Part I

Hello, I'm Ron Vale. I'm a Professor at the University of California, San Francisco and an Investigator with the Howard Hughes Medical Institute. In these three lectures, I would like to tell you about biological motility. Now, there are some types of biological motility that everyone is familiar with, for example, the contraction of your muscles or maybe the movement of sperm or the beating of cilia and flagella. But it's also true that the interior of all eukaryote cells is teeming and bubbling with all kinds of intracellular motility.

[Slide] Let me take you here, into the interior of a squid giant axon, seen in real time through a video microscope, very much like the original experiments of Bob Allen, Scott Brady and Ray Lasik. What you can see here is a tremendous amount of motility – all kinds of small organelles which are traveling between the nerve cell body and the distal nerve terminal. These long snake-like things are mitochondria that are also moving through this very dense cytoplasm. Indeed, if you turn a microscope to any eukaryotic cell, you will see lots of intracellular motility like this.

[Slide] Here is another prominent example of intracellular motility: the process of cell division. Here the DNA is in green and the microtubules are in red. The DNA congresses to the middle, and then you have the physical separation of the saer chromatids to equally partition the genetic material. Now, all of this fantastic intracellular motility is driven by biological machines called molecular motors, which is going to be a major subject of these lectures.

Just to get you in the right spirit for thinking about these molecular machines, I'd like to show you an animation that was developed by XVivo, in conjunction with Harvard and Howard Hughes Medical Institute, which takes you into the interior of a cell, to show you a little bit about what these machines might be doing. [Video] What I hope you can

appreciate from that remarkable video is that the cell interior is packed with interesting machines. Maybe they don't work exactly as they're shown in the video, but the spirit of the video shows that the inside of cells is very complicated. There are lots of molecular machines that are working together to create all of this complex cell behavior which makes cells function and execute a number of complex behaviors and abilities.

[Slide] Our goal as scientists is to try to understand how cells work, how these machines work in cooperation. By analogy – and this is a very hard problem – let's imagine coming to this chemical plant here, also a very complicated enterprise, without a particular degree in chemical engineering or having the benefit of watching this chemical plant being constructed in the first place. This is a little bit analogous to what we're trying to do in biology, is to figure out how such complex systems work. So, what can we do? We can come up to this chemical plant and watch it. We can see people coming in and going out of the plant, maybe trucks transporting material, machines in action in this chemical plant. This is analogous to what we do as cell biologists with microscopes, which is to watch cell behavior and deduce how cells work through observation. We could also measure material flowing into this plant. We can see various chemicals coming in and going out of this plant, and follow the flow of material through this system, maybe a little bit like what systems biologists are trying to do these days. We could also take another route of disabling – we could get a sledge hammer out here and disable part of the machinery. From that, we can maybe deduce what parts of the machine in the chemical plant are performing what kinds of function. That's a little bit like what geneticists do.

[Slide] Indeed, in the third part of my lecture, what I'd like to do is show you how these three tools can be applied to understand one type of intracellular motility, and a very complex structure, which is the mitotic spindle.

[Slide] But in addition to studying this factory as a whole here, we can also appreciate that if we walk inside the factory, we'll find that it's made up of remarkable machines, such as this steam value here. Each of these individual machines is very precisely

engineered to exact specifications for its function and regulated in a number of important ways. It's the function of many of these machines that make this chemical plant work. In fact, we need to know the details of how each of these machines works if we're going to understand how this chemical plant functions as a whole.

[Slide] Indeed, that's another important part of biology. In the second part of this lecture, I'll give you a tour of one fascinating molecular machine, which is the cytoplasmic dynein motor, and the tools we use to try to understand how this machine works.

[Slide] But it's in this part of the lecture that I'd like to tell you a little bit about these machines, which are molecular motors, and discuss their activities in a general introduction. There are three classes of cytoskeletal motor proteins – there is kinesin, myosin and dynein. These motor proteins use chemical energy from ATP and they use this energy to move unidirectionally along a track. These are going to be the main focus of my lecture, but I should also stress that there are many other kinds of molecular motors in biology.

[Slide] In addition to cytoskeletal motors, there are motors that move along DNA or RNA as well, like helicases or polymerases. There are also rotary motors – motors that spin around – such as motors that drive the bacterial flagellum. There is also a remarkable rotary motor inside of your mitochondria that spins around like a turbine and makes ATP; that's the engine that makes all the ATP in your cell, which is the F1-F0-ATPase.

[Slide] So what are these molecular motors? I thought I'd start off by comparing them to engines that you're more familiar with, for example, an automobile engine. Naturally, these protein motors are much smaller and they are truly nano-scale engines. Now, we often tend to use the term 'nano' to describe things that are small, but maybe not that small, like this iPod Nano. While small, it's really not 'nano' scale. But these molecular motors that I'm going to be talking about, such as kinesin, truly work at the nano-scale level. They are about 10 to 15 nm in size. Now, like your car engine, they also need a fuel

source. They use a chemical fuel, which is ATP, to produce their work, comparable to hydrocarbons for your automobile. They move at a few millimeters per hour, which might seem pathetically slow compared to your car moving on a highway. But if you actually do the calculation of how fast these motors are moving, relative to their own length per unit/time, they are actually moving several times faster than your car engine is on the highway. Now, for those of you who have filled up your gas tanks recently, I'm sure you would also appreciate another virtue of these protein motors, that is, they are much more work efficient than the pitiful combustion engine in your car which works at 10% efficiency. These molecular motors work at 60% efficiency – some even work at 95% efficiency or more. In fact, I think engineers who are trying to design better types of machines could learn a lot from how these biological machines work.

[Slide] So, why should we study these cytoskeletal motors? I would like to give you a few reasons. The first reason is – for me personally – it's a fascinating subject. One of the things that everyone appreciates about living organisms is that they are endowed with the ability to move. Scientists going all the way back to the ancient Greeks have developed theories about how biological motion works. I think you'll see in this and the next lecture how we finally have the tools to understand this very old question in biology. Second of all, these cytoskeletal motors work in so many aspects of biology, whether you're a neuroscientist, a developmental biologist, someone interested in signal transduction pathways or membrane trafficking. Invariably, some element of your problem involves some kind of cytoskeletal motor, which is involved in producing the activities that various scientists study. Finally – and I hope to illustrate the last part of this lecture – studying these cytoskeletal motors also has a number of pragmatic benefits and relevance to medicine. First of all, we know that mutations in molecular motors or molecular motor associative proteins, can give rise to transport defects that can cause disease, for example, neurodegenerative diseases and in other cases birth defects. In addition, we're also beginning to find that drugs directed against motor proteins, either to inhibit or enhance their actions, can have therapeutic benefit for human disease. This is something that I'd

like to illustrate at the end of this lecture and also at the end of the third part of this lecture as well.

[Slide] Let me now dig in a little bit and tell you more about the components of these molecular machines. Cytoskeletal motors work along tracks and there are two main tracks that are used to produce motion. One is the microtubule shown here. It's a cylindrical polymer, made up of repeating subunits of alpha beta tubulin. Another type of track is the actin filament, which I show over here. It is made up of a single protein called actin arranged in this helical polymer. If you want to know more about this actin filament, I encourage you to watch the lecture by Julie Theriot, as she discusses actin in much more detail.

[Slide] Now, these particular tracks here are what these molecular motors run along. The actin track serves as a substrate for myosin. This is a cryo-EM with the myosin motor domain decorating actin. Microtubules serve as the tracks for two classes of motors, which are kinesin and dynein. You can also see that these tracks are segregated in different regions of the cell. The actin filament is surrounding the outside of the cell and the microtubules are more interior, such as is shown in this mitotic spindle. One other thing you need to know about these tracks is that they are polar structures. Each of the subunit proteins that make up actin and microtubules are themselves polar – they are asymmetric. The subunits polymerize in a head-to-tail manner. That results in a net polarity of the whole filament. In addition, these filaments are organized with uniform polarity in the cell. So for example, with microtubules, let's say the minus end of the microtubule is located at the pole of this mitotic spindle, whereas the plus end is extending out to these blue chromosomes over here.

[Slide] Now this polarity is also true in interphase cells as well. Here is just a generic fibroblast with microtubules extending all throughout the cell, but in an organized fashion. The minus ends of these microtubules are found at the centre, at a place called the centrosome, and then the plus ends of these microtubules extend out to the periphery

of the cell. I should also say that the motors recognize this polarity and a given motor will only move in one direction along this track. So, the combination of having polarized filaments that are organized in this uniform manner in the cell, in conjunction with motors that move in one direction, creates a fantastic transport system for the cell, which allows certain cargos to move to the distal periphery of the cell and other type of cargos to concentrate in the cell centre.

[Slide] Let me now introduce you to the motor proteins and show you a little bit more what they look like. Here is a kinesin motor that is moving along a microtubule track, and the functional engine of this motor is shown here in purple. It's these purple domains of the kinesin molecule that are churning up the ATM and moving along this microtubule track. Beyond this so-called motor domain, the rest of the molecule is referred to as the tail domain, and you can see here in the case of kinesin, part of the tail domain is an alpha-helical coil and that dymerizes two kinesin polypeptides together, which is very common for many motor proteins. At the far end of the kinesin motor is another part of the tail domain that docks this motor onto a particular cargo in the cell. Here we see kinesin docking onto a membrane organelle, to specifically transport this cargo inside of the cell.

[Slide] Now one thing: when I refer to kinesin, dynein or myosin, I'm not referring to one motor but actually a big class of related motor proteins. For example, kinesin is not one motor, but in the human genome, there are 45 different kinesin genes. The reason why there are so many is that these different kinesins are specialized for different types of transport activities. That's why there are so many of these kinesins, because they perform a whole variety of different transport functions. Some kinesins are involved in moving intracellular membrane organelles, like I showed you in the squid giant axon movie. Other ones are moving RNAs and proteins, and yet others are transporting building blocks up to the tips of cilia/flagella, and that helps these structures grow. Yet other kinesins are involved in signaling pathways, and whole other groups of kinesins are

involved in creating the mitotic spindle, as I showed you in that earlier movie from drosophila embryo and in moving chromosomes as well.

[Slide] These many kinesins actually have a variety of different architectures. They have a similar motor domain here, shown in blue, but if you scan over these images, the tail domains of these kinesins all look very different from one another, and that allows them to attach to different cargos and also be regulated in a number of different ways inside of the cell.

[Video] The topic that I'd like to finish this part of the lecture with is how these motor proteins actually work; how do they produce motion? Here, for example, is a kinesin motor transporting plastic beads along a microtubule. We'd like to know in detail how this works. How is it that a very small protein molecule is able to convert chemical energy into this remarkable unidirectional motion?

[Slide] For a start, I could tell you that these motor proteins are enzymes. They hydrolyze ATP and use that chemical energy to produce the work that you saw in that last video. For example, for motor protein here, it binds to an ATP molecule, it hydrolyzes it and then it releases the products in a sequential manner. During this one ATPase cycle, the motor produces work and some kind of step along its cytoskeletal track. So for that to happen, the protein itself has to undergo some sort of structural changes during this enzymatic cycle, which allows it to walk along the track. The real goal in the field is to understand how all these transitions in this enzymatic cycle result in changes in the protein structure, and ultimately explain the motion that we see in these videos.

Now what I'd like to do is to take you to two animations that were made by Ron Milligan, Graham Johnson and myself. They recapitulate decades of work by hundreds of investigators studying this problem. This movie here shows the myosin motor and how it's thought to work. This is a myosin motor that might be found in your muscle. The goal of this myosin is to bind to this actin track and slide the actin filament so that your

sarcomere can contract, and your muscle can contract. I'll show you that at the very end of this lecture. But let's see how it works. [Video] Here's the motor domain. It binds to the track, it releases phosphate – actin causes phosphate release – and that causes a big rotation on the part of that motor, a 10 nm displacement. That is the part that causes the movement. Now ATP comes in and kicks the motor off the actin filament, hydrolysis occurs and recocks the motor so it can bind to a new filament and undergo a stroke. Here it is again, phosphate release, the stroke, ATP binding, detachment and recocking. And it's millions of these events that happen in your muscle – many of these cyclic binding/release events that result in the contraction of your muscle.

Now, myosin is made to work in these big ensemble arrays with millions of other myosin molecules causing muscle contraction, but other motors in the cell have to do it solo. This is a kinesin motor. It may be attached way up here somewhere to its organelle cargo. It's thought that even a single kinesin molecule or maybe just a few kinesin molecules are what are transporting organelles and mRNA proteins inside of cells. So the kinesin motor has to be very good, even acting solo, to move continuously along a track without letting go. This is very different from muscle myosin which can detach from most of its cycle. It can just produce a brief stroke and then release. So let's see how this works. [Video] First of all, here is the motor domain and it binds the microtubule – the microtubule kicks off ADP. ATP comes in here. Now, look at this yellow element here: it zippers up along the side of the enzyme. As you'll see in the next movement here, that zippering causes the rear head to be displaced from the rear binding side and to move to a forward binding side over here. It's the motion of what we call the neck-linker here that pulls the rear head from this forward binding side, it moves around randomly, locks onto a new binding side. In this way, the motor can work in what we call a hand-over-hand manner to move continuously along a track. In fact, it can move for hundreds of ATPase cycles without letting go, due to this coordinated action of the two heads of the kinesin motor protein. So, these animations recapitulate a lot of data. I should say that we should always be careful with models like this because, no doubt, they're not correct in every detail, and

scientists are still working out many of the detailed mechanisms of both kinesins and myosins. These models are continuously being refined.

[Slide] But how do we get to a model like this in the first place? How do we collect data that even gets us close to thinking about a protein at this level of detail? That's what I'd like to tell you about. How do you study the mechanism of a molecular motor? Well, there are several tools that we can use – very powerful tools that we're endowed with in modern biophysics right now.

[Slide] One thing we'd like to know is what the motor looks like and we'd like to know this in incredible detail, at atomic resolution detail, so we know exactly where all of the amino acids and the side chains are positioned in this motor. That's a job of x-ray crystallography. Here is an x-ray crystal structure of a kinesin motor domain, and what you can see is the ATP in the active side – this is a ribbon diagram. Over here is a space-filling model. This was done by our lab in conjunction with Robert Fletterick's lab at UCSF. This is the detail that we really need to know in order to begin to understand protein structure and how it changes, during this enzymatic cycle.

[Slide] But in the case of kinesin, it also gave an answer to a very old question in our field, which was: are kinesin and myosin at all related? Before the kinesin crystal structure, I think the answer that everyone thought was *no*, and it made sense. Kinesin is a microtubule motor; myosin is an actin-based motor. If you look at them, they're totally different in size. Kinesin is shown here in red and the myosin motor domain is much bigger as you can see here. In addition, if you asked a computer to line up the sequences of these motors, the computer came back and said that there was no sequence similarity between kinesin and myosin at all. Aha! Well surprise, we got the crystal structure of kinesin and looked at it in detail compared to myosin, and in fact, there was a remarkable structural similarity in the core part of these molecules surrounding the nucleotide. And this shows beta strands of kinesin and myosin in yellow and green. You can see that there is a remarkable overlap between the beta strands and these two motor proteins. In

addition, all of the helices you see here for kinesin overlap with similar helices in the myosin motor as well. This is no accident. This must indicate that the kinesin motor protein and myosin evolved probably very distantly in evolution from some kind of common ancestor. That made a huge difference to the psychology of the field. People always thought: if I'm studying kinesin… myosin is another kind of motor that I don't really need to pay much attention to – or vice versa. This information indicated that the myosin field and the kinesin field were really about studying variations on a common kind of motor that evolved very early in evolution.

[Slide] Not only that but the structures of the motors also revealed a surprising similarity to a whole other class of proteins, which are the G proteins here, which people don't even think about as conventional motor proteins. But they also bind nucleotide, GTP, hydrolyze it to GDP, and they need to change their shape in order to act as switches in single transduction cascades and other kinds of activities like that. Indeed, looking at the structures here in yellow, there is a common core element surrounding the nucleotide that was similar in these proteins.

[Slide] So we have some idea now about how all these machines evolved, probably very distantly in evolution. There was a great invention, a nucleotide switch, something that learnt to bind nucleotide, hydrolyze it – but not only learn to hydrolyze it. It learnt to change its shape when it went from 3 phosphates to 2 phosphates, like ATP to ADP, or GTP to GDP. This gave rise to two different kinds of machines: the G protein family and some motor precursor that may still exist in biology or may have been lost. It probably evolved really early in evolution. The motor precursor then evolved into two different branches: one motor that learnt to walk on microtubules, and another one that learnt to walk on actin. Wow! This was such a great invention that evolution then produced many subclasses of these motors, a whole bunch of kinesins and a whole bunch of different myosins.

[Slide] Now, in addition to getting some idea about the history of these motors, we also just learnt a lot by comparing these structures to one another. I don't have time to go into this in great detail, but what seems to be the most ancient and common theme that is shared between myosin, kinesin and even G proteins, is how these proteins bind nucleotide, hydrolyze it and then change their shape when the protein goes from an ATP to an ADP state or GTP to a GDP state. The nucleotide switching mechanism seems to be very similar in all these proteins, this core element here shown in blue. But, onto this core switching part of the motor of the enzyme – nature has plugged in a bunch of different mechanical elements. Here is the myosin mechanical element: this big, huge lever arm that you saw rotate. And in kinesin, there is this much smaller peptide that threw the partner head forward. So the mechanical elements have learnt to work with this kind of common switch unit, and in fact, even in the myosin and kinesin families there are a number of different variations on the types of mechanical elements and how they work.

[Slide] Now, the one thing you can't get from crystal structures yet – people have been trying – but we haven't had a crystal structure of a motor with its track. So all the information we have of that has been derived from a cryo electron microscopy, here shown a cryo electron micrograph reconstruction from Ron Milligan's lab with the microtubule in gray over here. This microtubule is decorated with a whole bunch of kinesin motors, a special type of kinesin motor called Ncd. From this, we can get lower resolution but very valuable information on how the motor interacts with its tract, particularly valuable if we combine this with x-ray crystallography.

[Slide] Here we see this web-like structure which is the information from cryo-EM, and one can dock into that the atomic structure for this kinesin motor protein. These two in combination gives us a very good idea of exactly how this motor protein is interacting with the track, and how the track might be influencing the behavior of this motor.

[Video] Now, we can't get everything from cryo-EM and x-ray crystallography because these are static techniques. We just get a snapshot of the motor. But we know that motors

are very dynamic. Here they are transporting beads along microtubules. So, we need to study the dynamics of how they produce motion, and we have great assays for doing that. In fact, we can take these things out of a cell and reconstitute motility in a test tube. We can purify the motor protein, purify the track, add some cargo – like the bead here, add ATP and the system takes off. You can see that here. In fact, this was the original bead motility assay that gave rise to the purification of kinesin in the first place in the mid 1980s.

[Video] Here is another type of assay. In this assay, the motor protein is attached onto a glass slide, so the motor proteins are fixed. The microtubules are added, ATP is added, and then these motor proteins grab hold of the microtubule and they transport these microtubules along the surface. If you follow any of these microtubules here, you can see them crawling along the glass surface, as these motor proteins are grabbing hold of the microtubule and transporting it like a log. This assay is also still used today.

[Slide] Now, another great tool that emerged out of these in-vitro motility assays was the ability to study motility at the level of individual molecules, single molecular motors. This is a work that Joe Howard, Jim Hudspeth and myself did a number of years ago, where the density of these kinesin motors on the glass surface was spread out to very low density, so that only one motor could grab hold of the microtubule track. The position of that motor shown right here and when the microtubule reaches the end of that kinesin molecule, it then dissociates off into the solution. So the motion of the microtubule that you see here – and we can show this through statistical arguments – is driven by a single kinesin molecule.

[Slide] Now subsequent to this time, there are a number of other advanced techniques that allow us to understand how molecular motors work, such as optical traps, which I will show you in the next lecture. But here is classic work from Steve Block's lab which uses an optical trap to measure individual steps by a single molecule. Here it shows a kinesin transporting a bead and it's stationary from *here* to *here*, and then it takes a jump

– boom! That's one step, another step, another step… And all of these steps here are 8 nm in size, very small. This 8 nm dimension corresponds to the distance between these stepping stones along the microtubule, the distance between the alpha-beta tubulin monomers.

[Video] There are other cool assays that one can use too. Here is an assay where a single fluorescent dye molecule is added to a motor protein – a kinesin here. You can use a very specialized microscope, which I'll show you in the next lecture, which can track these single motor proteins labeled with a single fluorescent dye. These are kinesins that you can see traveling down this microtubule track, again in real time.

[Slide] Okay, so we have great assays and great tools. But for us to really understand how motors work, I think we have to get control of them. We have to be able to manipulate them, design and make new motors. I really like this quote here from Richard Feynman ("What I cannot create, I do not understand") because I think it also illustrates what we're trying to do in the motor field, to understand how they work. And that is to actually engage beyond evolution to try and design our own motors.

[Slide] So, we can take information on motors – we know their sequences, we know their atomic structures and from that we can say: Ah, I think this is how the motor protein produces motion. And then I could imagine that if that hypothesis was right, then I should be able to design a motor in this way and then produce some other kind of unique outcome – maybe change the direction in which that motor moves, change its velocity or other kinds of parameters. We have the ability to make these motors in bacteria or other systems, and then redesign these motor proteins – do protein engineering – and then purify and test them using all these assays, like the in-vitro motility assays that I showed you. But there are a lot of other great assays. We can look at their chemical cycles in great detail by enzyme kinetic measurements, or look at their conformational changes by techniques by fluorescence energy transfer. There is a whole bunch of assays that we can use. And then we can see from our hypothesis if the motor behave as expected. Well,

maybe not quite. So we go back, re-think our hypothesis, design another kind of motor and then test it again. And it's through this cyclic mechanism that you see here that we can begin to understand how these machines work.

[Slide] Now, in addition to understanding the fundamental nature of how these molecular motors work, there are a lot of things that we can do which could have pragmatic benefit for mankind. One thing – which I'll show you right now – is that it's possible to manipulate small motors using small molecules in ways that will allow us to modulate motor activity in a way that's beneficial for improving certain human diseases. I'll show you that in this lecture and also in the last lecture as well. It's also possible that we might be able to engineer motors in ways that allow them to deliver drugs inside of cells, or produce new types of cell fate, or even outside of cells. We could design motors that might be useful for certain types of nanotechnology.

But let me just talk about this right now. I'd like to tell you a brief story. This was not done in my lab; this was done in a company which is called Cytokinetics. I should mention that I'm a co-founder of this company, a shareholder and also currently on the SAB. I would like to illustrate this story, because I think it's a great story of how you can manipulate a motor in a way that is useful for medicine. What I'm going to tell you is a story on the ways in which one can use small molecules to make myosin motors in the heart actually work better, to improve contractility and produce cardiac output for patients that have heart failure, where their cardiac output has been compromised. But first let me tell you a little bit about the science here in this video. I'm going to walk off the screen here, but what you'll see is a heart beating and then we're going to go down to the cellular level and you'll see the sarcomere, the basic unit of myosin contractility, where myosin filaments are pulling in actin filaments to make the sarcomere contract, and that results in the contraction of the heart. You'll also see these floating little speckles which are calcium ions, and those are the signals which control a regulatory complex on the actin, made of tropomyosin and troponin, that allows the myosin to engage in the actin filament. It's these cyclic flows of calcium that turn on the myosin, the myosin

works, starts contracting and that causes your heart to contract. [Video] Okay, well, that's how the heart is supposed to work. But there are some people where the heart is not working properly, where their cardiac output is compromised, the contractility of these ventricles is not working as it should, and that can result in severe health problems and also mortality. So, we need to think of strategies for improving the contractility of the heart. There are several drugs that are out there on the market already that work through the signaling pathways. They work through controlling the output of calcium. Now, that's a reasonable strategy, but these drugs also have side-effects because working through the signaling pathways can also be very complex. So Cytokinetics, led by Fady Malik, who was my very first graduate student that I had at UCSF, who became a cardiologist and then moved on to start this cardiac program in Cytokinetics, has led an effort to develop ways in which one can activate the myosin to make it work better. Now this is a pretty dramatic, bold strategy because most pharmaceutical companies develop drugs to inhibit enzymes and I can't really think of any other example of a drug that activates a particular enzyme. At least, it's a rare type of drug.

[Slide] This was a bit risky, but they did this project… First of all, to find these drugs, what they had to do was reconstitute the motility system out of the heart and in a test tube. They did this by purifying actin, myosin, troponin, tropomyosin and reconstituting this whole system in a test tube. Then they measured myosin ATPase activity, and what they were looking for were small molecules like this one that I'm showing you here, that would activate the ATPase of the myosin. In other words, make it cycle faster which should result in increased contractility of the heart when the natural calcium signal was signaling. Now, this was not an easy effort. It involved doing biochemical screens, and screening for hundreds and thousands of molecules to find one that showed this type of activity. After that, there was a lot of chemistry that had to go on to refine the affinity and the pharmacokinetic properties of these molecules, to make them suitable drugs to put into people. But that did happen, and before that happened, they tested this.

[Slide] Here is how they think it works. It increases this particular step of the myosin cycle, that step where the actin is tickling the myosins in some way, to force the phosphate out of the actin side and cause the power stroke. And these drugs facilitate this particular step of the myosin cycle. So, the knowledge of the biochemistry here has been essential for the drug discovery process.

[Slide] Here's what this drug looks like in a dog model. Here is an echo cardiogram showing the ventricle of the heart in a dog contracting. Here is the same heart after giving this particular myosin activator. You can see that this heart is now contracting much more vigorously, resulting in increased cardiac output and improved blood flow from this heart. So this is pretty dramatic. This drug has also been in phase I clinical trials in people, and I can say that echo cardiograms of people show exactly this type of effect. Now, the real issue is will this help people who's hearts are failing, and will it result in improved health for these individuals and less mortality. So, that's what is happening right now. These drugs are in phase II clinical trials and we'll probably find out if this program did result in helping these individuals in a couple of years.
I hope that in this first lecture you can see that these molecular motors are fascinating machines. We have great tools for studying them, and understanding these machines also have pragmatic outcomes for human health and disease. In my second lecture, I'll talk about current work in our lab on the dynein motor, and the last lecture will be on understanding motility in the context of the process of mitosis. Thank you very much.

Part II

[Video] Hello, I'm Ron Vale. I'm a Professor at the University of California, San Francisco and an Investigator with the Howard Hughes Medical Institute. In the first lecture, I told you about this motor over here, which is the kinesin molecule, shown here walking along a track. This is work from our lab, but many investigators, over a couple of decades, have led to the development of a model for how kinesin works. Now what I'd like to do is tell you about a much less well-understood molecule – a motor protein called

dynein. This is very much work-in-progress, where we have yet to produce such a detailed model. But I'll tell you where we are in this process.

[Slide] Now, there are many kinds of dynein molecules. Some of them are involved in beating in cilia and flagella. There is one type of dynein here called cytoplasmic dynein, also a very important protein molecule. In most interphase cells in eukaryotes, it's involved in producing virtually all of the trafficking towards the minus end of the microtubule transport system – organelles, RNA, viruses. Many types of molecules are trafficked by cytoplasmic dynein. In mitosis also, it plays a critical role in forming the spindle, and also in the interactions between kinetochores and microtubules.

[Slide] Dynein is a lot more complex than kinesin and myosin. In fact, shown in just a linear diagram of the polypeptide, it encodes one of the biggest polypeptides in the genome. Even the minimal motor domain, shown in yellow, is much bigger than the minimal motor domain here in myosin and that in kinesin. Also, myosin and kinesin just have a single ATP binding domain; dynein has four. So it's a much more complicated beast.

[Slide] Shown in an even more complicated fashion is our model – not a real crystal structure – of what the whole dynein model looks like, with its associated polypeptides. It's about a one-and-a-half megadalton complex. The motor domain is this ring structure over here. It extends out this long stalk, and at the tip of that stalk is where it interacts with the microtubule. Beyond this is the tail domain, which has all of these associated proteins that interact with cargo, and very likely regulate the dynein molecule.

[Slide] Now, dynein is also a very different beast because it's not evolutionarily related to kinesin, myosin and G proteins, as I showed you in the first lecture. In fact, it comes from a completely different evolutionary branch. It's related to another group of proteins that are called AAA proteins. Dynein, in this family, is a bit of a weird uncle, because most of the AAA proteins are involved in unfolding polypeptides. Some AAA proteins unfold

proteins to stuff them into proteases. Some other AAA proteins are involved in breaking apart and destabilizing a very stable protein-protein interaction. So, dynein is somewhat unique in having evolved a motor function out of this particular lineage.

[Slide] What I'd like to tell you about are our efforts to understand how dynein works. To understand such a complicated molecule, you really need a team of very talented people – and also a group of people with very complimentary talents. This work was started by a very talented post-doc, Sam Reck-Peterson, who is now starting her own lab. She came from a yeast cell biology background, which was critical for getting this project started. She was then joined by these individuals: Andrew Carter is a very talented protein-chemist structural biologist, involved in doing a lot of the protein engineering of dynein; Ahmet Yildiz and Arne Gennerich come from physics backgrounds, and they applied their physics knowledge to do single molecule studies of dynein, which I'll show you later.

[Slide] First of all, we can ask: why don't we have a good model for dynein? There are a number of reasons for that; the primary one is that it's big. It's much bigger than the kinesin molecule. That makes it very difficult to express – you can't express it in bacteria. As I showed you in that earlier figure, it's also very complicated. There are many associated molecules with dynein, and we have relatively little structural information; there are no crystal structures right now.

[Slides] So what do we need to do? We need ways to express the dynein, so we can manipulate it and do protein engineering on dynein. We need to make simpler motor constructs; we can't always work with this big one-and-a-half megadalton beast. We need simpler proteins to study the mechanism of motility. And we need to develop assays in-vitro to study this mechanism. Finally, one day hopefully we'll get some crystal structures but at least I'll tell you our efforts in the first three categories here.

[Slide] So, as a system to make dynein, we turned to yeast. The primary function of dynein in yeast is less than in many other eukaryotic organisms. Its main job is during cell division of budding yeast where it pulls daughter nuclei into the emerging bud for cell division. In dynein null mutants, where there's no functional dynein, this process often fails, and you get both nuclei in the mother. Fortunately for us, this process doesn't happen – this failure doesn't happen all the time. It only happens 30% of the time. 70% of the time, the yeast muddles through and makes it through cell division. That allows us to take the genomic copy of dynein and we can manipulate it in different ways, that are good for us to express the protein, but maybe bad for dynein function in yeast. But, these yeast that are harboring all the engineered dynein molecules that I'll be describing to you, still live, still grow, and they produce the dynein protein that we want to study. By homologous recombination, we can introduce various tags into the dynein gene in yeast, to make dynein molecules that we can manipulate and study.

[Slides] For example, we can put certain tags on the dynein molecule that allow us to do affinity purification of dynein and purify it out of a yeast system. In other cases, we can introduce other kinds of tags that allow us to put fluorescent dyes in particular locations on the dynein molecule – and I'll show you why that's very important for our research to understand how dynein works.

[Slide] Here is an example right away. Here we have a tag that we introduced onto dynein – it's called a halotag. This is a particular inactive enzyme that will covalently react with a special modified version of a fluorescent dye called tetramethyl-rhodamine. This allows us to put one fluorescent dye molecule per dynein polypeptide chain, and know exactly where we're placing the fluorochrome. So, with this type of assay and a special kind of microscopy called total internal reflection fluorescence microscopy, that you'll see more about later, we can now do single molecule motility assays and ask a very old question, which we wanted to know. Is dynein processive like kinesin? In other words, can it bind to the microtubule and then take many steps along the microtubule like kinesin? Or does it bind and release quickly like muscle myosin?

[Video] And indeed, the answer is that it's a highly processive motor. You can see that in this video here, a time-lapse video of dynein. All these individual spots that you see moving along in these linear lines here represent single dynein motor proteins moving processively along the track.

[Slide] Now one thing we can ask is: how does this processivity work? In this complicated molecule, what are the features of this molecule that are required for this processive motion? One thing you obviously notice is that this is a dimer. It has two motor domains here and it also has all these other associated chains. We can ask: do you need the dimer to produce processive motility? Do you need all these other associated chains?

[Video] So, with protein engineering, one thing we can do is make a very simple dynein polypeptide that is truncated at the tail and is monomeric. So it's just one motor domain. This motor domain is functional in an in-vitro microtubule gliding assay. Here, the dynein motor is attached on the glass surface, as I showed you in the first lecture. These dynein molecules are stuck, they grab hold of microtubules and they push them along the surface of the slide. Now under each of these microtubules are many of these monomeric dyneins. But, if we attach a fluorescent dye to this monomer and see if it can move processively along a microtubule, we find that it can't. We see no processive motility at all, suggesting that you need two motor domains to produce movement.

[Slide] But that's kind of a negative experiment. We'd like to come up with a more dramatic demonstration that you need the two motor domains to produce processive motility. And here's a nice protein engineering experiment that demonstrates that. So, in one yeast strain we took a dynein motor domain and joined it to a small protein called FRB. In another yeast strain, we took a dynein polypeptide and coupled it to another protein domain called FKBP. If we examine these things with the single molecule fluorescence, we saw no processive motility. Now, the reason we did this was because

there's a special drug, a natural product called rapamycin. Rapamycin binds to both FRB and FKBP, and joins these two domains together. And with this small molecule, this chemical, we can induce an artificial dynein dimer. [Video] When we add this small molecule, the motor goes from no processive motility to beautiful processive motility, like you see here in this video. So now we can turn processive motility on and off, simply by adding this drug, demonstrating that you need a dimer to create processive motion.

[Slide] Going one step further, we'd like to get more of the finer details and really see how this motor steps along the track. That could give us more detailed information on the mechanism. A way to do this was developed by Ahmet Yildiz, who is now in our group, but previously when he was a graduate student in Paul Selvin's lab, he developed a very successful way of tracking motor stepping by following a fluorescent dye attached to the motor with great precision, even a couple of nm. So, the way this works is that the fluorescence from a single dye spreads out by diffraction when it's collected by the microscope, with a width of about 300 nm. But, computationally, you can determine the centre point of this fluorescence very accurately down to a couple of nm. Then when you make a movie of these motors, in each frame you do this computational fitting and determine the centre point of that fluorescent dye. Then you go onto the next frame, determine the centre point again, and you build up a lot of this positional data to follow how this motor is moving along the track.

[Slide] Now, to aid this, it also helps if you have a super-bright fluorescent dye. A new strategy that really helped with this is using a new kind of fluorescent structure, a small semi-conductor particle called a quantum dot. We can attach this quantum dot in particular locations of the dynein molecule. This quantum dot is very bright; it doesn't photo bleach, so we can collect a lot of information for tracking.

Now I'd like to introduce Ahmet Yildiz. He's actually the person who developed this technique and he'll describe to you this tracking program himself, doing a live experiment at the microscope and explaining this approach.

[Video] Hello, this is Ahmet Yildiz. I work at Ron Vale's lab. This is a single molecule total internal reflection microscope. The light is coming from three different lasers here – red, green and blue. They are all aligned through a single path and then they come through to the microscope. Then laser light is reflected back on the glass water interface, and the fluorescence is collected through the very sensitive camera which collects 92% of the incoming photons. Here is the live image of molecular motors coming from a single fluorophore and they are walking on the tracks of the microtubules. If we look at the screen, we can easily see that the motors are walking on these tracks.

[Slide] Ron Vale: Okay, that was a live experiment done by Ahmet. Now let's look at some of the data that comes out of these experiments. Let's first start with what this technique shows for kinesin stepping, the motor we described in the first lecture. This is a quantum dot attached to one of the two motor domains of kinesin, and what you can see here is that each of these little dots is the centre point of that quantum dot per frame. This is the displacement versus time, over here. You can see how many of these dots cluster in one position, but then they jump up to another point. That's a step taken by the motor. It then jumps again, again and again. You can see this beautiful detail of stepping by the kinesin motor protein. Even at a big picture here, the two things you can appreciate are that the stepping is very regular and it's unidirectional for very long distances.

[Slide] Now, let's look at dynein. You can immediately appreciate that it's a very different kind of beast. Here we're putting a quantum dot on the centre point of the dynein molecule. You can see that in many cases, the centre point moves in these 8 nm steps, the distance between the lines. In many cases the steps are quite big and sometimes they're backward steps as well. The stepping mechanism is not identical to kinesin.

[Slide] We learn additional information by comparing stepping when we put the quantum dot in different locations of the motor. In one case, the case I just showed you, we placed the quantum dot at the centre point. Then we measure many steps of this

motor, collect lots of data and then make a histogram of the step sizes. You can see that there's a primary peak of step size at 8 nm. Remember, 8 nm is kind of the magic distance between adjacent alpha-beta tubulin binding sites along the microtubule. However, if we put the quantum dot on one of the motor domains here, the peak in this histogram shifted over from 8 nm to 16 nm, although again you can see the bigger steps and also some backward steps as well. How do we explain this?

[Slide] Here is a model that we think might explain the data. We think the way the dynein walks along the track is the way the two motor rings are shuffling past one another as this machine moves along the microtubule track. Let's just show you that for example. Here, we're looking face down on the microtubule; this is a top-view of the rings. Here is a dye attached to the centre point of the dynein molecule, and it takes an 8 nm step, an 8 nm step and another 8 nm step. But look at what's happening to the individual rings. Here is a dye attached to just one of the two motor rings, and in this step, it shuffles past its partner head over to this position, taking a 16 nm step. But in the next step here, it is stationery and it waits for its partner head to shuffle past it. Then, in this final step here, it's the turn of the first motor ring, which shuffles forward. So, while the centre of the mass is moving 8 nm, one individual ring is moving 16 nm, 0 nm and then 16 nm.

[Slide] Another way to look at the dynein motor is to measure its forces. We can do this using an optical trap. In this particular experiment, we have the dynein here, we fuse it to a GFP molecule – we make this by protein engineering and express it – and then we take a one micron bead, attach GFP-antibodies to it and then we can couple this molecular motor onto the bead. Then, using a special device called an optical trap, we can measure forces. I'd like now to introduce Arne Gennerich, who is a post-doc who has been using the optical trap to study the dynein motor.

[Video/00:19:00] Hi folks, my name is Arne Gennerich. I am a post-doc in the Vale lab and I am working with an optical trap set up, a trapping microscope to analyze the stepping behavior and the force production of yeast cytoplasmic dynein. The main

component of the optical trapping microscope is a near infra-red laser source. We guide the beam along an optical pathway and steer directly into the back focal plane of a high numerical aperture objective. The laser beam is focused to a tiny spot, and this allows us to trap beads that are coated with motor molecules. We can image the position of the bead via CCD camera and also we map the image of the bead on the surface of the photo diode. This allows us to determine the position of the bead with a high precision. We see here the bead trapped in the laser centre. What we see is brownian noise, and eventually the motor that is attached to the surface of the bead binds to the microtubule and moves along the microtubule. At this moment, the precision between the bead and the trapped side increases, and while the motor is moving against an increasing force, it starts to slow down. Eventually it reaches a stall at the first level and it releases. Here we see single runs and when the motor reaches a stalled level, it detaches and the bead turns back to the centre of the laser light.

[Slide] Ron Vale: So that shows how the experiment is done. Here is some of the data that Arne gets with this system. He follows the dynein molecule in the optical trap. Again, this is measuring the force of the dynein as a function of time. These are individual runs by the dynein. The dynein starts off in the centre of the trap against low force, and it's a little bit like pulling against this cord over here. As the dynein molecule starts at the beginning, it's working against the low load, but eventually it gets to a point where the pull from the optical trap equals the force that the dynein molecule can exert, and the motor can't move anymore. It's stalling at this particular point. And that's the maximum force that this motor can produce, about 7 pN. You can also see, even against these very high loads, the dynein motor holds tenaciously onto the microtubule. Occasionally it lets go, as you can see here, but it can hold on for many seconds of time.

[Slide] All of these features here may be very important for the biology of how this dynein motor works in yeast. As I mentioned before, dynein pulls one part of the nucleus after division into the bud over here. The way this happens is that dynein is in the daughter cell over here. It grabs hold of a microtubule and then it starts pulling on that

nucleus, trying to move it up into the bud. And there aren't that many microtubules in yeast; there aren't that many dynein molecules. So probably once dynein grabs hold of a microtubule, it doesn't want to let go. It wants to keep pulling it in very tenaciously. It's pulling a very big object, a nucleus, into this narrow structure over here. For that activity, again, it probably needs to produce very large forces.

[Slide] So as I said, we're really just at the beginning of understanding dynein. I just want to illustrate that there are fantastic problems left to solve here. I mentioned at the beginning that there are 4 ATP binding sites in dynein in this motor ring here. We know that one is the main ATP binding hydrolytic site, but we really don't understand what the other ATP binding sites are doing in the dynein mechanism. I also mentioned that microtubules bind at the end of this very long stalk. The stalk is so big that you can put two kinesin molecules head-to-tail in the stalk structure alone. How does information from ATP hydrolysis in this ring get communicated through these long distances to control affinity at the microtubule binding site? We don't know the answer to that.

[Video] And we don't know exactly what the conformational change is that produces movement. There is some very nice EM work done by Burgess, Knight, Oiwa and colleagues. By EM, they found two different conformations of dynein. Here is the microtubule binding stalk and here is another element that they call the linker. If they look in two different nucleotide states, no nucleotide and an ADP phosphate-like state, they can see differences here. This is just a movie superimposing these two states. The difference between these conformations kind of looks like a power stroke that could be driving motility, but we have yet to find the answer to that.

[Slide] In a cell, dynein is not alone. It has many other associated proteins, and these associated proteins must be regulating dynein activity at the level of its motor activity as well as its cargo binding. We have yet to understand much about how these associated proteins work.

[Slide] So, we'd also like to use our knowledge of motors to produce some kind of pragmatic outcome. In the case of dynein, there are kinds of strategies for thinking about possible medical benefits with dynein. For example, dynein is involved in transporting viruses, and if we could interfere with that process there may be clinical benefits for producing novel anti-viral therapies. In addition, we could think about engineering dynein to carry new cargos inside of cells, such as drugs, or potentially transporting DNA molecules inside of cells. But, we still know very little about the dynein molecule and we need to get a lot more knowledge to begin to tackle these kinds of difficult problems.

Part III

[Video] Hello, I'm Ron Vale, a Professor at the University of California, San Francisco and an Investigator with the Howard Hughes Medical Institute. In the first two parts of my lecture, I talked about molecular machines and studying these machines at single molecule level. Now I'm going to a very different scale, at the level of whole genomes, trying to understand very complex structures made up of many proteins, in this case, the mitotic spindle here.

[Slide] Now, mitosis is a wonderful problem. It's one of the oldest problems in cell biology. In fact, it dates back to biologists from the 19th century. Here is an image by Flemming from the 19th century. It's a remarkably accurate depiction of the mitotic spindle, showing the chromosomes and even these fibers we now know are microtubules.

[Slide] The problem of mitosis is also very important for medicine as well. First of all, failures in the assembly of the mitotic spindle can give rise to chromosome mis-segregation that results in aneuploid cells. Aneuploid cells are often a precursor for cancers and uncontrolled cell division. In addition, there are many drugs used in cancer chemotherapy that work by targeting the mitotic spindle, as I'll describe to you at the end of this lecture.

[Slide] So, the question that I'd like to discuss with you today is: what are the molecules and molecular interactions that are needed to build this remarkable assembly of the mitotic spindle? Here we see microtubules in green, chromosomes in blue and the poles of the mitotic spindle marked here in orange.

I would like to primarily focus this lecture on the first step of mitosis: the building of the mitotic spindle. [Video] Here is tubulin GFP emanating from the centrosomes, and at the onset of mitosis what you'll see is the nuclear envelope break down and these microtubules form into the mitotic spindle. It's that formation of the mitotic spindle that we'd like to understand and what I will discuss in this particular lecture.

[Slide] There are many questions that remain in this field, even though it's been the subject of extensive study. First of all, there are lots of microtubules that build the spindle, and we'd like to know how those microtubules are assembled. There are specialized structures in the spindle: the centrosome found at the poles, kinetochores at chromosomes that interact with these microtubules. We'd like to know how these structures are formed. We'd also like to know how this characteristic football shape of the spindle is formed and organized. So, there are lots of questions that we need to understand.

[Slide] The approach that I'm going to share with you today is one that was done in our lab, to find new proteins involved in creation of the mitotic spindle, using a whole genome RNAi screen in drosophila S2 cells. This work was executed by a very talented group of people. The leader of this project was Gohta Goshima, who has now started his own laboratory in Nagoya. Nico Stuurman, who is a senior research scientist in the lab, developed a lot of the technology infrastructure to make this happen. The follow-up work after the screen was performed by Nan Zhang and a new student, Sarah Goodwin. Another key person for this work was Roy Wollman, working in John Scholey's lab. Roy did a lot of the computational analysis that I'll share with you later. As you'll see, it's possible to do a whole RNAi screen even with a relatively small group of individuals.

[Slide] Why do an RNAi screen? Well, I would submit to you that you do this to try and discover something new. I'll even use this analogy of how early explorers went on expeditions to find new things in the world. This shows an early world map where much of the world is very poorly defined. There are large land masses that have yet to be defined. This is very analogous in many ways to 20th century biology. Even as a graduate student, I had very little idea of how big the genome was and what was in there. At that time, even in the mid-1980s, you could still discover a big protein super family, like the kinesins. A lot has changed, both in world geography and in biology – world geography over centuries, whereas in biology, over a short span of a couple of decades.

[Slide] Now the world looks more like this. In biology, the genome has now become a finite entity through all the sequencing effort and work by thousands of investigators. We know a lot of the components that are present in the genome. But I do want to stress that there are still a lot of undiscovered genes out there that we need to understand. This RNAi screen that I'll show you is a good way of making new discoveries.

[Slide] Let me tell you a little bit about RNAi. It's a technique that allows you to destroy mRNA corresponding to one gene. As a result of the destruction of that mRNA, it results eventually over a couple of days in a depletion of that particular protein. By this method, you are able to deplete a particular protein and look at a particular outcome on the cell. The way this works – at least in drosophila – is that you can first make a double-stranded RNA, corresponding to a particular gene. You'd like to use a particular piece of the gene that is unique to that gene, and not found in other genes in the genome. That double-stranded RNA is then taken up by cells. Then there is a special enzyme called dicer that chops this RNA into 21 base pair fragments, which then interact with a specialized protein complex called the risk complex, which then unravels these siRNAs. It then base pairs them with an mRNA in the cell, and after base pairing, results in the degradation of that mRNA. Then, as I said, over a couple of days it results in the depletion of the protein.

[Slide] We've been using RNAi in this drosophila S2 cell line. We've chosen this cell line for a number of reasons. First of all, it's very easy to culture – you can even grow it at room temperature. You also get very efficient RNAi knockdown in these particular cells. We've worked out methods that make these cells very good for microscopy, which is critical for the screening I'm going to tell you about. Then, if you want, you can also follow up with genetic manipulations in flies.

[Slide] The screening that we did was a visual screen. What we wanted to do was to knockdown mRNA corresponding to each gene in the drosophila genome, of which there are over 14,000 genes, and then look at the effects on the morphology of the mitotic spindle. We did this by imaging the spindle after RNAi of each gene. There's a lot of information you can get from these images: you can see the overall shape of the spindle; you can measure the length; you can see if the chromosomes are aligned properly; you can measure a very special protein that defines the poles called gamma-tubulin, that I'll tell you more about. You can also see if there are two poles as there should be in a normal spindle or if the poles are split or fragmented. So, there's a tremendous amount of information that you can get from direct imaging. So, we knocked down over 14,000 genes of the fly genome, and in this screen, we analyzed over 4,000,000 spindles.

[Slide] Let me just tell you a little bit about the technical details of how this screening was done. First of all, you need to develop an RNAi library against the entire fly genome. This takes a lot of careful bioinformatics to design double-stranded RNAs that are unique for each particular gene, so you don't get knockdown of multiple genes, for example. This bioinformatics effort was done by Nico Stuurman in the lab. In fact, this library is now available at reasonable cost from a company called Open Biosystems.

[Slide] Then what we did is we treated these drosophila cells with double-stranded RNAi corresponding to each gene. We arrayed these S2 cells in 96-well plates, each with a unique double-stranded RNA. To cover a whole genome, one had to use nearly 150 such

96-well plates. We also did a double-RNAi with another molecule called the anaphase promoting factor. This is a protein that's involved in the transition from metaphase to anaphase. By knocking down this protein, more of the cells are arrested in this metaphase spindle, which was the structure that we wanted to investigate.

[Slide] Now, after we did all this RNAi, we had to collect all the images of these mitotic spindles. This was done through a high-throughput microscope. We first transferred the cells to glass-bottomed 96-well plates and then put them into this robotic microscope. You could place all these plates into this robot here and grab and place these plates on the microscope stage automatically. The microscope will go around and automatically focus on each well. Then it will take a number of different images, again, totally automatically around the well of these cells.

[Slide] We get a tremendous amount of image data and what we image here are the DNA, microtubules, the special protein gamma-tubulin – which I'll tell you more about later – and another marker called phospho-histone, which is used as a marker to know that the cell is in mitosis.

[Slide] So, out of this, we get a tremendous amount of image data, far too much for any person to manually go through. Here's where computation proved to be very valuable. This was co-written by Roy Wollman who wrote MatLab code to find these mitotic spindles automatically in these images. After the computer found these images, we did two things with them. First of all, this computer code arrayed all these mitotic spindles into this big gallery that you see here. The next day, after the computer collected all these images, Gohta Goshima could wake up in the morning, come into the lab and open up these files with all these galleries of the mitotic spindles. For example, this is 200 mitotic spindles found from one well of one RNAi knockdown. He could look at these images and just visually assess whether there was any defect in the shape or architecture of the mitotic spindles.

[Slide] In addition to this human analysis, Roy also wrote code that would segment this mitotic spindle and then analyze a number of features of the spindle that a computer could analyze, such as the intensity of the stain, the length of the spindle or the number of poles. So, the combination of a human looking at the data and a computer analyzing this quantitatively proved to be a very effective way of analyzing a whole genome RNAi screen.

[Slide] The results of the screen were that we found about 200 genes, that when their protein levels were knocked down produced some kind of defect in the metaphase mitotic spindle. A lot of these different phenotypes are shown in this gallery here. First of all, I should say that we knew going into this screen that there 49 genes that we knew produced mitotic spindle defects in these cells. Through this blind whole genome screen, we found 45 of them, which is really quite good. In addition to those 49 known genes, of this list many of the genes that gave spindle defects are components of multi-subunit complexes, like ribosome, spliceosome, proteosome and RNA polymerase. Interestingly, the knockdown of these large protein complexes produced quite interesting and in fact unique spindle phenotypes from one another. In addition, we found about 60 knockdowns of unknown or unexpected genes that were previously not known to have any role in the mitotic spindle.
[Slide] This is the list that we spent a lot of time investigating. Now, I should say that all of this data is publicly accessible. It's on a website (http://rnai.ucsf.edu/mitospindlescreen) and you can actually scroll through and find any gene that we analyzed in the genome, and all the details of the images and phenotypes that we found for that particular gene.

[Slide] So what can one learn from a screen? Of course, what you get is a big list of genes. You can arrange these genes – as many people do – into a pie chart, where you separate them into cytoskeletal or signaling proteins. Indeed, this list is somewhat useful. It gives you an overview of the most important proteins involved in spindle assembly. But I would argue that this list itself really does not, on its own, provide insight into how

the mitotic spindle really works. To do that, you have to go beyond this list of genes and do more experiments.

[Slide] What you're trying to find out of the screen is some unexpected result, not just a big list of genes. You want some unique finding here that can give you some new insight in how this spindle is formed.

[Slide] To track down an unexpected result, as I said before, you have to do a lot more work. We then did a bunch of secondary assays. If we found a novel protein, we wanted to know where it was located in the cell, for which we had to do GFP tagging. To better understand the phenotype, instead of having a fixed image of the cell, as we did in our whole genome screen, we wanted to analyze these phenotypes by doing time-lapse imaging. In addition, we also then custom designed a number of specific other experiments to try and understand the phenotype, using additional RNAi knockdown approaches or by adding drugs or doing other kinds of localization experiments to better understand the mechanism of how these genes work.

[Slide] We did find a number of interesting results from the screen – far too much for me to talk about in this short lecture. I would like to focus on one story that came out of the screen, and that is how you make microtubules to build the spindle. [Slide] Let me just tell you the conventional view that you'll read in a textbook on how these microtubules form. There are two main ideas. One is that these microtubules emanate from centrosomes here, and another is that microtubules are nucleated at the chromosomes themselves.

[Slide] This first mechanism, centrosome-nucleated microtubule nucleation, was really pioneered by Kirschner and Mitchison in the 1980s. It is a very important part of building the mitotic spindle. These microtubules grow out of these centrosomes; these microtubules are growing and trying to grab hold of chromosomes. Eventually some of

these microtubules make connections with chromosomes. These microtubules become stabilized into the mitotic spindle.

[Video] Indeed, you can see this by using a special tag that just binds to the very tip of the microtubule, a special protein called EB1 that just tracks along the microtubule plus end, which is here tagged with GFP. This is a very good mechanism for following new microtubule nucleation. If we look more carefully in this blow up, we can see the centrosome here. We can see these little comets of EB1 GFP kind of emanating out of the central area. And all of the little comets that you see growing are new microtubule that are nucleating, extending out and trying to reach chromosomes.

[Slide] Now, we know the main microtubule nucleator there. It's a complex that's called the gamma-tubulin ring complex, which has been studied extensively. It forms this ring-like structure here. And from this multi-subunit complex, this forms a nucleating base from which a microtubule can be nucleated and extend. This gamma-tubulin ring complex docks onto the centrosome. At the very interior of the centrosome is a pair of centrioles. There are a lot of proteins coating the centrioles, and some of these proteins are then responsible for docking the gamma-tubulin ring complex. One of those key docking proteins is a protein called centrosomin. This centrosome can then nucleate this spiny array of microtubules.

[Slide] However, we also know that spindles will form without centrosomes – so they're not essential. For example, we know that plant cells and germ cells in animals don't have centrosomes, and yet they form mitotic spindles. Khodjakov and Reider have shown that you can oblate the centrosome in normal animal somatic cells and the mitotic spindle will still form.

[Slide] So we know something about how that centrosome independent mechanism occurs, and that's through nucleation of microtubules around chromatin or DNA. This was really beautifully demonstrated in an experiment by Rebecca Heald and Eric

Karsenti. They just put beads coated with DNA into a mitotic extract from frog eggs, and the DNA-coated beads – not even normal chromosomes – would nucleate microtubules, and the microtubules would reorganize themselves into a spindle-like structure.

[Video] This is also shown in these S2 cells here. I'm showing you the centrosomes here. Here is the nucleus. You'll notice right at nuclei envelope breakdown, right over here, you can see chromosomes – these dark bodies – and then you can see these flashes of new microtubule growth right around these chromosomes. Then, these chromatin-mediated microtubules are then integrated into the body of the spindle.

[Video] However, the question is: are these the only two mechanisms that are responsible for nucleating microtubules. Here we did this experiment that produced this surprising result that suggested there may be an additional mechanism. What you see here is a spindle without any functional centrosomes. So, we've knocked out the centrosomes completely. What you can see here is that there are no centrosomes in the cell, but most of the microtubule nucleation is not happening from the chromosomes – in fact it's happening throughout the body of the spindle over here, including at the poles. And these new microtubule comets are moving from these pole regions out to the chromosomes. So this suggested that there may be some other kind of mechanism at play, something that we really did not completely understand, but which we got some insight into from this big whole genome RNAi screen.

[Slide] And that's when we looked at genes that affected the localization of gamma-tubulin, this critical nucleator of microtubule assembly. So, normal gamma-tubulin staining is shown over here, and this is superimpositioned with DNA and microtubules. Most of the gamma-tubulins are found at the centrosome here, but there are also gamma-tubulins found in the body of the spindle itself – dimmer, but nonetheless present. From the screen, we found the known gene, centrosomin that knocked out gamma-tubulin localization to the pole, but still preserved this gamma-tubulin localization to the body of this spindle. This was a known gene, but then we found four totally unknown genes,

which we called dim gamma-tubulin genes, that produced the exact opposite effect. They still allowed gamma-tubulin to localize to the spindle poles at the centrosome, but they completely knocked out the localization of gamma-tubulin to the spindle.

[Slide] And this suggested that maybe this gamma-tubulin localization to the spindle involved a specific set of docking factors, perhaps these new proteins that we found. If this was the case, that these new proteins are involved in docking gamma-tubulin to these spindle microtubules, then we would expect that these novel genes that we found would be localized to the spindle themselves.

[Slide] Indeed, when we tag these proteins with GFP, we found that these new proteins were localized to the spindle. If we knocked out the microtubules with a drug called colchicine, that localization of these Dgts went away. So, these new proteins called Dgts are in the right spot in the spindle to be specific gamma-tubulin docking factors.

[Slide] Now we can ask this question: is spindle localization of gamma-tubulin by these Dgts important? Now we had a very specific tool; we had ways of knocking out gamma-tubulin localization at the centrosome. That we could do by eliminating centrosomin. Or we could knock out gamma-tubulin localization to the spindle with these new proteins. The results here were quite dramatic.

[Video] Here is a normal spindle. We're looking at GFP tubulin in green and chromosomes in red. You can see that this is a wild-type spindle – perfectly normal. The chromosomes are aligned normally. In this next video, we're going to look at a cell that has been depleted of one of these Dgts. It's unable to localize gamma-tubulin to the spindle. [Video] What you see here is the spindle very much perturbed. The chromosomes are misaligned; the spindle microtubules are very weak. This is a very abnormal spindle, indeed, and quite different from this other wild-type cell.

[Slide] Let me just summarize what we learned here. We learned that there are two different pathways for localizing the key microtubule nucleator gamma-tubulin. There is one pathway that localizes gamma-tubulin to the centrosome. Through this screen and work from other labs as well, we know that a whole pathway of components from the centriole through other components in the pericentriolar material, that dock gamma-tubulin to this pole structure. [Slide] But there is a separate set of components, including these novel Dgts, which dock gamma-tubulin onto the spindle.

[Video] And surprisingly, the spindle localization of gamma-tubulin appears to be more important than the centrosome, at least in drosophila. We can knock out gamma-tubulin localization to the centrosome, and the spindle forms just fine and the chromosomes will segregate. But if we knock out gamma-tubulin localization in the spindle, as I already showed you, we get these very abnormal spindles.

[Slide] All of this work has led to at least a new hypothesis on how you build a mitotic spindle. I emphasize this is a very new study and hypothesis, and we'll have to see how it translates in the next few years. The idea is that the well-known mechanisms of the centrosomes and chromatin constitute the important way of getting the new microtubule start at the build of the spindle. You can nucleate new microtubule growth at centrosomes or at chromosomes, and that allows you to quickly build microtubules to make a spindle.

[Slide] But after the spindle forms, possibly one of the more important mechanisms for maintaining the spindle is that the spindle has a way of self-propagating itself. It has a way of bringing the microtubule nucleator via these Dgts onto the spindle microtubules themselves, and this complex generates new microtubules that help to maintain microtubules in the spindle, and interaction with the chromosomes.

[Slide] As I mentioned at the very beginning of this lecture, understanding the mechanism of the spindle assembly also has a lot of important medical implications. [Slide] Many of the drugs that we currently use to treat cancer work by interfering with

mitotic spindle function. Some of these drugs like taxol, vincristine, vinblastine and vinorelbine all work by affecting the microtubules and interfering with mitotic assembly. And they're very effective anti-cancer drugs. They block cell division of tumor cells and they interfere with tumor growth. However, these agents here not only affect tubulin in dividing cells but also tubulin in all cells of the body. In addition to affecting other dividing cells like bone marrow, there are also a number of neurotoxic effects of these agents, because they interfere with microtubule transport in neuron cells as well. So maybe the future is to try to develop more strategic specific ways of affecting the mitotic spindle to stop cell division. And there are a number of other potential targets besides the microtubules, ones that are much more specific to dividing cells.

[Slide] An example of this is a special class of kinesin motors called kinesin 5. These kinesins are only used in mitosis. They have no function in nerve cells or other cells. Their specific role is to help build a bipolar mitotic spindle. We know from studies in many organisms that if you interfere with the function of these kinesin 5 motors, you get a very abnormal spindle indeed. You get what is called a monopolar spindle, where instead of having microtubules coming from two poles they are coming from a single pole; the microtubules are spreading out in this radial way. The chromosomes, instead of aligning in the centre of the spindle, are in fact aligned as a ring, such as shown here in blue. This ring of chromosomes simply cannot divide equally to two daughter cells. This results in a massive failure in mitosis.

[Slide] Again, I should say that Cytokinetics is a small biotech company in South San Francisco, of which I'm a co-founder, shareholder and also on the SAB. Cytokinetics took a strategy to develop inhibitors against this specific kinesin, to try to develop a new strategy for blocking tumor growth. And indeed, through in-vitro assays developing small molecule inhibitors against this motor, using biochemical tools and high-throughput assays, refining these small molecules then testing them in animal tumor models, they developed an inhibitor called Ispinesib, which is now in clinical trials in humans. In this tissue biopsy of a tumor from a patient after treatment with this drug, we can see a stain

of the DNA. You can see that there is a characteristic ring-like pattern of the DNA, indicating that this anti-kinesin drug is working in people just as it is in-vitro and in animal models.

[Slide] Currently, these drugs against mitotic kinesins are now in phase II clinical trials. They've shown activity for patients with breast cancer, and over the next couple of years, we'll see how these mitotic kinesin inhibitors develop, whether they will join the armament of anti-cancer therapies.

[Slide] So, in addition to these mitotic kinesin inhibitors, there are likely other candidates and new targets involving the mitotic spindle that might be next generations' strategies for cancer chemotherapy. Maybe the Dgts that I described in this lecture that so profoundly affect the spindle, at least in fly cells, might be candidates to look for in human cells and in human cancers, to see if they might be interesting targets for new cancer chemotherapeutics. So I've hopefully illustrated that the connection between the study of basic molecular machines, at the level of even single molecule assays, cell biological studies such as I've shown you here with whole genome RNAi, not only provides insight into mechanism but also gives us new routes of thinking for new kinds of medical therapy.