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Pioneering the Xenopus Oocyte and Egg Extract System

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Getting Started: Selecting *Xenopus* Oocytes as a Model System

A Reflections article provides an opportunity to focus on key findings during a career from a personal and historical perspective. As a graduate student in the late 1960s at Berkeley, I was introduced to the *Xenopus* oocyte by John Gerhart, who shared his fascination with the work of John Gurdon, who had pointed out the unique and powerful features of the oocyte and egg, particularly the ability to microinject biochemically significant amounts of material into them while they carried out complex biological events. This approach using the oocyte as a living biochemical test tube long predated the age of cloning and gene transfection. Later, the synchronous, hormone-stimulated transition of the oocyte from G_2 to M phase during maturation encouraged a focus on cell cycle control. Key events over the years include identifying changes in protein phosphorylation as the initiating signal for oocyte maturation and, more universally, for the action of maturation-promoting factor (MPF) to drive cells into M phase. A crucial advance came from developing methods to produce large quantities of concentrated egg extracts that could carry out MPF-dependent cell cycle transitions, DNA replication, and other complex processes *in vitro*. M phase phosphorylation analysis led to purification of p90RSK and the discovery of the MAPK pathway, which underlies many biological events, including the metaphase arrest of the unfertilized egg. A seminal breakthrough came from purifying MPF from egg extracts and showing that it was a complex of the Cdc2 kinase and cyclin B, uniting the genetic and biochemical approaches to cell cycle control. The oocyte and egg extract system continues to provide new insight into many areas of biology, including the action of other mitotic kinases and centrosome duplication. The unique and powerful features of the *Xenopus* system hold great promise for more work and insight into fundamental problems of biology and medicine.

Early Education

As an undergraduate in biochemistry at Cornell University, I found that I liked both organic chemistry and biology. I had to work my way through college and knew that I wanted to go to graduate school in an environment totally different from upstate New York and in a program where students would have full support for their studies. I had heard about the new field of molecular biology and was accepted into one of the first programs in that area, at the University of California, Berkeley. I arrived in Berkeley in early 1969 just after the People's Park demonstrations and was entranced by the high intensity of life, science, politics, and food and the beauty of the mountains and the sea.

After doing rotations in several laboratories, I realized that nearly all students in the department were working on the properties of a single molecule isolated from a bacterial cell, and I wanted to find a laboratory or project with more relevance to actual cell physiology. At that time, John Gerhart, famous for discovering feedback inhibition of aspartate carbamoyltransferase from bacteria, had just returned from visiting John Gurdon in Oxford, who had introduced *Xenopus* oocytes and eggs as a new system to study embryology and the stability of the differentiated state,

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especially using nuclear transplantation. Gerhart had just decided to change his research interests from bacterial enzyme regulation to developmental biology. *Xenopus* was particularly attractive because the oocyte, egg, and embryonic cells were large enough that biochemically significant amounts of material could be microinjected into them. Furthermore, unlike most amphibians, oocytes and eggs could be obtained year-round from laboratory-maintained animals. I agreed to join the Gerhart laboratory and worked to get the *Xenopus* system established there.

We considered the system to be an *ex vivo* approach to studying the effects of a single molecule introduced into a single cell. Gurdon had shown early on that quiescent brain nuclei injected into *Xenopus* oocytes or eggs would change their activities to conform to those of the resident nucleus, suggesting that egg cytoplasm had the ability to dictate nuclear behavior (1).

Just after I joined the laboratory in early 1971, Yoshio Masui published a most influential paper using oocytes and eggs from another amphibian, *Rana pipiens*, that also showed that cytoplasm could have a dominant effect on the nucleus. In particular, small catalytic amounts of cytoplasm taken from progesterone-treated oocytes maturing from G_2 prophase arrest to meiotic M phase caused the same progression rapidly when microinjected into other G_2 prophase-arrested oocytes in the absence of hormonal stimulation. Masui called this activity "maturation-promoting factor," or MPF (2). Because the recipient oocyte subsequently contained at least 100-fold more MPF activity than had been microinjected, there appeared to be an autoamplification of pre-MPF in each injected oocyte, leading to breakdown of the oocyte nucleus (germinal vesicle), condensation of oocyte chromosomes, and spindle assembly for the meiotic divisions. Later work by many laboratories would show that an activity with the same properties appears in every kind of M phase, meiosis or mitosis, in cells from yeast to human.

In the 1971 paper (2), Masui also showed that the cytoplasm of mature oocytes and unfertilized eggs, which are arrested at metaphase of meiosis II, contained an activity distinct from MPF that could cause metaphase arrest in dividing blastomeres of embryos. He termed this activity "cytostatic factor," or CSF, and proposed that it was responsible for the unique metaphase II arrest of the mature oocyte or unfertilized egg. Because Masui's original studies used leopard frog oocytes, my first goal was to obtain *in vitro* maturation of *Xenopus* oocytes from G_2/prophase arrest to M phase of meiosis I, signaled by germinal vesicle breakdown, and then ultimately to metaphase of meiosis II, equivalent to an unfertilized egg laid by a frog.

There were no regularly scheduled meetings in Gerhart's laboratory, and I found it attractive that students had a high degree of independence and the freedom to pursue approaches they thought best to advance their thesis projects. I was fortunate that, during my first year, a very good technician, Michael Wu, was hired, and he helped with my experiments, particularly in the establishment of needle-making and microinjection technology that Gerhart had observed in Gurdon's laboratory.

At that time, it had become clear from work in *Rana* by Masui and several other laboratories that the effects of gonadotropins on the follicle to cause maturation were mediated by steroidogenesis in the adherent follicle cells to produce progesterone (3). My initial task was to develop culture conditions in which follicle cell-free, G_2-arrested *Xenopus* oocytes obtained by manual dissection from the ovary and then treated with progesterone would undergo meiotic maturation *in vitro* all the way to metaphase of meiosis II. At the same time, I would assess whether and when MPF and CSF activities appeared during maturation in *Xenopus*. It turned out that the defined medium conditions for *Xenopus* oocytes were substantially different from what had been reported for *Rana*, and I was able to demonstrate that oocytes matured *in vitro* with progesterone could be fertilized to produce viable embryos. Importantly, MPF and CSF activities were produced with different but reproducible kinetics during the process using the same microinjection assays developed by Masui with *Rana* oocytes. From a cell cycle perspective, this system offered an *in vivo* model for the G_2-to-M transition, as judged by germinal vesicle breakdown, which presented as a sudden prominent white spot on the pigmented animal pole of the oocyte. As with budding or fission yeast, it was therefore possible to ascertain the stage of the cell cycle merely by looking at the cell in the microscope. The high synchrony of the process derived from the single initiation point of progesterone addition enhanced the ability to monitor biochemical activities going on during maturation.

The second part of my thesis work was to gain some insight into how MPF might be mediating M phase. We found that MPF activity was largely present in the 0–34% ammonium sulfate fraction of yolk-free supernatants that had been dialyzed in buffers containing β-glycerophosphate and ethylene glycol tetraacetic acid, and that became the standard MPF preparation for some years. The choice of β-glycerophosphate was initially made on the basis of extensive work reported for the first protein kinase to be highly characterized, phosphorylase kinase,

which was greatly stabilized by β-glycerophosphate (4). EGTA was included because MPF activity disappears at fertilization in response to an increase in free calcium. Work with protein synthesis inhibitors showed that MPF activity did not appear after progesterone treatment if new synthesis was blocked, but microinjected MPF could cause germinal vesicle breakdown even in the presence of cycloheximide. This suggested that the mechanism of action was a post-translational process and that there might be some sort of initiator protein synthesis after progesterone treatment.

I was influenced at this time by a paper from the Morrill laboratory that showed that total protein-bound phosphate was increased in unfertilized eggs compared with resting G_2 oocytes of *Rana* (5). We therefore set out to measure protein phosphorylation in progesterone-treated maturing *Xenopus* oocytes incubated with [^{32}P]phosphate. Protein-bound phosphate was measured as acid-stable, alkali-labile phosphate, and remarkably, a dramatic 3-fold increase in the total amount of protein phosphate occurred just before germinal vesicle breakdown with progesterone treatment or immediately after injection of partially purified MPF. We found that this burst of phosphorylation occurred even in the presence of cycloheximide when oocytes were injected with partially purified MPF. When metaphase II-arrested eggs were activated with calcium ionophore to exit M phase, mimicking fertilization, the level of phosphorylation returned to the lower interphase level. The tight correlation between increased phosphorylation and MPF and M phase was confirmed in a variety of other systems by several laboratories. Therefore, the main conclusion of this work and of my thesis was that MPF might be a protein kinase or an activator of a protein kinase (6).

Role of PKA in Oocyte Maturation

In considering where to undertake postdoctoral training, I believed it was important to continue pioneering the *Xenopus* oocyte and egg system and to develop further the possible link between protein phosphorylation and MPF. I also truly enjoyed Northern California living, which did not then suffer from the traffic congestion, pollution, and overpopulation it does today. I ended up joining the laboratory of Edwin G. Krebs, who was the Chair of Biological Chemistry at the University of California, Davis, fifty-five miles from Berkeley. Ed had a long history of working in the field of glycogen metabolism and had characterized the first protein kinase, phosphorylase kinase, for which he later won the Nobel Prize. While at Davis, he and Don Walsh had discovered the cAMP-dependent protein kinase as the activator of phosphorylase kinase in response to adrenalin, defining the first protein kinase cascade (7). I decided his laboratory would be a good one in which to learn more about protein phosphorylation. I was able to rent an old farmhouse on three acres ten minutes from the laboratory, which allowed me to enjoy the fantasy of being a gentleman farmer, and I also rented, with some other postdoctoral fellows, a winter ski cabin in Lake Tahoe, a two-hour drive away.

It is hard to believe now that, at the time (1975), phosphorylation was widely believed to be important only for glycogen metabolism. Ed had a large laboratory of twelve composed almost entirely of postdoctoral fellows from around the world, each of whom had a different project related to some aspect of glycogen metabolism or cAMP regulation (Fig. 1). Each postdoctoral fellow was largely self-directed and spoke at a laboratory meeting only about once or twice a year.

Despite Ed's busy travel schedule and the demands on his time as department chair and associate editor of the *Journal of Biological Chemistry*, he maintained a strong interest in everyone's projects, and he expected each of us to exhibit passion for our work. His keen intellect was evident at laboratory meetings and in one-on-one meetings in his office. His philosophy of laboratory management was a hands-off approach focused on providing resources to a sizeable laboratory with diverse projects. This model was broadening for postdoctoral fellows and was consistent with my experiences at Berkeley; it formed a model for how I eventually ran my own laboratory. Ed suggested projects for newly arrived postdoctoral fellows, and most projects studied the regulation or properties of one or another purified enzyme involved in glycogen metabolism.

When I arrived, I was the laboratory's first cell biologist, and I was unusual because I brought my own project and system to his laboratory that really had nothing to do with glycogen metabolism but might prove interesting as a system to study protein phosphorylation. Ed was intrigued by the idea of evaluating the properties and effects of purified molecules introduced into the living cell and was also impressed by the fact that oocytes, like skeletal muscle, are 8% glycogen by dry weight. Also impressed was the Muscular Dystrophy Association, which funded a postdoctoral fellowship application that proposed using oocytes to study calcium signaling.

In Ed's laboratory, I first learned about large-scale purification of proteins by observing with Floyd Kennedy, his longstanding chief technician, the purification, via several column chromatography steps, of phosphorylase kinase

FIGURE 1. **Laboratory of Ed Krebs in Davis in 1976.** Shown standing (from left to right) are Peter Bechtel, J. L. M., Ed Krebs, Joe Beavo, Bruce Kemp, Toby Dills, Yutaka Shizuta, and Jackie Vandenheede. T. S. Huang and Ramji Khandelwal are kneeling.

and other enzymes from several kilograms of skeletal muscle from thirty-five rabbits. There was not enough muscle on the front legs of these restaurant-grade rabbits to be useful for purification, so as soon as several hundred were accumulated, one or another postdoctoral fellow in the department organized a laboratory rabbit leg barbecue. Academic enology was just beginning at Davis at the time, and some postdoctoral fellows who had developed an interest in winemaking taught me the subtleties of pinot noir.

Subsequently, I learned how to pack columns and perform my own purification of phosphorylase kinase. It helped that phosphorylase kinase is 1% of total supernatant protein in rabbit skeletal muscle. Phosphorylase kinase was of particular interest to me initially because the key buffer component in the purification was β-glycerophosphate, without which yields and activity were low (4). EGTA also was present because phosphorylase kinase is a calcium-activated enzyme. Ed once remarked that the use of β-glycerophosphate dated back to his early days in the Cori laboratory at Washington University, where it was selected as a buffer because standard phosphate buffers interfered with enzyme assays. It is not clear why β-glycerophosphate has proven so beneficial for protein kinase purification; it is probably not acting as a phosphatase inhibitor because at least 50 mM β-glycerophosphate is necessary to have a stabilizing effect, far above the level of any contaminating phosphatase. In fact, purified phosphorylase kinase is inhibited by β-glycerophosphate (8). However, β-glycerophosphate had proved essential to the stabilization of crude MPF, and MPF activity was autoamplified in each injected oocyte. Because phosphorylase kinase undergoes autoactivation by autophosphorylation (8), it occurred to me that there might be a connection between phosphorylase kinase and MPF. With this in mind, my initial efforts concentrated on attempting to take phosphorylase kinase and various other protein kinases from rabbit skeletal muscle available in concentrated form in the Krebs laboratory and test them by microinjection for effects on oocyte maturation, hoping that one of them might have MPF-like activity or reproduce the phosphorylation burst. The results were not what I expected. None of the protein kinases had MPF activity to cause germinal vesicle breakdown, but it was soon evident that microinjected PKA was a potent inhibitor of progesterone-induced maturation, whereas injection of the heat-stable PKA inhibitor PKI or the regulatory subunit of PKA induced maturation in the absence of progesterone (9). Subsequent studies revealed that there was indeed a rapid drop in cAMP in oocytes treated with progesterone (10), and our results suggested that this drop was both necessary and sufficient for the initiation of maturation.

This work was rapidly accepted by the *Journal of Biological Chemistry* as a "most interesting paper" and, at the time, was the first example of a biological response involving altered cAMP levels that could be fully accounted for by a change in the ratio of dissociated PKA subunits and thus the activity of the cAMP-dependent protein kinase. Subsequent studies from a variety of laboratories have revealed that a drop in cAMP is a general feature of the

initiation of oocyte maturation, including starfish and mouse oocytes (11, 12). High cAMP levels in *Xenopus* oocytes appear to be mediated by elevated adenylyl cyclase, whereas inhibition of phosphodiesterase activity in mouse oocytes keeps cAMP high (13, 14).

These results suggested that a phosphoprotein was responsible for the prolonged G_2 arrest of the oocyte. This stable arrest is evident for perhaps the two years an oocyte in a *Xenopus* ovary is competent for maturation, but in humans, for example, a woman is born with all her oocytes already present and arrested in G_2. Even forty years later, some of these same oocytes can undergo maturation and ovulation to ultimately be fertilized and produce a complete human being. This remarkably durable cell cycle arrest mediated by PKA has given me a longstanding interest in trying to find the substrate of PKA that might be responsible.

For a time, it was proposed that a kinase-independent activity of PKA might be responsible (15), but that possibility has now been excluded by further studies with completely dead and nonfunctional PKA (16). Of course, early on, the usual approaches of overexpressing PKA or PKI in oocytes incubated with ^{32}P and then looking for changes in radiolabeled proteins on two-dimensional gels were evaluated. Nothing of note was revealed by these studies, most likely because of PKA involvement in complex feedback loops with other kinases and the reality that only very abundant substrates can be identified by this route.

In more recent times, my laboratory has reinvestigated the problem using chemical genetics, the so-called Shokat approach (17). This protocol involves expressing a mutant form of PKA (F80G) that has the unique ability to use a bulky ATP analog, in our case, N^6-benzyl-ATP. This allows only direct substrates of PKA to be identified, as wild-type PKA is unable to utilize the analog. It is fortunate that analog-sensitive PKA retains ~50% of the specific activity of wild-type PKA and is fully capable of regulating the initiation of oocyte maturation by progesterone. After microinjection of our own laboratory-synthesized, ^{32}P-labeled N^6-benzyl-ATP along with F80G PKA, followed by progesterone treatment, two-dimensional gel electrophoresis, autoradiography, and mass spectrometry analysis, one interesting candidate emerged as a potentially relevant target: a protein known as breast cancer suppressor candidate 1 (BCSC-1) whose sequence is highly conserved between the *Xenopus* and human genomes.

This tumor suppressor is mutated in a large fraction of various human tumors (18), but its sequence has not revealed anything about how it might work to control cell cycle progression or cell growth. Overexpression of BCSC-1 in oocytes by mRNA injection with or without mutation of conserved consensus PKA phosphorylation sites has not shown a consistent effect on progesterone-induced oocyte maturation to date, which might be expected for a tumor suppressor already expressed at high levels in the cell. To evaluate a role, loss-of-function approaches are needed, such as immunodepletion of *Xenopus* BCSC-1 from oocyte extracts that remain arrested in G_2 in the presence of cAMP (19) or development of inhibitory antibodies. Further studies in cancer cell lines and mouse models are also highly appropriate if this candidate is to be fully evaluated. It seems likely that the essential substrate of PKA responsible for *Xenopus* oocyte G_2 arrest will be of general significance and also mediate PKA-dependent G_2 arrest in mammalian oocytes.

It is disappointing that, during my career, I was unable to resolve this now classical question in the oocyte maturation field. I have told many recent postdoctoral fellows that whoever solves this longstanding question about the mechanism of PKA action will undoubtedly have a high-impact factor publication and likely a successful job search.

Membrane Action of Progesterone

Midway through my postdoctoral years, Ed Krebs accepted a position as a senior investigator with the Howard Hughes Medical Institute (HHMI) at the University of Washington, which also included serving as the chairman of the Department of Pharmacology. All of the Davis postdoctoral fellows who were not at the end of their postdoctoral training elected to move with Ed to Seattle in 1977, thereby becoming instant pharmacologists, at least from the point of view of future job searches.

The support that the HHMI gave to the very few senior investigators ever appointed appeared to be essentially unlimited, as this period coincided with the initial expansion of the Institute after the ruling by the Internal Revenue Service that the Institute had to spend much more of its assets on medical research. I can remember Ed receiving phone calls from the HHMI asking whether he could spend a lot more money in the next two weeks. It was thought at the time that the HHMI support would continue for the rest of Ed's career, and the HHMI encouraged long-range projects that might otherwise not be completable within the time and fiscal constraints of National Institutes of Health (NIH) grant support.

I found it sad to leave Northern California and the special home my Davis farmhouse had become. It also was hard to leave the sun; it rained for the first thirty-four days I lived in Seattle, and I became skeptical that Mount Rain-

ier would ever be visible from downtown Seattle as I had been promised. But few places in the world can compare with the beauty of Seattle on that rare clear day when Mount Rainier, the Olympic Mountains, and the Cascades are all visible.

During my time in Seattle, I continued to try to monitor the activity of PKA during oocyte maturation by measuring cAMP levels, evaluating phosphorylation of injected synthetic peptide substrates of PKA, or performing column chromatography to monitor the state of dissociation of the PKA holoenzyme (10, 20, 21). At the same time, I began searching for assistant professor positions, including ones in departments of pharmacology. I hoped to continue living in the West, and I was successful in obtaining a position in pharmacology at the University of Colorado School of Medicine, which was chaired by Norman Weiner. Although the medical school in general was not ranked very high nationally, Norm had built pharmacology into one of the top departments in the nation with a particular focus on neuropharmacology and cell signaling. The startup package in 1978 seems laughable by today's standards (about $25,000 spread over two years), but I had written my first NIH grant while still at Seattle, and it was funded on the first round with a good budget for equipment and setup available soon after my arrival.

My initial work as assistant professor was to investigate how the level of cAMP was modulated by progesterone in the oocyte. It had been evident for some years that progesterone acts at the level of the plasma membrane to initiate oocyte maturation because microinjection of progesterone into oocytes did not have any discernable effect on overcoming the G_2 prophase arrest (22). Work done by my first graduate student, Susan Sadler, advanced the concept of membrane action of progesterone significantly by showing that isolated, manually dissected plasma membranes exhibited adenylyl cyclase that could be inhibited directly in a GTP-dependent fashion by progesterone addition *in vitro* (13). Other laboratories obtained similar results (23). The actual G protein involved has never been characterized fully but is probably different from G_s or G_i because progesterone can inhibit membrane cyclase even when G_s has been activated by cholera toxin or when G_i has been inactivated by pertussis toxin.

In later years, the question of whether this inhibition is receptor-mediated was partially resolved by a collaboration with Peter Thomas that identified a *Xenopus* membrane progesterone receptor whose modulation affects oocyte maturation (24). Peter had earlier discovered this novel class of steroid receptor in fish and had shown that one or more isoforms are widely distributed in fish, amphibians, and mammals (25). Membrane receptors for different steroids are now a hot area of current hormone research.

However, in oocytes, classical nuclear (intracellular) progesterone receptors are also present and can affect maturation when overexpressed (26). Some studies have suggested that these classical receptors may have both genomic and non-genomic effects, so the relative contribution that the membrane and intracellular progesterone receptor classes make to the initiation of *Xenopus* oocyte maturation is still unresolved. In mouse oocytes, which are also maintained in G_2 arrest by high cAMP, the mechanism is different: cAMP phosphodiesterase activity is inhibited by cyclic guanosine monophosphate transport from the follicle cells (14), whereas in *Xenopus*, phosphodiesterase activity is not affected by progesterone (27).

p90RSK and Discovery of the MAPK Pathway

The concept that MPF was responsible for the burst of phosphorylation suggested the hypothesis that, if the proteins that became phosphorylated in the burst were identified, then they could be used as substrate in assays for protein kinase purification, potentially leading to MPF. We called this approach in the laboratory "working backward up the pathway." In a collaboration with George Thomas, then in Basel, the first protein identified as becoming phosphorylated in the burst was ribosomal protein S6, which George had worked on for many years. Because of ribosomal gene amplification, each oocyte has over a trillion ribosomes, and all S6 becomes maximally phosphorylated at five sites during oocyte maturation (28). Purification of the kinase(s) responsible was undertaken by a very talented technician, Jo Erikson, and led to the identification of a 90-kDa protein as the relevant kinase (29),which was eventually named p90RSK for ribosomal S6 kinase (RSK).

Unfortunately for the MPF hypothesis, microinjection of active RSK into oocytes did not induce oocyte maturation. However, we noticed that the protein underwent a pronounced upward electrophoretic shift when activated, and moreover, this shift could be reversed by treatment with the catalytic subunits of either PP1 or PP2A that correlated with loss of kinase activity *in vitro*. This suggested that there was an upstream kinase responsible for RSK activation, which could be termed an S6 kinase kinase in accordance with the earlier literature with phosphorylase kinase and the discovery of PKA as phosphorylase kinase kinase (7).

A variety of kinases available in the laboratory or obtained from other laboratories were evaluated for their

ability to phosphorylate and reactivate phosphatase-treated, deactivated RSK. Success was elusive until a chance encounter with Tom Sturgill from the University of Virginia at a Gordon Conference. Tom had identified and purified, from 3TL-L1 cells treated with insulin, a protein kinase that phosphorylated microtubule-associated protein 2 (MAP2) *in vitro*. We found that this MAP2 kinase was able to phosphorylate and at least partially reactivate deactivated RSK, forming the second known protein kinase cascade (30).

Subsequently, we renamed MAP2 kinase mitogen-activated protein kinase, or MAPK, and discovered that it also was deactivated by phosphatase treatment, validating the concept of working backward up the pathway and suggesting the existence of a MAPK kinase (31). This kinase, MEK1, ultimately turned out to be activated by yet another upstream kinase, Mos, in the *Xenopus* oocyte system (32). It later turned out that MAPK and MEK1 had yeast gene homologs implicated in the pheromone signaling pathway (33).

Our *Xenopus* biochemical work identifying a new protein kinase cascade has encouraged studies of MAPK in many different fields that reflect the widespread importance of the pathway in cellular function. The discovery of the MAPK pathway stands as one of the major achievements of my laboratory and provides another example of the power of the *Xenopus* system for the study of signaling and protein phosphorylation.

Unfortunately for the MPF hypothesis, microinjection of purified MAPK did not induce oocyte maturation. In later years, it became evident that both p90RSK and MAPK become rapidly dephosphorylated and likely deactivated after microinjection into G_2-arrested oocytes. When phosphatase-resistant, constitutively active forms of either kinase were generated years later, they were found to be capable of stimulating oocyte maturation when overexpressed even though progesterone can cause progression from G_2 arrest to germinal vesicle breakdown (but not beyond) in the absence of MAPK or RSK activity. However, MAPK/RSK activation proved critically important for the metaphase arrest of the fully mature, unfertilized egg in meiosis II, known as CSF arrest. Work over many years in my laboratory and others shows that p90RSK activation is necessary and sufficient for CSF arrest (34, 35), and later studies showed that it functions to cause metaphase arrest by modulation of an inhibitor of the anaphase-promoting complex (APC), now known as Emi2 (36). The stable metaphase II arrest of the vertebrate egg is an essential element in ensuring successful fertilization, cleavage, and normal development.

Development of Egg Extracts and MPF Purification

By 1985, I had obtained a second R01 grant and an American Cancer Society grant and had been promoted to associate professor with tenure. Although the backward signaling approach had been enormously successful in defining a major new pathway of signal transduction, the question of the nature and identity of MPF remained a major outstanding problem in the field and in cell cycle regulation generally.

A number of laboratories devoted substantial effort to purifying MPF via the classical Masui oocyte microinjection assay, but success remained elusive. Besides the skill and time required for microinjection, one of the reasons was the all-or-none character of the assay. The oocyte has only one germinal vesicle (nucleus), and it either undergoes breakdown, or it does not. Moreover, even with partially purified preparations of MPF, there is a concentration effect such that the concentration of active MPF that is injected is very important; a mere 2-fold dilution of an active preparation might not cause germinal vesicle breakdown even if the entire amount is injected (37).

The situation changed with the arrival of Manfred Lohka in my laboratory in 1983. Manfred had worked as a graduate student with Masui, and using *R. pipiens* eggs, he had shown that it was possible to assemble nuclei from demembranated sperm chromatin in extracts from activated eggs, whereas condensed chromatin and spindles were produced in extracts from metaphase-arrested eggs (38). After his arrival, we set out to develop extracts from *Xenopus* eggs that would mimic the transition of an unfertilized egg from M phase to interphase or of the activated egg in interphase back into M phase in response to MPF, monitored as breakdown of nuclei assembled *in vitro*. The laboratory had to be retooled for these studies with new centrifuges, compound fluorescence microscopes, multiple gel apparatuses, FPLC equipment, and a large increase in the size of the frog colony. With a hearty diet of crickets, mealworms, and fourteen-day chick embryos, frogs could be reused for egg production every two to three months. It then took over a year to develop methods to generate *Xenopus* egg extracts that could be stimulated either to exit M phase in response to calcium or to enter M phase in response to the addition of partially purified MPF (39).

A key element in our success based on the precedent with *Rana* eggs was preparing extracts by crushing packed eggs with centrifugal force rather than making homogenates. Crushates are highly concentrated cytoplasm and retain many properties of cellular processes that are inac-

tivated by homogenization and dilution. In a biochemical sense, extracts or homogenates are usually thought of as trying to maintain the state of the cell before homogenization. Crushates, which are now routinely called egg extracts, do this also, but more importantly, they are capable of performing dynamic changes in the cell cycle and other processes upon receiving the appropriate signal. Two other features of the system also are powerful. First, large amounts of crushate can be prepared daily. Typically, 10–20 ml of packed eggs from three to five frogs generates 1–4 ml of pure cytoplasm that is only minimally diluted by the buffer in the interstitial space between the eggs before crushing. However, the presence of β-glycerophosphate in the buffer still greatly stabilized the M phase properties of the extracts. Second, the extract is amenable to depletion or reconstitution of purified components before dynamic processes are initiated. We liked to think of the depletion system as a biochemical knock-out in which the knock-in could be performed the same day using the same extract. Important for assaying MPF, candidate samples could be added up to a 1:1 ratio with extracts containing a large number of newly assembled nuclei. The percentage that underwent nuclear breakdown and chromosome condensation was dependent on the amount of MPF added, and intermediate levels of MPF activity could be detected.

The ability to obtain the events of mitosis *in vitro* proved difficult to publish; reviewers were skeptical that such a complex process as mitosis could be reconstituted *in vitro* and demanded additional evidence that the nuclei were really nuclei, that chromosomes were really chromosomes, and that spindles were really spindles. Eventually, they were convinced (39). The development of this system for making *Xenopus* egg extracts stands as one of the greatest achievements of my laboratory over my career because so many fundamental biological processes have been and continue to be obtained in the extracts. The system now enjoys widespread use in biomedical research.

In the first important use for our work, we found that the egg extract semiquantitative assay for MPF could be employed to study MPF action *in vitro* and possibly evaluate fractions from columns for activity causing nuclear breakdown and other mitotic events. Although the approach was intended to be an unbiased one, one of our first efforts was to show that, in addition to the morphological events of mitosis, the extracts recapitulated the burst of phosphorylation seen in oocytes entering M phase in correlation with MPF activity. Two-dimensional equilibrium and non-equilibrium gels of phosphoproteins showed a number of changes that correlated with MPF and M phase (40). A most prominent change was in a 42-kDa protein that contained both phosphothreonine and phosphotyrosine in M phase and became dephosphorylated in interphase. Reviewers demanded that we take out any suggestion that this protein was related to a 42-kDa protein reported by Tony Hunter and others to become phosphorylated in many growth factor-stimulated systems. Later, it turned out that this protein was MAPK, which, as described above, is the activator of p90RSK and critical for CSF arrest in the mature M phase-arrested egg.

I knew it would be foolish to propose MPF purification in an NIH grant application because the long history of failed attempts by others would likely diminish study section enthusiasm. However, I felt adequately funded for a period, and Manfred and I decided it was worth a shot to devote the last year of his postdoctoral work to try to purify MPF using the egg extract assay. It was an exciting effort. From my work in the Krebs laboratory and Jo Erikson's rich experience with purification of p90RSK, we knew which types of chromatography should be tried, and we also knew that MPF was very labile. Therefore, it was important to start with a large amount of biological material, to stabilize initial activity, and to pool fractions from multiple preparations as the purification proceeded so that the final steps, representing material from over a kilogram of eggs, could be fractionated on the low-capacity but high-resolution columns of a newly available and expensive instrument, the FPLC.

In addition to using β-glycerophosphate, EGTA, and magnesium in the buffer around the eggs before crushing, it proved important to have phosphatase inhibitors present initially, including α-naphthyl phosphate and particularly thio-ATP, as thiophosphorylated proteins are often resistant to dephosphorylation. We assumed that the high level of M phase phosphoproteins would be important for maintaining MPF activity. Because of the lability of MPF activity, it was also essential to devise a scheme in which fractions could be separated quickly on columns and assayed directly in the egg extract without dilution and without dialysis before immediate application to the next column.

The behavior of MPF activity was first characterized on a range of chromatographic resins, and over the course of a year, a purification scheme was developed using a combination of anion and cation exchange, gel filtration, hydrophobic interaction, heparin, and other columns. In the end, dialyzed concentrated ammonium sulfate preparations from unfertilized eggs were separated on six columns over about sixty hours, requiring only two assays and one dialysis step. The result was a near-homogeneous preparation of MPF that caused not only nuclear break-

down in egg extracts but also germinal vesicle breakdown when injected into cycloheximide-treated oocytes (41). The peak fractions of the preparation contained only two major bands on a silver-stained SDS-polyacrylamide gel of 34 and 45 kDa; autophosphorylation analysis of the preparation suggested that MPF was a complex in which the kinase subunit was the 34-kDa protein and the 45-kDa subunit was a substrate. Histone H1 was a very good exogenous substrate *in vitro*.

This was a monumental achievement that reflected the effort and perseverance of Manfred, as well as the advantages of using the egg extract assay. This advance resolved a key question in the oocyte maturation and mitosis field that had festered for over seventeen years. The achievement was worthy of publication in a high-profile journal. However, we elected to publish it in the *Proceedings of the National Academy of Sciences of the United States of America* after Ray Erikson, who appreciated the significance of the system from our work together on p90RSK (42), agreed to obtain reviews and communicate the paper to the journal.

Manfred began an active search for tenure-track assistant professor positions and, in early 1988, accepted one here at the University of Colorado School of Medicine in the Department of Cell Biology.

MPF Is a Complex of Cdc2 and Cyclin B

Obviously, we knew it was going to be very important to determine the identity of the two subunits of MPF seen on the SDS gel. Our assumption at the time was that we would need to repeat the purification to generate sufficient material to get partial peptide sequences, design probes, and screen a library to clone the genes.

It had not escaped our attention that mitotic entry in both budding and fission yeast had been associated with activation of a gene encoding a 32–34-kDa kinase known as Cdc2 in fission yeast and Cdc28 in budding yeast. Although Paul Nurse had reported that *cdc2* gene function in fission yeast could be supplied by either the human or budding yeast Cdc2 homologs, there were essentially no biochemical studies of the *cdc28* or *cdc2* gene products. Moreover, it had been mentioned at meetings that overexpression of Cdc28 did not increase the level of MPF activity in budding yeast extracts using the oocyte microinjection assay. Our assay and approach to purifying MPF had been an unbiased one, and therefore, it seemed more appropriate to prepare larger quantities of pure MPF for cloning work than to guess about its identity.

However, when we spoke with Nurse about our purification, he mentioned that he had an anti-peptide antibody to a universally conserved sequence in Cdc2 homologs, the PSTAIRE sequence. He agreed to send us the antibody, and a newly arrived postdoctoral fellow in my laboratory, Jean Gautier, was given the task of checking whether this antibody recognized anything in the purified MPF preparation. The rest is now history, as the anti-PSTAIRE antibody was able both to Western blot the 34-kDa subunit of purified MPF and to immunoprecipitate the pure MPF complex with retention of protein kinase activity (43).

This work was quickly accepted by *Cell* because its resolution of a longstanding problem in the field united the genetic and biochemical approaches to the control of cell cycle progression into M phase. It showed the value of having a diverse set of experimental systems to study a fundamental biological problem, and for a brief moment, everything seemed clear. The significance of our work was enhanced even further by concurrent evidence from Dunphy and Newport that MPF activity in extracts could be reduced by depleting Cdc2 along with other proteins (44). In some ways, the discovery was similar to the identification of the MAPK pathway via the study of p90RSK. Ice bucket biochemistry using *Xenopus* egg extracts yielded a purified protein that turned out to have a yeast gene homolog implicated in a process but lacking definition of how it fit into a pathway.

For MPF, the way forward to attempt to understand how MPF activity is regulated and what substrates it phosphorylates was clear. Answers to the former question were advanced by showing that the other subunit of MPF besides Cdc2 was cyclin B, using antibodies provided by Tim Hunt from Cambridge (45). The cyclin B identification took longer than expected because cyclin B runs at 45 kDa in the Laemmli SDS gel system used in my laboratory, but it runs at 60 kDa in the Anderson gel system Hunt and others used for earlier cyclin B studies. Also, purified MPF contains at least two antigenically distinct cyclin B isoforms. Direct sequencing and cloning of the subunit also showed cyclin B to be a component of purified starfish MPF (46).

We then discovered that Cdc2 itself is regulated not only by complexing with cyclin B but also by inhibitory phosphorylation at Tyr-15 (47). This phosphorylation event turns out to be the target of many checkpoint pathways that regulate cell cycle progression, and the pre-MPF in G_2-arrested oocytes is a maternal store of Tyr-15-phosphorylated cyclin B-Cdc2. When purified MPF was analyzed biochemically, it was found that β-glycerophosphate was a potent inhibitor of the enzyme, not unlike what had been found earlier for phosphorylase kinase (8).

Progress on MPF substrates has been much slower; the two best characterized substrates are the phosphatase Cdc25 and the kinase Wee1, two enzymes that directly regulate MPF activity and contribute to a positive feedback loop that governs the switch-like abrupt entry into mitosis (48, 49). Using the Shokat approach with analog-sensitive Cdc28 in budding yeast extracts, many substrates of varying stoichiometry have been identified, but these have not revealed how MPF is able to cause the seminal events of mitosis, such as nuclear breakdown, chromosome condensation, and spindle formation (50). It appears that MPF acts to induce mitosis pleiotropically via a complicated network of feedback loops rather than as a workhorse kinase that directly catalyzes the events of mitosis.

Recognition of MPF Characterization

It is fair to say that my laboratory's purification of MPF and identification of its subunits as Cdc2 and cyclin B revolutionized the study of the cell cycle because it united the genetic and biochemical approaches to the problem and focused future efforts on the regulation and activity of the complex. A large number of awards and prizes eventually were given for this breakthrough in understanding of the cell cycle. Masui shared the 1992 Gairdner Award and the 1998 Lasker Award with Lee Hartwell and Paul Nurse, an indication that the discovery of MPF activity and its universality was considered at least as important as genetic analysis of a conserved gene important for mitotic control. The Lasker Award citation itself says the most important advance "still was the observation that Masui's MPF contains a protein that is like the yeast proteins discovered by Hartwell and Nurse."

In my opinion, it is unfortunate that Masui and MPF were not recognized with the ultimate award, the Nobel Prize, because without the initial concept of MPF and the work that finally characterized it, there would be no understanding of how Cdc2 and cyclin B are involved in entry into mitosis. They would just be two genes known to be important for mitosis in some poorly defined, indirect way, and our understanding of the network of mitotic control would be far less than it is today. There might not even have been a Nobel Prize for the discoverers of Cdc2 and cyclin B without the purification and characterization of MPF. However, it is gratifying that Masui and MPF continue to be featured prominently in textbooks of biochemistry and cell biology and also that our paper showing that MPF contains Cdc2 is featured as one of the forty-two most important papers in the history of cell biology (51).

Mitotic Protein Kinases and Centrosome Duplication

In the years following the breakthrough of MPF purification and characterization, in addition to work on regulation of the phosphatase Cdc25 that underlies initial MPF activation at the G_2/M border (48), my laboratory began studying other mitotic protein kinases that play key roles in mitosis. We also embarked on work in a new area, centrosome duplication, a fundamental problem for over a hundred years that still represents a largely unknown frontier. It turns out that the two mitotic kinases we have studied in depth, Polo-like kinase 1 (Plk1) and Aurora A, have roles in both multiple mitotic events and centrosome function. Plk1 is a key contributor to initial activation of Cdc25C at the G_2/M border, and a major question in mitosis has been what is responsible for initial activation of Plk1.

Using the backward-up-the-pathway-signaling approach described earlier for p90RSK, Yue-Wei Qian, a postdoctoral fellow working with Jo Erikson, purified from egg extracts a novel protein kinase that phosphorylated and activated Plk1, which we termed Polo-like kinase kinase 1 (Plkk1) (52). This kinase kinase could phosphorylate the T-loop and activate Plk1, forming the third known protein kinase cascade.

However, later work studying the activation of both kinases in oocyte extracts using immunodepletion approaches showed that *Xenopus* Plkk1 was a downstream element and substrate of Plk1 in a positive feedback loop pathway (53). In principle, any element of a positive feedback loop pathway can be isolated as a promoting element for another component upstream or downstream in a pathway. In fact, it seems logical that the purification of MPF could have led just as easily to the isolation of activated Cdc25C rather than Cdc2/cyclin B, as both behave similarly in the oocyte microinjection and egg extract assays, and they form a potent positive feedback loop (48). *Xenopus* Plkk1 accounts for only ~5% of the total Plk1-activating activity in a CSF extract. The dominant mechanism of activation of Plk1 by an upstream kinase remains an important area of current and future work.

A similar backward-up-the-pathway approach was taken to investigate the activation of Aurora A. In work by two recent postdoctoral fellows, Pat Eyers and Frank Eckerdt, a protein known as TPX2 was purified as the principal activator in maturing oocytes, and it links Aurora A function to the Eg5 kinesin and to Plk1 activity (54, 55). TPX2 is overexpressed in many cancers and appears to account for Aurora A activation on the polar regions of the

spindle. The identity of the Aurora A activator on the centrosome has remained elusive.

One of my main interests in the last few years has concerned centrosomes and centrosome duplication in particular. This has been a great unsolved problem for well over a century. Until recently, it was studied mostly by microscopic observation in egg systems. Like DNA replication, there are tight controls on centrosome duplication to ensure that it occurs once and only once during each cell cycle. Moreover, it now has become evident that overduplication of centrosomes is a primary cause of aneuploidy in human tumors, making their study even more exciting and important (56). One of the attractions of studying centrosome duplication in *Xenopus* is that S phase-arrested *Xenopus* embryos exhibit multiple rounds of centrosome duplication (57).

To advance the analysis in a more molecular direction, I developed an important collaboration with Kip Sluder at the University of Massachusetts Medical School in Worcester, a major centrosome and microscopy laboratory. We used polarization microscopy in *Xenopus* egg extracts arrested in S phase to show that they recapitulated the phenomenon of centrosome overduplication in the embryo and made the discovery that Cdk2-cyclin E is crucial for this repeated overduplication (58). Later, my laboratory identified short modular sequences in cyclins E and A that move Cdk2 to the centrosome during the replication process (59). The cyclin binding partners on the centrosome include MCM5, which, independent of its role in DNA replication, is able to control overduplication when expressed in mammalian cells (60).

In *Xenopus*, as in most vertebrates, the centrosome is paternally inherited, and we found that a specific Plk isoform, Plk4, controls overduplication in both activated eggs and activated egg extracts (61). Plk4 has been reported to reside on the centriole and is implicated in overduplication in *Drosophila* and human cells as well. Interestingly, in unfertilized eggs, we discovered that the MAPK pathway not only causes metaphase II arrest via CSF activity but also functions to prevent maternal centrosome neogenesis in the egg, thereby ensuring paternal inheritance of the centrosome (61). Whether, like metaphase arrest, the MAPK-dependent suppression of centrosome formation in the egg is entirely due to activation of p90RSK is worthy of further investigation.

Envoi

Looking back over my career, I can appreciate how important it was to have trained in good places. As Jonathan Slack notes with tongue in cheek in his book *Egg & Ego: An Almost True Story of Life in the Biology Lab*, "good" people train at "good" places and publish "good" work that is accepted at "good" journals (62).

At Cornell, I remember Efraim Racker as an imposing figure who could reduce very complex issues to something simpler. My enjoyment of chemistry was sparked by lectures by M. Sienko, R. Plane, and H. Scheraga. At Berkeley, I was exposed to a wide range of truly outstanding scientists. Besides my mentor, John Gerhart, famous for discovering feedback inhibition in allosteric regulation of aspartate carbamoyltransferase, next door down the hall on one side was Gunther Stent, who had just written about the end of the golden age of molecular biology when I arrived to get a degree in molecular biology; on the other side was Harry Rubin, who provided my first example of a large laboratory and demonstrated the importance of having substantial funding and a good experimental system that everyone worked on.

At Davis, the Krebs laboratory exemplified the model of having a large laboratory of postdoctoral fellows from different nations where everyone had a different project that he or she was free to pursue without undue oversight. This hands-off model in which a mentor facilitates diverse projects reflecting his or her different interests turned out to be the way I ran my laboratory over the years and is quite stimulating. Of course, not all projects work out as well as others, and supporting a variety of projects requires maintenance of good funding and attention to developments in different areas. From a training perspective, it can be argued that, in some cases, particularly right now, it would be more efficient but perhaps less stimulating to assemble a team that possesses different skills to focus on exactly the same project to progress more rapidly, generate more publications, and ensure continued funding. It may be that the era of large, open-ended, uncertain projects carried out individually is at an end. At one point, someone suggested that if my entire laboratory moved to concentrate solely on the Plk field, I could become the preeminent world laboratory in that field. However, I did not heed this advice to narrow the laboratory's focus and effort to a single area.

Nearly all of my work has been with the *Xenopus* system that I helped to establish, and I have no regrets about staying with the same system for my entire career. It is customary in the guidelines for a Reflections article to identify the two or three most important achievements or advances and any new disciplines that emerged from one's work during a career. One such achievement has to be the development of *Xenopus* egg extracts as a model system. It still presents tremendous opportunities for studying

almost any biological process, and fundamental discoveries in egg extracts have proved almost always to be of general significance for mammalian cells. Besides being used to study the regulation of MPF activity, the system is now widely used to study spindle assembly and microtubular motors, as well as controlled protein degradation by the APC. Egg extracts revolutionized the study of the mechanism of eukaryotic DNA replication and its checkpoint regulation and continue to be a major model in that field that complements more indirect genetic studies in yeast.

The second most important advance that came from my work, which relied on the egg extracts, was the purification of MPF and identification of its subunits as Cdc2 and cyclin B. Besides the convergence of genetics and biochemistry that it provided, the work spawned the now very large new field devoted to the study of multiple Cdks and cyclins that control a myriad of events in the life of the cell. It moved protein phosphorylation squarely into the cell cycle and mitosis field and came not long after the discovery here in Colorado that Src was a kinase moved the study of protein kinases far beyond its original glycogen metabolism roots (63).

Besides the MPF advance, a key achievement over time has been the characterization of CSF activity and its regulation by the MAPK pathway in unfertilized eggs. Because of efforts by a bevy of postdoctoral fellows, including Olivier Haccard, Stefan Gross, and Junjun Liu, there is a new understanding of how the MAPK target, p90RSK, inhibits the APC/cyclosome and keeps the egg in metaphase II arrest receptive to the sperm and also how this arrest is removed by the coordinated action of Plk1 and Ca^{2+}/calmodulin-dependent protein kinase II during fertilization.

The pioneering work done in *Xenopus* on CSF has, for the most part, been shown to apply as well to the arrest of mouse eggs, and it is thought that failure to establish or maintain CSF arrest contributes to reduced fertility in older women. Although most work in both *Xenopus* and mice has focused on the regulation of the APC/cyclosome by the MAPK/RSK pathway, equally important but largely unexplored aspects of CSF arrest include the formation and stabilization of the meiotic metaphase II spindle and the inhibition of centrosome formation.

It is remarkable that the bulk of my career has been devoted to exploring the nature of MPF and CSF activities discovered by Masui over forty years ago. These activities have turned out to encompass large questions central to biology and medicine and still guide the path of future research.

The egg extract system we developed has been modified and expanded by others in many ways. In particular, if extracts/crushates are made under a light oil after CSF release by calcium, increasing the protein concentration by ~20%, the extracts cycle spontaneously between M phase and S phase, more or less like in the early cleavage embryo (64). This modification allows the study of cell cycle oscillators and timers and facilitates analysis of the establishment of and recovery from various checkpoints that halt the cell cycle, including DNA damage checkpoints that have been the focus of work in my laboratory by Chris Conn, Aimin Peng, and others in recent times. Ironically, such spontaneously cycling extracts would not have been suitable for purifying MPF or for analyzing regulation of CSF activity.

My forty years spent studying mitotic control have been a good time to be in science. I arrived at Berkeley not long after Sputnik fears had led to massive funding of nearly every grant submitted. It made it relatively easy for John Gerhart to change fields completely and to obtain continuous NIH funding with a long hiatus in publications, something that would be unlikely to succeed today. Krebs' work in glycogen metabolism and signaling by protein kinases was pioneering, and his grant applications and fellowship applications to work in his laboratory were almost always funded. Once HHMI support began, funding issues never arose at all. My work on MPF came out of modest support from NIH grants and was possible also because Weiner's pharmacology department had a broad view of what pharmacology is; the faculty were diverse in their interests but all good at what they did. In 1990, I was appointed an HHMI investigator, a position I retained for twenty years until my voluntary retirement in 2010.

HHMI support came as a complete surprise. The phone call came near the beginning of my one and only sabbatical, which I spent in Helen Piwnica-Worms' laboratory at Tufts University in Boston, where I was learning the baculovirus expression system. In those days, there were no competitions, and appointment was made by a private committee of distinguished scientists assembled by the Institute from time to time. It is hard to imagine an institution more supportive of research than the HHMI. Although I had considered myself reasonably well funded with two NIH grants and an American Cancer Society grant, the department and medical school received little support from the state (and receive even less now!), and the facilities were antiquated. HHMI support was robust, initially refitting and remodeling the laboratory and then ensuring consistent long-term funds to recruit a large number of postdoctoral fellows, many of them foreign and

ineligible for most fellowships. The size of the laboratory increased by 3-fold, reaching twenty-three people at its peak. The HHMI also ensured that I could continue to support the efforts of two highly experienced but expensive senior technicians, Jo Erikson and Andy Lewellyn, who had different skills that proved essential to the long-term function of the laboratory as postdoctoral fellows and students came and went.

The HHMI enabled me to adhere to the model of having a wide range of projects going on at the same time, each carried out largely individually. It seems to me that a laboratory composed primarily of postdoctoral fellows is advantageous for staying at the forefront of an evolving field. The need to incorporate new technology into a laboratory effort is often met most easily by recruiting a postdoctoral fellow already trained in that area. Examples in my laboratory included molecular cloning and confocal microscopy in the 1990s. A large contingent of postdoctoral fellows is also helpful to graduate students in the laboratory, who have access to a wide repertoire of advice on a daily basis and often have projects related to those of the postdoctoral fellows.

Unlike at many elite institutions on the coasts, with the exception of Manfred Lohka and several others, few postdoctoral fellows applied directly to work in my laboratory, as Colorado was not on the map as a major scientific center of excellence in those days. It is hard work to get good people to come to Colorado. The bulk of my postdoctoral fellows came as a result of one of the most enjoyable aspects of science these days: travel, usually as an invited speaker, to small focused meetings abroad that often featured extended opportunities to interact with graduate students nearing the end of their thesis work. These meetings have taken me to every continent except Antarctica. At such meetings, I was able to assess not only English language skills but also the level of science that graduate students were conducting and their enthusiasm and passion for hard work in the laboratory. Generally, these students knew little about the *Xenopus* system or the advantages of doing a postdoctoral fellowship in Colorado or in an HHMI laboratory. They would never have thought about my laboratory for a postdoctoral fellowship if they had not met me in a remote meeting location. Those who were thinking about postdoctoral fellowships in America had thought only about New York and San Francisco as desirable locations. They generally did not have high-impact factor publications arising from their thesis work, but they were essential to the advances we made and confirm the truism that recruiting good people is an important element of a successful scientific career.

Several key collaborations also began at such small meetings. Our discovery of the MAPK pathway would not have happened without chance encounters at small meetings with George Thomas and Tom Sturgill. Travel also provides the opportunity to present one's work personally and get good feedback. In today's world, the literature is so vast as to be almost overwhelming; meetings can provide one of the best ways to keep abreast of what is going on in any field but especially in small fields like oocyte maturation and centrosome duplication. Of course, travel also leads to new frontiers in food, wine, and culture that help to contribute to an enjoyable career. I am very happy that there is now a large cadre of former students and postdoctoral fellows around the world that may form the basis for new travel adventures that advance the cause of science.

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