My Life with Dicty

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Preface

At a recent meeting, I was telling a story about the time when I first started working with *Dictyostelium*. My friend in the conversation was interested and suggested that I consider writing my memoirs of *Dictyostelium* so that the stories would not disappear when I did. We agreed that it might be best appreciated by those who also worked with *Dictyostelium,* an audience we could target on dictyBase. These stories are from my personal experience and point of view and are not meant to be an objective history of the field. Moreover, some memories may have faded or changed over 50 years. If any of you remember it differently, please let me know. Errors can be fixed in later editions. The present edition has been read by Margarita Behrens, John Bonner, Danny Fuller, Adam Kuspa, Rolf Olsen, Gadi Shaulsky, and Michel Veron, who kindly corrected some points that were in error.

Early days

In the summer of 1960, as a 19 year old college student, I had the good luck to have a job at the Marine Biological Laboratories in Woods Hole, Massachusetts. I washed laboratory glassware, prepared solutions, and had the run of the place. I cut up small sharks to learn their anatomy. I fertilized sea urchin eggs to see early development with my own eyes. I sat in on the lectures of the Marine Biology Laboratory Embryology course where I learned to get beyond the flood of bewildering names of tiny structures and focus on cellular processes. One of the instructors was Maurice Sussman who made a lot of sense when he explained how developmental processes should be studied in their simplest form such as in the social amoeba *Dictyostelium discoideum*. After some interesting discussions, Sussman asked one of his graduate students, David Sonneborn, to show me how easy it was to collect growing *Dictyostelium* amoebae and initiate synchronous development. For the next 24 hours my attention was totally focused on the developing cells as they

aggregated, formed slugs, and culminated into fruiting bodies. I was enamored but did not realize for several years that it would be a life long affair.

The following year a paper by Francois Jacob and Jacques Monod was published in the Journal of Molecular Biology that showed how some gene products regulate the expression of other genes and can shape the transcriptional landscape. They had worked with the bacterium *Escherichia coli,* where they were able to use microbial genetics to generate and manipulate mutant genes, but they pointed out that similar mechanisms might account for the differentiation of cell types during embryogenesis. I was excited and convinced that they might be right, although it required a leap of faith to consider that "what is true of *E. coli* might be true of elephants" as Jacob had quipped. I accepted it and wanted to test it by applying detailed genetics to multicellular development. Back in the 60's, genetic techniques were limited to a few model systems. *Dictyostelium* was one of them.

Two of the exceptional instructors in the MBL Embryology course at Woods Hole, Ed Zwilling and John Saunders, described their analyses of the processes involved in chick limb formation. They were able to show that morphogenetic signals emanated from the Zone of Polarizing Activity (ZPA) near the arm pit and the Apical Ectodermal Ridge (AER) near the tip of the hand. Together they established the anterior posterior axis and determined the identity of the digits. I was impressed with the elegance of the experiments but saw no way to characterize the signals nor how to determine the mechanisms of cellular response since all the studies were in chick embryos where it is impossible to do any meaningful genetics. I decided to follow vertebrate embryogenesis as a spectator rather than an actor. It took hundreds of labs using cutting-edge techniques over 30 years to determine the molecular components of the signal transduction pathways in chick and mouse embryos. These studies were only possible because of advances made in simpler model systems.

The genetics course that I took at Harvard was almost exclusively concerned with chromosomal behavior in *Drosophila*. Mapping genes on the basis of recombinational frequency was all very well, but what did it tell you about the functions or interactions of the gene products? Flies were considered as collections of phenotypes rather than the products of successful embryogenesis. I don't think I ever looked at a

developing larvae in the lab part of the course. Luckily, *Drosophila* geneticists, including Ed Lewis, Walter Gehring, Yanni Nusslein -Volhard, Eric Wieschaus, Tom Kauffman, Mike Levine, and Bill McGinnis, forged ahead and 20 years later defined master genes that regulate development in almost all animals. Many of these scientists became my good friends, but I never really related to flies despite the fact that their genetics was so elegant.

I majored in Biochemistry and spent much of my time in the laboratory of Max Pappenheimer, who patiently took charge of my education. In his lab I worked on oxidative phosphorylation. I also got to talk with Jim Watson and Wally Gilbert who worked in their lab down the hall. They had ideas about mRNA and DNA control that were far ahead of the text books. Further down the hall Julius Marmur and Paul Doty were getting the first indications of nucleic acid reannealing that led to the techniques of DNA hybridization. Those were exciting times. The summer of 1961 I spent as a technician for Dave Bonner who had just moved from Yale to La Jolla, California to start the Biology Department at UCSD. Dave was an unusual man and an exceptional scientist who worked on the tryptophan synthase gene in the bread mold *Neurospora crassa*. He showed how biochemical genetics could further define the "one gene, one enzyme" hypothesis and provide surprises along the way. I grew *Neurospora* and purified tryptophan synthase for the lab. The best thing about being in the Bonner lab was being treated as their graduate student. Although I was still fascinated by embryogenesis, I thought that mastering microbial genetics first would make it much easier to confront multicellular organisms. I chose MIT for graduate school to be able to study bacterial gene regulation with Boris Magasanik.

Boris had been unravelling the complexities of catabolite repression for several years when I joined his lab in 1962. He had found that when the flux of catabolites generated from sugars exceeded the availability of nitrogen compounds needed to convert them to amino acids, a variety of catabolite enzymes were repressed. I decided that the *lac* operon of *E. coli* provided the best characterized regulatory unit for further studies. Jacques Monod had found that when bacteria are presented with medium containing both glucose and lactose, they first metabolize the glucose and then the lactose. They do not even express β-galactosidase from *lacZ* until all the

glucose has been used up; as a result they show diauxic growth with two different growth rates. Monod teamed up with Francois Jacob to isolate a series of mutant strains that expressed *lacZ* constitutively and characterized them using partial diploids constructed with extrachromosomal plasmids. They defined the *i* gene as encoding a repressor protein that bound to a genetic element at the start of the *lacZ* gene where it could block transcription until a ligand was produced from lactose. I wanted to see if catabolite repression could account for diauxic growth by regulating *lacZ* expression in a manner independent of the *i* gene. If we could show combinatorial control of the *lac* operon, it would present a much more versatile model for regulation of complex embryological processes than a simple on-off switch. Boris supported my proposals and provided continuous encouragement and brilliance throughout the 3 years that I worked in his lab.

After a bumpy start, the results finally settled down to give a clear answer: the *lac* operon is controlled by two independent systems, one of which is mediated by the inducer-repressor system acting at the cis-operator and the other regulates the basal levels of expression of the *lac* operon as well as the fully induced or constitutive levels of expression. Mixing and matching these independent control systems allows for a wide range of outputs.

Throughout the time that I was getting proficient in genetically manipulating *E. coli* I followed the literature on yeast and *Dictyostelium*. Lee Hartwell was a graduate student in Boris' lab during this period and we often discussed the best way to understand complex processes. When he set up his own lab at the University of California Irvine a few years later, he started using *Saccharomyces cerevisiae*. He particularily liked the "awesome power of yeast genetics". The trouble with yeast, in my opinion, was that it never became multicellular and so could not shed light on developmental processes. On the other hand, Lee realized that he could study control of the cell cycle using conditional mutations in yeast and that what ever he found had a good chance of being universally relevant to all eukaryotic cells. He was right and in 2001 he received the Nobel Prize together with Paul Nurse and Tim Hunt.

Developmental mutations are innately conditional in *Dictyostelium* since fruiting body formation is not an esssential part of the life cycle. Strains can be passaged as

either spores or amoebae. I was convinced that *Dictyostelium* could become a good genetic system when we learned how to efficiently generate mutations and cross strains. I started looking around for a good lab to learn the tricks of *Dictyostelium* as a postdoc. In the early 60's there were only 5 major labs actively working with *Dictyostelium*: Bonner, Gerisch, Takeuchi, Raper, Sussman. I considered each one.

John Bonner (no relative of Dave Bonner) established his lab at Princeton University in 1947 immediately after finishing his PhD. in the laboratory of William (Cap) Weston at Harvard. His thesis was a continuation of the work of Ken Raper characterizing the development of *Dictyostelium*. Bonner set out to prove that the cells aggregated by chemotaxis rather than by using contact guidance as suggested by the eminant embryologist Paul Weiss. Bonner designed and carried out ingenious experiments showing that the cells secreted a diffusible chemical that controlled the direction of movement of surrounding amoebae. For the next 20 years Bonner's laboratory at Princeton gradually defined the nature of the chemoattractant. In 1967, while Bonner was at his summer house in Nova Scotia, one of his graduate students, David Barkley, and a visiting scientist, Theo Konijn, realized that cAMP fit the bill for the chemoattractant. They got hold of some and found that it worked beautifully even at very low concentrations. When they phoned Bonner with the exciting results, he went to find out what cAMP might be and immediately recognized the importance of the finding. His lab was soon able to show that cAMP was the natural chemoattractant. They went on to partially characterize the enzyme that makes cAMP, adenylyl cyclase, the enzyme that breaks it down, cAMP phosphodiesterase, and the surface receptor for cAMP. These studies hold a central place in understanding *Dictyostelium* development.

In the 50's and early 60's Bonner's lab clearly showed that growth and differentiation were separate in *Dictyostelium*, thereby greatly simplifying the analysis of changes in cell types. He used vital dyes to show that prespore and prestalk cells sorted out in slugs such that the faster prestalk cells were at the anterior. He also showed that culminants produced a gas, most likely ammonia, that repelled the stalks of fruiting bodies forming nearby. Many of his early experiments were summarized in his influential book "The Cellular Slime Molds" that was published in

1959. While there was no question that John Bonner was a pioneer in the field of social amoebae, I wanted to extend the studies into biochemistry and genetics.

Gunter Gerisch only started publishing studies on *Dictyostelium* in 1959 but over the next few years put out a series of highly interesting reports of development in shaken suspension where the conditions were more uniform and the differentiation more synchronous. The only trouble was that all these papers were in German. He also made some time -lapse movies available that were highly informative. It seemed clear that he was aiming in the right direction, but had the drawback of working in Tubingen, Germany. The Harvard/ MIT conceit at that time was that the only meaningful biology was being done in Cambridge, Massachusetts and the laboratories of a few friends. Germany was not on the map at that time.

Ikuo Takeuchi suffered from the same problem, since his lab was in Japan. He had been a graduate student of John Bonner and got his PhD from Princeton in 1960. He then did a postdoc with Jim Ebert at Carnegie Institute in Baltimore before returning to Japan to set up his own lab at the University of Kyoto. Early on Takeuchi published several important papers on biochemical and immunological studies of differentiation in *Dictyostelium* and analyzed the effects of metabolic poisons on slug formation. Although I was very interested by his quantitative studies on the changes in specific enzymatic activities, I never really considered working in Japan.

Ken Raper was the patriarch of *Dictyostelium discoideum*. He was the one who isolated the first sample from Little Butts Gap near where he lived in North Carolina. He described the development of *D. discoideum* in brilliant detail as part of his thesis with William Weston at Harvard in 1936. His early work set out a whole series of important questions that have kept dozens of labs busy ever since. He was elected to the National Academy of Sciences in 1949 and many of us in the field assumed it was in recognition of his seminal work on *Dictyostelium*. It turned out that the Academy was recognizing Ken's war time efforts to isolate and culture strains of the mold *Penicillium notatum* that would produce more of the wonder drug, penicillin. A culture isolated from a moldy cantaloup near his USDA labortatory in Peoria, Illinois turned out to produce over a hundred times more penicllin that the 1928 culture studied by Alexander Fleming in London. Thanks to Ken Raper and others, plentiful

supplies of penicillin were available by D-day, June 6, 1944. After the war, Ken went back to work on social amoebae at the University of Wisconsin. Among other things, his laboratory studied the sexual cycle that produces macrocysts, phototaxis of migrating slugs, and stalk formation in *Dictyostelium*. It was all good work but seemed a bit like old-fashioned mycology to me. That left the Sussman lab as a possible choice.

Maurice Sussman was a microbiologist having trained with Sol Spiegelman at Washington University in St. Louis. He received his PhD. in 1950 and established his own laboratory at Northwestern University in Evanston Illinois. Maurice was always looking for the big breakthroughs that would affect how cellular physiology is understood. He chose to study *Dictyostelium* because it showed clean separation of growth and development and had the potential for microbial genetics. Together with his wife, Raquel, he quickly developed techniques for isolating mutant strains that grew normally but showed aberrant morphogenensis. He found that when he mixed some of these strains together, they synergized; that is, they formed fruiting bodies when developed in mixed populations but not when incubated separately. Clearly, cells of these strains were communicating with each other. In 1958 Maurice was offered a professorship at Brandeis University in Waltham, Massachusetts and moved his lab East. He had always been interested in how synthesis of new proteins directed cell differentiation and morphogenesis. He was able to make a first step towards this goal when his lab found an enzyme activity that was responsible for making a specialized polysaccharide. UDPgalactose polysaccharide transferase was the first well defined developmentally regulated protein of *Dictyostelium*. Having a quantitative assay for this activity opened up many avenues for further exploration. This was just the approach I was looking for and I decided to apply for a post-doctoral position in his laboratory. In the summer of 1965 I drove out to Brandeis from Cambridge, a distance of about 12 miles.

At the beginning of the interview I don't know who was more nervous, Maurice or me. He seemed to want to impress me and I was already convinced that his lab was the best fit for me. He explained what was going on in his lab by showing me a series of slides from a recent seminar he had given. The more I heard, the more I liked it. I

summarized some of my graduate work and tried to explain how similar approaches might be applied to *Dictyostelium* development. We soon found that we had similar interests and aspirations. He invited me to join the lab as soon as I finished my graduate work. I accepted.

1965 to 1975

As soon as I returned to MIT I started writing up my thesis work and thinking more about what could be done with *Dictyostelium*. I was confident that I could manipulate *E. coli* and generate almost any genotype I wanted. I loved working on the *lac* operon and it was hard to suddenly shift to a field where I was new. First I had to finish my thesis and pack up.

At the end of the summer I moved from Cambridge to an eighteenth century farm house in Weston that was about 2 miles from Brandeis. Maurice returned from Woods Hole where he had taught in the MBL Physiology course. John Ashworth, a new post-doc from England, had joined him there. Just before coming to the US, John had finished his PhD. work in the laboratory of the eminent microbiologist Hans Kornberg at Leicester University. He had studied the control of the glyoxylate cycle by isocitrate lyase in *E. coli* and made significant advances. John wanted to understand the biochemical mechanisms that eukaryotic cells could use to take on specialized roles and had decided that *Dictyostelium* provided the most promising test material. We had lots in common and quickly established a close and lasting friendship.

One of the first things that John did at Brandeis was pour an acylamide gel and electrophoretically separate proteins. This was a standard technique in biochemistry but had not been previously applied to *Dictyostelium*. I remember John showing me a gel with a series of lanes with extracts he had prepared during development. The proteins were separated on the basis of size and then stained with a dye. The patterns changed slightly every 4 hours but there were no sudden changes. It seemed that 1D gels did not provide the resolution to routinely identify individual proteins. The problem was solved 15 years later by using 2D gels. Nowadays, these techniques are supplemented by identification of isolated proteins by mass spectroscopy.

I shared an office with John at Brandeis and always looked forward to the discusssions we would have while waiting to collect the next developmental time point or protein purification step. Many of the experimental lines we talked about were never successful or did not come to a successful conclusion for many years, but that did not diminish the intellectual excitement of the moment. One dream that led

almost immediately to a joint project was to show that specific genes were responsible for developmental morphogenesis. Keep in mind that this was at least 7 years before cloning techniques were worked out to isolate DNA regions in bacterial plasmids. Our experiments had to be indirect. Raquel and Maurice had recently shown that actinomycin D blocked synthesis of all RNAs other than tRNAs in *Dictyostelium*. They also showed that it blocked accumulation of UDPgalactose transferase if added before the slug stage when the enzyme accumulated. We decided to add the drug at two hour intervals throughout development and characterize the terminal structures for intercellular adhesiveness, stalk formation, spore formation and pigment accumulation. We took turns staying up all night as we carried out repeats of this experiment. The results clearly exposed the role of RNA synthesis on morphogenesis at different stages. Maurice was the first author of the paper and greatly enjoyed it that we had contradicted some statements that Barbara Wright had recently published. More about this feud later.

In the fall of 1965, we were joined by another postdoc, Kai Yanagisawa, who had just completed his graduate work at Columbia University in New York working on the genetics of the T-locus in mice. A series of confusing t-alleles had been isolated that affected embryogenesis and distorted the sex ratio in offspring. Kai's work was aimed at understanding the molecular basis of the effects. After finishing his thesis, he wanted to work with a more tractable system that was cheaper and faster. *Dictyostelium* seemed to be the answer.

Kai was somewhat older than John or me and a rather formal Japanese man. He grew up in the mountains to the West of Tokyo before the war. He once took me to his ancestoral village where he showed me the temple in which he had been given his name. He then showed me "his dam". Towards the end of World War II, as a young teenager, he had been forced to work on building a dam to help irrigate the fields in central Japan. It was hard for me to understand how he had been able to come all the way from rural, war-ravaged Japan to the molecular biology laboratories of the East coast of the United States. But Kai was an exceptional man - very smart, very hard working, very determined. He was also very nice.

Kai and I started thinking how to improve genetic techniques in *Dictyostelium* and soon focused on the choice of mutagen. Maurice and Raquel had found that treatment with strong UV irradiation increased the frequency of mutations, but it was known that UV irradiation caused a high proportion of insertions, deletions and other chromosomal abnormalities that could affect more than one gene. I had used N-methyl-N'-nitro-N-nitrosoguanidine (NTG) extensively to mutate *E. coli* at MIT and brought some along with me to Brandeis. Kai and I found that NTG was a remarkably potent mutagen for *Dictyostelium* and proceeded to isolate a series of mutant strains. They were characterized by the developmental stages that they could reach before morphogenesis was arrested as well as by the developmental behavior of the marker enzyme that Maurice had discovered, UDGgal polysaccharide transferase. We found that mutants blocked before the stage when transferase normally accumulated failed to accumulate the activity while strains blocked at later steps accumulated transferase at the usual time to the usual level. Some of the strains that failed to make normal aggregates when incubated as pure populations formed fruiting bodies and accumulated transferase activity when incubated as mixed populations with each other. In other words, they synergized at both the morphological and the molecular level. This was the first time that cell-cell interaction among mutant strains was shown to regulate developmental gene expression.

The results made a nice balance to parallel studies that I had carried out with Maurice, which showed that morphogenesis could be blocked with EDTA following the initiation of aggregation without blocking the expression of transferase. Cell-cell communication does not seem to be essential for expression of transferase after 8 hours of development which is several hours before the enzyme starts to accumulate. The stage was set to determine the signals necessary for transcriptional control in development. I am still working on it to this day.

Maurice was always stimulating to be with in the lab. He would sit down in the middle of any discussion and take over the conversation. He was full of ideas and opinions. He loved to be outrageous as well as brilliant and usually succeeded in both. He had a bawdy sense of humor that was often at the expense of undergraduates but could also target colleagues, competitors, friends, enemies, or unwary post-docs.

Luckily, his enthusiasm and innovative ideas about carefully crafted experiments overshadowed his quirks. He patiently taught me how to determine the number of cells by counting in a hemocytometer; how to spread the cells as a uniform layer on nitrocellulose filters; how to check that the cells were developing normally; and how to help when they weren't developing well. He taught me everything that I had wanted coming to his lab. However, after about 8 months he asked me one day when I was going to get a job. I was surprised that he was encouraging me to move on so soon. Nevertheless, I got in touch with the Biology Department at UCSD where I knew they were going to be hiring quite a few new faculty. In the spring of 1966 I was invited to give a job seminar and flew out to La Jolla.

I gave a talk on my analyses of feedback loops necessary for glucose/lactose diauxic lag in *E. coli*. In my "chalk talk" the next day I tried to outline how this style of thinking could be applied to more complicated problems in multicellular development. I guess I was at least partially successful since the Chairman of the Department, Cliff Grobstein, offered me a position as Assistant Professor. Could I start in two months? I told Cliff that I was thrilled at the prospect but that I did not want to start for about 6 to 8 months since I needed to finish up some experiments that were underway at Brandeis. Cliff understood the problem and granted me a leave-of-absence before I had even started. I returned to Brandeis with plans to finish the experiments and to start writing a grant application to the National Science Foundation to allow me to purchase supplies and equipment for my lab at UCSD.

One of the unfinished lines of experimentation was aimed a developing a nutrient medium that could support the growth of *Dictyostelium* amoebae. Many experiments would benefit from being able to grow amoabae axenically in suspension. We would not have to worry about the residual bacterial components that were inevitably carried over when we washed up amoebae from bacterial growth plates. We could get cells to incorporate radioactively labelled compounds during growth without competing with the superior uptake mechanisms of bacteria. We might even be able to isolate auxotrophic mutant strains that required specific nutrients for growth. Maurice had successfully grown the related social amoeba *Polysphondylium pallidum* axenically but had not been successful with *D. discoideum*. He had no idea why he had fail and

seem to have lost interest. I picked up the challenge and tried all manner of media while I was in the Sussman lab. I tried standard bacterial media, yeast media, a diet drink for humans, almost anything I could think of. The flasks were innoculated with about a million amoebae and incubated for months in hopes that one would grow out. None did. Finally, just before leaving for La Jolla, I tried out a specialized medium that W. Balamuth had perfected for amoeboflagellates that was being used in the lab next to ours for growing *Naegleria*. I went next door and Chan Fulton gave me a flask of Balamuth's which I innoculated and put on the shaker. When I left a few weeks later, the amoebae had not grown, but they had not died either. In most of the previous media the cells died after a few weeks. I do not know what happened to that flask of Balamuth's after I left, but I suspect that something started to grow, perhaps one in a million cells, because a few months later word got to me that Maurice was having some luck growing a mutant strain of *Dictyostelium discoideum* axenically. The paper describing the medium and mutant strain Ax1 was published by Raquel and Maurice in October 1967, less than a year after I left their lab.

In December 1966 I packed up the car and, together with my wife Janet, drove across the country. We had planned to stop in Aspen, Colorado to ski for a few days, but the road between Vail and Aspen was closed by a huge early winter snow storm. We were stuck in Vail where we had 3 glorious days of skiing. Since the road was still closed, we had the mountain almost to ourselves. We then continued West and arrived in La Jolla before Christmas. On a previous trip to UCSD I had bought a small house near the beach in La Jolla. We got settled in and became acquainted with many at UCSD. The campus was so new at that time that you could get to know faculty in all the departments. It was very collegial.

My grant application had been approved at NSF and I had a budget that I could use to purchase essential equipment. One of the first pieces was a Zeiss spectrophotometer. I still have it in the lab where it is used in preference to more recent digital machines. I also filled the lab with incubators and a variety of microscopes some of which are still there. Every day was like Christmas since packages were being constantly delivered and unwrapped. I also started teaching since the Department suddenly needed someone to teach biochemistry. Luckily, there

were only 25 students in the course because they had to be my guinea pigs as I gradually learned to teach. For the first few years I made the mistake of trying to teach biochemistry the way I would have liked to have been taught myself. I did not realize that the students were not all like me and some of them wondered why they even had to take biochemistry. Over the years I have adapted various courses to the changing student body, but I have always enjoyed teaching the most eager and smartest students the most.

In my NSF grant I proposed to explore the possible roles of gases in establishing cell type proportions as well as use the marker enzyme UDPgalactose polysaccharide transferase as a molecular marker of progression through development. After several false starts, a few experiments started working. The assay for transferase was always cumbersome and I wondered if it could be replaced with an assay as easy as the one for β-galactosidase that I had used in my graduate work on the *lac* operon. All you had to do was lyse the cells and add a nitrophenol derivitized sugar as substrate. The bright yellow product could be seen with the naked eye and its rate of production quantitatively measured by absorbance at 410 nm at high pH. I went to the catalogs of the chemical supply houses and ordered every nitrophenol derivitized substrate they had for sale. There were about a dozen. Within a few weeks I was able to test extracts of vegetative cells, aggregating cells, slug cells and culminating cells for activity in hydrolyzing these substrates. I found 6 enzyme activities that changed aburptly at one stage or another of development. These "yellow enzymes", as we called them in those days, were purified, partially characterized and the assays optimized. Most of them were lysosomal enzymes that were maximally active under mildly acidic conditions. Over the years they have been the object of attention not only in my lab but also in labs of my colleagues. They provide quantitative traits when there are no changes in visual phenotypes. They have been more important as developmental markers than as components in physiological pathways.

I soon had more promising lines of work than I could follow on my own. Luckily, I was joined by several exceptional graduate students who took up one or another of the yellow enzymes. They included Bruce Coston, who studied the isozymes that hydrolyze p-nitrophenyl- β-glucoside, Randy Dimond, who studied N-

acetylglucosaminidase, β-galactosidase and α-glucosidase, and Steve Free who studied α-mannosidases. I worked on alkaline phosphatase together with my technicians. Papers presenting these marker enzymes were published during the 70's.

While the biochemical work was going on efforts were also directed at making *Dictyostelium* a more convenient genetic system. Back at Brandeis, John Ashworth and I had become aware that Raquel and Maurice had been able to isolate a diploid strain of *D. discoideum* only as the result of heroic efforts to screen thousands of large cells. Only one or two diploid strains had ever been isolated. This was no way to do genetics. When I told John that I would be going to La Jolla at the end of 1966, he suggested that he might come out and work in the lab the following summer. He had been awarded a Harkness Fellowship in England that required that he work in two independent labs, perferably at different institutions. One was Maurice's lab at Brandeis, the other could be mine at UCSD.

When John arrived in La Jolla, we set out to isolate heterozygous diploids from genetically marked mutant strains. We noticed that some of the survivors of NTG mutagenesis were barely able to grow and made very small clearings in the uniform lawn of bacteria on which they had been placed. Wild type cells formed large clearings, called plaques, within a few days. John had the insight to realize that each of the small plaque strains probably resulted from a mutation in a different gene and that most of these mutations would be recesssive. Therefore, a diploid formed between two independent small plaque strains would grow at the wild type rate and form a large plaque that could be easily recognized on a plate with mostly small plaques. We systematically generated and characterized a set of *minute* mutants and crossed them with each other. Almost all pairs reproducibly gave rise to large plaques of diploids. Upon further subculturing these heterozygous strains gave rise to haploid progeny expressing the *minute* phenotype. Establishing the exact frequency of haploidization was complicated by the large growth advantage of the haploid segregants. But it was a promising start.

It was a wonderful time for us in the lab. We also enjoyed exploring the still wild parts of southern California. One weekend in the fall of 1967, John and his wife Ann joined Janet and me on a "surfing safari" in Baja California. I was learning to surf and

had heard about the uncrowded waves along the beaches in Baja California. We drove down to Mexico, crossing at Tijuana, and continued about 200 km down the peninsula to where a dirt road led off to Punta Cabras. We camped in the dunes just back from the beach and had the place to ourselves. The waves were small but fun to surf on. A few weeks later John had to return to England to take up a Lectureship at Leicester University. We agreed to stay in close contact and visit each other as often as possible.

It was not until two years later that I could arrange to go to Leicester. We went for Christmas since Janet's family lived nearby. I worked for about two weeks in John's lab but they had turned off the heat at the start of vacation and by the time I left the lab it was freezing. During the intervening years I had focused on generating mutant strains that were temperature sensitive for growth so that they could be used to select for diploids that did not have a growth advantage over haploids at the permisssive temperature. I had described these mutants to John and sent him isolates. He agreed that they were much better than the *minute* strains for selecting diploids and went on to characterize the segregation patterns of multiple markers. John compared the results to the parasexual cycle described for *Aspergillus*. I compared the genetic system that we had established to somatic genetics of mammalian cell lines. His term was picked up by subsequent *Dictyostelium* geneticists including Peter Newell and Keith Williams and seems to have stuck.

We had both contacted Maurice Sussman at the end of 1967 to ask for a copy of the mutant strain that he had isolated for axenic growth. When we had not heard anything six months later, both John and I decided to isolate our own axenic strains. John selected for a spontaneous mutant while I selected for a mutant from a NTG mutagenized population. We both succeeded. The strain isolated in Leicester was called AX2 and the strain isolated in La Jolla was called AX3. I brought my axenic mutant with me when I visited Leicester in 1969. We compared the growth and development of AX2 and AX3 side-by-side and did not see a clear advantage to one or the other strain. About 10 years later Keith Williams found that the mutations responsible for the ability to grow axenically were in the same genes in strains AX2 and AX3, raising the possibility that they had a common ancestor that could grow

axenically. Perhaps one or the other strain became contaminated by the other. If so, in subsequent years there must have been many further genetic modifications to explain the differences recognized when the full genome sequences were compared. In any case, they have proven useful for all sorts of studies.

In the fall of 1968 I was invited to present a seminar at CalTech. The development of *Dictyostelium* was still a novelty at that time and all I had to do was describe its unusual approach to multicellularity and present a few ideas about how these might be carried out. The seminar was well received, but was memorable mostly because a young graduate student came up to me afterwards and told me it was the most exciting story he had heard in biology. It was Rick Firtel. At that time he was a graduate student in James Bonner's lab at CalTech working on plant chromatin. He told me that he would like to come down to La Jolla and work on *Dictyostelium* in my lab. We soon agreed that he would come down for a 6 month stay at the beginning of 1969. When Rick arrived, I showed him how we grew strain AX3 axenically and could collect billions of exponentially growing cells with ease. He also learned how to develop the cells synchronously and recognize the different stages just by looking in the dissecting microscope. He proceeded to isolate and characterize both DNA and RNA from large populations at various stages of growth and development. Charactrerization of the nucleic acids mostly consisted of measuring the kinetics of reannealing and hybridization using absorbance and elution from hydroxyapatite and nitrocellulose. Even early on, Rick was ambitious and energetic. Nothing would stand in his way.

Rick returned to CalTech and finished his thesis with a lot of advice and help from the exceptional molecular embryologist Eric Davidson who had his lab nearby. They did cutting edge studies and laid the ground work for much subsequent progress. In my opinion, the results were always interesting but a bit noisy. In 1972 he published an excellent paper entitled " Changes in the expression of single-copy DNA during the development of the cellular slime mold *Dictyostelium discoideum*." which introduced molecular biology of nucleic acids to the field.

Rick subsequently was a postdoc in the laboratory of Harvey Lodish at MIT. Harvey was a molecular biologist who I knew from my days working with *E. coli*.

His lab had focused on translational control for several years, but by 1971 he wanted to shift attention to the regulation of gene expression during differentiation of *Dictyostelium*. He asked me how to get started. I suggested that he get Rick to postdoc in his lab. They worked together for 2 years and published an important set of papers on the synthesis of precursors of messenger and ribosomal RNA *in vivo* and *in vitro*. Ten years later I spent an enjoyable and productive sabbatical leave in Harvey's lab. Stories were still being told about the time when Rick was in the lab. One of them recounted how he managed to have the ultracentrifuge available whenever he needed it. The day before he would fill the head with empty tubes and run it until he needed it. That way he did not have to bump anyone out of the machine. There were more stories.

One of the nice things about working at the University of California is that sabbatical leave accumulates at the rate of one quarter off for every two years teaching. By 1970 I had been working for 4 years at UCSD and thought a short sabbatical in Europe would be a good idea. By then we had two daughters, Kate and Emily, and it would be nice to introduce them to their English grandparents, aunts, uncles and cousins. It would also give me a chance to see if there was an experimental system better suited to my needs than *Dictyostelium*. Many people thought that *Dictyostelium* was so evolutionarily removed from mammals that it could not be useful as a model system for any aspect of human biology. The new data on chemotactic motility and transcriptional logic argued against that point of view and showed how certain aspects of development in *Dictyostelium* illuminated similar processes in more complex organisms. But it would always be an uphill effort to convince embryologists and members of the biomedical community that *Dictyostelium* studies were relevant. Perhaps it would be better to work directly with mammalian cells.

I was increasingly convinced that one of the best ways to unravel complex processes was to use mutational genetics to collect the pertinent genes in an undirected, unbiased manner. The trouble with this approach is that the National Institutes of Health only funded hypothesis driven research. However, when mutants

had been collected and characterized, their phenotypes led naturally to testable hypotheses.

So what would be the best system to work with? Humans carry interesting mutations that turn up at clinics when they are disabled. However, you had to be part of the biomedical community to have access to these patients. And even then you could not possibly control their behavior for genetic studies. It did not sound like this was for me.

On the other hand, mouse genetics was well developed with inbred lines and some interesting phenotypes. Heterozygous lines carrying embryonic lethal mutations could be bred such that a quarter of the embryos were homozygous mutants showing the desired trait. It wasn't efficient but it was possible. However, no one was sytematically generating embryonic lethals in mice because it was so laborious and expensive. It would be many years before the techniques for manipulating embryonic stem cells to generate mutant mice would be developed. If you wanted to use mutational genetics in mammals in the 70's, you had to use established cell lines.

Since I was more interested in cell differentiation than in growth or metabolic processes, I looked through the literature for cell lines that could be induced to differentiate one way or another. I found that David Yaffe had generated a rat cell line at the Weizmann Institute in Israel that would grow exponentially as single cells and differentiate into multinucleated myotubes when they became confluent. Their differentiation was rapid, robust and easily monitored by visual inspection of the colonies. With a little more detective work in the library, I found that there was a lab in Paris that was working with Yaffe's L6 line of rat myoblasts. It was sounding better and better.

I wrote to Francois Gros whose lab at the Institute Jacques Monod was working with L6 cells, wondering whether I could sabbatical in his lab. He described some of the work that Denise Luzzati was doing with him on the relationship of biochemical differentiations to morphological differentiations in the myoblasts. After some further discussion, I enthusiastically agreed to join the lab in early 1971.

After settling in Paris, I started work with the L6 cells. As soon as I was comfortable growing the cells, I killed large numbers with the mutagen NTG that I

routinely used with *Dictyostelium*. A high proportion of the survivors showed temperature sensitivity of either growth or differentiation. Others would not differentiate at any temperature. Neither fusion into myotubes nor accumulation of any of the muscle specific proteins occurred at the non-permissive temperature. We had established that biochemical and morphological differentiations go hand in hand in these mammalian cells just as they do in *Dictyostelium*. As I was wrapping up the experiments I came to realize that all I had done was treat L6 cells just the way I treated *Dictyostelium*. However, even with the wonderful help of Denise Luzzati, I had been able to do far fewer myoblast experiments. Working with cultured mammalian cell lines was just intrinsically slower and far more expensive than working with *Dictyostelium* cells. I spent the month of August in a white-washed house on the mountainside above Nerja, Spain, that Claudio Guillen, a Spanish writer and professor of literature at UCSD, had lent us. Emily took her first steps and Kate played in the waves at the beach. I decided that myoblast differentiation was beautiful and an excellent experimental system but that *Dictyostelium* was better for me. I returned to La Jolla with renewed commitment to learn as much as possible about *Dictyostelium* development.

The lab at UCSD was in good shape with graduate student Sheng-Shung Pong characterizing the RNA polymerases during development, post-doc Ken Poff defining the photopigment responsible for slug phototaxis, post-doc Paul Farnsworth characterizing the extracellular matrix by electron microscopy and a graduate student, Hud Freeze, characterizing the sheath biochemically. Randy Dimond and Mike Brenner were developing screening techniques for mutant isolation. I resumed teaching, writing grant applications and directing research. I also arranged to spend two months at the MBL in Woods Hole the following summers to provide a little variety.

I arranged to rent a small house on Buzzards Bay next to Stony Beach in Woods Hole. When we arrived, I found out the house had once been owned by Ed Conklin, one of the pioneer embryologists who had established the MBL in Woods Hole. I felt privaledged to use his house and garden. In a small upstairs office I found a copy of

Thomas Hunt Morgan's 1928 book "The theory of the gene". It had an inscription that I have never forgotten "To Ed from Tom". I felt I was in the presence of giants.

I shared a lab with Gary Borisy who was also visiting the MBL for the summer. He was studying microtubules and could purify large amounts of the tubulin subunits from marine organisms. One afternoon he rushed into the lab and showed me a microscope slide on which he had placed a drop saturated in tubulins. They were polymerizing into microtubules! I was probably the third person to see microtubules grow under the microscope.

I had brought equipment from La Jolla for growing *Dictyostelium* so that I could continue studies on the lysosomal enzymes. I wanted to used the "yellow" substrates to directly stain clones derived from mutagenized cells to isolate strains lacking the activity. The problem was that *Dictyostelium* cells do not stick to glass or plastic very well and are very motile. As a result they move around the surface of a petri dish and do not remain as pure clones. I found a solution to this problem by culturing them in the wells of microtiter plates. These plates were commercially available and used by immunologists for serial dilution. I put them to a completely different use. We learned how to efficiently fill each well with medium and inoculate them with a single viable cell as well as replica-plate the clones once they had grown up. We could then lyse the cells, add the substrates and score the activity in each well. Clones of interest could then be recovered from the replica plates. These techniques were soon picked up by other labs that recognized the utility of carrying out hundreds of independent biochemical reactions in a convenient format. Multitest plate technology is now taken for granted in most labs.

Within the first few weeks in the lab at the MBL I had hundreds of 96 well plates incubating mutagenized *Dictyostelium* cells. By the end of my stay I had found several good candidates for mutations affecting α-mannosidase. Back at UCSD I passed them over to a talented graduate student, Steve Free, who isolated several more mutations affecting α-mannosidase and fully characterized them. They were all shown to fall in the structural gene encoding the developmentally regulated enzyme.

Although I could walk home for lunch every day and play with the children in Woods Hole, I had time on my hands as I waited for the clones to grow up. For a

while I had thought that it would be useful to bring together all the varied results and ideas on *Dictyostelium* development in a book. I started to prepare an outline for a monograph on the subject and wrote some preliminary drafts of the chapters. It was wonderful to sit at my desk overlooking the narrow stretch of water separating Woods Hole from Naushon Island and slowly bring the book to life. By the end of the summer I had a thick folder to bring back to La Jolla.

Paul Farnsworth had been a graduate student in Lewis Wolpert's lab in London and came to La Jolla with all the confidence that might be expected for someone from the leading group in theoretical embryology. He was also a highly skilled electron microscopist and fully appreciated the power of a picture. When I returned from Woods Hole, I convinced him to take a series of electron micrographs of cells throughout *Dictyostelium* development to illustrate the book I was working on. The final images made a very significant addition to the book and are still some of the best available. The book also benefited from Paul's critical reading and his often repeated question "Bill, What are you trying to say here?"

1975 to 1985

A book can focus attention on an aspect of the field that had been previously taken at face value. I was aware that Barbara Wright had been working throughout the '60s to measure the enzymes of carbohydrate metabolism and changes in the concentrations of their substrates. Similar studies were also carried out in the laboratories of Elmon Coe, John Ashworth and Maurice Sussman; unfortunately, the results were not always the same as those reported by Barbara Wright. Partly because of the disdain that Maurice Sussman had for her, I did not worry about the discrepancies. I did not want to get into the feud between them which often got heated when they were at the same meeting. Barbara Wright openly questioned whether physiological changes required synthesis of new enzymes, let alone transcriptional regulation. She had the idea that metabolic flux, the flow of molecules down a given branch of the metabolic map, was sufficient to account for cellular differentiations and that the flux pattern was controlled by gating the substrates rather than changing the relative strength of the enzymes. She also questioned whether enzymatic parameters, such as substrate affinity, measured with *in vitro* assays really represented the *in vivo* properties of the enzyme of interest. These ideas unsettled molecular biologists but attracted those who liked outsiders.

Starting in 1968 Barbara Wright published a series of papers presenting and expanding on kinetic models of carbohydrate metabolism in *Dictyostelium* development. The analyses were elegant but close to impossible to judge because they consisted of coupled differential equations that could only be solved numerically with the help of a computer. I had incorporated her conclusions in the 1975 book but had not tried to independently confirm her results or conclusions. After the book was finished, I had some time to revisit the question and started to reread her papers. To my dismay I found that different values were used for concentrations of the intermediates of carbohydrate metabolism in successive papers and that the enzymatic parameters were sometimes changed without mentioning it. I started to get angry when I found that the basic flow path was quietly changed from one model to another. How could anyone know if the kinetic models had been confirmed or not?

I decided to repeat the whole study myself using published measurements of the small molecule intermediates that I trusted and enzymatic parameters that I confirmed in the lab. I could write out metabolic pathways and describe them with Michaelis-Menten kinetic equations, but I couldn't run the simulations because I did not know how to program. Luckily an energetic, bright, computer savvy undergraduate, Steve Thomas, came by during this time and asked if he could work in the lab to get some research experience. He wondered if there were problems that could benefit from computer assistance. I laid out the problem for him. I was also lucky that a neurobiology colleague in the lab directly above mine, Al Selverston, had just bought a new PDP-11 computer to model a neuronal ganglion. He generously allowed us to use it evenings and weekends. This rather impressive DEC machine had several innovative features making it easier to program. Steve Thomas initially used the programing language FORTRAN but subsequently rewrote the final program in BASIC. Gradually I learned how the machine worked and could read the code, although I never learned to write it. I rather enjoyed picking up the stack of punch cards and carrying them to the compiler at the University mainframe where the final output was graphed.

As soon as we had caught and removed all the bugs in the program we found that the published data on the changes in the critical enzymes accurately predicted the developmental changes in small molecules and polysaccharides. Equations using the *in vitro* measured activities and Km values faithfully reproduced the measured physiological changes. When I challenged Barbara Wright with these results, she dismissed them on the basis that I was not as good a biochemist as she was. I came to the conclusion that Maurice had been right all the time and that she was damaging to the field. Over the next few years I successfully worked to exclude her from further interactions in the *Dictyostelium* community. She moved to the University of Montana in 1982 where she studied evolution and was an avid kayaker. I am glad to say that Barbara is the only scientist that I have consistantly confronted in my career.

But that does not diminish my respect for her proposal that metabolic flux is an important aspect of differentiation. Steve Thomas and I explored various aspects of our flux balance model of metabolism in wild type and mutant strains and were

impressed by the importance of substrate concentration on pathways that shared one or more substrate. We also found that when we simulated cells in which one of the critical enzymes failed to accumulate in a wild-type manner, the physiological changes were affected. The accumulation of sugars and carbohydrates was all screwed up. We also found that success of the simulation required that we assign substrate affinities close to those that had been measured *in vitro*. Both of these results directly contradicted Barbara Wright's conclusions. She became known in the lab as Barbara Wrong.

One especially satisfying outcome of our simulation studies concerned the enzyme that synthesizes cellulose, cellulose synthetase. At that time no one had been able to accurately assay the enzyme nor determine its developmental time course in *Dictyostelium*. However, it was clear that substantial amounts of cellulose accumulated during culmination of fruiting bodies. To make the equations for other carbohydrates and polysaccharides work we had to make guesses for when the gene for cellulose synthetase was expressed and how much activity accumulated. Twenty-five years later I found a mutant strain in which a plasmid had disrupted the cellulose synthetase gene which could then be easily cloned. Northern blots showed that cellulose synthetase mRNA appeared during development and accumulated exactly as we had predicted.

Another subject that was reviewed in depth in the book concerned the expression of a set of about a dozen developmental enzymes and other developmental markers in wild type and mutant strains. We had characterized 4 mutants that all looked identical in that they completely failed to aggregate. We found that they could be distinguished by the patterns of the early enzymes thereby defining a linear sequence of early stages. Characterization of mutants that aggregated but arrested morphogenesis before culmination defined later stages. I came to realize that further study of these mutant strains might give strong support for a dependent sequence of stages underlying development. I encouraged my students Sally White and Randy Dimond to collect the missing data and put together a paper. I remember enjoying the animated discusssions about how best to present the ideas and felt the paper turned out well.

While we were fairly sure that the cells had to signal each other to progress through the dependent sequence, we did not know what the signals might be. A talented graduate student, Laura Grabel, developed an assay to purify a quorum sensor that triggered expression of the marker enzyme N-acetylglucosaminidase (NAG). This early developmental enzyme required conditioned medium for expression in low density populations. Grabel managed to purify a low molecular weight, heat resistant component that was secreted by the cells and induced NAG. Further purification was not possible due to the difficulty of the assay. However, many years later, Richard Gomer characterized a protein which he called CMF (conditioned medium factor) which he showed gave rise to peptides with the activity Grabel had described. In recent years, the list of known intercellular signals has continued to increase, but even 35 years later, the hunt is not over.

One day I had a visit from Rich Lerner who worked at The Scripps Research Institute (TSRI) up the road in La Jolla. He wanted to know more about *Dictyostelium* because he was interested in defining membrane proteins using monoclonal antibodies that he was raising in his lab. I was more than happy to talk with him since I realized that well characterized membrane proteins would, at a minimum, provide an additional set of developmental markers that could be added to the analyses on dependent sequences. Rich asked me if I would train his postdoc, Chi-Hung Siu, in growing and developing amoebae. It was "the beginning of a beautiful friendship" as Bogart said to Claude Rains at the end of the movie "Casablanca". For several years we worked in close collaboration. Even after Chi-Hung moved to Toronto, we continued collaborating on the mechanisms of cell-cell adhesion.

The first conference dedicated to *Dictyostelium* was held in the spring of 1977. The year before Harvey Lodish and I had arranged for a conference on microbial development that was held at the Cold Spring Harbor Lab in New York. Most of the people we knew who worked on *Dictyostelium* development were present at that meeting but we also invited some who were studying related processes in yeast, fungi or algae. The 1977 meeting was organized by John Ashworth and Piere Cappuccinelli in Porto Conte, Sardinia, and it was pure Dicty. Thereafter, meetings were held every few years and annually after 1981. Meetings were arranged in interesting places that

alternated between European and American sites, with an occasional expedition to Japan. The Sardinia meeting certainly qualified as an interesting place. Piere Cappuccinelli happened to be from Sassari, Sardinia and knew many in the regional government. While he was a postdoc in John Ashworth's lab at the University of Essex, he arranged for funding from EMBO and the local Sardinian government to fund the meeting. We flew from Rome and managed to land in Sassari which was known to have one of the most dangerous airports in Europe. We then proceeded to a lovely hotel in Porto Conte set among dry hills and rocks. The swimming pool was freezing but the food was excellent, complete with suckling pigs with apples in their mouths.

A photograph has survived of those who attended the meeting in Sardinia. I will use the pictures of the leaders in the field to jog my memory. One thing stands out immediately: all of us had longer hair 40 years ago. In 1965 when I entered the field there were 5 major laboratories. Ten years later there were more than 30; it was clearly a growth industry. I will give a short synopsis for those I can recognize in the picture.

First International Dictyostelium Meeting. Porto Conte, Sardinia - April 12 - 16, 1977

Recognized Leaders
Top row: Hiroshi Ochiai**;** Günter Gerisch; Albert Goldbeter; Jeff Williams; Dieter Malchow
Second row: Salvo Bozzaro; Theo Konijn**;** Michel Veron**;** Julian Gross**;** Robert Kay**;** Keith Williams; David Soll**;** David Ratner; Tony Durston; Yasuo Maeda; David Garrod
Third row: Ikuo Takeuchi **;** Jim Gregg**;** Kenneth Raper **;** Peter Newell**;** Rick Firtel; Maurice Sussman**;** Harvey Lodish**;** Bill Loomis**;** Piere Cappuccinelli; Reg Deering
Front row: Ellen Henderson**;** Philippe Brachet; John Ashworth**;** Claudette Klein**;** Irene Hames**;** David Hames

Cast of Characters

Hiroshi Ochiai (top row, near the middle of the picture) In the 1970's Hiroshi Ochiai and Masaki Iwabuchi worked together at Hokkaido University in Northern Japan to bring advanced biophysical approaches to the study of ribosomes of *Dictyostelium*. He later worked with **Gunter Gerisch** on Contact Sites A in cell-cell adhesion. I visited his lab in Hokkaido in 1996 when he was a major player in the Japanese cDNA project.

Gunter Gerisch (below Ochiai with dark glasses, partially hidden) One of the first generation of leaders in the field, Gunter was working at the Biozentrum in Basel, Switzerland at the time of the meeting. He presented beautiful data on the oscillations in cGMP and cAMP in entrained populations of *Dictyostelium*. One of his early students, **Dieter Malchow**, can be seen at the far right of the top row.

Albert Goldbeter (dark glasses; just to the right of Gunter) He studied non-equilibrium thermodynamics with Nobel Laureate Ilya Prigogine and became interested in the properties of oscillatory processes. For over 40 years he has been a leader in theoretical analyses of signal relay in *Dictyostelium*. At various times, he and I shared common interests but often came to different conclusions. I have greatly enjoyed knowing him all these years.

Jeff Williams (next to last in the top row) He learned how to analyze polyA+ mRNAs while working with Sheldon Penman at MIT, an old friend of mine. When he returned to the ICRF Mill Hill Laboratories near London, he collaborated with **Rob Kay** on a molecular study of globin mRNA during the life cycle of the South African clawed toad, *Xenopus laevis*. When Rob showed him *Dictyostelium*, he decided this was the system for him. He came to Sardinia to learn all about it. I remember talking with Jeff while we walked to a three thousand year old Phoenician tower on a nearby promotory (see rear left of the photo). He told me he was going to bring modern

biology to *Dictyostelium*. For almost 40 years he had an illustrious career dissecting cell type specific transcriptional regulation in *Dictyostelium*. He retired from the University of Dundee last year.

Dieter Malchow (last in the top row) Dieter participated in most of the early landmark studies from the **Gerisch** lab. Later in his own lab at the University of Konstanz he pursued the role of calcium ions in chemotaxis and oscillations for more than 25 years, often collaborating with physicists.

Salvo Bozzaro (second row, on the far left of the picture) He joined the Gerisch lab shortly before the Sardinia meeting. In Basel he was working with the related species *Polysphondylium violaceum*. He was particularily interested in cell-cell adhesion. In his own laboratory at the University of Turin in Italy he has pursued a wide range of questions in *Dictyostelium discoideum* for more than 25 years. He also served as Dean of the Medical School.

Theo Konijn (second in the second row) He did his graduate work with **Ken Raper** in Wisconsin, receiving his PhD. in 1961 and then returned to Holland. While working at the Hubrecht Lab, he showed that extracts of *E. coli* contained material that attracted *Dictyostelium* amoebae. The material later turned out to be cAMP. In 1966 he took a leave of absence to work on the nature of the chemoattractant in the laboratory of John Bonner. They were able to show that the chemoattractant was cAMP. For the next 20 years he brought out more and more of the details of cAMP signaling. He also trained **Peter van Haastert** and **Pauline Schaap** who have continued this line of work up to the present. In 1969 he helped to recruit **Tony Durston** (white shirt in second row of the picture) to the Hubrecht Lab where he subsequently trained **Kees Weijer**, another long time *Dictyostelium* worker.

Michel Veron (second row with a beard) Michel spent almost his whole career at the Pasteur Institute in Paris. I got to know him when we both worked on *Dictyostelium* in the laboratory of **Philippe Brachet** (front row). When I was on sabbatical in Paris

in 1972, Philippe and Luiz Pereira da Silva came to discuss development of *Dictyostelium*. I gave them advice and cultures of the axenic strains and my best wishes. Five years later I spent a sabbatical year at the Pasteur and got to know Michel Veron very well. Many years later Michel's student, **Christophe Anjard**, came to my lab in La Jolla and stayed for 12 years.

Julian Gross (just to the right of Michel) By the time that Julian Gross started working with *Dictyostelium* in 1975 he was already a highly respected microbiologist who had made major contributions to understanding mechanisms of bacterial genetics and DNA repair. When he moved to the ICRF at Mill Hill, he joined forces with **Rob Kay** to try to understand the signals necessary for prestalk and prespore differentiation. Among other things he was interested in the responses to ammonia, calcium and pH. I always found him charming and stimulating in discussions of science or philosophy. He retired in 2000.

Robert Kay (just in front of Julian) Rob worked with rat liver nuclei before moving to the ICRF Mill Hill with **Julian Gross** and John Cairns in 1973. His initial studies in collaboration with Julian were aimed at defining the minimal conditions necessary for stalk or spore formation in *Dictyostelium*. In 1984 he moved to the MRC Laboratory in Cambridge where he continued to make breakthroughs concerning intercellular signals. Over the years we have had many interesting discussions and collaborations. It is always a pleasure to be with Rob.

Keith Williams (the bearded one behind Ken Raper) Keith carried out sophisticated genetic analyses of *Dictyostelium* in the laboratory of **Peter Newell** (third row center) at Oxford University in England. Most of these studies were in collaboration with a postdoc in the lab, **Rich Kessin**. Peter Newell and Rich Kessin had met in **Maurice Susssman**'s lab at Brandeis before moving to Oxford in 1972. Keith returned to his native Australia in 1976 where he had a position in the National University in Canberra.

David Soll (the one with a mustache just in front of Hiroshi Ochia) Although he started out at Brandeis in the laboratory of Chan Fulton, David worked with **Maurice Sussman** on *Dictyostelium* before starting his own lab at the University of Iowa. He initially studied the regulation of developmental timing and the phenomenon of erasure during dedifferentiation. Throughout his long carrier he tackled a broad range of developmental processes using highly quantitative computer assisted techniques. For over 20 years David and I collaborated on the analysis of chemotactic motility in a series of well defined mutant strains. We had many adventures.

David Ratner (with a smaller mustache to the right of Albert Goldbeter) David had joined **Peter Newell**'s lab at Oxford just before the Sardinia meeting. He continued many of the genetic studies of **Keith Williams** and **Rich Kessin** before starting his own lab at TSRI. When he arrived in La Jolla, I suggested that he might want to hire Wayne Borth, who had been an outstanding technician in my lab for several years. Together they perfected separation of prestalk from prespore cells on Percoll density gradients. He was only in La Jolla for a few years before moving to a position at Amherst College in 1984, but we became long time friends.

Tony Durston (just in front of David Ratner) Three years after Tony was introduced to *Dictyostelium* at the University of Chicago by **Anthony Robertson**, he moved to the Hubrecht Lab in Utrecht where he continued to study chemotaxis. Starting in 1980, he increasingly focused on signaling during *Xenopus* development. In 2005 he moved to the University of Leiden. I remember intense theoretical discussions with Tony when I visited the University of Chicago in 1968. I miss them.

Yasuo Maeda (suit and tie to the right) Yasuo was a student of **Ikuo Takeuchi** (far left of third Row) and received his PhD. in 1971. Takeuchi had been studying *Dictyostelium* biochemistry for more than 10 years at the University of Kyoto when Yasuo arrived and carried out some studies using high resolution electron microscopy. He was a lecturer at Kyoto from 1967 to 1982 when he was appointed to

the faculty at Tohoku University in Sendai. He retired in 2006. I was lucky that several of his graduate students became postdocs in my lab.

David Garrod (mustache with white shirt) He was a graduate student in Lewis Wolpert's lab in London and studied *Dictyostelium* for about 15 years while at the University of Southhampton (1972 - 1989). In a collaboration with **John Ashworth** (front row center) David Garrod showed that cells grown in the presence of glucose had an advantage in making spores when competing with cells grown in the absence of sugar. He also became very interested in cell-cell adhesion. When he moved to the Department of Medical Oncology at University of Southhampton in 1978, he had been told to drop the experiments with *Dictyostelium*. He told me that he was very sorry to leave the field, but he had no choice.

Ikuo Takeuchi (seated far left of third Row) Another of the first generation of leaders in the field, Ikuo Takeuchi had a position in Botany at the University of Osaka until 1971 when he became a professor in the Department of Botany at Kyoto University. He developed a quantitative assay for prespore cells that depended on antibodies to spore coat antigens of *Dictyostelium*. He addressed many important problems of patterning and cell type proportioning over the years. One of his first students, **Yasuo Maeda**, can be seen in the second row of the picture. In 1979 Ikuo and I organized a Japanese-American meeting in La Jolla on development of *Dictyostelium*.

Jim Gregg (third in the third row) After returning from combat in Germany at the end of World War II, Jim entered graduate school at Princeton. In 1951 he joined the Faculty of the University of Florida where he stayed until his retirement in 1985. Throughout his long career, he studied development in *Dictyostelium* with emphasis on physical and microscopic measurements. Jim was also an accomplished artist who made large sculptures that were displayed outside his lab.

Ken Raper (third row, tie and jacket) The book that collected the talks given at the Sardinia meeting was dedicated to Ken Raper who was about to retire from his position at the University of Wisconsin. Everyone at the meeting recognized the immense value of his pioneering work with *Dictyostelium*. He was truly the father of the field. One of his first students, **Theo Konijn**, is in the second row of the picture.

Peter Newell (center of the row) Peter came to the laboratory of **Maurice Sussman** (just to the right in the picture) about a year after I left Brandeis for UCSD. We never overlapped but soon got to know each other since we thought about *Dictyostelium* development much the same way. In 1971 he returned to Oxford University to start his own lab where he continued to carry out important biochemical and genetic experiments in *Dictyostelium* development. Two of the many scientists who trained in his lab, **Keith Williams** and **David Ratner**, are in the second row of the picture.

Rick Firtel (checkered shirt) Rick was offered a position at UCSD in 1973. We have been colleagues in the Department of Biology for more than 40 years. Before coming to La Jolla he had been a postdoc in the laboratory of **Harvey Lodish** (two to his right). Throughout the 70's Rick's lab laid the ground work for characterizing the *Dictyostelium* genome and the expression of multigene families. Twice, Rick and I organized an annual *Dictyostelium* meeting in La Jolla.

Maurice Sussman (just to the right of Rick) Maurice was one of the four first generation leaders at the meeting; only John Bonner was missing. Several participants of the meeting had worked at one time or another in his lab. They included **David Soll, Peter Newell, Bill Loomis**, and **John Ashworth** who are all in the picture. In 1973 Maurice and Raquel took positions at the Hebrew University in Jerusalem. Maurice had to drive a taxi during the Yom Kippur War but relished living in Israel. In 1976 they moved to the University of Pittsburgh where Maurice was appointed Chair of the Department of Life Sciences. At the time of the meeting, Maurice was exploring the morphogenetic role of ammonia in *Dictyostelium* culmination.

Harvey Lodish (to the right of Maurice) I knew Harvey when he was a graduate student of Norton Zinder at the Rockefeller where they worked on the molecular biology of phage f2. In 1968 he started his lab at MIT and gradually shifted to the study of *Dictyostelium* development. The pace accelerated when **Rick Firtel** (two to the left of Harvey) joined his lab in 1971. Over the next 10 years Harvey trained a dozen excellent graduate students in the molecular biology of *Dictyostelium*. He has also been lead author of the textbook Molecular Cell Biology for many years.

Bill Loomis (to the right of Harvey) I was happy to be surrounded by so many old friends and colleagues at the Sardinia meeting. I have always felt that I belong to a village of Dicty workers that happens to be spread out over the world most of the time but aggregates periodically. It is always a pleasure to be together with so many who agree on what is interesting.

Reg Deering (two to the right of me) Reg is almost single-handedly responsible for what we know about DNA repair in *Dictyostelium*. Before shifting to *Dictyostelium* in 1968 he had built a strong reputation for studies of radiation sensitivity in bacteria. He showed that *Dictyostelium* cells were exceptionally resistant to UV and gamma radiation as the result of having highly effective repair mechanisms. He was also responsible for introducing **Dennis Welker** to genetic analyses of radiation-sensitive genes of *Dictyostelium*. Dennis went on to make many contributions to the genetics of *Dictyostelium*.

Ellen Henderson (far left of the front row) Ellen became familiar with biochemical studies in *Dictyostelium* in the laboratory of **Julian Gross** (second row) at the University of Edinburgh and with genetic studies in the laboratory of **Peter Newell** (third row) at Oxford. She continued these studies at MIT and later at Georgetown University concentrating on glycoproteins.

Philippe Brachet (third in the front row) Philippe and Luiz Pereira da Silva took the *Dictyostelium* cells that I gave them in 1972 back to their lab at the Pasteur Institute

and started a series of experiments on their growth and development. One study that amused me showed that 50 μM cannabinol inhibited both growth and development but THC did not. Unfortunately, they did not pursue this line of work. **Claudette Klein** (three to the right of Philippe in the front row) was a postdoc in the lab at the time of the Sardinia meeting but was about to leave for her own lab in the Department of Biochemistry at the St. Louis University Medical School. **Michel Veron** (second row) was recently returned from Seattle where he had postdoced with Ben Shapiro working on sea urchin eggs. He was settling into the Pasteur at the time of the meeting. Philippe Brachet was making significant progress in understanding the role of cAMP both in chemotaxis and in induction of cell differentiation in early development. I am grateful to Philippe for hosting me while on sabbatical at the Pasteur later that year.

John Ashworth (to the right of Philippe) At the time of the Sardinia meeting John was a Professor at the University of Essex in Colchester England. He became undersecretary for scientific affairs to the government in 1979 and Vice-Chancellor of the University of Salford two years later, effectively ending his involvement in *Dictyostelium* studies. **Piere Cappuccinelli** (to the right of me in the third row) was a postdoc in John's lab in Essex who returned to a position at the University of Sasari just before the meeting. **Irene** and **David Hames** (front row right) were students in John's lab. Sir John was knighted in 2008 in gratitude for his public service in education and government. We are all grateful for his work on *Dictyostelium*.

Before I went to the Pasteur Institute in the fall of 1977 to learn more about chemotaxis, I had been interested in the physiology of aggregation but not convinced that it was of sufficient general interest for me to work on it. The answers might only relate to *Dictyostelium* since chemotactic aggregation of individual cells to form an aggregate is only seen in the social amoebae. Moreover, Bonner, Konijn, Gerisch and others were making good progress in defining the mechanisms for synthesis, secretion and removal of cAMP as well as the response of cells to cAMP. I could wait and see what they found.

Discussions with Rick Firtel, Harvey Lodish, Julian Gross, Rob Kay and others changed my mind. I came to realize that cAMP not only controlled the direction of chemotactic motility but also regulated expression of aggregation stage genes. If I wanted to understand progression through the dependent sequence, I needed to know as much about cAMP in *Dictyostelium* as posssible. Philippe Brachet's lab at the Pasteur was involved in studying both chemotaxis and gene induction by cAMP and seemed like a good place to become familiar with the latest approaches and insights.

I decided my first challenge on this Paris sabbatical would be to purify adenylyl cyclase to homogeneity so as to be able to characterize it biophysically and raise specific antibodies. I had considerable experience and success purifying the marker enzymes and was confident that, with a good assay, I could purify any enzyme. Claudette Klein had just set up an assay for adenylyl cyclase in Philippe's lab based on an assay published a few years earlier by Salomon, Londos and Rodbell. For some unknown reason, she reversed the order of columns that separated cAMP from ATP but it gave reproducible results. I went back to the original Salomon assay and confirmed her enzymatic results. I also found that replacing magnesium salts with manganese salts stimulated the activity considerably. Using this assay, I partially purified adenylyl cyclase from aggregation stage cells but found that the solubilized activity was very unstable. I had to work fast. I tried to stabilize the enzyme with all sorts of treatments and additives but none of them worked. After several months of experiments, the most highly purified material was still associated with small vesicles. I never succeeded in getting pure protein with activity. Although many labs have tried hard to purify the enzyme since then, it has stubbornly rebuffed all biochemical approaches. Only the introduction of cloning techniques finally solved the problem in 1992 when the structural gene for adenylyl cyclase was isolated in the laboratory of Peter Devreotes at Johns Hopkins School of Medicine.

My confidence in biochemistry was severely shaken, but I stayed busy with the characterizations of cAMP phosphodiesterase and cAMP surface binding sites that were being carried out by Michel Darmon and Michel Veron in the lab. I also used a technique described by Gunter Gerisch where cells showed spontaneous cycles in light scattering triggered by cAMP pulses. After 5 hours of development, we could

observe oscillations in cAMP every 7 minutes. Using the improved enzyme assay, I could also measure spontaneous oscillations in adenylyl cyclase activity as the cells became entrained. I puzzled over these observations for the next 20 years before Mike Laub and I came up with a plausible explanation for entrainment of cells that can generate a 7 minute periodicity.

My sabbatical leave had convinced me that a satisfactory understanding of chemotaxis would not come from biochemical studies alone. We needed molecular genetics to come to the rescue. The moratorium on recombinant DNA experiments that had been established in 1974 was being lifted slowly but the techniques were a long way from being streamlined. It took 10 years for the Devreotes and Firtel labs to clone and characterize the cAMP surface receptors and components of the G protein signal transduction pathways. I returned to the problems of cAMP signaling only after the turn of the century.

One of the stories that was waiting for me when I got back to La Jolla was the characterization of the major proteins that make up the spore coats. David Cotter, a graduate student in Ken Raper's lab, had found out how to get spores to germinate synchronously. Using his technique, we could isolate the empty spore coats and then dissolve them in boiling SDS solutions. Electrophoretic separation in 1D gels was sufficient to see that the major protein species had molecular weights of 96,000 , 70,000, and 60,000 daltons. We named the proteins SP96, SP70 and SP60. They were the heros of a saga that was pursued in the lab for the next 15 years by a continuously changing group of graduate students and postdocs. The amino acid sequence at the N-terminus of each one was determined. Antibodies were raised to each spore coat protein and used for subcellular localization as well as cloning. Their genes were cloned, sequenced and mapped to a cluster on chromosome 2. Strains were isolated with mutations in one or more of the major spore coat proteins that helped to understand their roles in building the ellipsoid spore coats. They were also used in studies of transcriptional regulation that went on until well into the 90's.

The spore coat proteins came out of our general interest in membrane proteins during development of *Dictyostelium*. When we ran 1D gels of proteins solubilized from membranes isolated from cells late in development, we found that SP96, SP70

and SP60 bands could be recognized over the generally fuzzy background. Just before going to the Pasteur I suggested to Mike Orlowski that he purify spore coats and see if the same proteins were present there. Mike was a post-doc who had recently joined the lab after working on the dimorphic fungus *Mucor* with Paul Sypherd. While I was off in Paris, Mike became adept at purifying spore coats free of amoebae and indeed found them to have high levels of SP96, SP70 and SP60. Shortly thereafter he set up his own lab at Louisiana State Univeristy in Baton Rouge where he reverted to working on *Mucor*.

A postdoc couple from Ireland, Kevin Devine and Barbara Dowds, picked up the saga and added higher resolution protein characterization and heroic cDNA cloning. Kevin used the 2D technology that Jim Morrissey had established in the lab in which proteins are sequentially separated by isoelectric focusing and sized by gel electrophoresis. Jim discovered conditions that gave highly sensitive, reproducible silver staining of isolated protein spots. His technique is now standard world-wide. Using these techniques they were able to show that SP96, SP70 and SP60 are all coordinately synthesized by prespore cells after 14 hours of development. Moreover, SP96 is post-translationally fucosylated and accounts for most of the fucose incorporation that Jim Gregg had previously shown was restricted to prespore cells.

Kevin also collaborated with John Bergmann, a postdoc in Jon Singer's lab on the floor above us at UCSD. Tokuyasu and Singer had recently worked out a way to prepare frozen sections for electron microscopy that could be stained with antibodies coupled to tiny gold balls. With Tokuyasu's guidance, Bergmann and Devine used antibodies specific to the spore coat proteins to demonstrate that they are packaged in prespore specific vesicles seen only in prespore cells at the slug stage. During culmination these vesicles fuse with the surface membrane and release their contents to generate spore coats. This was state-of-the-art cryomicroscopy.

Kevin carried out several other interesting studies in the lab, including high resolution determination of expression of cell type specific proteins using 2D gels and molecular characterization of anterior-like cells. In 1985 he accepted a position in the Department of Genetics at Trinity College Dublin and returned to Ireland where he has stayed happily ever after.

Capitalizing on the specificity of the antibody to SP96 Barbara Dowds managed to clone a cDNA for this spore coat protein. She screened a cDNA library that Rex Chisholm and Harvey Lodish had made at MIT from cells developed for 20 hours. Four batches each containing 192 clones were used to select mRNA molecules by hybridization. The mRNAs were translated *in vitro* and the products characterized with anti-SP96 after gel electrophoresis. The batch giving the strongest signal was subdivided into 8 groups of 24 and the most positive sub-batch grown as 24 individual clones. A strongly positive clone was picked and shown to carry a piece from the 3' end of the gene encoding SP96. This was the first successful cloning of a specific cDNA in the lab. It was an heroic effort fraught with possibilities for failure. Thanks to Barbara, it worked. The cDNA clone was used to establish a restriction map around the gene that directed subsequent isolation of genomic fragments encoding the full length protein. Luckily, cloning the genes encoding the other spore coat proteins was more orthodox and straightforward. cDNA for SP60 was isolated by hybridization to oligonucleotides encoding the 6 amino acid sequence that is repeated four times near the N-terminus. cDNA for SP70 was recognized from clones that were enriched for expression in prespore cells. Both proteins carry signal sequences for export across membranes that are subsequently cleaved off giving the N-terminal sequence that had been previously determined.

It seemed that the lab was entering the cloning era, but I was accutely aware of the fact that I was only advising from behind my desk rather than at the bench. I realized I needed some hands-on experience to become completely at ease with the new approaches. I arranged with Harvey Lodish to spend a one-quarter sabbatical leave in his lab at the Whitehead Institute of MIT to learn cloning and sequencing up front. I thought I would try out several techniques and also indulge my nostalgia for Boston. I spent the fall of 1984 in Boston just across the Mass Ave bridge from MIT and walked to work every day.

When I got to the Whitehead every morning, I was greeted by loud rock-and-roll from Steve Cohen's radio. Steve would turn it down when others came into the lab. However, he continued to work at a frenetic pace all day. Harvey had asked him if he would show me the ropes and answer my questions. I wanted to see if I could clone

cDNA that encoded the cell adhesion protein gp80 using antibodies to screen cDNA expressed in λgt11 vectors. I had the antibodies with me and I soon was able to make a λgt11 library. Making iodinated second antibody was a bit stressful for me since the Geiger counter kept screeching from the high levels of iodine131 I had to use. It all went well and for a few months I could supply Harvey's lab as well as the next-door lab of Rick Young with this useful reagent.

Steve Cohen answered all my questions in a patient and through manner. His usual answer to why the ligation had not worked was "Use more ligase". It worked some times. I also watched closely as he sequenced portions of a genomic clone. He had found that this gene was expressed at high levels throughout most of development and made a perfect control for the normalization of expression of other genes. Slowly, the sequences of the flanking regions around the 1.1 kb coding region were finalized. I kept pushing Steve to put it in a computer that could translate the open reading frame, but he wanted to wait until it was complete. Finally, he translated it and it was clearly a member of the actin multigene family that Rick Firtel had recently characterized. "What a bummer". However, it was not one of the dozen or so actin genes that had been previously sequenced. The new one was designated actin 15.

Steve and I talked a lot about how the upstream regions might be used to express ectopic genes once cell transformation was a routine technique. By the end of the fall we had a plan in which Steve would come to La Jolla in the spring and finish his postdoc in my lab so that we could continue our enjoyable and productive collaboration. When he arrived at UCSD, he teamed up with Dave Knecht, a post doc in my lab who had learned to clone and sequence as a graduate student with Randy Dimond at the University of Wisconsin. After leaving my lab, Randy had been a postdoc in Harvey Lodish's lab (small world). Steve and Dave built a plasmid with the regulatory region of actin15 driving the neomycin phosphotransferase I gene that confers G418 resistance. When they used their plasmid for transformation, it worked like a charm. They went on to show that transcriptional regulation from actin15 only requires the proximal 270 base pair region.

We frequently discussed our results with Wolfgang Nellen who was a postdoc in Rick Firtel's lab at that time doing similar work on the actin6 regulatory region. We would all have lunch together at the cafeteria and talk about a little bit of everything. A few years earlier Hirth, Edwards and Firtel had described a plasmid carrying a G418 resistance gene that they reported would transform *Dictyostelium* cells to drug resistance. However, neither Wolfgang nor I could repeat these experiments. Wolfgang decided to make his own transformation plasmid using the actin6 regulatory region to drive neomycin phosphotransferase II. This plasmid worked very well. Wolfgang went on to show that the original plasmid carries a mutation that precludes it from functioning. It serves as a negative control in Wolgang and Rick's paper on transformation.

As we compared results over lunch, it became apparent that both the actin6 and the actin15 essential regulatory regions carry a sequence closely related to AATGGGATTTT, which is also found upstream of many other actin genes. Steve, Dave and Wolfgang targeted this sequence in a series of deletions and point mutants that showed that it was essential for expression of adjacent genes. This was the way to get to the gene regulatory network. Wolfgang and I have had a wonderful open scientific relationship ever since those days of excitement when transformation started to work.

1985 to 1995

Parasexual genetic techniques are sufficient to map specific mutations to defined loci on one of the 6 linkage groups by phenotypic chartacterization of segregants from heterozygous diploid strains generated from independent mutant isolates. However, we needed molecular genetic techniques to be able to get to the underlying molecular mechanisms of development in *Dictyostelium*. Wolfgang Nellen and Rick Firtel had shown in 1985 that antisense inhibition worked well in *Dictyostelium*. This is a technique that Hal Weintraub developed a few years earlier in which RNA complementary to a specific mRNA is used to inhibit translation. Wolfgang and Rick transformed cells with a construct in which a gene encoding the discoidin lectin was inverted relative to the promoter and showed that they had the same phenotype as discoidin-1 minus mutants. This technique looked promising for knocking down genes, especially those that were essential at a particular stage since we could use conditional promoters to drive the antisense. We hoped to develop a way to knock down most developmental genes and determine the consequences.

Knocking down the gene encoding myosin heavy chain, *mhcA*, sounded like a good place to start. The role of myosin was of great interest unto itself and the *Dictyostelium* genome was known to have only a single gene encoding the heavy chain. I asked my friend Jim Spudich who was studying non-muscle actin-myosin complexes at Stanford if he had a myosin clone. He told me he had recently gotten a cDNA from Leslie Leinwand that appeared to encode myosin in *Dictyostelium*. However, he was convinced that knocking out myosin would kill the cells. I told him about driving *mhcA* antisense with the actin15 promoter which was much less active in growing cells at low cell densities. I thought it might only produce antisense RNA when the cells were about to enter development. He wished me luck and sent the cDNA clone.

Dave Knecht picked up the clone and rapidly generated an antisense transformation vector. He put the transformed cells in petri dishes containing medium with G418. After several weeks, colonies could be seen on the bottom of most plates. If he had incubated the cells in suspension, as we usually did, he never would have gotten the *mhcA* knock-downs because we soon learned that the *mhcA* antisense cells

can not grow in suspension; cell division is impaired and the cells all become highly multinucleated and die. On the surface of petri dishes they can attach and pull themselves apart to give rise to daughter cells. It seems that *Dictyostelium* cells absolutely need myosin heavy chain to divide in suspension but not when they are attached to a substratum. It also seems that antisense successfully knocked down myosin heavy chain.

When we told the results to Jim Spudich, he was very pleased and excited. He wanted to send a graduate student down to learn how to transform cells so that they could repeat the experiments at Stanford. Arturo De Lozanne promptly appeared and Dave Knecht took him under his wing. Dave and Arturo made variations in the original antisense plasmids, changed the length of the region that would be transcribed in the reverse direction as well as prepared plasmids that would be transcribed in the sense orientation to use as controls. They recovered a whole set of new transformants and characterized them for myosin accumulation using antibodies specific to myosin that had been prepared in the Spudich lab. After a few months, Arturo felt he had learned enough to solo, and returned to Stanford. A few weeks later, I got an excited phone call from Arturo telliing me that he thought one of the transformants had undergone homologous recombination in the myosin strucutural gene. His restriction digests were messy but it looked as if the structural gene was disrupted. The strain was able to grow on petri dishes but not in suspension and so was likely to be a *mhcA* mutant. Was it really a case of homologous recombination with the transformation vector? A few days later Arturo called back with clean proof of homologous recombination. This was such an important advance in the molecular genetics of *Dictyostelium* that we agreed to hold off on publishing our antisense results until we could publish together back to back with Arturo and Jim. It was not until May 1987 that our papers came out in *Science* but the wait was worth it. For one thing, it made it very clear that antisense could be accurately aimed at a single gene with no off-target effects since the phenotypes of the antisense cells and the homologous knockout were identical. I remember Tom Cech being particularly taken by this evidence. He was convinced that much genetic specificity was mediated by RNA.

Jim Spudich and I were well aware that we had the good fortune to be the first to have non-muscle cells that were unable to make myosin. We knew that the cells could not divide in suspension, but what else besides cytokinesis might be affected? We recruited Peter Van Haastert and David Soll to help characterize both the antisense and the knockout strains. We found that chemotaxis, signal relay, actin polymerization, and expression of cell type specific marker genes were all essentially normal. Although the cells were still motile, they moved at about half the speed of wild type cells. The fact that these cells moved at all meant that some other motor system must be at work. Subsequent work, mostly in the laboratory of Meg Titus, characterized a dozen genes encoding unconventional myosins and showed that some of these played essential roles in amoeboid motion. Mutational based studies in *Dictyostelium* took the lead in this field.

These were not the only matters that interested me. I had a long term interest in the structure and evolution of genomes. I had come to appreciate that new genes mostly resulted from divergence of duplicated genes. This required a lot of duplication and deletion to keep the genome stabilized. However, deletion is inherently more dangerous than duplication because having more than enough of a gene product is seldom detrimental while deleting a gene is potentially lethal. If the rate of duplication is about the same as the rate of deletion and the positions of the ends are random, then there has to be a high proportion of dispensible DNA to provide neutral end points for many of the deletions. I realized that this thinking could explain what people were calling the "C-Value Paradox" - the observation that the total amount of DNA in different species did not correlate with the complexity of the species and that many species had much more DNA than necessary. I thought I might be able to convince people to consider the role of vestigial DNA by treating the problem mathematically. I got in touch with my UCSD colleague Mike Gilpin who is a mathematician interested in evolution. He considered the problem and soon told me that he could not solve it analytically but could write a computer program that would iteratively simulate a genome over 30,000 generations. The final artifactual genome was about the same size as a mammalian chromosome. Many genes were present in multigene families, much like the actin and Ras genes in *Dictyostelium*, and about 50

to 90% of the sequence was dispensable. The proportion of dispensable DNA depended on the relative rates of duplication and deletion. The analysis predicted that the rate of deletion was higher in the smaller *Dictyostelium* genome. It will be interesting when that prediction can be tested.

I was also interested to understand the relationship of *Dictyostelium* to plants, animals and yeast. Although classical phylogenetic systematics had tried to relate them on the basis of cellular morphology, there were not enough quantifiable traits to be conclusive and some put *Dictyostelium* with the plants while others put them with the animals. Carl Woese had come up with a new approach by comparing the sequences of the small-subunit ribosomal RNA in a large number of organisms. Mitch Sogin sequenced the ssRNA from *Dictyostelium* and compared it to similar sequences of plants, animals, yeast, and protists. In 1983 Sogin and Woese published that *Dictyostelium* was one of the earliest branching organisms, apparently leaving the line leading to plants and animals long before other eukaryotes. From what I knew about shared characteristics of cell biology, I seriously doubted their conclusion.

After thinking about the technique of sequence comparison for a while, I realized that Sogin and Woese could have been misled about the relative time of divergence of *Dictyostelium* because there was a big difference in the GC content of the ribosomal RNAs. The *Dictyostelium* genome had been known for some time to have one of the lowest GC contents ever encountered. This bias had spread into the rRNA sequences where compensatory mutations could maintain the essential secondary structure of stem-loop modules. Comparing a low GC RNA sequence to a high GC sequence would show a huge divide that would be interpreted as early divergence. However, comparison of proteins in *Dictyostelium* and their homologs elsewhere should not be affected by difference in GC content even though codon usage might differ. I was curious what the phylogeny would look like if a half dozen or so proteins with homologs in the major phyla were compared.

One of my colleagues at UCSD, Doug Smith, had become good at protein sequence comparisons and offered to help in establishing the proper position of *Dictyostelium* among eukaryotes. He searched the available data bases for homologs of one or more of the small number of *Dictyostelium* genes that were available in

1988. He needed a cluster of homologs that included at least one animal, one plant, one yeast and a *Dictyostelium* gene. If there was also a clear homolog among bacterial genes, all to the good for rooting an evolutionary tree. He found actin, myosin (head region), calmodulin, Ras, and four enzymes of pyrimidine biosynthesis that fit these criteria. Trees built by two independent techniques, distance matrix and parsimony, were found to agree to all extents and purposes for each of these 8 clusters. Plants and yeast were found to diverge first, followed by *Dictyostelium*, *Drosophila*, nematodes and ending with mammals. We concluded that *Dictyostelium* diverged shortly after the split of plants and animals, and was certainly not an "early diverging " species. This was important if we were to convince people that biological properties in *Dictyostelium* were sometimes relevant to biomedical problems. The phylogenetic arguments have improved over the years as the data bases have expanded, culminating in whole genome comparisons in 2005. It is now well established that *Dictyostelium* shared a common ancestor with animals more recently than plants and that it carries orthologs of many animal genes that are missing in yeast.

I think it was in 1988 that Adam Kuspa came into my office and asked "Would you like me to map every gene in Dicty?". Ofcourse, I said yes. Then I asked how he thought this could be done. Adam was a graduate student in Dale Kaiser's lab at Stanford, but had been an undergraduate researcher in my lab 5 years earlier. We were good friends. Adam explained that he was building a library of large inserts carried in yeast artificial chromosomes (YACs). Each YAC could carry up to 400 kb of insert and be replicated in a yeast host. Adam was building a five fold redundant library with inserts from the bacterial species that he had been working on in the Kaiser lab, *Myxococccus xanthus*. He planned on aligning them with each other on the basis on shared hybridization using several hundred probes from the bacteria. This should result in full coverage of the 9.5 Mb *Myxococcus* genome. He was about to finish up his graduate work and was going to return to my lab as a postdoc. He proposed making a *Dictyostelium* YAC library while still at Stanford and then bring the library down with him in a few months. It was the start of a collaboration on genetics and genomics in *Dictyostelium* that has gone on now for more than 25 years.

Adam brought the YAC libraries with him when he returned in a few months. Unfortunately, they did not survive the trip down from Palo Alto to La Jolla. Adam set out to make a new library and quickly succeeded. I had offered him a bottle of good champagne if he could generate a YAC clone with a megabase insert, but he never succeeded. It seems that the high AT content of *Dictyostelium* DNA puts an upper limit on the size of YAC inserts at about 400 kb. The average insert in the YAC library was 200 kb. Our mapping set of 1,000 YACs provided more than 5 fold redundancy for the 34 Mb *Dictyostelium* genome. We approached this project in an industrial manner, buying programable large flat-bed CHEF electrophoresis machines where we could electrophoretically separate large DNA fragments from 360 independent clones at a time. Each of the blots was sequentially probed with up to 10 different cloned fragments. By the end of the project we had archived at least 100 kilos of large sheet photographic film of the autorads. YAC contigs started to form when we found probes that connected a half dozen different YACs.

Some of the first genes that we positioned on the YACs were ones that had been previously assigned to one or another of the 6 chromosomes on the basis of segregation patterns from heterozygous diploids. This established the chromosome identity of the YAC contigs. The YAC inserts were also characterized by construction of long range restriction maps using rare cutting restriction enzymes. The pulse-field CHEF machines were essential for these studies as well. Some of the chromosomal contigs grew to be several megabases long.

Adam was tireless in keeping the machine going. Not every probe worked, but enough did that after two years we had about a quarter of the genome covered with YACs. I don't know why we were such fanatics but the challenge was clear and we just kept going. We decided that it must be that we enjoyed mapping for its own sake. There is something rather rewarding about a straightforward problem where the answer is known to be a simple one-dimensional string of loci. We just had to find the right sequence of loci. At other times, we joked that whatever we did, when we published it, we could only be wrong. But it would help get funding for the ultimate mapping - sequencing the genome.

Meanwhile, Adam was exploring other techniques that he had picked up from *Myxococcius* that might work in *Dictyostelium*. He had used a transposon to randomly disrupt genes when it hoped about in the *Myxococcus* genome. Dale Kaiser and Adam found that they could pick mutant clones, isolate the genomes and clone the sequences flanking the insert sites by digesting with restriction enzymes that did not have sites within the transposon. Adam tried randomly inserting a plasmid that carried genes for pyrimidine biosynthesis by selecting transformants from a *pyr5-6* mutant strain for growth in the absence of added uracyl. He could get transformants that carried the plasmid but very few showed any phenotypic abnormality. However, he picked a few of the apparent mutants and characterized their insert sites. The insertion site in one of them appeared to be a perfect EcoR1 site. That was curious because the plasmid had been opened with EcoR1 to generate linear DNA. I suggested that he might try leaving the restriction enzyme there next time and see if it increased the frequency of integration in EcoR1 sites. He thought this was not a particularly good idea because there was no evidence that enzymes entered cells during electroporation and, even if they did, EcoR1 might easily kill the host cell by cutting up its genome into small 400 bp fragments.

A day or two later I was browsing through PNAS and ran into a paper by Schiestl and Petes describing "illegitimate recombination" in yeast when they introduced the restriction enzyme BamH1 along with a selectable plasmid. More than half the insertions were at BamH1 sites. I gleefully brought the paper to Adam. Within the next few days he repeated the experiments with *Dictyostelium* and had immediate success: the frequency of recovery of transformants was increased more than 20 fold, about two thirds of the insertions were in sites recognized by the specific restriction enzyme that had been used, and 1 in 400 of the transformants showed noticably variant morphology. The plasmid was isolated carrying flanking regions from the mutant strain and used to recapitulate the mutant phenotype by homologous recombination. We named this technique for tagging genes REMI, which was an acronym for Restriction Enzyme Mediated Integration. Then we opened a bottle of Remy Martin brandy in celebration. For the last 25 years REMI has significantly

facilitated the study of *Dictyostelium* genes and has also been useful for the isolation of mutated genes in filamentous fungi and certain plant pathogenic fungi.

By 1992 it was clear that we could tile the 34 Mb genome with 200 kb inserts and generate as much detail as needed within selected YACs. However, it was going to take several hundred more probes to generate all the contigs and weed out the errors. One day, as we sat in the garden of a local pizza place, Adam and I faced up to the problem that finishing the map the way we were going involved more work than either of us really wanted to do. We recognized that what we needed was mapping at a scale intermediate between the whole chromosome resolution of parasexual segregation and the ordered YAC inserts. It suddenly occurred to us that the plasmid inserts resulting from REMI could be used to subdivide the chromosomes if the plasmid carried sites for restriction enzymes that rarely cut the genome. In a form of isogenic RFLP, we could determine when a gene probe recognized a smaller fragment in one of the the rare restriction site REMI strains than in the others by comparing the large fragments separated by pulsed-field electrophoresis on our CHEF apparati. It did not take too long to select 147 independently transformed strains with single plasmid insertions, digest them with ApaI, SmaI, SstI, or NotI and probe the sized fragments with labelled portions from 100 genes. In 1994 we published REMI-RFLP maps covering 22 Mb. We had more data that had not yet been analyzed but Adam had to leave to take up his position as an Assistant Professor in the Department of Biochemistry at Baylor College of Medicine in Houston where he has stayed while rising through the ranks. He is now Senior Vice President and Dean of Research at Baylor, but still talks to me.

Meanwhile, back in La Jolla I collected all the data on my dining room table and tried to finish the mapping puzzle. I had recruited Dennis Welker to map a half dozen orphan YAC contigs to one or another chromosome using strains of *Dictyostelium* independently isolated from nature. All of the other YAC contigs mapped to one of 6 chromosomes but there was some evidence in the literature for another chromosome. Convincing the field that there are only 6 chromosomes would be easier if Welker were part of the team because he had previously supported the 7 chromosome orthodoxy. Welker parasexually mapped markers from the orphan contigs and

positioned each of them on one of the 6 previously mapped chromosomes. There was no evidence for a 7th.

For several months I wrestled with the long-range restriction maps, REMI-RFLP maps, YAC contigs and chromosomal assignments. People have asked me what program I used to generate the final maps. I have had to say that it was all done in my head because the major differences in the types of information that had to be integrated were completely incompatible with any known program. Nevertheless, I made paper strips with the physical parameters of maps marked proportionally along the length that could be slid along other maps to try to align the markers. A lot of attempts ended in the waste basket. Finally, with the telomeres and centromeres delineated, I reached consensus maps for each of the 6 chromosomes with no glaring internal inconsistances. We published the integrated maps in 1995 with the admission that there were likely to be unknown errors in the order and spacing of genes. We could see how to check the maps and remove the errors, but it would entail almost as much work as generating the maps in the first place. We decided that the sensible thing would be to sequence the whole genome and find discrepancies that way. Moreover, it was becoming very clear that the genome sequence was so useful that *Dictyostelium* would not remain an organism of choice for cell and developmental biology if we did not sequence it within the next 10 years. Rather than doing any further mapping, Adam and I set out on a campaign to get the funding and expertise to sequence the 34 Mb genome.

In 1989 I was invited to speak in a Max Planck course on model systems in developmental biology. The meeting was designed to showcase organisms where modern techniques could be applied to problems of differentiation and embryogenesis. Graduate students from all over Europe were invited to attend so that they could make informed decisions for their future. The meeting was held in the Dahlem district of Berlin in the summer before the fall of the Berlin wall. It was a time of rising hopes and good fellowship.

Leading scientists were asked to present the strengths and weaknesses of their chosen experimental organism. Among others, Yanni Nusslein-Volhard defended *Drosophila*, Amar Klar championed fission yeast, David Cove spoke of the

excitement in mosses, Elliot Meyerowitz described how *Arabidopsis* makes flowers, and I made a pitch for *Dictyostelium*. One of the students in the course was an Israeli from the Weizmann Institute, Gadi Shaulsky. He was already a serious, driven scientist who trusted his judgment and ability to do important work. He was also charismatic and brilliant. I was immediately taken by him. He told me he was looking for an experimental organism where he could test his hypothesis that everything was determined by competition for survival. From his point of view, embryogenis was a matter of germ cells dominating somatic cells; or in *Dictyostelium*, a matter of prespore cells dominating prestalk cells. I agreed to some extent. By the end of the course he told me his choice had narrowed down to *Dictyostelium* with me or fission yeast with Amar Klar. I encouraged him to come to La Jolla.

After the course finished, I traveled to Rome to meet my daughter Kate who had been backpacking around Europe. We visited the monuments and trattorias of Rome before going to Florence for 4 days. Then on to Mill Hill, England where I gave a seminar at the ICRF and caught up on the latest advances being made in Jeff Williams' lab. Jeff and Keith Jermyn had recently used the regulatory regions of prestalk specific genes to drive bacterial β-galactosidase. The staining patterns clearly marked several types of prestalk cells that had never been distinguished before in slugs. Staining of earlier stages showed that prestalk cells differentiated in random spatial patterns and then sorted out. These were major steps in understanding development in *Dictyostelium*. We finished each day in the local pub where Kate met us and told us of her adventures.

Gadi wrote me the following year that he would like to come to La Jolla in 1991 after he had finished his graduate work with Varda Rotter on mammalian p53. He told me that one of the advantages of *Dictyostelium* is that it did not make p53 which he had come to loath. p53 did too many different things and the people in the field all had different ideas and very little tolerance for each other.

When Gadi arrived in La Jolla, I encouraged him to get used to the organism and the ways we treated it. He repeated many classical experiments to convince himself of the facts with his own eyes. He was particularily taken by one set of experiments: the old Raper microsurgical manipulations of slug tips and proportions of cell types that

had suggested that the tip might act as an organizer region and that lateral inhibition of prespore differentiation in uncommitted cells might establish the proportions. We tried to come up with a genetic selection that would allow us to isolate REMI mutants in which the cell type proportions were abnormal or in which tip dominance was compromised. Gadi suggested that expressing ricin A from cell type specific regulatory regions might kill the cell type from within. Since ricin A cannot enter cells without its heterodimer partner ricin B, there was no danger that release of the ricin from dying cells would kill adjacent cells or endanger us. Gadi made the vectors and tested them. Unfortunately, we never found conditions that gave sufficient selection such that we could get mutant strains. However, the strains expressing either the prestalk or the prespore specific vectors had very interesting phenotypes. Prespore specific expression of ricin A killed not only all the prespore cells but also the prestalk cells because they converted to prespores as the original population died. On the other hand, prestalk specific expression of ricin A resulted in the death of prestalk cells but did not affect the prespore cells. The results suggest that prespore cells compete with prestalk cells by producing an inhibitor of prespore differentiation to which they are insensitive.

Over the next few years Gadi teamed up with Adam in analyzing the first set of REMI mutants isolated in the laboratory. We collaborated with Joe Dynes and Rick Firtel in studying the effects of loss of the late adhesion protein LagC (subsequently renamed TgrC) and found that it was also part of an intercellular signaling pathway. We collaborated with Robert Insall and Peter Devreotes on the gene encoding the cytoplasmic regulator of adenylyl cyclase CRAC. The following year we collaborated with Jeff Segall on studying the consequences of loss of the MAP kinase ErkB which we later found to be a major player in the oscillatory circuit of *Dictyostelium*. Adam and Gadi also studied a prestalk specific transporter which is fused to a protease, TagB. Many years later Christopher Anjard was able to show the protease was specific for the precursor of the culmination signal SDF-1. Each one of these genes presented a set of challenges that required new ideas and new approaches. Gadi continued to add to the list of well characterized developmental genes for many years even after he left to establish his own lab in the Department of Molecular and Human

Genetics at Baylor College of Medicine in Houston. For me, this was the best of times as I looked forward to new results every day, along with stimulating discussions and general camaraderie.

During this period Kathy Fosnaugh was one of the outstanding postdocs in the lab. She had recently gotten her PhD. with Peter Greenberg at Cornell studying chemotaxis in spirochaeta bacteria and had become proficient in cloning and sequencing. She wanted to understand how cell type specific genes were transcriptionally regulated in a eukaryote. She picked up the spore coat protein story where Kevin Devine and Barbara Dowds had left it and generated all sorts of new clones. She finished sequencing the *cotA*, *cotB* and *cotC* genes and added about 1 kb to each of their upstream regions. She then subcloned different pieces of the regulatory regions and inserted them into expression vectors where they could drive β-galactosidase. Transformants were developed and the specific activity of β-galactosidase was measured every 4 hours throughout development. It became clear that there were independent *cis*-acting sites in the regulatory regions that worked additively without affecting the time of transcriptional initiation at 8 hours of development. She also found that barely functional regulatory fragments retained their cell type specificity; β-galactosidase activity was only found in prespore cells. Within each of these regions we could see a sequence closely related to CACCCAC. Kathy's results also clearly demonstrated that transcription as well as translation of the spore coat genes was coordinate under a variety of conditions, suggesting that these genes shared regulatory factors. One of her most interesting discoveries was that the cAMP dependent protein kinase PKA had to phosphorylate the transcription factor GBF to induce expression of the spore coat genes. These experiments were carried out in collaboration with Jeff Williams who had seen hints that this protein kinase was involved.

In 1993 Kathy accepted a plush position at Ligand Pharmaceuticals here in La Jolla. She has stayed in the private sector, applying her skills in genetic manipulation and high throughput screening while learning the business end of things. She is presently Director of Youtell Biochemicals and Associate Director of Marina Biotech

in Bothell, Washington. They target orphan diseases with nucleic acid based drugs. She has clearly gone a long way.

1995 - 2005

By 1995 molecular genetics in *Dictyostelium* had matured to the point where just about any genotype could be rapidly generated. We could knock out or modify specific genes at will. We could determine the subcellular localization of any protein of interest by fusing it to Green Fluorescent Protein. We could modify the stage of expression of a gene by driving it with an exogenous regulatory region. We could select for novel mutants from a REMI mutagenized population and have the genes cloned within a month. At last *Dictyostelium* was a fully accredited genetic model system. All that we needed was the sequence of the genome.

Craig Venter had successsfully sequenced the 1.8 Mb genome of the bacterium *Haemophilus influenza* in 1995 and the 140 Mb genome of the fly *Drosophila melanogaster* in 2000. The sequencing technology using automated ABI capillary sequencers and lots of computer power was well established. The 34 Mb genome of *Dictyostelium* with its detailed physical map looked quite feasible. But we needed a sequencing center and funding. Adam and I considered many alternatives and reached out to our international colleagues to form a partnership. The 1995 annual *Dictyostelium* meeting was organized by Michel Veron and Michel Satre in Dourdan just outside of Paris. At that meeting our friend Mineko Maeda told us that there was a rumor that Ikuo Takeuchi might have connections in the Japanese government that could help finance sequencing efforts. I clearly remember joining Takeuchi after lunch one day in Dourdan and suggesting to him that we collaborate on sequencing the genome. He was very interested but, like any responsible administrator, made no promises. After further discussions that fall in Japan, they decided to initiate the Japanese cDNA Project in which they would sequence up to 10,000 cDNA clones to establish the sequence of the expressed portion of the genome. We were excited to hear that this resource would be built, but the whole genome still had to be sequenced.

I had breakfast with Craig Venter at the La Jolla Beach and Tennis Club a few months later. He was staying there while giving a seminar at UCSD. Craig thought of himself as a successful chieftan and only reluctantly granted me a audience. However, we found we shared an irreverent sense of humor as well as a thirst for science that could lead to sequencing *Dictyostelium*. Craig was a little disappointed when I told him we had mapped the genome to high resolution. He said he would have enjoyed sequencing the whole thing blind just to show that it was possible without a map. In any case, he said that if I could put 3

million dollars on the barrel, he would sequence the genome within three years. When breakfast was over, we agreed that both of us would prepare grant applications and hope that one or both were funded.

As part of the effort to convince NIH to fund our sequencing efforts Doug Smith and I built a web site, DictyDB, where all the mapping and sequence data available in 1997 was openly available and searchable. Sequences from all of our REMI strains as well as cDNA clones were included. As genome sequences started to accumulate, open reading frames were inspected and translated to generate a protein sequence database for those who were using mass spectroscopy to analyze *Dictyostelium* proteins. Doug kept it up to date until the genome was completed in 2005 when Rex Chisholm took over with his team at dictyBase.

Our first efforts to secure an NIH grant were not successful. At about the same time we found out that Craig had not made any progress towards funding for *Dictyostelium*. Adam suggested that rather than sequencing with Craig Venter's Institute for Genomic Research (TIGR) we should use Richard Gibbs' sequencing center at Baylor College of Medicine. I flew down to meet with Gibbs and Adam so we could work out the details. In 1997 Gibbs was still in need of business, having set up the Sequencing Center at Baylor only the year before. He readily took on *Dictyostelium*. Adam and I wrote the application and agreed that Adam should be the PI because he was there at Baylor with Gibbs. I agreed not to request partial salary for myself so that all the funds could go to the sequencing. In hindsight, this was a mistake because some of our collaborators thought that I was not intimately involved.

The grant was approved and sequencing started the following year. It took longer than expected to complete the sequence of the genome, partly because it turned out to be impossible to clone large DNA fragments of the Dictyostelium genome in bacterial artificial clones (BACs), and partly because Gibbs had been so successful in getting other business that *Dictyostelium* reads were only scheduled when convenient. Luckily, we had been encouraging Angelika Noegel to lead an effort to get funding for collaborative work in Germany. She modified our application and submitted it to the Deutsche Forschungsgemeinschaft (DFG). They agreed to support sequencing of chromosomes 1, 2 and 3, and left responsibility for chromosomes 4, 5, and 6 to the NIH supported efforts at Baylor. The European Union and the MRC also provided support for bioinformatics and physical mapping at the Sanger Center in England. At last, we had an international

consortium that could do the job. Slowly over 5 years the sequences came in and were assembled into large contigs. The range of proteins available to *Dictyostelium* cells was analyzed by different groups all over the world and collected into a composite manuscript which was finally published in Nature in early 2005. It had been a decade in the making but was well worth it.

During all this time Gadi encouraged us to continue with the mapping and sequencing but did not want to waste his time actually being involved. He could rely on us to give him the sequence when we finished. Meanwhile, he wanted to explore the underpinnings of multicellularity in *Dictyostelium*. The idea was not just to accumulate important developmental genes, but to weave them into pathways and circuits that regulate morphogenesis. Almost every day at lunch, Adam and Gadi and I would dream up new ways that the cells might coordinate development. We would then try to come up with ways to test the models.

Gadi and Adam had shown that strains lacking either of the transporter proteases, TagB or TagC, were blocked at the mound stage and made almost no spores. Therefore, rare survivors that sporulated could be selected and the suppresssor mutation characterized. No matter what the suppressor gene turned out to be, it would help define the TagB and TagC pathways which were complete mysteries at that time. A single strong suppressor was isolated from a saturation mutagenesis and shown to result from inactivation of a previously unknown cytoplasmic cAMP phosphodiesterase, RegA. The extracellular phosphodiesterase, PdsA, had been known for years to play an essential role in cAMP signaling between cells, but no one had worried about controling cAMP within cells. Gadi's suppressor screen had uncovered a major player that probably would not have been found otherwise for many years, even by the best minds. Further inspection of RegA also established it as a member of a two-component system in that it carries a response regulator region near its N-trerminus. These regions accept phosphate that is relayed from a histidine kinase via a small H2 intermediate. It seemed likely that the phosphodiesterase activity was regulated by phosphorylation of the response regulator region. Since *tagB*$^-$ and *tagC*$^-$ null mutants sporulated almost as well as wild type cells if they also carried the *regA*- mutation, we proposed that internal cAMP bypassed the need for the transporter proteases possibly by activating PKA.

Different genes were being studied in the lab by different people. We had given a REMI mutant that appeared to have suffered an insertion in a gene encoding a histidine kinase to a promising graduate student, Nancy Wang. All we knew was that this gene, *dhkA*, was essential for sporulation and normal stalk formation and probably encoded a membrane embedded receptor. The amino acid sequence of DhkA suggested that it was able to autophosphorylate, adding a phosphate to a histidine moiety, and then transfering this phosphate to an aspartate further down its sequence. Nancy was having trouble with the biochemical techniques that could show this phosphorelay and was beginning to lose self-confidence. Gadi started helping her and soon the experiments began to work. As we hoped, DhkA was a true histidine kinase. We had no idea what its partner in a two component pathway might be, but we had a candidate with a response regulator region, RegA. We started thinking that DhkA might be keeping internal cAMP and PKA low by stimulating RegA. In support of this idea, Nancy and Gadi were able to rescue sporulation in *dhkA*$^-$ null cells by addition of cell permeant cAMP. Somewhat later, we were able to show that sporulation could be restored to *dhkA*$^-$ null cells by expressing constitutively active PKA. It was beginning to look as if we had been lucky.

If we could find the intercellular signal that was recognized by DhkA, we could flesh out the pathway. For several years I had been talking to Michel Veron and his graduate student, Christophe Anjard, about small molecule signals generated by prestalk cells that induced sporulation in cells that were partially constitutive for PKA. We decided to test the putative pathway with these factors. Although loss of DhkA had no effect on the ability of the phosphopeptide SDF-1 to induce sporulation, it completely blocked the ability of the peptide SDF-2 to induce sporulation. Moreover, production of SDF-2 was completely dependent on the presence of TagC. We were convinced that we were on the path of a specific pathway of developmental signaling. I invited Christophe to come to La Jolla to characterize SDF-2 and uncover other developmental pathways.

Ofcourse, I checked with Michel Veron before making the offer to Christophe. I also checked with Christophe Reymond and Wolfgang Nellen who had also had Christophe in their labs. They all told me that Christophe was brilliant but different and sometimes hard to understand. I suggested it might just be a problem with speaking a foreign language. They said he was difficult to understand in any language, including French, because what was

obvious to him might not be to others. Christophe ended up spending 12 years in my lab and we got to know each other very well. I agreed with the assessment of all his previous mentors. However, he also had the ability to remember detailed results from experiments he had carried out many years earlier. This strength turned out to be critical for our eventual success in figuring out many different developmental pathways.

Nancy Wang, Christophe and Gadi carried out high resolution site-directed analyses of DhkA and showed that SDF-2 bound to an extracellular domain flanked by transmembrane regions and established exactly which histidine and aspartate were phosphorylated. When SDF-2 bound to DhkA, the protein kinase became a protein phosphatase, removing phosphate from RegA and leaving it much less active. Christophe tried hard to purify sufficient SDF-2 for chemical determination but never succeeded. Finally, after 5 years, a genetic hint showed that SDF-2 was a peptide cut out of the well conserved metabolic protein AcbA. Christophe spent the next 5 years working out the surprisingly complex set of interacting pathways involving steroids, GABA and glutamate that fine-tuned the temporal control of sporulation.

Rick Firtel and Peter Devreotes were making rapid progress in understanding how gradients of exogenous cAMP direct movement of the cells into aggregates. Work in their laboratories had defined a set of G protein coupled receptors (GPCRs) that bound cAMP with different affinities and transduced the signal through PI3kinase, PTEN and TORC2 to the regulation of F-actin polymerization. They were able to account for the subcellular localization of the signal in a satisfactory manner but had not accounted for the observed 6 minute periodicity in the relay of the cAMP signal. I started thinking about possible circuits that could generate an oscillatory signal and focused on the feedback of PKA activity on the inhibition of RegA by Erk2. It had recently been shown that activation of the cAMP receptor CAR1 by ligand binding stimulated Erk2 activity. Ligand binding to CAR1 was also known to activate the adenylyl cyclase ACA to make cAMP more rapidly. I reasoned that inhibition of RegA would lead to the rapid accumulation of cAMP which would activate PKA activity. Feedback loops from PKA to RegA inhibition and ACA activation could then reduce the cAMP level and PKA activity would fall back to the unstimulated level. I was sure this circuit could account for the oscillations in cAMP. I tried to get some of my friends who were mathematicians and theoretical physicists involved, but they were not interested. An

exceptional undergraduate, Mike Laub, offered to help if I would teach him how to do experiments in the lab. Mike was at ease with MatLab programs and understood the power of numerical solutions. I wrote out seven differential equations describing the temporal changes in the activity of five proteins and the consequences to internal and external cAMP concentrations. After we had set the parameters, we found that the circuit generated pulses in external cAMP with a 7 minute periodicity. One of the reasons that the circuit worked so well is that the kinetic terms describing the inhibition of the regulated proteins, CAR1, ACA, Erk2, RegA and PKA, were all first order in the activity being considered. Activation terms were all zero order. Mike and I were convinced that we had uncovered the basic oscillatory machine. Mike then went to the bench and became an exceptionally talented experimentalist. He initially collaborated with a graduate student in the lab, Justin Stege, in studies on a set of REMI mutants that generated multiple tips on mounds rather than the usual single tip. Ricardo Escalente, a previous postdoc in the lab, later found that the "multitip" genes affect the ubiquitin pathway of protein modification.

In 2002 the annual Dicty meeting was held in Palermo, Sicily. I remember being excited when Mineko Maeda showed me a Western blot stained with antibodies to activated Erk2 that showed spontaneous oscillations in phase with oscillations in cAMP. Further discussions with Adam and Gadi uncovered that we had amassed a diverse set of experimental results in different labs that all had bearing on the Laub-Loomis oscillatory circuit. We decided to pool our results and write up a paper on the experimental support of the architecture and the parameters. It was published in Science in early 2004 and still holds up.

Thinking about PKA, I started to wonder what role it might play in motility of the cells during chemotaxis. My friend and colleague David Soll was the expert in computer assisted analysis of cell motility and he offered to analyze strains with constitutive PKA activity, resulting either from the loss of the phosphodiesterase RegA or with null mutations in the gene encoding the regulatory subunit of PKA. I sent the appropriate strains to Deb Wessels in his lab and waited patiently to hear if motility was affected or not. They used a series of tests for response to temporal waves, spatial waves, and natural waves. Single cells were imaged and computer analyzed for roundness, length, width, directional change, velocity and chemotactic index. Both the *regA*$^{-}$ null cells and the *pkaR*$^{-}$ cells were able to determine the direction of spatial gradients and could distinguish rising and falling concentrations of

cAMP. However, neither strain was able to suppress lateral pseudopods during the response to an increasing temporal gradient and, as a consequence, showed very poor chemotaxis in a natural wave. When PKA activity stays constitutively high, filamentous myosin does not localize to the cortex as it normally does. The oscillations appear to be required for proper localization.

David Soll tended to exaggerate many things but was always very thorough in his experiments. I enjoyed our boisterous interactions and suggested that we look at mutants lacking the adenylyl cylase, ACA, where PKA activity would be very low. He agreed and to our surprise found that these cells behaved in a manner that was almost identical to the *regA*$^-$ null cells and the *pkaR*$^-$ cells. They also failed to suppress lateral pseudopods in the front of a wave and so staggered around. Yet unlike the *regA*$^-$ null cells and the *pkaR*$^-$ cells, the *acaA*$^-$ cells had low internal cAMP. It seems that good chemotaxis depends on the Laub-Loomis circuit generating oscillations in cAMP and PKA activity.

Since 1990 I had been discussing chemotaxis and development of *Dictyostelium* with Herbie Levine, a theoretical physicist in the Physics Department of UCSD interrested in nonequilibrium dynamics. I have learned a lot from him. At one point Herbie tried doing experiments in my lab but soon found that he was not cut out for time consuming minutiae or attention to boring details. Meanwhile, he successfully modeled spatial patterns and brought further insight into the nature of spiral waves of aggregating cells. At some point we realized that if he were to make further significant advances we needed to know how the cells moved and sorted out. In 1997 Herbie, Jose Onuchic, Terry Hwa and I put together an application to NSF which had requested proposals for studying Complex Systems. We proposed studying *Dictyostelium* at many levels, both theoretical and experimental. Somewhat to my surprise, it was funded and for five years we had unexpected freedom.

One of the projects we had described aimed to understand how prespore and prestalk cells sort out. I had been using a strain that I got from Peter Devreotes which had the fluorescent protein GFP fused to the cAMP receptor CAR1. Initially I had been interested in the possibility that the receptor might be localized at the front of chemotaxing cells, but soon confirmed that it was uniformly distributed on the surface. This distribution might not explain the dramatic amplification of the chemotactic signal, but it could be used to delineate cells as they sorted out. It was possible to identify individual cells even in close packed

aggregates because they were outlined in bright fluorescence. We thought that time lapse movies of cells expressing cell-type specific markers would allow us to follow prespore and prestalk cells as they sorted out. All we needed was a good microscopist who was familiar with development in *Dictyostelium*. Luckily, Alistair Nicol answered our advertisement and was able to join us. He had previously been trained by David Garrod while studying cell sorting among four species related to *Dictyostelium*. He was up and running within a few weeks of arriving from England.

Almost from the start we realized that we would have to keep aggregates as thin layers to be able to image individual cells with sufficient resolution. Alistair put agar overalys on the cells and found that they could still aggregate and then move in large circles if he raised the agar slightly on little posts. The circling movement in aggregates and slugs had been observed years before by Kees Weijer who thought it resulted from chemotaxis to scroll waves of cAMP. However, Alistair found that mutant cells lacking ACA but expressing PKA constitutively would circle around each other in the monolayers even though they do not secrete any measurable cAMP. It looked as if the cells liked to move but also wanted to stick to neighboring cells. When Alistair waited a few more hours, he could observe prestalk cells sorting out from surrounding prespore cells. Once prestalk cells had sorted out into clumps, they often circled in the opposite direction from the surrounding prespore cells and would then suddenly take off for the edge where they would act like the tip of a slug. Sorting out certainly looked as if it involved chemotaxis but it probably was not chemotaxis to cAMP. We still have no idea what other molecules might be chemotactic in *Dictyostelium*.

Danny Fuller has been the secret to the smooth running of the lab for the last 30 years. He joined the lab as a technician in 1984 and soon learned most of the techniques in the lab. He could use genetics, biochemistry, microscopy, you-name-it, to study development of *Dictyostelium*. When new people joined the lab, it was usually Danny who trained them in the basics. Over the years, he went up the ranks until he was appointed as Specialist in 2002. It is a curious title for a master jack-of-all-trades. While I no longer know how to find most things in the lab, Danny does. He keeps us going.

In 1995 we hired Negin Iranfar to help us in sequencing the large number of REMI mutants that were being isolated in the lab. She had just graduated from UCSD and was a young and energetic Persian immigrant. She rapidly learned to be a master sequencer. When

funding from the NSF grant on Complex System started in 1998, I asked Negin if she would like to take responsibility for carrying out microarray studies on transcriptional patterns in wild type and mutant strains. The NSF application included a project to understand the complexity of transcriptional regulation by analyzing RNA isolated at stages throughout development by hybridization to immobilized cDNAs. After leaving my lab, Mike Laub had gone to graduate school at Stanford where he worked in the laboratory of Lucy Shapiro on *Caulobacter* differentiation. As part of his thesis, he arrayed several thousand *Caulobacter* genes on microscope slides and quantitated the level of hybridization from different samples of RNA. We had stayed in close contact so that I knew what he was up to and could appreciate his enthusiasm for microarray assisted RNA profiling. It was Mike who had convinced me to include such analyses in the NSF grant.

At Stanford, Mike had the benefit of advise on microarrays from the pioneers of the technique, Ron Davis, Pat Brown, Michael Eisen and Dave Botstein, who were all in the same Department. They helped him chose which robotics to use, how to hybridize and how to analyze the readout. He offered to help me over the hurdles of setting up such a system for *Dictyostelium* and to act as a sounding board for the results. We used a GenIII Molecular Dynamics robot to print PCR amplified portions of 330 genes and 690 cDNAs from the Japanese cDNA Project. Fluorescently labelled probes prepared at 2 hour intervals during development were hybridized to the slides which were scanned and analyzed using ScanAlyze. Roman Sasik and Terry Hwa acted as our statisticians, although they actually did much more. For instance, Roman and Terry invented a new way of clustering our data which was based on percolation theory. They also implemented a prior-knowledge based approach to the kinetic data that inferred the time of initiation of transcription for each of the genes that increase at some stage of development.

We not only analyzed developmental time courses but also differences between prespore and prestalk cells. The cell types were separated in bulk by density gradient centrifugation to allow us to make mRNA for the microarrays. All the previously known markers were recovered as well as many new markers. Most of the new cell type specific genes were confirmed using Northern blots to check the microarrays. Suddenly, we had a lot of excellent new molecular markers.

Over the next 5 years Negin organized and carried out microarray studies that defined the cAMP independent and cAMP- pulse dependent genes of early development as well as a feedforward loop that controls post-aggregation genes. She also determined the consequences of loss of a MADS box transcription factor in collaboration with Ricardo Escalante and the consequences of loss of the DNA binding factor CbfA in collaboration with Thomas Winkler. There was also a microarray study of the transcriptional profile in a strain lacking RasC with Deb Wessels and David Soll and one in a strain lacking a suppressor of the MAP kinase MEK with Rick Firtel and members of his lab.

Some of the most exciting results concerning transcriptional patterns came from an ambitious collaboration with Mineko Maeda. We were working with Mineko on the ERK 2/ PKA oscillatory circuit at that time and she often visited us in La Jolla. On one trip, she asked to see all the raw microarray data on cell type specificity with the idea of choosing several hundred of the best candidate genes for detailed *in situ* hybridization. The idea was to look for any changes in the spatial pattern of accumulation during development. When Ricardo Escalante was in the lab, he had optimized conditions for whole-mount *in situ* staining with cDNAs. Mineko proposed using his technique for each of the cell type specific genes. Negin downloaded the data for her and we wished her good hunting.

Over the following year, together with her Japanese colleagues, Mineko collected photographs of slugs and culminants stained with hundreds of different developmental genes. When I visited her lab at Osaka University in 2002, she dumped whole boxes of photos on the table in front of me. Many of the pictures were beautiful and showed the spatial pattern with high resolution. But there were too many genes and too much data to recognize general patterns. I remember getting up and going outside for air several times as we struggled with the results. After a few hours, certain recurring patterns became clear to me and I started clustering the genes on the basis of their cell type specificity at different stages. First, we separated the prespore and prestalk genes. There were only a few genes that started out in one cell type and then switched to the other. Most prespore genes gave simple patterns localized to the posterior cells. However, prestalk genes showed a wide range of different temporal and spatial patterns. Mineko and I spent the next day distributing the prestalk genes into 13 groups and choosing the pictures to illustrate the patterns we were seeing. It was

beginning to make sense, but it was also clear that *Dictyostelium* was not as simple a developmental system as it appeared.

The microarray work tapered off after that. We were running out of money and Negin was getting tired. It was a lot of work to amplify all the probes and we were using 6,345 cDNA and genomic targets by the end of the project. The robot was often eccentric and needed continuous care. We had gotten answers to most of the questions we had put to the microarrays. Although we focused on the 50 best developmental genes, we had to consider what happened to the other 6,000. In most of our studies, microarray data was used for high resolution, reliable, quantitative evidence for transcriptional progression through the developmental stages. It was somewhat like carrying out a large number of Northerns on well considered marker genes. We had highly reproducible results from our chosen set of highly expressed genes, but much more variable results from genes that were more weakly expressed. Gadi was carrying out very similar microarray studies at Baylor but had decided to consider the totality of the results rather than just the results from the best genes. Working with bioinformaticians from Slovenia, Gadi used statistical approaches to pattern recognition to establish developmental pathways. I was never satisfied by his explanation that the data was innately noisy and had to be treated as an ensemble. We talked about it for years but never agreed. Luckily, in 2008 RNA-seq came along and completely replaced microarrays for global transcriptional analyses. Gadi and I were thrilled to see the enormous dynamic range and reproducibility of RNA-seq data. We collaborated on repeating the time courses and cell type specificity measurements with the highest possible resolution. Every gene in dictyBase now has its quantitative mRNA profile posted for a wide range of growth and development conditions.

2005 - 2015

It is a bit strange to write a memoir of events in the recent past; it is more like a log or a diary. Most of the players are still on the stage and will have memories different from mine. But there might be people or ideas that were important to me that others knew little about. I will try to concentrate on them. I am not writing a history; I am remembering times past.

One of my last graduate students was not even a biologist. Rolf Olsen was getting his PhD. in the Department of Physics with my colleague Terry Hwa. The trouble was Rolf was driving Terry nuts with his convoluted answers to simple questions and simple answers to complicated questions. Rolf had been a physics major in college but had then quit academics and survived by driving a taxi. He liked to dream up big solutions and grand theories, but they did not pay the bills. After a while, he decided he really needed a PhD. and so enrolled at UCSD. He was an excellent mathematician and thought that statistical mechanics was great, but the good problems were in biology. He became interested in how amino acid sequences evolved in proteins of diverse organisms. This led logically to phylogenetic comparisons of homologous proteins and finally to a whole new way to measure the degree of similarity among proteins derived from a common ancestral protein. I was very interested in using proteins recognized in whole genome sequences to establish the phylogenetic relationship of *Dictyostelium* to other organisms. I encouraged Rolf to pursue his ideas and found myself getting deeper into his new approaches. Finally, he decided to move from Terry Hwa's lab to mine and I welcomed him.

Rolf continued to develop his multimatrix model of protein divergence and rigorously determined clusters of orthologs from organisms that last shared a common ancestor over a billion years ago. A cluster of 7 archaebacteria was used as the outgroup to root a tree of all eukaryotes. Twenty-three eukaryotes representing plants, animals, fungi, amoebozoa and early diverging species with well sequenced genomes were analyzed using several thousand clusters of orthologs. The final tree had highly significant nodes and reproducible branch lengths that have not been challenged or improved ever since. His trees grace both the Nature paper presenting the *Dictyostelium* genome and the PLoS Computational paper comparing the *Dictyostelium* genome to that of another Amoebozoa, *Entamoeba histolytica*. After the papers were finished, Rolf left to start his own company to develop

new ways to store wind and solar energy. It is an ambitious project that I hope works before global warming gets too bad.

A few years later, Adam arranged to have the genome of the related Dictyostelid, *Dictyostelium purpureum*, sequenced. *D. purpureum* cells develop in a very similar manner to *D. discoideum* cells and we expected them to be closely related. However, comparison of clusters of protein orthologs showed that the two amoeba diverged as long ago as fish did from the line leading to humans ie. about 400 million years ago. Yet both amoebae develop from aggregates, sort out their cell types, and form fruiting bodies that are almost identical to each other except that those made from *D. purpureum* cells are purple. We wondered whether their transcriptional programs might also be conserved. Gadi agreed to use RNA-seq to get the answer.

mRNA was prepared at 4 hour intervals throughout development from both species as well as from prespore and prestalk cells separated from the late slug stage. Millions of reads were made of cDNA copies of the RNA and mapped onto the genes. The number of reads for each gene was a measure of its level of expression. Transcriptional regulation of orthologs was found to be highly conserved with respect to time of development, level of abundance and cell-type specificity. It seemed that evolutionary constraints on progress through the stages limited both drift and variation in transcriptional control. The genetic networks controlling these genes must be robust and flexible to have lasted this long. These results took Adam, Gadi and me into new intellectual territory. We had lots of fun trying to reach consensus in evolutionary conjecture but realized that some of the questions could not be answered with present techniques.

One of the most significant outcomes from this collaboration was the posting of the transcriptional profile of each gene at dictyBase where anyone can see it. When it is necessary to chose a gene for further study from a set of similar genes, the decision can often be guided by choosing the *D. discoideum* gene with the *D. purpureum* ortholog that shows the most similar transcriptional pattern. Anything conserved over 400 million years of evolution is likely to be fundamental to the survivial of the species. Gadi says he routinely uses this approach.

Although the *Dictyostelium* genome carries genes for about 100 putative transcription factors, detailed changes in genetic networks have only just begun to account for the four

major transitions in transcription. Several DNA-binding proteins of the GATA family as well as a transcriptional factor related to Serum Response Factor (SRF) play essential roles at distinct developmental stages, but this is probably only the tip-of-the-iceberg. Gadi is eager and able to characterize the roles of dozens more transcription factors and we all cheer him on.

Somewhere around this time my own lab took a turn towards cell biology. Ofcourse, developmental biology is just a sequence of cell biological events but sometimes we focus in on the details and other times we pan out for the broader picture. The day that Vivek Malhotra walked into my lab with the plea that we knock out a specific gene shifted the balance towards cell physiology and away from multicellularity. Vivek is a card-carrying cell biologist who has made major contributions to our understanding of protein trafficking within cells. He usually has strong opinions and often voices them. He was convinced that his competitors were wrong about a protein named GRASP (Golgi reassembly and Stacking Protein). His graduate student, Matt Kinseth, had found that *Dictyostelium* carries only a single gene encoding GRASP while mammalian cells have two copies. He asked us to help Kinseth isolate a null mutant in the GRASP gene. We said we were happy to do so. In reality, it was Danny Fuller who knocked out the gene and characterized the transformants because Kinseth was being treated for a brain tumor and was either recovering from chemotherapy or about to go through another round of poisoning.

Cells lacking GRASP grew well and had no visible defects in their Golgi apparati, supporting Vivek's conviction that GRASP had little or nothing to do with reassembly of Golgi stacks. The mutant cells also secreted lysosomal glycoproteins at the same rate as wild type cells indicating that protein processing within Golgi was unaffected. Morphogenesis was normal but most of the spores turned out to be non-viable. This observation got the attention of Christophe Anjard. He applied his assays for the secreted spore inducing factors and found that, while the levels of SDF-1 were normal, there was no measurable SDF-2 either inside or outside the mutant cells. It looked like GRASP was somehow involved in secretion of this peptide signal.

Christophe had previously shown that SDF-2 was produced by a protease on the cell surface acting on a protein outside of the cell. The precursor is a housekeeping protein called AcbA (acyl coA binding protein). But there was a problem: AcbA lacks the N-

terminal signal sequence that is essential for conventional secretion. It must get out some other way. Using his highly sensitive SDF-2 bioassay, Christophe was able to show that GRASP mediates unconventional secretion by using components of the autophagy pathway to position AcbA within vesicles that subsequently fuse with the plasma membrane to release their cargo. Together with Vivek Malhotra and Suresh Subramani he was able to show that this pathway is also used in the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*. In both *Dictyostelium* and *Pichia* AcbA is secreted and processed into peptides that trigger rapid sporulation. The pathway also functions in mammals where the active peptide is called DBI, for Diazepam Binding Inhibitor. It is generated in the brain by proteolytic cleavage of AcbA and binds to GABA A receptors on neurons at the site where benzodiazepines, such as Valium, bind. This is one of the most well studied signaling pathways in biology because Valium is one of the most frequently prescribed medications in the world. When evolution discovers a signaling pathway that works, it holds onto it almost forever. Guided by our observations in *Dictyostelium,* we were able to show that steroid hormones, such as cortisol, induce secretion of AcbA from astrocytes. The AcbA/SDF-2 story shows how studies in a model system can bring unexpected light to bear on an important biomedical process.

Shortly after publishing these results, Vivek was offered the position of Coordinator of the Cell and Developmental Biology Programme at the Centre for Genomic Regulation in Barcelona, Spain. We have not collaborated as much since he moved, but I have visited his laboratory by the beach and can see why he likes it. Suresh Subramani has stayed at UCSD but went over to the Administrative side. He is now Executive Vice Chancellor of Academic Affairs with responsibility for the excellence of the research and teaching at UCSD. He is well liked and respected but usually too busy to just talk casually about autophagy or protein trafficking. Christophe Anjard was offered a professorship at Claude Bernard University in Lyon and returned to France in 2011 where he continues to study intercellular communication in his own lab.

In the winter of 2000 Eberhard Bodenschatz escaped the snows of Cornell by spending a sabbatical with Herbie Levine in La Jolla. Eberhard is an experimental physicist who likes to tinker in the lab and generate data as well as carry out quantitative analyses. Among his several strengths is a deep understanding of fluid dynamics at many scales. On

this sabbatical, he wanted to test out some microfluidic devises to see if they would produce useful chemotactic gradients. I showed him *Dictyostelium* while he showed me the wonders of laminar flow in microfluidic devices. Eberhard was always optimistic and boisterous; he was a pleasure to be with. He is now the Director of the Max Planck Institute for Dynamics and Self-Organization in Gottingen, Germany.

The first devices pumped buffer with different concentrations of cAMP in from both sides near the top creating a gradient across the chamber. *Dictyostelium* cells would respond and move across the flow to the side with higher cAMP. Just the simple fact that aggregation competent cells would chemotax in a flow device cut down the number of models of chemotaxis that we had to consider, including those in which the gradient was modified by membrane bound extracellular phosphodiesterase. We developed more and more complex devices and added better temporal control by using "caged" cAMP that was activated by strong illumination. During the next several years some of Eberhard's students including Loling Song, Danica Wyatt and Carston Beta came over to work in the lab for several months. We also collaborated with some of Eberhard's colleagues at Cornell who could fabricate microfluidic devices.

Our first studies were aimed at determining how shallow a gradient would still attract cells. The devices generated linear gradients which made the calculations of minimum gradient spatially dependent but gave some answers. The analyses improved when Alex Groisman joined the Physics Department at UCSD and started designing dedicated microfluidic devices for us. Alex had been a postdoc of Stephen Quake at CalTech and was strongly against using pumps for microfluidics. He liked changing the pressure of the various inputs by raising or lowering reservoirs. I gradually came to agree that this gave better control of flow rates. And it was much cheaper. Alex designed a device that generates exponential gradients such that the change in concentration of chemoattractant was a constant proportion of the ambient concentration all the way across the test chamber. With this device we could accurately establish that cells were able to respond to concentrations of cAMP far below the Kd of the CAR1 receptor and recognize gradients that generated less than 2% difference between the front and the back. Day after day Danny Fuller measured the trajectory of cells in different chemotactic gradients and built up a most exhaustive data set. We calculated that at the lower limits, only a few hundred more

ligand filled receptors were present at the front than at the back. Herbie Levine and his colleague in the Physics Department, Wouter-Jan Rappel, analyzed the data using mutual information theory to delineate the external and internal noise that limits chemotaxis. At the lowest concentrations, stochastic fluctuations in external cAMP limits the ability of cells to detect a gradient. We had complete confidence in our approach in which experimental work guided numerical modeling studies that, in turn, directed further experimental investigations. We decided that to continue we needed a joint grant that would fund our labs for at least 5 years. Wouter spearheaded the effort to prepare the application and submitted it. The large PO1 grant was funded by the NIH in 2007 for 5 years and renewed in 2012 for another 5 years. We have all benefited greatly and have a joint responsibility to continue to generate significant new insights into chemotactic motility.

I had been aware for some time that *Dictyostelium* cells are able to greatly amplify the information presented by a shallow gradient such that they move almost directly towards the source. We wanted to figure out the biochemical basis for this amplification. By 2008 work in the laboratories of Gerry Weeks, Rick Firtel, Peter Van Haastert and Peter Devreotes had convincingly shown that one of the first responses to cAMP binding to CAR1 was the preferential activation of Ras at the front of cells. This small GTPase is membrane bound and only active in the GTP bound form. The GDP bound form is inactive. Activated Ras can stimulate PI3kinase, TORC2 and other kinases upstream of F-actin regulation. A specialized exchange factor called GEF can lead to the replacement of GDP by GTP in Ras. The intrinsic GTPase activity of Ras converts GTP to GDP by hydrolysis of the terminal phosphate. However, this resetting of Ras is very slow in the absence of a specialized activating protein called GAP. When mulling this around, it occurred to me that if ligand binding to CAR1 activated not only the GEF but also the GAP, their opposing activities could give almost all-or-none responses depending on which activity was stronger at a given instant. I did not know it at the time, but this is an example of an incoherent feedforward loop. If the activating and inhibiting activities are balanced such that they cross each other when conditions change then the response can be ultrasensitive. This mechanism can account for signal amplification. Again, I did not know it at the time, but Albert Goldbeter and Dan Koshland had previously worked out the kinetics and

consequences for a case of a kinase and a phosphatase activating and inhibiting glycogen phosphorylase, respectively. The last step in building this intellectual construct was to get it to give directionality. I realized that if diffusion of the GEF within the cell were limited as a result of it being membrane localized and the GAP was a freely diffusing cytoplasmic protein then Ras-GTP would be stabily amplified in a gradient at the front of cells while Ras at the back would remain in the GDP inactive form. This was just a variant of the LEGI model that Peter Devreotes and Carole Parent had been proposing for a number of years. I was sure that it would work but now we had to prove it.

One of the first things we did when the PO1 money started to flow was to purchase a top-of-the-line Zeiss laser confocal spinning disc microscope with computer assisted optics. It has been a work horse for the last 7 years and generated all sorts of interesting results as well as beautiful color pictures. Danny spent a few months getting to know all the dials and switches and was then joined by a new postdoc, Monica Skoge, who had just finished her graduate work with Ned Wingreen and Ted Cox at Princeton. She was quite familiar with *Dictyostelium*, microscopy, and writing computer code. She was well prepared to acquire high-resolution quantitative live-cell images of cell polarity, chemotaxis and motility. She used a transformed cell line of *Dictyostelium* that expressed a GFP- coupled protein that bound Ras-GTP but not Ras-GDP. To follow the localization of Ras-GTP in the confocal microscope she found that she needed to take at least 5 sections in the vertical dimension or she would miss RasGTP held up in the cell. However, the necessary level of illumination for such imaging limited observation to less than 10 minutes; any more and the cells suffered significant phototoxicity. Monica got around this problem by constraining the cells to 2 μm vertically in a microfluidic device. She would inject cells on one side of a low ceiling chamber that connected a channel carrying buffer to another channel on the other side carrying cAMP. The cells would squeeze into these low ceiling chambers and proceed across, moving at about the same rate as free range amoebae. These flattened cells could be completely imaged in a single confocal slice. Images were collected every 2 seconds for 30 minutes to accurately define the position of RasGTP. There was no evidence for phototoxicity.

Patches of RasGTP were seen at the tip of all active pseudopods. Only when a pseudopod was being retracted did the patch of RasGTP disappear. Wouter Rappel, Herbie

Levine and one of his students, Inbal Hecht, formulated a model for amoeboid cell motion in which activated RasGTP determined the location of membrane protrusion, taking into account cortical tension and the availability of protrusion resources. Some of the parameters of the equations were constrained by direct measurements. For instance, the half-life of a RasGTP patch was less than 2 seconds when the gradient was rapidly turned off. When the gradient was re-established, the RasGTP patch rapidly reappeared indicating that both the RasGAP and the RasGEF were very active. The equations were numerically solved and coupled to an animation program that generated a little cell that ran around chasing cAMP gradients much as cells do in the real world. These simulations showed that the commonly observed splitting of pseudopods was the direct result of the dynamics of RasGTP patches.

Other parameters were established by carefully measuring the kinetics of membrane localization of RasGTP in response to global adddition of cAMP. These experiments were carried out by Kosuke Takeda, a postdoc in Rick Firtel's lab, using microfluidic devices that were designed and built by Micha Adler and Alex Groisman. As expected, cAMP induced rapid membrane localized of RasGTP followed by perfect adaptation back to the starting level. Herbie and Wouter modeled the results and showed that only an incoherent feedforward network could give perfect adaptation under our conditions. A set of ten differential equations was found to accurately account for all of the kinetic data. We suggested that RasGEF was activated slightly more rapidly than RasGAP but that RasGAP was somewhat more stable. These kinetics could account for the rapid formation of RasGTP just after addition of cAMP as well as its return to the gound state.

One of the unsolved mysteries of aggregation was accounting for the continuous forward movement of cells as they encountered a series of travelling waves of cAMP. As a wave passes over a cell, the direction of the gradient changes from front to the back, yet the cell keeps moving in the same direction for at least two minutes in the back of the wave. It seems that each cell has a memory of the direction it determined when the concentration was increasing which overcomes signals from the back when the concentration is decreasing. Alex Groisman designed a microfluidic wave machine so that we could characterize cells in cAMP waves of well defined periods and concentrations. Monica showed that persistant movement in the back of waves was a developmentally controlled

capability. Cells that had developed for 3.5 hrs were chemotactic but stopped at the peak of waves and just dithered around. On the other hand, cells that had developed for 5 hrs continued to move forward in the back of waves and showed uninterrupted progress in waves with 6 minute periods. As predicted by the mathematical simulations, the RasGTP patches disappeared in the back of the wave. Therefore, to account for the persistence of motility, we had to add a bistable memory module to the equations. We are presently trying to use molecular genetics of candidate genes to establish the components of this module. Preliminary data suggests that the protein kinase complex TORC2 plays a central role.

Experiments with the wave machine as well as other microfluidic devices with rapid gradient reversals produced a wealth of kinetic measurements that could be used to challenge the mathematical modeling. I could estimate the outcomes of the models in my head and was confident that our present version could account for the results from all the diverse experiments, but I was very happy to hear from Wouter that equations using the same set of parameters worked equally well for the kinetics of adaptation to global stimulation as for the kinetics of RasGTP patch persistence under changing cAMP gradients or even in the cAMP waves generated in our microfluidic machnine. Since one model could account for all these different behaviors, it was likely that the model captured the essence of the cell. It was also rewarding to see that the model produced the ultrasensitivity that could account for amplification. The model could be further tested by direct measurements of the critical biochemical parameters, but it might not be possible to determine some of the rate constants outside of their cellular context. Those aspects of the model will have to rely on molecular genetics for confirmation.

In the Spring of 2011 Herbie called me to give me good news and bad news: he had just been elected to the National Academy of Sciences and he had agreed to move to Houston to be the Director of the Center for Theoretic Biological Physics at Rice University. I congratulated him and berated him for leaving us. I knew that I would miss him when he left the following year.

It is impossible to consider the mechanical forces that result in cell movement without including the traction needed to transmit the work to the substratum. The prevalent opinion held that *Dictyostelium* cells, like mammalian mesenchymal cells, must generate traction

by using surface receptors that bind to immobilized proteins. It was well established that mammalian cells use heterodimeric combinations of integrins to bind to extracellular matrices. But I knew from searching the genome that *Dictyostelium* did not carry genes encoding homologs of integrins or the extracellular matrix proteins that they recognized. Something else must provide adhesion to substrates. I proposed that we explore the problem of cell-substratum adhesion but my colleagues were not very interested. I couldn't get Herbie or Wouter or any postdoc enthusiastic about solving this problem. Luckily, Danny Fuller thought we might be able to make some progress in this direction.

Alex Groisman agreed to modify a microfluidic device that he had designed for studies with yeast cells so that we could quantitatively measure the resistance of *Dictyostelium* cells to hydrodynamic shear stress. The device has 8 chambers in which the flow rate doubles from one chamber to the next. We let cells settle onto glass cover slips sealed to the bottom of the devices and then turn on the flow. After Danny made some adjustments to optimize the system, we found that cells were washed away in an exponential fashion over a 40 minute period. We imaged fluorescently labelled cells every minute and found that the rate of detachment was directly proportional to the shear stress. The surprising thing was that it didn't matter what surface they were on - naked glass, plastic, Teflon, silanized glass or protein coated glass. The cells seemed to have an innate ability to bind to just about anything. Having ruled out covalent or hydrogen bonding as well as ionic or hydrophobic interactions, what were we left with? A friend of Herbie's, Len Sanders, suggested that we consider van der Waals attractions. I went to the Internet to find out what was known about this force.

The biophysics of van der Waals attractions between surfaces has an intellectual history and theoretical underpining. However, it is essentially impossible to predict with confidence whether one surface will be more attractive than some other surface. On the other hand, it is possible to predict small molecules that can interfer with attraction between surfaces of known composition. We found that either monomeric sugars or amino acids were effective in reducing cell substratum adhesion indicating that surface glycoproteins generated the induced dipoles of the van der Waals attraction. The results indicated that *Dictyostelium* cells generate innate adhesion by van der Waals attraction although they do not rule out the possibility that receptor - ligand based adhesion might be added on top. We

are now extending these studies to a human lymphoma line after the cells have been induced to differentiate in a neutrophil manner. They appear to stick to surfaces by van der Waals attractions as well.

Since our results have considerable biomedical importance, we didn't want to be fooled by systematic errors in the assay and sought out an independent way to test cell-substratum adhesion. One day I was talking with Eberhard Bodenschatz at a meeting and he mentioned that a postdoc in his lab, Marco Tarantola, was trying to use an atomic force microscope to measure cell-substratum adhesion forces. Marco showed me his results at a Gordon Conference in the Swiss alps and we quickly recognized how our assays complemented each other. The following summer he came to La Jolla for a few months and essentially repeated all the experiments that Danny, Wouter, Alex and I had done with hydrodynamic shear assay. Marco's technique depended on his coating the tip of an atomic force microscope with a strong organic glue that attached to cells when it touched them. He would then lift a cell and lower it onto a clean area of the substratum before measuring the force necessary to dislodge it. This assay measures the resistance of a cell being pulled up rather than the resistance of a cell to being blown away. With various normalizations and standardizations the two techniques gave essentially the same results.

We then started looking at development in wild type and mutant strains previously shown to have reduced cell-substratum adhesion. Both assays clearly showed that cell-substratum adhesion decreases dramatically during the first 6 hours of development in wild type cells, such that by the time that they start to aggregate, there is no difference between the wild type and mutant cells. These results appear to rule out the involvement of surface receptors that might be controlled to regulate attachment to the substratum. We still do not understand how adhesion is maintained at the front while it is released at the back as the cells move along. But then, there is a lot we still don't understand.