Clockwork Genes: Discoveries in Biological Time
Lecture Three—PERfect TIMing
Michael Rosbash, Ph.D.

1. Start of Lecture Three (00:15)

From the Howard Hughes Medical Institute, the 2000 Holiday Lectures on Science…This year's lectures, "Clockwork Genes: Discoveries in Biological Time," will be given by Dr. Michael Rosbash, Howard Hughes Medical Institute investigator at Brandeis University and Dr. Joseph S. Takahashi, Howard Hughes Medical Institute investigator at Northwestern University. The third lecture is titled "PERfect TIMing." And now, to introduce our program, the president of the Howard Hughes Medical Institute, Dr. Thomas Cech.

2. Introduction by HHMI President Dr. Thomas Cech (01:05)

Good morning, everyone, and welcome back to the Holiday Lectures on Science. Yesterday, we had a really terrific introduction to how scientists have explored the very molecular nature of what a biological clock is. Joe Takahashi talked about how the circadian clock in mammals is located in a specific part of the brain, and Michael Rosbash talked about how he and his colleagues had cloned and sequenced the *period* gene, "p-e-r," from the fruit fly *Drosophila* melanogaster. Today, our speakers will continue their story. Michael will tell you how the *period* gene and its protein interact in a biological feedback mechanism. Then after a break, Joe will shift the focus from the fruit fly to mammals, and he'll discuss how all of this research might also have an impact on human patients. I wish I knew more about biological clocks when I was your age. During my first year of college, I would stay up until 2:00 in the morning and then stagger out of bed for my 8:00 class. I was a zombie, and it's amazing that I stayed awake, much less managed to do anything useful in science. Michael Rosbash has done pretty well in science, to put it mildly, and it's interesting to me that he started out his career doing research on RNA, just as I did. In fact, Michael and I have been colleagues for a long time in the RNA splicing field, and I've really enjoyed that relationship. Then, Michael expanded his focus to include circadian rhythms. My own research has branched out in new directions as well, as has Joe Takahashi's. Do you see a trend here? Let me make an observation that you might find useful when you consider what you want to study in college and beyond. As I look to the future, I think that most of the really exciting things that are happening in science are happening at sort of the intersection between different disciplines. For example, the interface between biology and computer science is giving rise to the hot new area of computational biology. To succeed in this ever-changing arena, you need to get a broad education, not just in different sciences but also in mathematics and in the liberal arts. I call it cross-training for your mind, and it will serve you well. Michael Rosbash will speak first this morning. Once again, we'll introduce him with a brief video.

3. Introductory interview with Dr. Michael Rosbash (3:34)

What do I like best about my job? I guess I would say that I'm very taken with two aspects of it. First, there's a very rewarding intellectual component. That is, you can really think meaningfully about something and make a contribution based on an idea. And secondly and equally importantly, I really like the human aspects of my job. The myth of the lonely scientist in the white coat is, I think, very much a myth. Biology is very much a team endeavor. Those personal relationships are important. I must say, to this day, I still like the informality and the attractive trappings of academics. I like hanging out in the lab. I like the informal nature of the interactions, the inquisitiveness. It's still to a large extent "follow your nose." Do more or less every day what you're interested in. A lot of the attributes it takes to be a good scientist are in common with the attributes it takes to be a good anything. This is my own view -- not

everyone shares it -- but I think to be a good lawyer, to be a good doctor, to be a good musician, it takes a lot of the same assets, skills -- namely, hard work, interest, ambition, and I would say an almost indispensable part of the job, to be really good at it, is to be curious. You have to really want to understand how things work. There certainly is the sentiment that scientists are nerds or geeks, and that can be off-putting. There are plenty of cool people who do this profession. They are just as fun, have just as good a sense of humor, like to socialize, enjoy things -- mountain climbing, skiing -- as anyone else. So if you think you might like this, go for it and try it. More specifically, again, with respect to science, I think the thing to do is to get into a lab somewhere and see what the research is like, see what it's really like to work at the bench and work next to somebody, and do experiments, and see if you find the craft appealing. The idea is to follow your star. If you're interested in something, go for it.

4. Summary of the fruit fly's circadian clock (06:12)

Good morning, and welcome to the second day of the Holiday Lectures. How many people here didn't get enough sleep last night? So I'm interested, actually, the only job I'm interested in, other than the one I've had, is Norv Turner's, so... maybe that will forward my career in that direction. So, if I might have the first slide, we'll begin by reviewing some of the salient points that I tried to cover yesterday about biological clocks in fruit flies. So first of all, there is this negative feedback loop in which the *period* protein is synthesized and then, with 24-hour periodicity, turns off its own transcription, which we believe is at the heart of the circadian oscillator. Secondly, there's a relationship between period and phase where these period mutants, if they're maintained in a light-dark cycle, actually turn out to be phase mutants, so that the time during the day at which they undergo locomotor activity is the same every day. It's fixed, but it is advanced with respect to the wild type or normal population of flies, much like advanced phase sleep syndrome -- a human sleep disorder that Joe will talk about in his second lecture. Third, there are new technologies that have emerged over the past few years in which we can make transgenic flies with reporter genes which contain the Luciferase protein, and those flies will glow with circadian periodicity as the reporter protein is synthesized and degraded in circadian fashion in a manner which mimics the normal circadian proteins. And then finally, these reporter-containing tissues or organs can be put in culture, and they continue to undergo circadian oscillation, which can be entrained by the light-dark cycle that's imposed on those organs, thus illustrating two points -- first of all, that there are light-sensitive clocks all over the flies, and secondly, that those light-sensitive clocks, at least in culture, can be independently connected to the environmental light cycle.

5. Using attached-X-chromosome screening to find *per* gene (08:35)

Now, the next slide reminds us of the initial screen that Konopka and Benzer reported 30 years ago in which they mutagenized male flies and then utilized the genetic technique that harkens back to 1915 in which they mated those males with a strain of females which have an attached X chromosome, and in this unusual kind of cross, which is still used to this day as the standard method in *Drosophila* genetics, the males inherit the X chromosome from their fathers, and this simplifies the analysis of genetic transmission. So all of these mutagenized X chromosomes end up uniquely in the paternal lineage, and by screening these males, Konopka and Benzer were able to identify these period-gene mutants and show that they mapped to the X chromosome.

6. Other approaches for finding new clock genes (09:40)

Over the subsequent 30 years and, in particular, over the past decade, new approaches have been used by the genetics community to find new clock genes. First of all, genes were screened for on the autosomes by more laborious, but more traditional genetic methods. That is, we can look not only on the X chromosome, but on all the autosomes, for clock mutants. Secondly, the fact that the period messenger RNA was discovered to undergo circadian oscillations suggested a way in which the community could

look for new clock genes by looking for genes whose products underwent circadian oscillations, a molecular screen that might lead to genes that are important for this basic principle. Third, the ability to construct these transgenic strains which produced light in a circadian manner suggested that that, too, could be used as a screen or a search for new cycling genes because those reporters could be inserted at different locations, and they might themselves lead to the identification of new genes which would be relevant to the circadian program. And finally, one can test candidate genes that come from genomics or from bioinformatics, and you will see in the course of my lecture and in the course of Joe's lecture how the mammalian genetic world and the *Drosophila* world have fertilized each other, and we have been able to follow their lead by looking for ortholog genes, genes which are present in both organisms and are then suspected to do the same thing.

7. *Drosophila* DNA microarray used to assay RNA expression levels (11:31)

Now -- just a glimpse into the future here -- this is an early-stage *Drosophila* microarray in which about half of the *Drosophila* genome, about 6,000 genes, are actually placed down on a microchip. Each of these little dots represents a second gene -- excuse me, a separate gene -- and one can hybridize messenger RNA, for example, taken from different stages of the circadian cycle to such a chip and look for genes which are illuminated, which have a high level of messenger RNA at one time of day and a low level of messenger RNA at another time of day. So the *Drosophila* genome project has and will continue to make enormous contributions to our field with these new emerging technologies.

8. Currently known *Drosophila* clock genes (12:24)

So, this kind of progress over the past decade has taken our single-period gene, the one-clock gene we knew about a decade ago, and has now given us 9 clock genes in flies, and I've arranged these clock genes into 3 categories, which reflects the general outline that the field superimposes on clock physiology. We think about a central pacemaker, that is, the oscillating feedback loop that I described yesterday, and these 5 genes appear to be involved in the dynamics of this 24-hour gene expression feedback loop cycle. There is one very important gene, the cryptochrome gene, Cry, which we believe is an input gene and actually acts as the photoreceptor protein which harvests light and transmits that information from the environment to the central pacemaker. And then there are a series of output genes which connect the central pacemaker to the physiological or biochemical or behavioral events that we actually measure. So the prediction is, if these genes are eliminated, then the particular output phenomenon is eliminated, but the clock will continue to run because the central pacemaker is not dependent on these output genes.

9. Central pacemaker genes: *clock* and *cycle* (13:54)

So the first two genes I want to describe in brief are *clock* and *cycle*, these two central pacemaker genes. Now, we screened for new rhythm mutants by using this apparatus I presented to you yesterday, and most of the *Drosophila* world has looked for new rhythm mutants by placing thousands and thousands of mutagenized flies one at a time in this apparatus and looking for altered behavioral program. And one of the mutants that we came upon, which was arrhythmic, we named Jrk. One of the nice things about *Drosophila* is that you can give your mutants names which remind you of some of your best friends... and this mutant not only was arrhythmic, but in this assay for transcription for gene expression using the luciferase reporter, we found that in this -- in the homozygous mutant, the mutant over mutant, which was arrhythmic, there was very, very little transcription from these flies. So we had the hint or the expectation that this mutant would connect to the transcriptional program that we were interested in, and it turned out that during the two years it took to clone this gene, a time period which should be shortened considerably by the genome project finishing over the past few months, Joe Takahashi cloned a mutant called *clock*, which you will hear about, and it turns out that this mutant Jrk turned out to be mutant in the transcription factor clock, the fruit fly ortholog of the mouse mutant that Joe and his colleagues had cloned just before.

And there's a second mutant called *cycle*, which we also cloned about the same time, with very similar characteristics of transcriptional effects, and these two genes form a heterodimeric partnership. They bind to the promoter of the *per* gene at an E-box, an element that has been described in many transcription-related studies to be important for turning on RNA synthesis, and these two clock proteins are very important for what is called the positive limb of the feedback loop for turning on *period* gene expression, and I have a brief animation to show you which illustrates this.

10. Animation: Negative feedback by *period* protein (PER) (16:26)

Now, this animation is similar to the one I showed you yesterday, except now we have the positive transcription factor CYC and clock which actually bind to the *per* promoter at this E-box and drive transcription turning on RNA synthesis, and here is the production of the *per* protein by the ribosome, the unstable pink proteins which are rapidly degraded, and then every other protein or so molecule is stabilized and accumulates in the cytoplasm during the evening. And when sufficient protein has accumulated, then the protein migrates into the nucleus near the end of the night, and *per* protein interacts with the *clock* and *cycle* protein, probably directly, and that interaction extinguishes transcription, and then in the morning, the *per* protein is degraded. It disappears slowly over the course of the morning until all of the protein is gone, and as the last protein molecule disappears, the Clock and Cycle transcription factors are activated, and transcription and the cycle begins anew.

11. *timeless* protein (TIM) and PER levels in a day (17:45)

So, the next clock gene that I want to describe is called *timeless*, and it was first identified as a mutant and then cloned by Mike Young and his collaborators. And what's shown here is a western blot of the *timeless* protein during a light-dark cycle, and shown on the top is a western blot of the *period* protein in which we directly look at the accumulation of this protein during the circadian cycle, and what you'll notice is that the *timeless* protein accumulates with kinetics and features which almost superimpose on the accumulation of the *period* protein. So both proteins begin to accumulate at the end of the day, and as lights turn off at nighttime, the proteins build up to high amount and then disappear in the morning. You'll notice two additional features. That is, first of all, the *timeless* protein really disappears almost immediately after lights are turned on, a phenomenon I will return to shortly, and you'll also notice that the *period* protein changes its mobility in this assay. That is, on this acrylamide gel analysis, the protein migrates here, appropriate with its molecular weight, and then not only does it accumulate in amount, but it changes its mobility, and that change in mobility is due to phosphorylation of the protein which occurs during the course of its accumulation.

12. TIM is degraded by light and stabilizes PER (19:17)

Now, the effect of light on the *timeless* protein, which I alluded to on the previous slide, can be seen directly here because by merely exposing the fruit flies to a light pulse of 30 minutes causes a very profound effect on the amount of the protein. It's degraded very rapidly to about one quarter its normal level by a brief exposure to light, and this was the indication that this is the clock component which appears to be directly light-sensitive or most light-sensitive. So it is the connector of this clock feedback loop to the external light environmental cycle. Now I think -- next slide, please. So it turns out that not only does the *timeless* protein accumulate in parallel with the *period* protein, but it turns out to be the *period* protein's partner. These two proteins interact and dimerize -- that is, that they actually form a one-to-one partnership in the cytoplasm, and this dimerization is important, critical for *period* protein stabilization during its accumulation phase. And I have a brief video to show that illustrates this point.

13. Animation: TIM causes PER feedback to be affected by daylight (20:45)

So here we have the *period* gene and the *timeless* gene, and they are both driven -- transcription is driven by clock and cycle which binds to both promotors. So these proteins accumulate, and RNA accumulates, is synthesized, and you'll notice that the pink protein disappears, but when the pink protein interacts with the *timeless* protein, then a stable heterodimer is formed. So the distinction between the pink PER protein which gets degraded, and the red *per* protein, which is stabilized, is an interaction with *timeless*. And so the heterodimers now move into the nucleus, so the form of PER that moves into the nucleus is a heterodimer, and it's the heterodimers which interact with clock and cycle and extinguish transcription. Now when the sun comes up, notice that that sunlight quickly causes the degradation of the *timeless* protein, and then, after *timeless* is degraded, the *period* protein slowly disappears, and that disappearance is then followed by the turn on of transcription of both genes, which is then followed by the production of RNA and the beginning of the next cycle.

14. Q&A: Can you affect the master clock by affecting the clock in the liver? (22:05)

And so at this point I think I'll stop and take questions, and start with a question from the house. Yes.

We discussed that the transcriptional feedback mechanism is essential for the clockwork system. So if a biochemical agent is introduced into the organ system such as the liver, which has its own clock, will it in turn affect the clock in the SCN, which is the main clock?

So the -- that precise experiment hasn't been done to the best of my knowledge. But I think we could make a guess at what the answer might be like. To a first approximation in mammals, the clocks in the periphery, like the liver, are downstream of the SCN, and so the guess would be that the SCN clock would continue unimpeded, unaffected by the changes which would take place in the liver. So the hierarchy works to a first approximation one way. But since the experiment hasn't been done, we have to be cautious about being too rigid about our predictions, and so it is conceivable that there are feedback loops from the periphery back to the central guy -- to the top guy in the hierarchy, and perhaps there are connections so that the SCN actually reads how effectively the periphery is responding and what the phase of the ticking in the periphery is and adjusting itself to accommodate changes out at the periphery. So I think the broad prediction would be no, but there may be some subtleties that the experiment, the actual experiment would reveal. So I think it's a good experiment to see what would happen. Let me try to put a spiral on this one.

15. Q&A: How does light degrade the protein? (24:12)

Next question in the house. Yes?

Do you know what it is about light that degrades the protein?

So, we actually don't know what it is about light that degrades the protein, but we can guess or take a hint from the work that's been done about degradation from our colleagues who study the degradation of proteins as biochemists. And the guess would be that light causes a conformational change in the protein, and then that protein is seen by the degrading enzymes of the cell as a favorable substrate. So a related view is that there are modifications which happen to the protein. For example, phosphates that are put on in special places by kinases, the enzymes which add phosphates to proteins, and that those phosphates, either directly or indirectly, make that protein a favorable substrate for the degrading system, either by directly changing its conformation or by perhaps recruiting another partner which likes those phosphates and then binds and then signals to the degrading machinery: "Over here."

16. Q&A: How many different RNAs do we know of? (25:28)

Next, a question from Fox Chase Cancer Center. Go ahead, Fox Chase.

Good morning, sir. My name is Matthew Barnashefsky. I'm from the Northeast High School in Philadelphia. I'm in -- I'm a freshman, ninth grade. My question for you is: how many different RNAs do we currently know of in modern society?

So every gene that we have makes at least one RNA, and fruit flies have about 13,000 genes, and so they make a minimum of 13,000 RNAs, but there are many genes which make multiple RNAs, different RNAs, through a variety of processes -- for example, differential splicing, as Tom alluded to. And so 13,000 is a very lower estimate. The human genome project -- rumors have it that the human genome project is coming in with a low number of total genes that we have, about 30,000 or so. And so that represents the lower estimate of the number of RNAs present in human cells. But probably these phenomena which produce more RNAs than we expect are actually -- are actually going to produce a large increase on that number of 30,000. So the short answer is tons and tons.

17. Q&A: Does light-related protein degradation happen in all cells? (26:52)

So, next a question from Moscow. Go ahead, Moscow.

Olga, Moscow Chemical Lyceum. Can you tell me, please, what you've been telling about the effect of light -- destructive effect of light on the protein? Does it take place in all cells and tissues of the fruit fly, or the process takes place only in some specific cells located, for instance, on the surface of the body of the fly?

That's a terrific question, and I'm going to expand on that point in the second half of my lecture. But the simple answer is that it takes place in all cells, in all tissues of the fruit fly, and that represents a substantial difference between fruit flies and mammals. And I'll hope to give you an explanation of why that is, a simple explanation which will make complete sense.

18. Q&A: Why is it necessary for two proteins to control the circadian rhythm? (27:48)

So next there is a question from East Lyme High School in Connecticut. Go ahead, East Lyme.

Hi. My name is Sarah Hughes, and I'm a senior at East Lyme High School. And my question is: why is it necessary for both the proteins to control circadian rhythms instead of just one?

So that's a terrific question, and of course I don't have a complete answer for you because it involves some speculation about why this came about, and of course we don't know really why it was designed that way. But I can -- we can guess that first of all one of these proteins really connects the system in a very intimate way with light, and that's the *timeless* protein. So it appears that *timeless* is really the light connector, and the *period* protein perhaps is more involved with the free-running oscillations of the system -- the fact that these oscillations persist under constant conditions -- and *timeless* produces the connection with the external world. In addition, there is the general notion in circadian biology that a multicomponent system makes the oscillator more robust, as it's called -- that is, that it can operate over a wider range of conditions and continue to tick away, with almost invariant properties, this 24-hour rhythm at different temperatures, under different conditions, and perhaps the multiple components give it properties which allow it to do this despite changes in the external world.

19. Q&A: Do any other external factors affect the system? (29:25)

So the last question is from the house. Yep?

Are there any other factors, external factors, that would affect the way that the cell works, or the production?

So the answer is -- the answer is almost certainly yes, and some of them we really don't know about. So for example, there may be -- when you eat, how you eat may well have an effect. There may be subtle feedback. But the big one, the one that we do know about other than light, is temperature. So in an organism like fruit flies which are not kept at 37 degrees like us, whose metabolism varies with the external temperature -- temperature has a big effect on the clock, but in terms of setting the clock, not in terms of changing the periodicity. So the period, as I mentioned, is temperature-independent or temperature-compensated, but if you experience a temperature shift and don't experience a change in the external light environment, you can then reset the clock with that temperature change, just like the warming of the day as the sun comes up or the cooling off of the day as the sun sets represents a signal to the animal that it's time to reset the time of day. So I'm not sure if I can do this overhand up there all the way. Let's see how close we get. Not quite.

20. The role of the kinase Doubletime (DBT) (31:01)

So with that, let's continue into the second half of the lecture. And I'll now add another component to the central feedback loop, the protein kinase doubletime, which was initially identified as a mutant and cloned in Mike Young's laboratory. Now, the doubletime kinase -- this doubletime mutant is related to a human -- a mammalian enzyme called casein kinase 1 epsilon, and you will hear about the connection between this gene, this fruit fly gene, and that enzyme with its mammalian equivalent in Joe Takahashi's talk. Because it turns out that this protein not only impacts and is a clock mutant in fruit flies -- just like the transcription factor clock, it is also a clock mutant in mammals. So this modifying enzyme adds phosphates to the *period* protein and affects the metabolism of this protein both in the cytoplasm during this accumulation phase here and also most prominently, in the fruit fly at least, in the nucleus, where this doubletime kinase and these modifications on the *period* protein signal the protein for degradation and contribute to this degradation phase here. So this is the one, two, three -- this is the fourth central clock gene that we have added to this picture. Next slide, please.

21. Cryptochromes: The light-sensitive protein as a candidate for circadian light sensor (32:47)

So now I want to tell you a brief story about what we believe is the photoreceptor in fruit flies, the protein which actually harvests light and which transmits that light information to the central pacemaker. And this is a story about a protein called *cryptochrome*, and this is a protein family -- the photolyases or cryptochromes, which had been studied both in bacteria and in plants. In bacteria, the parent protein, the one that had been studied in most detail, was shown to be a blue-light dependent DNA repair enzyme, so this protein actually is involved in repairing UV damage to DNA and functions in a wide range of organisms, and the fact that it exists in E. coli as a repair enzyme and in other higher organisms attests to the fact that the protein has been around a long time doing the same job in these diverse organisms. And it has cousins -- that is, related proteins -- that function as blue-light photoreceptors in plants to carry out a number of developmental functions. And now we even know that these proteins also contribute to circadian light harvesting in plants. And so when the plant data emerged and when there was even a paper published on the presence of these proteins in humans, we were very interested in exploring the idea of whether these proteins might be the light harvesters, the actual photoreceptors in fruit flies. And we had known for 30 years or more that blue light were the wave lengths that were most effective in phase shifting the circadian clocks in flies, but we had no idea for all this time, well before I became involved in the field, of what was the real molecule which was the photoreceptor.

22. Are transgenic flies that have a double dose of the *chryptochrome* gene sensitive to light? (35:00)

And so with this possibility that cryptochromes which were blue-light sensitive proteins might do this job, there appeared, through the magic of the *Drosophila* genome project just at the time we were thinking about this -- there appeared a *Drosophila cryptochrome* as a sequence about which there was nothing known. So we cloned this gene using the information from the database, and the first experiment we did was to insert this gene into fruit flies, wild type copies of the gene, extra copies all over the fly and all of the fly tissues, just giving the fly a lot more of this protein. And we wanted to ask, would this make the flies more light sensitive? Would it make their circadian system more light sensitive? And what's shown here in blue are the flies which have extra copies of the gene, and what's shown in yellow are the flies which are normal, which have the normal one copy of the gene. And this, what was assayed was a phase shift at two different times of day in response to a light pulse. So normal flies experience a phase delay if one gives them a light pulse at this time of night, and they experience a phase advance if one gives them a light pulse at this time of night. And much to our disappointment, when we took the overexpressed -- the flies with extra *cryptochrome* genes -- and did the same experiment, we found little or no effect. And then someone in the lab had the bright idea, no pun intended, that maybe we should assay these flies at lower light intensities, because perhaps when one exposes the flies to an intense shock of light, that the normal amount of *cryptochrome* was sufficient to gather all the light that was there, and having extra Cryptochromes wouldn't make any difference. But if there was limiting light so you just give low-intensity illumination, then the normal flies would be limiting for the amount of light harvesting protein that they had, and the transgenic flies would respond more robustly to this low level illumination. And that's exactly what happened. At low levels of illumination, both at this time of night when there is a delay in response to the light pulse and at this time of night when there's an advance in response to a light pulse, we saw a very dramatic change in the response, suggesting that *cryptochrome* really was the light harvesting protein.

23. *cryptochrome* mutant showing no circadian oscillations (37:43)

So remarkably, at the same time, we were screening for new mutants in fruit flies using this strategy of sprinkling this *period* gene, *luciferase* reporter, around the genome and looking for locations in which this would cycle, indicating that the locations where the reporter gene had landed were actually potential clock genes. And one of these locations turned out to be flat in a particular mutant. So that the mutant failed to undergo these oscillations, and it turned out that this mutant was in the same gene, the same *cryptochrome* gene that we had cloned. And here's an example of this response of the *timeless* protein to a light pulse. Here's the degradation I showed you previously, where the wild type *timeless* protein disappears, and here is the Cry mutation called Cry-b, named by my colleague Jeff Hall after the rock 'n' roll song "Crybaby." And you'll notice that in this mutant, a 15-minute light pulse or even a one-hour light pulse fails to degrade the *timeless* protein. So the reporter gene fails to cycle, and the reason that it fails to cycle is because it can't get any light information. The flies are light blind and, therefore, don't undergo circadian oscillations.

24. Constant light does not suppress the circadian rhythm of *cryptochrome* mutants (39:29)

So, it also turns out, remarkably, that this *cryptochrome* mutant strain is also resistant to the effects of constant light. So, it turns out that all organisms that have been tested need some period of darkness for their circadian clocks to function properly. If you maintain a mouse or a fruit fly in constant light, then these animals' circadian systems respond adversely -- they don't like to be in constant light. And two things happen. Under low intensity illumination, the period alters. So, instead of being the natural period of 24 hours or 24 1/2 hours, the period may lengthen to 27 or 28 hours, and, in some organisms, the period actually shortens in response to low intensity illumination. In response to high intensity illumination, however, many organisms, including fruit flies, actually go arrhythmic. So the clock actually stops when the animals are exposed to continuous illumination. And yet, these mutant flies that have little

or no *cryptochrome* protein, these mutant flies continue to be rhythmic, and only a small portion of them are arrhythmic, and the period of these mutant *cryptochrome* mutant flies is pretty decent. It's very, very close to the wild type period. So even the period lengthening doesn't take place.

25. Animation: DBT and *cryptochrome* protein's effects on circadian rhythms (41:07)

And so I have, I think, next an animation which now illustrates the contribution of both *doubletime* and *cryptochrome* to this cycle, which we're building in a more and more complex fashion. So here's the Doubletime kinase, which puts phosphates on the *period* protein, and here's the Cryptochrome, the light harvesting protein. And so now the cycle starts out as it did before, but we see that the Doubletime kinase, this Casein kinase 1 epsilon, is actually the agent which contributes to the degradation of the *period* protein which kills the pink proteins. And if the *period* protein which is produced gets together with this partner Timeless and forms a heterodimer, then the heterodimer is resistant to the effects of the Doubletime kinase. So, that's really the distinction between the protein which is degraded and the protein which is accumulated. Then the heterodimers get together and connect, and here is the conversion of Cryptochrome from an inactive to an active form, and it goes and kills Timeless. And then the Doubletime kinase goes and kills the *per* proteins, turns over the proteins, and transcription becomes anew. So, this has added two elements to the story. First, the Doubletime kinase is actually the agent which turns over the *period* protein or assigns the protein as being a substrate for degradation in the manner that I referred to previously. And second, Cryptochrome is actually the light harvesting protein, and Cryptochrome, when it's converted from an active -- from an inactive to an active form by light, Cryptochrome actually then leads to that very rapid degradation of the *timeless* component, which then begins the day anew, leads to the turning over of the *period* protein, and starts the cycle once again.

26. DBT mutant strain showing a long period (43:15)

So, this is an indication -- this gives you a flavor for really a piece of biochemical data which shows how a Doubletime mutant actually changes the molecular profiles of these proteins. So, here is the wild type protein profile I showed you previously. This is the Timeless protein, which accumulates over the course of the night, and this is the *period* protein, which also accumulates over the course of the night and then, in the morning, experiences the slow disappearance, this degradation phase. And here is a doubletime mutant strain, actually a heterozygous strain. This is an allele, a mutant that we uncovered in our screen, in our laboratory, and this strain has a long period. It has a period of 27 or 28 hours instead of 24 hours. And note the fact that in the morning, this protein is disappearing with kinetics which take much, much longer than the wild type protein. So, the wild type protein is effectively gone 5 to 7 hours after the lights have turned on, whereas this protein hangs around for a long time. It's ineffectively degraded, and as a consequence, the whole profile is extended, and the 24-hour cycle actually takes 27 or 28 hours. Now, if I might have the next slide, please. I think we go now to the animation.

27. Animation: DBT mutant kinase makes circadian period longer (45:04)

So, this now gives you a flavor for how the doubletime mutant protein, this mutant kinase which doesn't work very well, compares with its wild type counterpart and the effect of that mutant on the cycle. So, here's the Doubletime kinase destroying the *period* protein. And notice that it's having trouble functioning. It takes a couple of shots to kill the pink protein as compared to the normal one shot which the wild type protein is able to effect. Yet the accumulation in the cytoplasm in flies accumulates -- occurs pretty normally, and so these heterodimers accumulate in fairly normal fashion. They migrate into the nucleus. They make contact with the positive transcription factors, extinguish transcription. And now here's the conversion of Cryptochrome to the active form. And now watch what happens. *Timeless* disappears very quickly, and now the nuclear form of the kinase starts to degrade PER, and the mutant form has trouble keeping up. It's working more slowly. This takes longer to go away, and as a

consequence, the turning on of transcription the next day occurs more slowly in the mutant strain, the strain with the mutant doubletime kinase, as compared to the wild type strain. So, that's actually a fairly realistic depiction of how that mutant actually lengthens the protein.

28. Description of some output genes, particularly *pdf* (46:38)

So, now I'll just say a word about output genes. I should say that there are a couple that we know about, vrille and takeout, which I won't have time to talk about. One of these is a transcription factor, and one of these actually connects to food and feeding behavior. But I will mention the pigment disbursing factor, this hormone that's important for locomotor activity rhythms. And we discussed this briefly yesterday, this protein. I told you that this neuropeptide is present only in 8 cells, and those are the 8 neurons in the brain which have very prominent, very intense, very high levels of the period and *timeless* proteins. And here's a picture of these 8 cells which are stained with a fusion protein of PDF. And these 8 cells are stained, and also their processes are stained. And these cells make contact with the eye. They also make contact to the same 8 cells on the opposite side of the brain, and they make connections up here to interneurons, which appear to be important for the locomotor activity program. So, this neuropeptide is important for locomotor activity rhythms, but it also represents a tool that we can use to ask and answer an important question.

29. Experiment indicating the lateral neurons as the *Drosophila* pacemaker (48:12)

We can take advantage of technology that was developed over this past decade by Norbert Perrimon and his colleagues, and we can take the control region of this PDF gene, and we can use it to express a transcription factor only in the cells in which that control region is programmed to express. And I told you that that gene is only expressed in those 8 cells important for locomotor activities. This then allows us to express the cryptochrome light harvesting protein only in those 8 cells by restricting its expression using this particular technology developed for fruit flies. And if we express this cryptochrome protein only in these 8 cells, we can now ask if the effects of the mutant, the strain which is missing the cryptochrome protein, is rescued by having this protein only expressed in those 8 cells. And it turns out that all of the locomotor activity defects that exist in the strain, in the mutant cryptochrome strain, are rescued by expressing this protein only in those 8 cells. So, this allows us to draw an important conclusion, and that is that these 8 cells, these lateral neurons, as they're called, or pacemaker cells, are really the fruit fly equivalents of the suprachiasmatic nucleus as far as locomotor activity is concerned because all the elements that we need to drive a clock -- both input elements, the cryptochrome protein; the 5 pacemaker components -- the clock, cycle, PER, TIM, and doubletime -- are present in these cells. And even an output component, a neuropeptide which is sent down the axon which connects to the next motor centers, are present in these 8 cells. And if these elements are functioning in these 8 cells, then we have a viable clock. And, of course, the fact that we can rescue light harvesting by only expressing this protein in these 8 cells tells you that these brain cells are directly photosensitive. So they connect directly to light, and these represent, then, deep brain photo receptors, and they're really the first example of this kind. Of course, you'll appreciate -- and something to think about both for questions and in general is the fact that our skull, of course, prevents a comparable biological solution to the problem for mammals.

30. Summary of the *Drosophila* clock system (50:51)

So this is -- this is a contemporary view, a pretty sophisticated contemporary view of the clock system in flies. And so this brings me to a summary, where I have told you how these 5 proteins are arranged in this feedback loop that generates 24-hour oscillations, cryptochrome is the light harvesting protein which connects to *timeless*, which is the light sensitive, the light relevant central clock component, and in work I just didn't have time to explore in any detail at all -- I'll simply tell you that there are output genes, genes which connect directly to behavior, which are related to this transcriptional feedback loop in a very

similar way. They're regulated by the same sets of genes, and they exhibit 24-hour periodicity in their gene expression, and that connects to behavior. So, with that, I'd like to thank my lab members, the people in my lab who were responsible for this work. I'd like to also thank a lot of ex-lab members, as well as my good colleague, friend, and collaborator, Jeff Hall, who really brought me into this field 18 years ago and has taught me an incalculable amount of work -- amount of information about biology. And finally, I'll leave you with just 3 brief sentiments. First of all, I hope I've given you some feeling for the remarkable progress that the field has made in understanding these timing mechanisms in the brains of all animals and the relationships between clocks in flies and other organisms like ourselves. Secondly, the importance that genetics has played both in general and in *Drosophila* as an entree into a biological problem which otherwise turns out to be quite mysterious if not really opaque, and last, but not least, how much fun you can have in pursuing your dream in this kind of an enterprise. Thank you very much.

31. Q&A: Have you substituted heat or other cues for light? (53:06)

So, a question from the house. Wow

Have you tried substituting heat for light or maybe different combinations of darkness but with a warmer temperature?

That's a great question. We've really not done that in fruit flies much. It's been done in other animals and in particular in neurospora by Jay Dunlap and his colleagues, and it turns out that you really can try to play off heat and light and ask which is more important. It's tricky because it's not completely clear how to play with the intensities to try to get a sense of who plays the dominant role. Our sense is that in flies, light is more important than temperature, but that's a very soft conclusion because the proper experiments haven't been done yet. Yes?

32. Q&A: How does a circadian clock sustain the 24-hour cycle in the absence of light? (54:00)

How do circadian clocks sustain the 24-hour cycle in the absence of light, which is necessary in the feedback mechanisms of the *per* and TIM cycle?

So, it appears for reasons which are not completely clear that these oscillators, at least in their central locations, the oscillators in those 8 cells, as well as in the brain of mammals in the suprachiasmatic nucleus, can keep ticking away without any external influence, and that distinguishes them from the oscillators which are present elsewhere. And it's not completely clear what that distinction is.

33. Q&A: Do the pacemaker genes have a similar sequence to a mammalian gene? (54:36)

There is a question from Miami. Go ahead, Miami.

Hello, Doctor. I'm Julian Amarancy from Miami Northwestern senior high school, and what I would like to know, Doctor, is do the pacemaker genes that you mentioned in the fruit flies have a similar DNA sequence to the pacemaker genes of a mammal?

So, that's a very good question, and the answer is -- the answer is they have a very similar DNA sequence, not an identical DNA sequence. We believe that most of those differences are due to what are called neutral mutations, which don't have very much impact on what the gene does. They just represent the drift of the DNA as fruit flies and mammals have separated, but it could be that some of them really are functionally distinct and affect the gene's function in subtle ways. So, I'm told that it's time to go, and I want to thank you all very much -- both here and at these remote locations for your attention. Thanks again.

34. Closing remarks by HHMI President Dr. Thomas Cech

Thanks, Michael. You talked a lot about feedback. So, now let me give you some feedback. That was a really terrific lecture, and I particularly like the way that you showed how early models were not wrong but they were just incomplete, and then latter data allowed to you get a more complete model, something that happens so often in science. We're going to take a break now for half an hour. When we return, Joe Takahashi is going to take us from the fruit fly to the mammals and tell us about how mouse genetics and genomic databases have allowed us to learn more about circadian rhythms in complex creatures. He'll also tell us a bit about how this research might be of use to human patients. See you then.