot of other chemistries by one-

electron transfers, as well. And what's happening in the phagosome with the cell, if you want to look at that, the [INAUDIBLE] review article I gave you spent a lot of time thinking about these kinds of reactions. And sort of think it's beyond the scope of what we need to talk about.

So here what we have happening-- so here now I'm just going to-- I'll tell you what R is in a minute, but what we're going to do is have one-electron transfer. And so instead of reducing this two electrons at a time, we're going to do two one-electron transfers, OK? So the system has to be set up. You have to have the right RH. You need to know what the redox potentials of these are-- determined by the ligands. All of that stuff, you need to think about. So now we're doing one-electron chemistry, so this is another possibility. And this again depends on how much chloride you have around. It's a potent oxidant, so it can be rapidly reduced, depending on whether you have an RH around that's going to actually do the reduction. And so what you generate then-- is you reduce the cation radical, and you form an R dot.

And then the next step, which you can do, is a second one-electron reduction, so you're doing two one-electron reductions. And this driving force is not as large-- 0.97 volts-- but again it's one-electron. And you're back then to iron III in water. I'm being sloppy about where the protons have come from. And you produced another R dot. So what could these R dots be? One of these R dots is ascorbate, vitamin C. So one of the RH's, which can-- the ascorbate can form radicals. I'm not going to go through this in any detail. Another RH could be tyrosines. So this is the amino acid tyrosine, and you form tyrosyl radicals. Has anybody ever seen that before in our department? Anybody seen use of this before?

**AUDIENCE:** Apex?

**JOANNE STUBBE:** Yeah. So, Apex. So this is the technology that is the basis. She doesn't use myeloperoxidase, but King's Lab-- it uses a ascorbate peroxidase, which catalyzes a similar reaction. So your R dot then becomes a phenoxide radical. And that can then do for the chemistry. Anyhow, so what you're generating, most people believe the key bad player in all of this, but I'm just telling you it's more complicated than that, is the hypochlorous acid, which clearly gets formed, and can be evidenced by the chlorination reactions you see of all your proteins. So you can isolate chlorinated aromatics out of the phagosome.

So if we go through here-- let me just give you this one first. So I've written this out for you in some detail. Again, it's two electrons, one electron, HOCl is two electrons. And that's thought to be the predominant species, but let me just tell you that-- so this is the one we're talking



about in the handout. This is the major reaction, but you can do a lot of other reactions. And so what you need to look at to see if these are important-- just like with superoxide, you need to look at the kinetics of the reaction under the conditions where these molecules find themselves-- to figure out what's really going on, and how much these other pathways contribute. [INAUDIBLE] actually just-- there's a paper online in the annual reviews of biochemistry, where she talks about the neutrophils in a lot of detail, and the complexity of all this radical chemistry.

So what I want you guys to take home from this is that we're working pretty hard with the NADPH oxidases, to engulf a bacteria. We're using reactive oxygen species, superoxide and hypochlorous acid to try to do in the bacteria inside the phagosome. So that's all I want to say about this section of reactive oxygen species, and now what I want to do is talk about something we've already talked about, because we've gone over this-- because we've gone over this in recitation. We spent a couple of recitations on the Carrol paper. And so I told you-- when I was introducing this, I gave you an outline of where we're going. We're going to reactive out of control versus controlled hydrogen peroxide superoxide production and signaling. So again, it's this thing all about-- it's all about homeostasis.

Just need to get my act together here. So where are we going with this? This is the outline of where we're going to go, and so I'm not going to write the outline, because it will take me too long. And I really want to get-- you can read the outline on my PowerPoint. But what I want to do very briefly is give you an overview of how these reactive oxygen species are thought, in general, to play a role in signaling. A lot of people working on this-- there are many, many proteins involved. We're only looking at one of these proteins, the epidermal growth factor receptor, which we talked about in recitation. I also want to sort of give you an overview of the importance of cysteines in general in the proteome, and the role they play in this signaling process. We'll see.

We are looking at one small modification, sulfenylation, but we'll see that there are many other modifications. And so I think one of the things for the future is figuring out, like the Carrol paper did, how biologically important are all these modifications that we can now identify because of the amazing power of Mass Spec and the creativity of chemists to figure out how to generate reagents. And so then I'll specifically introduce you very quickly to NOX and growth factors in NOX2-- the big family of NOX2. And then I'm going to talk about the general principles of signaling, what's required. And this is true of all signaling, not just with NOX, but

I'll use NOX as an example. And then I will probably spend no time on this at all.

The last part is how do we know all of this? We spent two recitations on this, so I'll tell you the key things I want you to remember. But you've now read papers. You've thought about this. And hopefully you can go back and think about it again, and it will all sort of now make more sense to you. OK, so where are we going?

And so what I want you to see is sort of the big picture-- so again, the overview. And the overview now is not of the bad radicals that we're talking about, but they are still the bad radicals-- but controlling them in a way. So in the radical systems we're going to be looking at-- we're not going to be looking at all of them. But the signaling agents that we're going to be looking at-- so this is an overview of signaling. The signaling agents we're going to be looking at are superoxide, hydrogen peroxide, and NO. And we've already talked about the fact that we're not discussing NO, but in our department in biomedical engineering, Tannibaum's lab has been a major player in figuring out how to look at the modifications of cysteines by NO. It's not by NO. It's by a metabolite of NO that then reacts with the cysteines.

So this is a very-- and he does that by Mass Spec, so this is a very active area of research. So now we're looking at signaling, not bad stuff. And one of the things that-- where have we seen this before? We've seen, although I don't think we realized it-- we were looking at iron homeostasis. This is what happens when you get up at 4:00 in the morning. OK, homeostasis. Homeostasis. And we have two proteins, the iron responsive binding protein one and two. And what do we know about iron responsive binding protein one? It has an iron sulfur cluster. And remember it has to go from the apo state to the iron cluster state, so we have IRP 1, and we go from the apo to the 4 iron 4 sulfur cluster. And it's believed, but it has not been very well studied, that this can be a sensor of oxidative stress, and so both NO and reactive oxygen species, such as hydrogen peroxide, can cause the metal center to be destroyed. And somehow you get to the apo state, and that's an active area of research.

And then we know what the signals are. You have translational control by iron responsive elements at the five prime or the three prime end. The same thing happens with IRP 2. So I'm just trying to tie things together, but does anybody remember what the sensor was with IRP 2? Anybody remember what it did?

**AUDIENCE:** Ubiquitin ligase?

**JOANNE STUBBE:** The what?

**AUDIENCE:** Is it ubiquitin ligase?

**JOANNE STUBBE:** Yeah, so we had a little ubiquitin ligase remain with the sensor that binds in ways we still don't understand, so again this is something that's very much an active area of research. You have iron in oxygen-- sorry, LBX L5, leucine-5, domain of the ubiquitin ligase. I'm not going to draw all of that out. So again, these are all tied-- with what happens with iron and oxygen, it's all tied to these reactive oxygen species. So what we've already talked about-- so this is the major focus, our growth factors. And I'll show you using PowerPoints, but this kind of signal is signaling is also involved in cell proliferation and cell differentiation. So it's widely used in the example that we chose to use, because it was one of the ones that's been most carefully and recently characterized as EGF receptor. EGF receptor is also of great interest, because it's a target. It's the target for successful drugs used clinically in the treatment of cancer.

So we then have another. So there are two other important signaling pathways that are proposed to be involved. One is called the antioxidant pathway, and there is a transcription factor. Some of you might have heard of it. Has anybody heard of NRF2? No. So if you're more biological or-- have you heard of the antioxidant? You're biological. Have you heard of NRF2?

**AUDIENCE:** No.

**JOANNE STUBBE:** No. OK. So anyhow, we have an antioxidant pathway, and NRF2 is a transcription factor. And it turns out-- I'm not going to go through the details of this, but for those of you who want to read about the details of this, this is a cell signaling review article published, where they go into all of the proposed mechanisms of how these reactive oxygen species connect to signaling. We're going to focus on epidermal growth factor receptor, but I think you need to know the picture is much bigger. It turns out that NRF2 is in a complex with an E3 ubiquitin ligase, and part of that ligase-- it's a multi enzyme complex-- is Keap. Keap has a huge number of cysteines on it. These cysteines get oxidized by some kind of reactive oxygen species. In that one, you want to turn on your antioxidant defense.

And so what happens when Keap cysteines get oxidized. It dissociates from NRF2, and NRF2 can go into the nucleus, and it turns on a whole bunch of genes. So I mean you're just getting the idea. I'm not going to go through any details, but it plays a central role. And this system, if you google it, you'll find there are hundreds of papers on this system. This is a very interesting system. I keep waiting for them to get to some stage where I can really talk about the

biochemistry, but we aren't at that stage yet, in my opinion. And then the other thing is DNA damage and repair.

And if you have DNA damage, or you're starting to get oxidative stress, and these things are out of control-- hydroxide radical is reacting with your nucleic acids-- you need to do something. So you turn on a signaling pathway, and this is controlled by a kinase. I'm not going to go into this in detail, but some of you might have heard of this. This is called the ATM pathway, and what you see-- actually, you'll see, I think, in the next five years if you remain biochemist-- all these acronyms for all these pathways, you're going to get it, because now we're seeing them over, and over, and over again. And at first, it was hard to see how this all fits together, but we're getting there. It's all fitting together, I think, in an interesting way. So all I want you to get out of this-- we're going to be talking about growth matters, but this is also a huge area, just like we just saw with oxidative stress trying to kill our bacteria.

OK, so the next slide is one I took out of this article. I'm not going to go through this in detail, but we've already been through the iron responsive binding protein. So this is a summary of that, and we spent a lot of time talking about this. This is what we're going to talk about now-- the role of sulfenylation, in controlling kinase activity, and phosphatase activity. That's what we spent the last two recitation sections talking about. This is a generic approach to that, and I'm going to show you there are many, many growth factors that are thought to go through the same pathway. And so I just want you to remember that.

So this is a big area, and so now the next thing is-- so there's another sort of overview picture I want you to get. So there's a second overview picture that I think is also important, and that cysteines really are playing a major role in all of these modifications. They are the easiest to oxidize, and so I think cysteine modifications are important. And there are many, many modifications. The question is, do they happen inside the cell? Do they happen inside the cell in a way that we can connect them to some interesting biology? And then what triggers off? Ultimately, what triggers off these modifications?

So we've been talking about the kinases, and we've been talking about sulfenylation. That's what we spent two recitations on, so that's one important thing. But somehow, we're going to see that one of the important things that I've tried to stress in recitations was these reactions need to be reversible, so ultimately sulfenylation-- some way, come to this later. But it's going to be able to be converted back into the reduced state. We've seen that you can form sulfonic

acids. This is also-- if you look at the Carrol paper carefully-- we didn't talk about this very much-- it's also reversible. There are sets of enzymes. Hydrogen peroxide can do the oxidation-- the back reaction that people have discovered an enzyme. They can do the back reaction. And then there's an irreversible step. So people don't know, but because it's irreversible this is likely not physiologically important.

So in addition to these states, if you start reading the literature, or you read any literature now, we have glutathione that we've talked about. Glutathione is this tripeptide with glutamylcysteine glycine. It is able to convert the sulfenic acid into a glutathionylated protein. We're seeing these all over the place. Is this the signaling pathway? How is it controlled? What's going on with these? I think we don't know the answer to that. So you can actually have glutathione react to give SSG. You can also have other kinds of proteins that I think-- so here they have RSH. This could be a thioredoxin protein, which if we get to deoxy nucleotide formation, there are hundreds of thioredoxins inside the cell that do the same kind of thing. And so one can also go from here, so you can have a little protein called thioredoxin, and it has two cysteines. And it can convert this back into the SH, and it itself can become oxidized. And there's a way of cycling the thioredoxin. So you're getting the idea, OK.

Over here in this model, we're not going to talk about this, because I decided not to talk about reactive-- not on nitrogen species, but nitrous oxide can get converted into peroxynitrite. Peroxynitrite is able to actually catalyze formation of molecules like this, which is thought to be involved in signaling. They're also controlled reversibly by thioredoxins, and again this is what the Tenenbaum Lab studies. And these things can also cyclize to form these kinds of structures. So these are called sulfenamides. People have found them. They have X-ray structures of them. Are they important in signaling? I don't know, but you can see that you have many, many kinds of modifications. That's the takehome lesson from this, and then the big question is how important are these in terms of controlling homeostasis?

So what I want to do now is briefly look at the players. I think I'm going to raise this. But briefly look at the players we've already started to look at, and make a few points, and make the general points about the signaling process, using epidermal growth factor receptor as an example. And so I'm hitting you over the head with this again, because we have already looked at this a couple of times. So we have an overview of EGFR and NOX. So we just looked at NOX. This is also NOX2. It's the same protein. They're found in different places. And so remember with NOX2 we had all these factors that I told you, if we're involved in the

phagosome, you had a GTPase, you had phagosome oxidase. Now you have, in some cases, similar factors-- in other cases, additional factors-- that play a role in these multi enzyme complexes that allow it to do something else, OK? So nature reuses, over and over again, these different factors.

So this is the cartoon picture you guys have seen before. We use this in recitation. This is where I started to have you think about recitation 11, when we started this, to try to introduce you to the system again. And so I just want to make a couple of points about this, but here's our epidermal growth factor receptor, which you all know now is a tyrosine kinase. Here is the NOX protein, and here you can see we have Rac1. If you go back, and you look at your notes from last time Rac1 is a GTPase. You can control its activity with little proteins that can bind to it and inhibit it. And the funny thing about this, and people were asking me questions about this, is that the chemistry, the tyrosine kinase, is in the inside of the cell. NOX1, the NADPH-- and going to NADP is in the inside of the cell just like we just talked about-- but where is-- because of the predisposition of the flavin and the two hemes, where is superoxide produced? On the outside of the cell.

That's rather bizarre. This is still rather bizarre to me. This is the model people have in the literature, but you're reducing equivalents from NADPH to convert oxygen to superoxide, which rapidly disproportionates to form hydrogen peroxide. And so then the question is we're saying hydrogen peroxide is the key signaler that's doing sulfenylation. How does it get into the cell? So the model then is it gets into the cell through an aquaporin. And is this aquaporin just moving around in the membrane, or is this some organization within the membrane? So this is going to be useful. We've already talked about the fact that hydrogen peroxide is not very reactive.

So one way you can get something to be more reactive is by increasing its concentration. So nature does this all the time. So if you can somehow stick things together and generate something, and it's generated right adjacent to where you're going to react, it has a greater probability of reacting here than over here. And where have you seen that before? Any of you thought about that? Graduate students should know this.

**AUDIENCE:** DNA templated synthesis?

**JOANNE STUBBE:** DNA what?

**AUDIENCE:** DNA templated synthesis?

**JOANNE STUBBE:** No. So, yeah. No. So you do, but I mean in terms of all of these reactive oxygen species. The Ting Lab, that's what she does. She generates these things in the middle of the cell, and it's all dependent-- how long can this go? Remember we talked about this diffusion question. How far does it go before it actually reacts? So the idea, and the question you need to ask yourself is, if you generate this, are these organized? Do you remember from the recitation? Are these guys organized? What did we learn the last time in recitation?

So we talked about-- we had this cartoon, and we talked about this. You looked at the data. What did the data tell you? Are these guys organized in some way, so that this hydrogen peroxide can actually do sulfenylation reactions? So what was the evidence for that? Does anybody remember? I mean, so if you don't remember this, you need to go back, and you need to read the paper again, OK? And I have all of this stuff on a PowerPoint, but I'm not going to go through it again. Yeah?

**AUDIENCE:** There was colocalization between, like, the NOX.

**JOANNE STUBBE:** Right. So there was colocalization between the NOX2 in the growth factor, epidermal growth factor. And what else was there colocalization from? It's not shown here, but there was also colocalization of a phosphatase-- which that's not shown in here, but it's going to be shown in the next slide-- plays a key role in controlling the phosphorylation state. OK, so this idea of-- I'm going to write this down because I think this is a central idea in biology-- is how you localize things to make them more reactive. Whether this makes it reactive enough-- it does make it reactive enough, because we can clearly sulfenylate, but are we missing something on top of it, to make it reactive enough to be able to do what we need to do? So what we see with this system is EGF, the Growth Factor, causes EGF dimerization. That's what I've shown you in the cartoon over there. I'm not going to draw out the cartoon, because you've seen this cartoon a bunch of times.

And what does that do? The tyrosine kinase activates itself by phosphorylation, and we're going to come back to this. So one way again, everybody has seen phosphorylations. Whether they activate or inactivate, you need to study. So here's the tyrosine kinase domain. When they come together, it has activity that it can phosphorylate itself, so you get into this form which then triggers signaling cascades.

So the other thing we need to think about in this paper is-- so here we are going from the tyrosine to the phosphorylated tyrosine. You should write down that this is active. How do you

activate a phosphorylated tyrosine if this is active? You use a phosphatase. So this, also, we saw in this paper. And in this paper, this Carrol paper, she identified, or she claims to have identified-- you can make your own judgment on that now-- the phosphatase, in the cell type that she looked at, that played a key role in the tyrosine kinase activity. So you're converting it from an active form to an inactive form, which is what you see by phosphorylation, dephosphorylation all the time. I'm going to come back to this in a minute.

So here's a protein tyrosine phosphatase. So we have a protein tyrosine phosphatase. And so while I didn't write that out, these ends, there are lots of different kinds of phosphatases. But they have a thiolate in their active site, and these things is attached to a tyrosine on a protein. So here's our tyrosine kinase. So what happens again is you use covalent catalysis in two steps. So you phosphorylate, and then you hydrolyze. So you phosphorylate, and then you hydrolyze. So you end up then with your tyrosine and the kinase, and you end up back with ES minus.

So again, there are many different kinds of phosphatases, but all the ones involved apparently in these signaling processes-- if you go back and look at the Carrol paper-- all have cysteines in their active site. You've seen this, covalent catalysis with cysteine, over, and over, and over again now at this stage. So this is going to be a key control. This is the active state, so this form is the active state. And over here-- sorry. So if you take this now, and you treat this with hydrogen peroxide, this becomes sulfenylated, and this becomes the inactive state. So sulfenylation, just like with the tyrosine kinase that we talked about in recitation, can become activated. The sulfenylation in this case becomes inactivated, so that's what it's all about in these post translational modifications.

And the question is-- I'm sorry, I'm over again. But the question is are these models correct? So what we'll do next time is spend a little bit of time talking about the six general principles of post translational modification, in general, and what the expectations are using what you've already-- which we've already seen in recitations 11 and 12. And then we're going to move on to the last module on nucleotide metabolism.