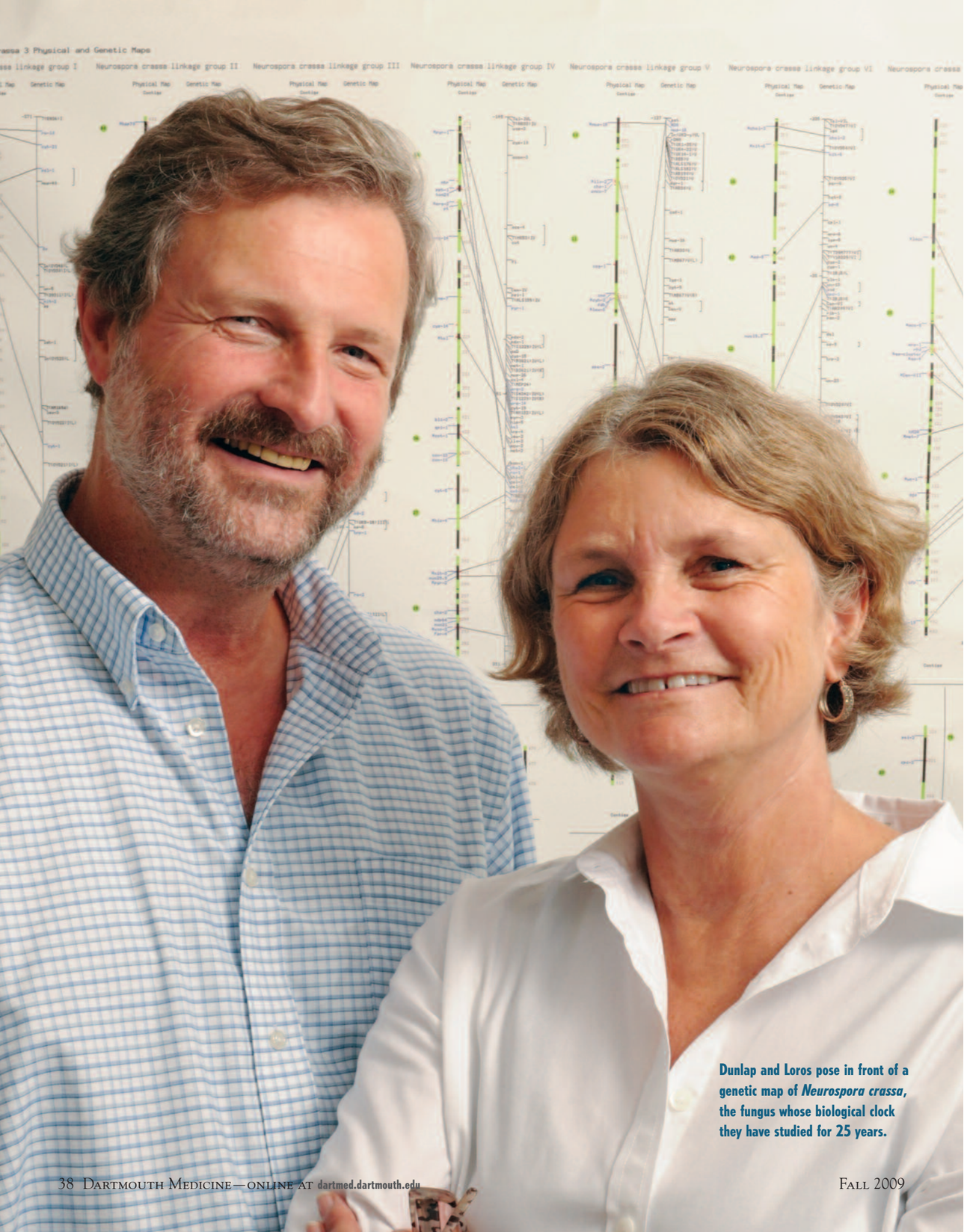


Dunlap and Loros pose in front of a genetic map of *Neurospora crassa*, the fungus whose biological clock they have studied for 25 years.

Clock-wise

Photographs by Jon Gilbert Fox Text by Amos Esty

This pair of Dartmouth researchers has become increasingly wise to the ways of the circadian clocks that control the daily routines of virtually every organism on Earth—from human beings to the simple fungus that they study.

For the past 25 years, a pair of Dartmouth researchers has been trying to figure out how a fungus tells time. Along the way, Jay Dunlap, Ph.D., and Jennifer Loros, Ph.D., have helped to explain one of biology's fundamental mysteries—how organisms, including humans, have adapted to the 24-hour cycle that we call a day.

During the time it takes for the Earth to rotate on its axis once, the average person experiences a host of physiological changes. Your body temperature rises from a low of about 97 degrees to a high of about 99 degrees, then falls again. Your short-term memory peaks just after you wake up, while your athletic performance improves in the late afternoon. And after midday, as drowsy workers around the world can attest, a siesta starts to seem very appealing.

These changes, and many more, are regulated by an internal circadian clock ("circadian" comes from the Latin words for "about a day"). This clock is powered by a series of biochemical reactions that take place within individual cells. In other words, Dunlap says, "you wake up in the morning because of things that are going on inside single cells."

Long before the field of circadian biology existed, close observers of nature realized that many organisms exhibit daily rhythms. In 1729, French scientist Jean Jacques d'Ortous de Mairan wondered why the leaves of a plant in his garden—a heliotrope—opened during the day and closed at night. The obvious explanation was that the heliotrope was responding to the sun. But when de Mairan dug up the plant and placed it in a dark room, he found that it continued to follow the same regular pattern, despite the lack of light. So the sun couldn't be causing the changes. Charles Darwin, too, was intrigued by leaf movement, a topic that he discussed at length in an 1880 book titled *The Power of Movement in Plants*.

The fungus *Neurospora crassa* (pronounced nuh-RAH-spuh-ruh CRASS-uh) is the focus of much of the research carried out by Dunlap and Loros. As do humans and heliotropes, it exhibits a number of regular daily rhythms. The most obvious example is its growth cycle. To study *Neurospora*'s development, researchers in the Dunlap-Loros lab introduce it into thin, 15-inch-long glass containers called race tubes and provide it with enough food to fuel growth. The fungus spends most of the day spreading down the length of the tube, a few millimeters per hour. But around midnight, it starts to lay down cells that can produce spores. Over the next several hours, as *Neurospora* continues to grow horizontally, these cells will simultaneously grow vertically, producing orange spores that rise above the mass of the fungus on the bottom of the race tube. By mid-morning, the spores have matured and in natural conditions could be picked up by a breeze. At this point, *Neurospora* stops producing spores, growing only horizontally until the clock again strikes midnight.

If left for a week, *Neurospora* will continue this same pattern, spreading horizontally for most of the day and then producing spores for several hours early each morning. At the end of the week, the race tube will contain seven orange spots where the spores are clearly visible, each separated by an inch or so of horizontal growth.

Like the heliotrope, *Neurospora* exhibits this cycle even when it's kept in the dark. The length of the cycle changes slightly—from 24 hours to about 22 hours—but the rhythm remains the same. And that raises the question: how is this simple organism able to tell time?

Dunlap now expounds enthusiastically about this question, but circadian biology was not a field that he originally intended to enter. As an undergraduate, he majored in oceanography. He then enrolled in graduate school at Harvard, planning to get a degree in biochemistry and apply it to marine life. Early in his study of biochemistry, he came across tiny creatures called dinoflagellates. Some species of these single-celled organisms can produce flashes of light—something like seafaring fireflies. Their bioluminescence is the result of a reaction between a number of proteins, and these proteins are present in much

Amos Esty is the managing editor of Dartmouth Medicine.

Grad student Chen-Hui Chen is one of many students and postdocs who have worked in the Dunlap-Loros lab.

The DMS researchers concluded that circadian clocks work as a negative feedback loop. An example often given of a negative feedback loop is a household thermostat. Too much heat causes the furnace to turn off, which allows the temperature to drop, which makes the furnace turn back on.

greater amounts at night than during the day, when the flashes of light would be overpowered by the sun. Somehow, these dinoflagellates are able to schedule their use of bioluminescence to maximize its effectiveness.

Dunlap wondered how these organisms could possibly know to expend energy to generate light only at night. No one he talked to had an explanation. "It was clear this was a problem where no one had any clue as to how it worked," Dunlap says. "And, as far as I could tell, they weren't even pursuing it in the ways in which you'd have to pursue it in order to get an answer. So I saw it as a great problem."

Dunlap decided to take on circadian rhythms, but in *Neurospora*, an organism that is widely used for genetic studies. He went on to a postdoctoral position in the lab of Jerry Feldman, Ph.D., at the University of California at Santa Cruz. Feldman had earlier identified a *Neurospora* gene called *frequency* (*frq*). Feldman found that strains of *Neurospora* with a mutated form of *frq* display abnormal circadian rhythms, indicating that the gene must play some role in the organism's clock.

As a postdoc, Dunlap encountered new tools made available by the revolution taking place in molecular biology—tools that made it easier to dissect the inner workings of cells. It was also while he was a postdoc that he met Loros.

Jennifer Loros was a graduate student at UC-Santa Cruz, and, like Dunlap, she started her scientific career with a very different focus from circadian rhythms. "I'm a gardener," she says. "That's actually how I got into science." She'd planned to study hormones in plants, but, she says, "I ran into sort of a wall with my first Ph.D. project and ended up changing laboratories to work in circadian rhythms. And I've been doing it ever since."

Early in 1984, Dunlap took a position as an assistant professor at Dartmouth Medical School. "They were looking for someone who was doing something different," he recalls. At the time, circadian rhythms certainly fit that description; in fact, some scientists still weren't convinced of the merits of circadian biology and didn't think studying a fungus could lead to a better understanding of circadian clocks in humans. There was another factor in the decision, as well. "It was the only job I got," Dunlap says. "That made the choice easy."

For a time, Loros remained in California to finish her dissertation. But that fall the two married, and in January 1985 Loros joined Dunlap's lab as his first postdoc. They have since shared lab space, as well as authorship on most of their papers. Within a few years, Loros became a fellow member of the faculty. Now, their offices lie on opposite ends of the same large lab and they run lab meetings together, but they are both independently funded principal investigators.

"The original plan was not necessarily to work together the rest of our lives," Loros says. "But I helped set the lab up while he was writing grants." In those grant proposals, Dunlap outlined three bold lines of research. He wanted to know how circadian clocks work at the molecular level, how clocks are affected by external cues (such as light), and how the clock shapes *Neurospora*'s behavior by regulating gene expression.

A second postdoc, C. Robertson McClung, Ph.D., soon joined the lab. "When I came to Jay's lab, I wasn't necessarily thinking that I would become a chronobiologist," says McClung, who is now a Dartmouth College professor of biology. "But the problem, as Jay explained it to me, was just beautiful."

Working together, Dunlap and McClung succeeded in cloning the *frq* gene, opening up new ways of investigating the role of the gene in the circadian clock. The breakthrough earned them a spot in the prestigious journal *Nature*.

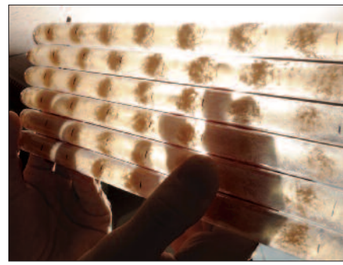
"It was tremendously exciting," McClung says. "You could see detectable progress, and there were 'eureka' moments."

Cloning *frq* allowed Dunlap and Loros to try to nail down the gene's exact role in *Neurospora*'s circadian clock, which turned out to be a big step toward answering the first question posed by Dunlap—how the clock runs at the molecular level.

Working with postdoc Benjamin Aronson, Ph.D., Dunlap and Loros discovered that the expression of *frq* is turned up at some points during the day and down at others, following a regular 22-hour cycle. Simplified, there are two basic steps in gene expression: transcription, in which DNA is transcribed into RNA, and translation, in which RNA is translated into proteins. The researchers found that frequency proteins limit the production of *frq* RNA—in other words, the translation part of the process feeds back to regulate transcription. When *frq* expression did not fluctuate—for example, when *Neurospora* was manipulated so that *frq* RNA levels remained constant—the fungus no longer followed the 22-hour cycle of horizontal growth and spore production. Instead, it produced spores constantly. Clearly the cycling of *frq* expression was central to the proper functioning of the clock.

Based on their findings in *Neurospora*, and the work of other researchers in fruit flies, Dunlap and Loros inferred that circadian clocks work as a negative feedback loop. An example often given of a negative feedback loop is the regulation of household temperature. If a thermostat is set to 68 degrees, it will turn on the furnace when the temperature drops below that point, producing heat. Once the heat raises the temperature above 68, the thermostat turns off the furnace, until the temperature again drops below 68. With help from the furnace and the thermostat, heat regulates itself—too much heat causes the furnace to turn off, which allows the temperature to drop, which makes the furnace turn back on to produce more heat, and so on. The expression of the *frq* gene plays a role in *Neurospora*'s circadian clock akin to that of heat in keeping a house at the right temperature.

In 1994, Dunlap and Loros published the results of this research in another top journal, *Science*, outlining the basic steps of the negative feedback loop. They also pointed out similarities between their findings and the work of scientists who were studying the circadian clocks of fruit flies. The similarities, they wrote, suggested that negative feedback loops "may be a universal feature of circadian oscillators." Studying a fungus was by then looking like a very promising way to find out exactly how circadian clocks work in more complex organisms.

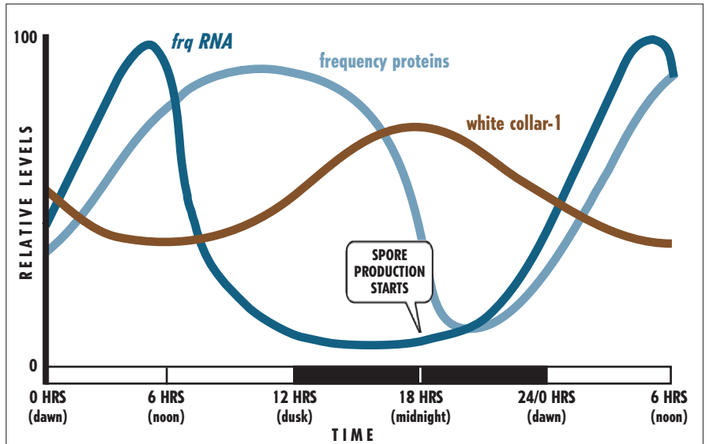


Above, these tubes show *Neurospora*'s growth over the course of a week; the spots mark the few hours each day when the fungus produces spores. At left, this illustration from a book by Charles Darwin shows early awareness of circadian rhythms; the leaves of this plant, *Cassia corymbosa*, are open during the day (left) but curl up at night.

In 1994, Dunlap and Loros published their results in another top journal, *Science*, noting similarities between their findings and the circadian clocks of fruit flies. Studying a fungus was by then looking like a very promising way to find out how circadian clocks work in more complex organisms.

Over the past 15 years, Dunlap and Loros have continued to add more pieces to this puzzle, resulting in a detailed understanding of *Neurospora*'s circadian clock. Along with *frq*, two other cellular components form the heart of the negative feedback loop—the proteins white collar-1 and white collar-2 (named for the effect that mutations in these genes have on *Neurospora*'s appearance). Together, white collar-1 and white collar-2 form a transcription factor for *frq*, meaning that when they bind to that gene, they turn it on, leading to the production of *frq* RNA.

In the hours after midnight, this transcription factor gets to work, prompting the expression of *frq*. By mid-morning, there's an abundance of *frq* RNA available in *Neurospora*'s cells. Eventually, the *frq* RNA is translated into frequency proteins. These proteins provide negative feedback by blocking the transcription factor from binding to the *frq* gene. As a result of this obstruction, the production of *frq* RNA slows down in the late morning and early afternoon. The *frq* RNA that has already been produced continues to be translated, which means that protein levels reach their peak in the mid-afternoon or early evening—hours after



This graph shows fluctuations in *Neurospora*'s relative levels of *frq* RNA, frequency proteins, and white collar-1 proteins over the course of a little more than one circadian cycle; day and night are indicated by the white and black portions of the bottom bar.

Say a *Neurospora* cell catches a morning flight from Paris to New York. When it arrives in mid-morning, the cell feels as if it's mid-afternoon; if it had stayed in Paris, its frequency proteins would be starting to decline. But the extra hours of daylight in New York adjust its circadian clock.

the amount of *frq* RNA goes into decline. Over time, frequency proteins are broken down by enzymes, diminishing their numbers. And with no remaining reservoir of *frq* RNA waiting to be translated, protein levels continue to decline.

Here's where it gets complicated: in addition to blocking the expression of the *frq* gene, frequency proteins also promote the translation of white collar-1 RNA into white collar-1 proteins. (White collar-2 levels remain constant through the day, but white collar-1 fluctuates.) The more frequency proteins there are, the more white collar-1 proteins are produced. But when frequency protein levels are high, it's hard for white collar proteins to act as a transcription factor, leaving all that white collar-1 and white collar-2 sitting around waiting for something to do.

Late at night, this process reaches a tipping point. There are no longer enough frequency proteins to block the white collar proteins from binding to the *frq* gene, which means *frq* RNA will again be produced, leading to the production of frequency proteins. The cycle starts all over again.

By identifying these pieces and putting them together, Dunlap and Loros proved that it is indeed possible for a single cell to contain all the components of a circadian clock. (In fact, there are several other pieces that add still more complexity to the picture.) But this explanation does not account for one of the fundamental properties of circadian clocks: when an organism is kept in constant darkness, its circadian clock does not stick to a strict 24-hour cycle. In *Neurospora*, the cycle reverts to 22 hours; in humans, it's between 24 and 25 hours. A related issue is the fact that, given a few days, it's possible to adjust to very different day-night cycles, such as when traveling across an ocean.

The second challenge posed by Dunlap in his early grant proposals—how circadian clocks are affected by external factors—targets these questions. Again, he and Loros deserve much of the credit for finding the answers.

Progress in this area resulted from the work of Susan Crosthwaite, Ph.D., a postdoc in Dunlap and Loros's lab, and Allan Froehlich, Ph.D., who worked in the lab as both a graduate student and a postdoc. Crosthwaite's research examined how light affects the expression of *frq*, and Froehlich built on her findings by outlining the "nuts and bolts," as he puts it, of how light resets *Neurospora*'s circadian clock at the molecular level.

Essentially, white collar-1 plays the role in *Neurospora*'s clock that eyes play for humans—it recognizes light. When that happens, the white collar proteins become more active as a transcription factor, triggering the production of *frq* RNA, regardless of the time of day.

As an example, say a *Neurospora* cell catches a morning flight from Paris to New York. When it arrives, it will be mid-morning in New York, but the cell—still on Paris time—will feel as if it's mid-afternoon. If it had stayed in Paris, the cell's level of frequency proteins would be starting to decline, reaching a low point about eight hours later.

Instead, the cell is exposed to several extra hours of daylight in New York, and its circadian clock starts to adjust. White collar-1 senses light, prompting the white collar proteins to continue producing *frq* RNA. As a result, frequency proteins are produced for several hours more than would have been the case had the cell remained in Paris.

If a *Neurospora* cell travels in the opposite direction, leaving New York at night and arriving in Paris at dawn, the exposure to light earlier than expected will cause the white collar proteins to ramp up their activity several hours ahead of schedule, again resetting the clock. This same process happens on a small scale every day, lengthening *Neurospora*'s innate circadian rhythm from 22 to 24 hours to keep it in line with the Earth's rotation.

But what if *Neurospora*'s clock couldn't be reset—if it consistently followed a 22-hour growth cycle? At first, it might not make much difference. But within about a week, the fungus would produce spores at noon, when the sun is most intense. The spores are vulnerable to ultraviolet rays and so less likely to survive if left to bake in the sun, which would reduce *Neurospora*'s ability to spread.

Producing spores costs *Neurospora* in energy expenditure, but the spores are an essential part of its life cycle. So it makes sense that, over evolutionary time, *Neurospora* has developed a rhythm that enhances the odds that spores survive. Dunlap cautions that this explanation for *Neurospora*'s development pattern hasn't been proven experimentally. But he notes that studies in other organisms, including plants and cyanobacteria, have shown that adhering to a cycle in tune with the Earth's rotation does provide fitness advantages.

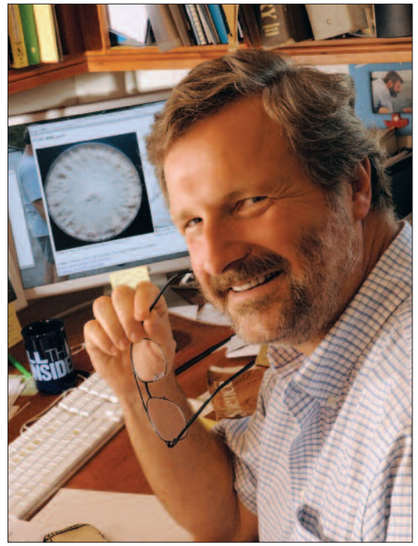
This leads to Dunlap's third challenge—figuring out which parts of *Neurospora*'s physiology are regulated by the circadian clock and how the clock exerts this control. Those are questions Loros has devoted much of her career to investigating, ever since she was a postdoc.

While Dunlap and McClung worked to clone *frq*, Loros tried to identify genes that are controlled by the clock. She used both a normal strain of *Neurospora* and a mutated form—called frq^7—that has a 29-hour cycle. She measured the amount of RNA present in *Neurospora* cells at different times of day and found two genes whose expression follows the same rhythm as *Neurospora*'s internal clock. Unlike the *frq* gene, these newly identified genes were not part of the clock itself; they could be knocked out without affecting the functioning of the clock.

In 1989, Loros published this research in *Science*, coining the now widely used term "clock-controlled gene" (ccg) to describe such genes. In subsequent years, she worked with postdoc Deborah Bell-Pedersen, Ph.D., to try to figure out what those genes do. They found that *ccg-2* produces a protein that coats the outside of spores, making them repel water. In moist environments, this protein helps spores survive. In strains with an inactive *ccg-2* gene, the spores clump together, making it harder for them to be dispersed by the wind.

Loros and Bell-Pedersen also identified a number of additional clock-controlled genes not related to *Neurospora*'s growth cycle. Bell-Pedersen says that these findings added to the evidence for the importance of circadian rhythms. "We'd known for a long time that the clock regulates development, but it wasn't clear what other processes in the cell were affected by the clock, if any," she says.

There are now more than 180 *Neurospora* genes confirmed to be under the control of the clock, but there are still many questions about exactly how the clock regulates genes. In some cases, Loros says, the white collar proteins might directly determine whether another gene is expressed. In other instances, they might be the start of a longer cascade, triggering the expression of one gene that in turn causes the expression of another. But whatever the exact mechanism, it has become clear that circadian clocks affect a wide range of behaviors. "In *Neurospora*, like in people, the clock controls an awful lot of the physiology of the cell," Dunlap says.



Dunlap, at left, is a member of the National Academy of Sciences, the premier U.S. scientific body. He and Loros—who is pictured above with their dog, Fiona—collaborate 24/7. They were married in '84, she became a postdoc in his lab in '85, and they've shared lab space ever since, though they're both now independently funded investigators.

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Some of those effects have implications for human health. In 2006, António Pregueiro, Ph.D., then a graduate student, cloned a mutation in the *Neurospora* gene *period-4*, which is a homologue of *checkpoint kinase-2*, a tumor suppressor gene in humans. *Checkpoint kinase-2* stops the cell cycle when DNA damage occurs.

Pregueiro's finding linked circadian clocks to the cell cycle, showing that damage to a *Neurospora* cell's DNA can reset that cell's circadian clock—just as changes in light exposure can. Many cells divide only at a specific time of day. If DNA dam-

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Clock-wise

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age occurs, resetting the clock might keep a cell from dividing at a time when a mutation is likely to result, thereby preventing mutations that could lead to tumor growth. Earlier this year, postdoc Joshua Gamsby, Ph.D., reported similar results in mouse cells, proving that in mice, as in *Neurospora*, DNA damage can reset the clock.

These findings add to the growing recognition of the relationship between the circadian clock and cancer. "We are, as a field, now understanding that the clock is very important in a number of different cell-cycle events," says Loros, "and that misregulation in clocks often results in cancer."

In one study, mice lacking the gene *Per2*, a key clock component in mammals, were more likely to develop tumors. Another study, done in Denmark, found that women who work at night had a greater risk of breast cancer than woman who work day shifts. And a number of studies have shown that timing chemotherapy to take advantage of circadian cycles can improve outcomes.

The complexity and importance of the circadian clock explains why it's held Dunlap and Loros's interest for so many years. "Although I've worked on clocks my whole life, I feel like I'm constantly being thrown into other areas, completely new areas of science, because clocks affect such a broad spectrum of an organism's biology," says Loros.

"Most of physiology is at least indirectly under clock control," Dunlap adds. "It bears on all of biology, and you work on it at the molecular level. So it's the perfect mix."

Dunlap, who chairs DMS's Department of Genetics, has earned numerous honors over the years. He was recently elected to the National Academy of Sciences, the country's premier scientific body. He's also received many awards, including the Honma Prize, an international award for biological clock research, and the George W. Beadle Medal of the Genetics Society of America.

McClung says the field owes much to Dunlap and Loros. "For a long time, only the work of Jay and Jennifer was what was pushing ahead *Neurospora* as a molecular genetic model for clockwork," he says. "They pretty much have been the key players in keeping *Neurospora* at the absolute cutting edge of clock research."

Today the study of circadian rhythms no longer qualifies as "something different," in part due to all the researchers who have passed through the Dunlap-Loros lab. Bell-Pedersen, now a professor of biology at Texas A&M, says Dunlap and Loros take a strong interest in promoting the careers of their grad students and postdocs. They "gave us a lot of freedom to really test ourselves and make sure that we were cut out for this field," she says. "It was a great experience."

Froehlich, a senior scientist at a biotech company, also credits Dunlap and Loros for helping develop new generations of scientists. "The training in that lab set me up well for what I'm doing now," he says.

Giles Duffield, Ph.D., spent time in their lab as a postdoc before leaving to join the biology faculty at Notre Dame. "When people leave the lab, they know how to do good science," he says. Of course, he adds, "many people don't want to leave, because it's such a nice environment."

Providing the researchers in their lab with the freedom to develop their own ideas has been a conscious decision for Dunlap and Loros. "We give people an enormous amount of independence," Loros says.

"We rarely tell people what to do," Dunlap agrees. "We'll suggest projects when they get here if they don't come in with something they want to do, but almost always within some months they have either developed entirely their own ideas or developed additional ideas that are their own."

Loros points out that much of their own success is due to the hard work of these up-and-coming researchers. "I feel very grateful for all the people I've worked with over time," she says. "Good science is very often a collaborative effort."

Of course, each one's most important coworker has always been the other. "In science, people constantly talk about how it's important to have colleagues at the institute, somebody that you can talk about your work with," Loros says. "Well, Jay and I just have a built-in colleague." Together, they have put together the puzzle of *Neurospora*'s circadian clock, constantly learning more about how significant circadian rhythms are to the well-being of virtually every organism on Earth. Given their track record, more findings are sure to come. It's only a matter of time. ■