How does a species get the ball rolling when it’s time to evolve? The change has to begin somewhere, and studying a tiny worm may show us the source.

When Charles Darwin visited the Galápagos Islands in 1835, he beheld many strange and wonderful creatures found nowhere else. He also collected a variety of small birds—some with long, pointed beaks for supping from cactus flowers, others with deep, wide beaks for crushing seeds, and still more with beaks of intermediate sizes for eating insects or fruit. Poring over his specimens later, he realized that they were all finches, and would write in *The Voyage of the Beagle*, “One might really fancy that, from an original paucity of birds in this archipelago, one species has been taken and modified for different ends.” The finches steered Darwin to the idea of evolution by natural selection, and in November 1859 he would publish *On the Origin of Species*.

Now, on the sesquicentennial anniversary of Darwin’s masterwork, we are on the verge of figuring out the molecular basis for species diversity. For example, scientists at Harvard and Princeton have discovered two signaling proteins that shape birds’ beaks. Plying chicken embryos with too much of one protein produced hatchlings with long,
slender beaks like the cactus finches; an oversupply of the other led to poultry with broad, oversized bills. In a normal chick, the two proteins are in balance, so how does nature nudge them apart? Everything has to start somewhere, and the answer may lie in a mathematical model created at Caltech by then-graduate student Claudiu Giurumescu (PhD ’08) when he applied a handful of differential equations to a tiny, transparent, soil-dwelling roundworm. It turns out that there is hidden variability in the network of molecules that determines what each cell in the worm becomes, even though its development is controlled with the mechanical precision of a self-winding Rolex. (The paper appears in the April 10, 2009, issue of PLoS Computational Biology.)

This worm, a nematode called Caenorhabditis elegans, is about the size of the comma that follows its name. C. elegans, as it is affectionately known, is right up there with the fruit fly in developmental biology’s stable of model organisms. Its short lifespan and transparent body have allowed scientists to track each individual cell as it sprouts forth, from the single-celled egg through the larval stages to the 959 body cells of the mature adult. Beginning in the mid-1970s, generations of grad students—including Paul Sternberg, now Caltech’s Morgan Professor of Biology and an investigator with the Howard Hughes Medical Institute—spent their academic careers hunched over microscopes, straining their eyes at squirming squiggles, painstakingly tracing the pedigree of each cell one division at a time. They found that the process is genetically hardwired—absent intervention by inquisitive biologists, those 959 cells will appear in the same place and in the same order in each and every worm.

In the 1990s, the worm’s full genetic code was deciphered—the first multicellular species to have that honor—and a loose collaboration of research groups sprang up around the world to work out what every protein specified in the code does. Many of these proteins proved to be signaling molecules that coordinate the construction of the worm by telling cells when to divide and what to become—nerve, muscle, intestine, or what have you.

Over the last 20 years, Sternberg’s and other labs have charted the entire network of proteins involved in creating C. elegans’s vulva, the opening in the skin that leads to the ovaries. The budding vulva is ideal for this, because you can tamper with the signaling molecules to your heart’s content without lethal consequences. (A vulvaless worm will grow up just fine, but try raising nematodes without digestive tracts.)
The nematode’s vulva begins to form when the anchor cell in the worm’s developing uterus begins secreting a molecular signal (blue diamonds) that diffuses away into the intercellular fluid. This molecule binds to receptors on the surfaces of six cells, numbered P3.p through P8.p. The closer the cell is to the anchor cell, the stronger the signal. This, plus a second signal (orange) between adjoining cells, tells each cell whether its progeny should form an opening (1° fate, yellow), a lip (2° fate, blue), or part of the surrounding tissue (3° fate, gray).

**BANDING A BETTER MODEL**

Giurumescu didn’t set out to monkey with roundworms. His PhD advisor was Assistant Professor of Chemical Engineering Anand Asthagiri, whose lab specializes in tissue engineering. Most of Asthagiri’s work is done with mammalian cell cultures, where the ultimate goal is to do such things as coax a patient’s own cells to cling to a surgically inserted man-made scaffolding and grow into functional tissues, such as blood vessels. Hence the interest in signaling networks—when they function properly, cells will spontaneously self-organize. (When they go awry, the cells run amok and turn cancerous. But that’s another story.) Says Asthagiri, “If we could predict how multicellular structures form, we could design them from scratch, and perhaps one day grow entire organs.” Of course, it’s not that easy. As Sternberg says, “The bioengineers and synthetic biologists on campus like to say, ‘To engineer is to understand,’ but in practice, when you try to engineer, you immediately realize what you don’t understand. And that drives further experiments, when you realize what’s missing.”

And in the study of human signaling networks, there’s quite a lot missing. So Giurumescu decided to lower his sights and create a computer model of a model organism’s model organ, for which he enlisted Sternberg’s assistance. Says Sternberg, “Anand understands biology. Claudiu understands biology. I would just correct them if they were getting facts wrong. So there was a really seamless integration of the modeling and the understanding of the biology, versus having someone who’s a modeler, and someone who’s a biologist, and they’re trying to figure out how to talk to each other.” The worm literature is full of models, Sternberg adds, but, “Anand and Claudiu pulled out the critical parts of the circuit in a way that makes sense in terms of the molecular biology. It’s a tradeoff between being simple enough to be able to analyze, but also capturing the interesting complexity, so it’s a good model.”

*C. elegans* goes through four larval stages over a period of about three days. Early in the third stage, the vulva starts forming from a string of six skin cells named P3.p through P8.p. (These are part of a group of cells called P1.p through P12.p, but the others play no part in this process.) P6.p, the central cell, chooses the so-called primary fate, in which its eight progeny form the channel leading to the uterus. P6.p’s neighbors, cells P5.p and P7.p, select the “secondary fate,” and their daughters form the vulva’s lips—seven cells per lip, or 22 cells all told. The three outermost cells—P3.p, P4.p, and P8.p—opt for the “tertiary fate,” in which their offspring fuse to the surrounding skin cells. A specific arrangement of cells is called a “phenotype,” and nematode biologists use a shorthand that lists the fates numerically in ascending order. Thus the normal, or “wild type,” pattern is called 3°3°2°1°2°3°, meaning that cells P3.p and P4.p choose the tertiary fate, P5.p the secondary, and so on.

Two competing signaling systems determine what each cell does. One system, named the EGF pathway after its human equivalent, a substance called Epithelial Growth Factor, is based on a water-soluble protein. This protein is secreted into the intracellular fluid by a cell called the uterine anchor cell and diffuses away in all directions, so that its concentration falls off smoothly with the distance from the source. The anchor cell adjoins cell P6.p, which thus gets a huge dose of the EGF-like stuff, inducing it to pick the primary fate; cells that get little or no soluble signal default to the tertiary fate. The second system allows neighboring cells to talk to each other via interactions between two proteins called...
Every chemical interaction in this web of events has its own rate constant, \( k \). The action begins when a molecule (the blue diamond) of the soluble signal, a protein called LIN-3, binds to its receptor, a protein called LET-23, on the surface of cell \( i \). (To further confuse things with another layer of nomenclature, this is called an inductive signal, \( I \), because it induces a change in the developing embryo.) The binding event triggers the conversion of a molecule named MAP kinase from an inactive form (mpk\(_i\)) to an active version (mpk\(_i^*\)). This active MAP kinase in turn stimulates cell \( i \) to produce the Delta signaling protein, called LAG-2, which binds to the Notch receptor, LIN-12, on the surface of the adjoining cell, cell \( i+1 \), to send that cell a lateral signal (lat\(_{i+1}\)). This lateral signal proceeds to deactivate the MAP kinase in cell \( i+1 \), causing that cell to be less sensitive to the soluble inductive molecules (I\(_{i+1}\)) binding to its surface. This also causes cell \( i+1 \) to produce fewer Delta molecules of its own, meaning that it sends a weaker inhibitory signal back to cell \( i \). Meanwhile, back in cell \( i \), the activated MAP kinase molecules are causing the Notch receptors to get sucked back into the cell, deafening it. In addition to this crosstalk between cells, each cell also contains enzymes that steadily deactivate the MAP kinase, ensuring that a continual influx of the inductive signal is needed to keep things humming along.

The model encapsulates all this biochemistry to predict the internal state for each cell—that is, the level of active MAP kinase and the strength of the lateral signal that each cell will ultimately arrive at, given a specific set of initial conditions. “Now the challenge is to translate this biochemical state into a prediction about what the cell will actually do,” says Asthagiri. “Will it become 1°, 2°, or 3°? We set up a simple mapping scheme based on the most conservative interpretation of the experiments. Maybe the dividing lines shouldn’t be perpendicular, or even straight, but it’s a way to start thinking about it.” The map is divided into four quadrants:

A high MAP kinase signal and a low lateral signal cause the cell to choose the primary fate. A low MAP kinase signal and a high lateral signal lead to the secondary fate. And cells with a low MAP kinase signal and a low lateral signal default to the tertiary fate. The fourth quadrant, where the cell experiences a high level of both signals, is called the “mixed” outcome. While it has not been found in nature, it is a theoretical possibility that the model predicts will occur if specific tweaks are made to the biochemical circuit. Asthagiri and Sternberg plan to make these tweaks and see what happens.
Notch and Delta that sprout from the cells’ surfaces. This lateral signal is responsible for triggering the secondary fate and, under normal conditions, ensures that only one cell chooses the primary fate by shutting down the EGF pathway in its neighbors. (For a closer look at the eye-crossing details of this process, see the opposite page.)

The EGF and Notch-Delta systems recur throughout the animal kingdom wherever tissues are forming. Their molecular mechanics have been exhaustively dissected, so Giurumescu was able to abstract how the two systems interacted. His model reduced the whole shooting match to eight parameters that allowed the concentrations of key molecules to be calculated within each cell as the vulva develops. He could then set each parameter—such things as the steepness of the soluble signal’s gradient ($\Delta l$), or the inhibition strength of the lateral signal ($\chi$)—as he pleased, and watch how the cells responded.

Giurumescu began by seeing how well the model mimicked published laboratory studies. For example, one well-known mutant, the vulvless $3^{\text{e}}3^{\text{e}}3^{\text{e}}3^{\text{e}}$ phenotype, is usually made by zapping the uterine anchor cell with a laser, destroying the EGF fountainhead. Would dialing down the soluble signal’s peak level ($l$) in the model have the same effect? It did.

At the other extreme, the $2^{\text{1}}2^{\text{1}}2^{\text{1}}2^{\text{1}}$ phenotype—a nematode hedonist’s dream with not one, not two, but three sets of naughty bits—is created by cranking up production of the soluble signal, which any

<table>
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<tr>
<th>Parameter</th>
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<tr>
<td>$l$</td>
<td>Level of the soluble signal at the gradient’s center</td>
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<td>$\Delta l$</td>
<td>Steepness of the soluble signal’s gradient</td>
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<td>$\chi$</td>
<td>Strength of the lateral inhibition of the soluble signaling pathway</td>
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<td>$\lambda$</td>
<td>Base level of the lateral signal</td>
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<td>$\phi$</td>
<td>Stimulation of lateral signaling to adjoining cells by the soluble signal</td>
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<td>$\theta$</td>
<td>Suppression of the lateral-signal receptors by the soluble signal</td>
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<td>$\kappa_c$</td>
<td>Threshold of the lateral signal needed to inhibit the soluble signaling pathway</td>
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of a number of mutations will do. The model reproduced this effect. It also predicted that preventing the gradient from tapering off—exposing all six cells to the same dose of the EGF–like molecule, whatever that dose might be—would give the same result. At the same time, researchers from the Howard Hughes Medical Institute discovered that another long-known mutation leading to the $2^{\text{1}}2^{\text{1}}2^{\text{1}}2^{\text{1}}2^{\text{1}}$ phenotype actually does work by causing other nearby cells to join the uterine anchor cell in secreting the soluble signal, in effect flattening the gradient. (The seven-person team, headquartered at the University of Colorado at Boulder, included Min Han, a former Sternberg postdoc, as well as Sternberg and Byung Joon Hwang, then a Caltech postdoc.)

Says Asthagiri, “If our model can predict which specific interplay of signals leads to each known phenotype, it opens up a broader set of questions. Can we manipulate the network to discover what is evolutionarily possible? Like Darwin’s finches, what types of diversity is the system capable of generating?” There are six cells in the nascent vulva, and four possible fates per cell: the primary, secondary, and tertiary ones found in the lab, plus one dubbed the “mixed” outcome that could occur if the two signals—EGF and Notch—were both cranked up. So in theory there are 4,096 (i.e., $4^6$) possible phenotypes. “But are all of these outcomes accessible? Can you get everything under the sun, or does the network itself constrain what’s possible?” To find out, Giurumescu began dialing each of the eight parameters up and down in all possible combinations. In the lab, you might have a family of mutations that sets a protein’s production to OFF, LOW, NORMAL, HIGH, and YIKESI!, but the computer can specify very subtle differences in protein levels. Setting and verifying those protein levels experimentally would entail measuring them inside the cells of a living worm—a nearly impossible feat. Most importantly, the computer examines hordes of worms beyond the dreams of the most masochistic grad student, as the model encompasses some 214,000,000 possible mutations. Says Giurumescu, “It’s like looking at 214,000,000 strains of worms at once. Each one is a data point.”

Giurumescu’s final tally was some 560 phenotypes—only about 14 percent of the possibilities, but waaay more than the handful that have been bred in laboratories. Says Asthagiri, “This suggests that the way that connections are made within the network does constrain what’s possible, but not to the level where the known phenotypes are the only possible outcomes. There’s a lot more out there to explore.”

WE’RE NOT IN KANSAS ANY MORE

It turns out that not all phenotypes are created equal. Giurumescu compiled an exhaustive map of the phenotypic landscape, an eight-dimensional cartographic nightmare in which he plotted the value of each parameter on its own axis and noted which phenotype occurred at that point in

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8-D space. “Separating all the phenotypes and seeing how we could actually get from one to the next by doing single or double mutations took months,” recalls Giurumescu. “I had two Pentiums running nonstop from January through March.” Earlier, experiment-based phenotype maps were constructed on pretty sparse data. They tended to look like the state of Kansas, with each phenotype plotted as a city or town—a tiny dot on a vast prairie of empty space. People knew that each dot would have some size if you zoomed in on it enough, but nobody knew how big these municipalities really were. Says Asthagiri, “We demonstrated that the wild type, and in fact all the phenotypes that have been described in the literature so far, are more like nations on a globe than cities on a map. They occupy widely varying amounts of real estate. We can move along some axis of the model, sometimes for a considerable distance, and still stay in the same phenotype. Then at some point we cross a border, and we wind up in another phenotype’s territory. This has fundamental implications for how we think about species diversification. It suggests that the network itself doesn’t have to change to produce new phenotypes; new signals and feedback loops don’t have to be added or deleted. All you have to do is vary the connection strengths in the existing network sufficiently, and you will get new phenotypes.”

Some phenotypes, like the wild type (3°3°2°1°2°3°), take up huge tracts of land, while most look more like Liechtenstein. Interestingly, the wild type is not nearly the biggest thing on the map. The vulvaless variant 3°3°3°3°3°3° sprawls through 54 percent of the 8-D volume—the equivalent, in terms of territory, of Asia, Africa, and Europe combined. Then comes the lipless 3°3°3°1°3°3°, which at 13 percent is slightly larger than Russia. This is followed by 12 other phenotypes, including five with at least one cell in the mixed outcome, before the wild type checks in at number 15. At a mere 0.2 percent, it occupies about as much real estate as Italy.

A certain amount of balkanization is a good thing. If the map were just one huge blob, then the species would have no capacity for change, because no matter how you jiggered the network’s parameters, the phenotype would stay the same. This is a recipe for extinction. On the other hand, if the map looked like a Jackson Pollock painting, the species wouldn’t be stable—the slightest jostling could push it into a new, and probably less fit, phenotype.

When Giurumescu plotted the size distribution of phenotypes on a logarithmic scale, he got a roughly bell-shaped curve. This means that the vast majority of phenotypes are mere postage stamps in parameter space, while the few in the right-hand tail (including the wild type) take up most of the room. This is the key to a species’ stability, as Asthagiri explains. “You can poke and nudge the parameters quite a bit. You can make lots of small biochemical changes within the cells, or have them be in somewhat different environments, and still get a normal worm. The network doesn’t have to be perfectly tuned to produce the wild-type pattern; instead, the worm has some wiggle room.”

The phenotypes to the right of the wild type in the bell curve’s tail are even more stable. “Once you arrive at one of them, you really have to flail around to get out,” Asthagiri says. Since they’re so big, they should be observable—any mutation you make has pretty good odds of pushing the worm into one of them. On the other hand, making one of the phenotypes found under the middle of the bell would be a very tricky proposition indeed. You’d have to overcome the natural biochemical variabilities that occur from cell to cell in a real worm, and the process of inducing a mutation is far from precise. And

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Top: Tweaking the network can cause big changes in the organism. Here, various settings for $I$, the level of the EGF-like signaling molecule, cause the three species (colored lines) to select different phenotypes at various points.

Bottom: That's because each species occupies a different region within the wild type's portion of the eight-dimensional parameter space. The wild-type region is relatively large, but the phenotypes adjoin ing it are not, so where you end up depends on where you begin.

indeed, all of the phenotypes that have been seen so far lie in that right-hand tail. The model predicts that there are 25 phenotypes that should be easy to get to experimentally, 15 of which have actually been made.

IN THE WILD TYPE THERE ARE MANY MANSIONS

But here’s the kicker—not all of them occur in C. elegans. This little fellow is just the most famous member of its genus; two of its brethren, C. briggsae and C. remanei, are almost as beloved by worm biologists. In the wild, all three critters share the $3°3°3°3°3°$ phenotype. In the model, however, Giurumescu and Asthagiri discovered that each species occupies its own territory within the wild type’s parameter space. Under the right conditions, giving each species the same push can thrust it into a different phenotype from its fellows. To return to cartography on yet another scale, the wild type might be Texas in a map of the United States. C. briggsae might live in the vicinity of Amarillo, where traveling some 250 miles east and a bit north would put the worm near Oklahoma City. C. elegans could be a denizen of the Dallas–Forth Worth area, and making the same phenotypic jump would land it in the vicinity of El Dorado, Arkansas. And finally, C. remanei might hang out at the Austin city limits, where that same journey east and north would end in the bayous somewhere south of Baton Rouge. The worms all set out from one state but ended up in three different ones, yet they all took the same trip—it all depends on the starting point.

This remarkable conclusion was sparked by results from a lab nine time zones east of Pasadena. While Giurumescu and Asthagiri were mutating model roundworms in a computer, Marie-Anne Félix, a former postdoc of Sternberg’s, was embarking on a set of parallel experiments with real nematodes at the University of Paris. (She left Caltech just before Giurumescu arrived.) Félix studied the effect of the soluble-factor signal on C. elegans and 10 close relatives, but we’ll just stick with the Big Three.

As expected, Félix found that low levels of the soluble signal caused all three species to assume the $3°3°3°3°3°$ phenotype. (Attentive readers will have noticed that there are now only five cells. That’s because in C. briggsae, the P3.p cell does not participate in the process. From here on it’s just P4.p through P8.p.) At normal concentrations, the wild type naturally resulted. But at an intermediate level that was less than normal, each worm produced a different phenotype—C. elegans made a lipless vulva ($3°3°1°3°3°$), C. remanei grew lips in the normal positions but did not form the central opening ($3°2°3°2°3°$), and C. briggsae created a third lip between the normal two ($3°2°2°2°3°$). When Félix cranked up the signal beyond normal levels, C. elegans and C. briggsae diverged again. C. elegans produced any of three outcomes: $2°2°1°2°1°$ and $1°2°1°2°1°$, both of which had been seen in labs before, plus a new one, $2°2°1°2°2°$. However, C. briggsae opted for another previously undiscovered phenotype, $2°1°1°1°2°$. And finally, when doused in the EGF-like molecule, C. elegans and C. briggsae went all-primary (1°1°1°1°1°). At the same time, Giurumescu and Asthagiri discovered that the model assigned all of these outcomes reasonable amounts of space.

Which brings us back to the question of the origin of species. All these nematodes use the same network for the same end, so how do the various species wind up in different regions of the wild-type space? The connection strengths must be different—there's the same arrangement of levers and gears, if you will, but perhaps \textit{C. remanei} has evolved a really stiff spring on one pivot. To confirm this, Giurumescu and Asthagiri plugged Félix’s results into the model and worked backward, retracing the routes from the low-level mutants back into the wild-type space. It turns out that the \textit{C. remanei} 3°2°3°2° phenotype requires a higher value for a parameter called \( f \) than does the \textit{C. elegans} 3°3°1°3°3° phenotype. This means that in \textit{C. remanei}, the soluble signal produces a stronger lateral signal between adjoining cells than it does in the \textit{C. elegans} on which the model was based. The model also gave \textit{C. remanei} a lower threshold concentration \( (k_M) \) at which the soluble signal turns on the lateral signal. But, says Asthagiri, “We can say anything we like about a bunch of Greek letters, but that isn’t a testable prediction—we have to come up with a specific array of cells. And the true test for a modeler is, can you predict the result for an experiment that’s never been done?” Félix had not tried the high-side experiment on \textit{C. remanei}, providing the perfect opportunity.

Working forward from the \textit{C. remanei} portion of the wild-type space and using the adjusted connection strengths, the model predicts that cranking up the soluble signal should lead, in ascending order, to 2°2°3°2°2°, 2°3°3°3°2°, and eventually 3°1°1°1°3°. Of these, only 3°1°1°1°3° has been seen before. (And, of course, at very high levels, our old friend 1°1°1°1°1° should eventually result.) Asthagiri and Félix met in July to plan a round of experiments that will provide the clincher. Stay tuned.

\textbf{Mapping Traffic Flow}

When you alter the connection strengths, “the outcome may be the same, but the paths by which the network gets to the answer are different,” says Sternberg. In other words, the various species all grow normal vulvas, despite their hidden diversity. As he explains it, “You might say that the best way to get to downtown L.A. is on Huntington Drive, and somebody else might say, ’No, it’s the 110 Freeway.’ In reality, the answer depends on the traffic. But let’s say there’s a parallel Pasadena where it’s always better to go down Huntington, and a third one where it’s always better to take the 110. Then people could do a really clear experiment—if we tried to drive down Huntington and found a big chasm, we’d realize, ‘OK, we can’t go this way.’ But since the answer really depends on traffic patterns, we might get different answers because one person did the experiment at eight in the morning, and someone else did it at two in the afternoon. But everyone always ends up in the same place—there’s an organ formed, and it happens perfectly. So Claudiu and Anand figured out all the possible routes and then learned about them by changing the parameters. Now they can say, ‘Well, if the Dodgers are playing a home game, we can show that taking the 110 is not going to work.’"

Biologists can’t look at every inch of pavement in the real process, so they have to guess which intersections have the critical stoplights that control the flow, and then figure out how to sample the appropriate molecules. Sometimes the researchers are just limited to whatever molecules are easy to detect—being at the mercy of wherever Caltrans put its traffic cameras, as it were. But in the model, all the dynamics are fully accessible. You can vary all of the connec-
So, 150 years after the publication of On the Origin of Species, it appears that, at least in the genus Caenorhabditis, the wild-type space contains the jumping-off point for many phenotypes. Different species live in different parts of that space, so each may have ready access to a different set of phenotypes; thus the worms might, over time, evolve modified body parts to exploit various niches in their environment. But in a striking display of economy, all of these forks in the evolutionary road can be produced without rewiring the underlying network of molecular signals—simply altering the connection strengths, a much easier feat in evolutionary terms, will suffice. And if it works that way in the 959 cells of the nematode, it probably works that way in fruit flies, finches, and us.

Tomita is now a postdoc in a worm lab at UC San Diego, but Sternberg, his grad student Paul Minor, and Asthagiri are building a model of another signaling pathway called WNT. Not all of the players in the WNT pathway have been identified, so Minor’s first job is to work out the relationships between the various parts. Says Sternberg, “The Notch-Delta, WNT, and EGF systems are three of the big food groups of signaling pathways, and they all work together both in the vulva and the hook. But beyond that, they’re in every organ. They’re involved in all different tissues in worms, flies, and mammals. The way they’re coupled might be different, and that’s another reason to look at the general case—what are the general ways you can couple these pathways and tweak them? They can be working cooperatively, or antagonistically, or in parallel. Or they may be unrelated. But they’re going to be used in all those ways somewhere.”