What makes the cell cycle tick? a celebration of the awesome power of biochemistry and the frog egg

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ABSTRACT **The cell cycle, a 19th century discovery of cytologists, only achieved a satisfactory biochemical explanation in the last 20 years of the 20th century. This personal retrospective focuses on how biochemical studies of the frog egg helped identify the cyclin-based mitotic oscillator and how this approach quickly merged with genetic studies in yeast to establish the basic mechanism of the eukaryotic cell division cycle. The key feature that made this a cyclic process was regulated protein degradation, mediated by ubiquitin, catalyzed by a massive enzyme machine, called the Anaphase Promoting Complex.**

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I struggle a bit to understand what a retrospective should be and why it might be worth writing, and especially, worth reading. A retrospective is not a review, which should describe the state of a field today. Neither should it be a history, objectively describing the order of events in an important period of the past; history, in any case, is generally best written by historians, not participants. Rather, a retrospective is meant to be a *personal* reflection that seeks an understanding (*Verstehen),* not just an historical recital of events (*Erklären*). I came to the cell cycle, while emerging from my PhD in 1971 at the University of California, Berkeley, with an inspiring physical biochemist, Howard Schachman. Like many of my contemporaries in biochemistry, I knew a lot about proteins and chemistry but was woefully ignorant of genetics and the emerging field of molecular biology. I also did not know exactly what I wanted to do. But I made a brilliant nondecision when I wandered into the lab of John Gerhart, a young associate professor at Berkeley, who at that time was also wondering what he should do further in science. John would be, and still is, the most important scientific influence on my life. The cell cycle beckoned to both of us as an obviously important subject that seemed to have barely advanced beyond E. B. Wilson's description in his first edition of *The Cell in Development and Inheritance* in 1896, which John and I used to say, "reads like a recent review article in the field." In fact, my reading of E. B. Wilson's book was a strong impetus for my studying the cell cycle (Wilson, 1896). I wondered why, having mastered a clear understanding of metabolic circuits, we should still be having difficulty understanding something like a *cell* cycle. During my last year at Berkeley, I attended a lecture by Lee Hartwell, where he explained his new approach to the genetics of the cell cycle in yeast. I was impressed. His thinking was not unlike mine, namely, why should the cell cycle not be like the Krebs cycle, just with different genes involved? In a metabolic pathway, the product of one step becomes the substrate for the next. In the cell cycle, the product of DNA replication could be mitosis and the product of mitosis could be DNA replication, of course with an unknown number of intermediates. Nevertheless, trained as a protein chemist, I wished to work on the actual proteins that themselves carried out the reactions, not the genes that encoded them.

There were important insights on the cell cycle that preceded my entry into the field. In 1951, the eukaryotic cell division cycle was described as a cycle of G1,S, G2, and M and then back to G1 in the pioneering labeling and autoradiography experiments by Howard and Pelc on the bean plant (Howard and Pelc, 1951). They demonstrated that mitosis and DNA replication did not happen at the same time and that there were two gap periods when neither occurred. For those of us interested in the regulation of the cell cycle, the masterly cell fusion experiments of Rao and Johnson in 1970 suggested that the nucleus might be a passive responder, rather than a regulator; the cytoplasm, it seemed, contained signals that could drive a G1 nucleus into S phase or an S phase nucleus into M phase (Rao and Johnson, 1970). The limitation of the cell fusion experiments was that you were mixing the contents of the two cells.

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Abbreviations used: APC, anaphase promoting complex; MPF, maturation promoting factor; UCSF, University of California, San Francisco.

You could not put a G1 nucleus into an infinite pool of S phase cytoplasm; it always brought its G1 cytoplasm with it. That problem had been effectively circumvented 2 years earlier, by John Gurdon, who showed that microinjection of brain cell nuclei into frog oocytes at different stages of the meiotic cycle would drive these G1 nuclei into DNA replication or mitosis (Gurdon and Woodland, 1968). These elegant experiments in the frog got less notice than they should have, but I found them very compelling. There were other experiments with the same theme, for example fusions of the syncytial plasmodia of the acellular slime mold, Physarum at different stages of the cell cycle, which also argued for cytoplasmic signals driving nuclei into mitosis or DNA replication (Rusch *et al*., 1966).

Experiments in cell culture seemed interesting but impractical for biochemistry. I tried Physarum and in making my first and last extract I learned quickly why they were called *slime* molds. Experiments on the cell cycle were a biochemist's nightmare because there was no simple way of isolating cells at different stages of their cell cycle. This was well appreciated and why so much of the genetics in budding and fission yeasts involved the use of morphological phenotypes, which could be evaluated at the single cell level in the microscope (Hartwell *et al*., 1970; Mitchison, 1963). The problem of cellular heterogeneity in theory could be addressed by synchronization but the outcome of such experiments is often ambiguous. If you synchronize a cell by drug arrest at mitosis or G1/S, not everything stops on a dime, nor does it immediately resume when you remove the block. But the genetic mutants created much less ambiguous forms of synchronization with very specific points of arrest. In the earliest days of cell cycle studies in fission yeast in Murdoch Mitchison's laboratory, where Paul Nurse got his start, and in budding. yeast in Lee Hartwell's laboratory, the focus was on morphological phenotypes. But though there were limitations, there were also early hints that genetic approaches would be fruitful for understanding the cell cycle in yeast. This was particularly well illustrated by the *wee mutants* in fission yeast, which sped up passage through the G2/M arrest point in the cell cycle but did not speed up growth, thus producing small cells; "wee" in the Scottish language means "small" (Thuriaux *et al*., 1978). Ultimately it would be Lee Hartwell in budding yeast and Paul Nurse in fission yeast and their students who would perfect genetic systems powerful for studying the core elements of the cell cycle.

A different biochemical approach to the cell cycle was to use a single cell that was big enough to be analyzed biochemically. The Xenopus oocyte (1.2 mm in diameter) contains about a million times more nonyolk protein than a typical somatic cell. In frogs and in humans the oocyte is naturally arrested at prophase of the first meiotic division. It will stay for years in that arrested state. Secretion of Lutenizing Hormone from the pituitary causes the follicle cells in the ovary to secrete progesterone, which causes the immature oocyte to exit its prophase block, finish the first meiotic division, and arrest again, as an unfertilized egg at metaphase of the second meiotic division. It is a robust experimental system where cells are naturally arrested at one stage of the cell cycle and where a physiological chemical stimulus, progesterone, drives them to a natural arrest at a different stage of the cell cycle-all without artificial synchrony! Both Smith and Ecker and Masui and Markert found a cytoplasmic activity, which they called "maturation promoting factor" (MPF) (Masui and Markert, 1971; Smith and Ecker, 1971), which appeared in the oocyte after stimulation by progesterone. When injected into an immature oocyte this factor on its own (without the hormone) would drive maturation through the meiotic cycle. Since it could be assayed biochemically, it was in theory open to systematic biochemical investigation; it just awaited purification of MPF. John Gerhart and I were very interested in this way to approach the cell cycle and the existence of MPF was in the back of our minds throughout the period that the cell cycle came together. But purifying MPF proved very difficult and by the time that MPF was finally purified by Fred Lohka and Jim Maller (Lohka *et al*., 1988) the general mechanism of the cell cycle had already been laid out by other means.

This was the state of things when I entered the field in 1971. There was room for everyone: cell biologists, geneticists, endocrinologists, and developmental biologists and even biochemists. I stayed at Berkeley in John Gerhart's lab for a very short postdoc of about a year. While I was there, I went to see Dan Mazia, a storied professor in the Zoology Department at Berkeley, who was an affable proselytizer for the cell cycle. Mazia worked on mitosis in sea urchin eggs. He was very encouraging but through our long conversation he gave me no hint of what he thought I should work on. Then at the end of our discussion, perhaps feeling some responsibility as a senior advisor, he changed the course of the conversation and said that he thought that my idea of going after MPF would be too risky and that I should pick a topic like the mitotic spindle (which he worked on). He mentioned that Ed Taylor's group at the University of Chicago had recently purified a protein called tubulin, which he thought that might be a good biochemical entry point to the cell cycle, or at least to mitosis (Borisy and Taylor, 1967). I took his advice. It would be a complete detour, diverting me from studies of the cell cycle in frog oocytes for my entire 6 year career at Princeton until I took a sabbatical and moved to UCSF. I can honestly say that I do not regret this diversion at all; microtubules are fascinating and served as my bridge from physical chemistry to cell biology.

In 1978 John Gerhart and I decided to take sabbaticals together at the old Hubrecht Laboratory in Utrecht, Holland, hallowed ground for classical Xenopus embryology. The plan was to go back to problems of developmental biology, which we had worked on together during my summer escapes to Berkeley from the heat and humidity in Princeton. John and I spent 3 mo together in Utrecht, where we worked out how the dorsal/ventral axis is established in the fertilized egg (Gerhart *et al*., 1981). When John left, I spent another 5 mo working on what the cytoskeleton might be doing in those early morphogenetic movements in the egg. But in the end what I really accomplished was making one movie of the frog egg that changed my vision of the cell cycle field (Hara *et al*., 1980). It was a totally serendipitous observation. Koki Hara at the Hubrecht was a master time lapse cinematographer, using the best cameras and lighting and 16 mm black and white film. When I exposed fertilized eggs to nocodazole or vinblastine, microtubule poisons that easily penetrate the egg, some early morphogenetic events were inhibited and others were not, but we kept cameras on overnight because no one wanted to come in and turn them off. When we received the developed films 3 d later, there was a striking set of images more than an hour after fertilization and proceeding for several hours. The eggs soaked in microtubule poisons of course did not cleave but instead went through periodic cortical contractions timed precisely with the 30 minute cell cycle in normally cleaving eggs. I later did an experiment of severing the fertilized egg into two separate cells; the fragment that contained the nucleus divided normally; the enucleated fragment heaved up and down, keeping time with division of the nucleated fragment. The prevailing view of the cell cycle was that it was process driven by mitosis and DNA replication, where the completion of one allowed the other but these timed contractions of the enucleated fragment showed that some timer completely independent of the nucleus or the mitotic spindle oscillated. It was much like Edgar Alan Poe's story, "The Telltale Heart"; the heart beat tormented the mind of the murderer, even though the victim was long

dead. The egg cell cycle, without DNA or a mitotic spindle should have been dead but the cell cycle kept beating. Ron Laskey, a Professor at the University of Cambridge was excited about these findings and invited me to a cell cycle session at an international Cell Biology meeting in Berlin in 1980. I showed my movie and no one asked me a question or talked to me during the rest of the meeting. In retrospect, the cell cycle session was only about DNA replication and mitosis; no one there was thinking about regulation. None of the new thinkers of the cell cycle, biologists like Lee Hartwell or Paul Nurse attended. It was a moment of time when our understandings were about to change but had not yet changed.

After my sabbatical in Holland I moved to UCSF and it was now possible for me to personally collaborate with John and his technician Mike Wu throughout the year. I knew the thing to do was to see if it was MPF that was oscillating, even though MPF was still not purified and had to be assayed functionally. I drove to Berkeley about every week or two for an experiment that typically lasted 16 h. This was a very difficult assay to make quantitative. First it was a highly concerted endpoint dilution assay; there was no proportional signal, one had to do many dilutions to find the point where the signal went from all to nothing. Each sample from an uncleaving activated egg was carefully diluted to different concentrations, loaded into injection needles and each dilution injected into 5 oocytes to see if they would mature. The results were unambiguous. The mitotic egg, whether blocked in division or not, generated peaks of MPF at the time of each nuclear division in a control egg (Gerhart *et al*., 1984). The process required protein synthesis. Our paper was published in 1984 and I remember that one of the reviewers wrote, "This is too important a paper to be published in the Journal of Cell Biology." In 1982, John gave a talk at the Marine Biological Laboratory in Woods Hole the same summer that Tim Hunt discovered cyclin and discussed our unpublished findings with Tim. Tim had done a very careful experiment on protein synthesis in the sea urchin egg by incubating the egg in radioactive methionine; he did a time course rather than just an endpoint. Most protein bands accumulated over time but one band increased and disappeared and then increased again over and over. If, instead of applying the label continuously, Tim used a pulse label, the same protein band was labeled but the signal remained constant over time. Tim's conclusion was that the protein must be continuously synthesized and periodically degraded. He named the oscillating protein cyclin (Evans *et al*., 1983). John Gerhart showed him the protein synthesis dependent oscillations of MPF activity and mentioned the periodic contractions of the frog egg timed precisely with the cell cycle. This was the first connection between oscillations of a protein and oscillations of an activity driving the cell cycle. It was the beginning of the idea, at that time completely unproven, that the cell cycle was a cyclin-based oscillator and not a linear pathway driven by mitosis and DNA replication.

At this time another feature of the cell cycle was coming together in budding yeast in Lee Hartwell's lab and in fission yeast in Paul Nurse's lab. The genes that regulated the G1/S and G2M transitions, cdc28 in budding and cdc2 in fission yeast were found to be protein kinases (Lörincz and Reed, 1984). In 1982 David Beach and Paul Nurse showed that the budding yeast gene (cdc28) could replace the fission yeast(cdc2) gene, a remarkable degree of conservation for over 500 million years of evolution (Beach *et al*., 1982). The key point of arrest for budding yeast was the G1/S transition, called "Start" by Hartwell, while in fission yeast the major growth regulated transition happens at G2/M. Partly for that reason workers on these two systems initially focused on different steps of cell cycle regulation. As the transition in frog oocytes was a form of G2/M control there was greater similarity between the work in frog and in fission yeast and that included genes involved in regulating cdc2 by phosphorylation and dephosphorylation. In fact, as important as the first collection of cdc genes in budding yeast was, the fission yeast/frog discoveries, focused on the G2/M transition, are what launched the great coalescence of ideas around the mitotic cell cycle engine.

Following from the in vivo studies of Rao and Johnson and Gurdon, we and Lohka and Masui began to test the capacity of the cytoplasm to drive mitotic events, such as nuclear disassembly and assembly, in extracts made from different stages of the frog cell cycle (Lohka and Masui, 1984; Miake-Lye and Kirschner, 1985). As we improved frog egg extracts to study the self-assembly of the nucleus (Forbes *et al*., 1983), the self assembly of the mitotic spindle (Gard and Kirschner, 1987), and the progression of the cell cycle and MPF activation in vitro in the oocyte (Cyert and Kirschner, 1988), our lab was less dependent on microinjection experiments and more committed to in vitro reconstitution and biochemistry. The hypothesis that cyclin actually drove the cell cycle, rather than simply responded to the cell cycle, was the key issue for the field. A new postdoc, Andrew Murray, one of several extraordinary people who braved the arduous migration from yeast genetics to frog biochemistry in my lab (exhausted by the ordeal, they inevitably reverted to yeast genetics when they left my lab), exploited the uncomfortable interface where the biochemistry is difficult and genetics at that stage was hard to interpret mechanistically. With extraordinary generosity from Tim Hunt's lab, which had just cloned sea urchin cyclins and provided them to us before publication, Andrew set out to test the importance of cyclin in the cell cycle. Inspired by in vitro systems developed in our lab and by Lohka and Masui, which carried out some cell cycle steps, Andrew built an in vitro system from frog eggs that spontaneously oscillated between mitosis and interphase, as measured by the mitotic kinase activity, chromosome condensation and decondensation, and nuclear membrane breakdown and reformation around added sperm nuclei. The extract actively synthesized proteins and synthesis was required for the oscillation, as we had shown previously. This extract also oscillated in MPF activity as assayed by injection into frog oocytes and in cyclin protein abundance. Andrew then destroyed all the mRNA with nucleases and not surprisingly the cell cycle stopped. When he added back mRNA of cyclin, the cell extract resumed cycling (Murray and Kirschner, 1989). Therefore, cyclin synthesis, driven by its mRNA, was both *required* and *sufficient* to drive the entire mitotic cell cycle as measured by all biochemical and morphological assays, including the interphase-mitotic conversion of nuclear and chromosome morphology. In the companion paper, Andrew made a mutant version of cyclin lacking 90 amino acids at its N-terminus (Murray *et al*., 1989). This mutant could still drive interphase extracts into mitosis, but there they were stuck. This led to the undeniable conclusion that cyclin synthesis and accumulation were required to get into mitosis, but that cyclin degradation was required to exit mitosis and progress into the next cell cycle. Cyclin degradation was a critical part of the cell cycle. Without cyclin degradation, there was no cell cycle.

The mechanism controlling the degradation of cyclin was an intriguing biochemical reaction. At that time there was something known about the degradation of damaged proteins, but little or nothing was known about degradation as a regulatory step. We seemed to have a perfect system to investigate this. We had a substrate, cyclin that was not degraded in an interphase extract. By adding nondegradable cyclin to that extract we could drive it into M phase where full-length cyclin would be degraded. Michael Glotzer, a graduate student in the lab at the time, was ultimately persuaded

to give up ideas of fancy genetic approaches to this problem and took a brutally straightforward biochemical direction. When he added trace amounts of radioactively labeled cyclin to an interphase extract, it was stable. When he added it to an extract that had previously been driven into mitosis by nondegradable cyclin, it was degraded. But there was something else. For some reason, after doing his experiment, Michael did not come back to the lab for several days to develop his gels. When he finally developed them, the cyclin bands were massively overexposed, but that overexposure revealed a faint ladder of intermediates in the degradation process of labeled proteins at *higher* molecular weights than the original protein (Glotzer *et al.*, 1991). The higher bands were separated by 7 kDa, which made us very suspicious that during the degradation process, the cyclin was first modified with ubiquitin. Further experiments proved this to be the case.

At this point, I moved to Harvard to set up a new cell biology department and part of my motivation, frankly, was that it would allow me to have a larger cold room and buy some professional biochemical purification equipment. Randy King, an MD/PhD student in the lab, agreed to come with me to Harvard, and Jan-Michael Peters joined as a postdoc. They worked brilliantly as a team and purified the protein machine that degraded cyclin in mitosis. The Anaphase Promoting Complex (APC) is an E3 ubiquitin ligase that transfers ubiquitin to cyclin and other substrates (King *et al.*, 1995). APC is a highly conserved, very large protein complex of 1.7 MD that is now known to have 13 distinct subunits, some present as two per complex (Chang *et al.*, 2015). The purification of APC was aided by a suggestion of Kim Nasmyth to contact Phil Hieter, who had antibodies against two human homologues of yeast proteins that seemed to have some role in mitotic protein degradation in yeast. These antibodies recognized APC subunits in the frog APC, and this allowed us to confirm our early purification steps and at the same time establish the universality of APC in regulating cyclin degradation and the exit from mitosis. Hershko's group identified a related complex from surf clam eggs and named it the cyclosome (Sudakin *et al.*, 1995). We next identified two substrate-binding proteins that conferred substrate specificity (Fang *et al.*, 1998a). We showed that APC was the target of the spindle damage checkpoint (Fang *et al.*, 1998b) and how APC integrated the mitotic phase with the S phase (Rape and Kirschner, 2004). We also identified several previously unknown and critically important new substrates such as securin (Zou *et al.*, 1999), which regulates chromosome separation at the metaphase/anaphase transition, and geminin, which helps assure that DNA is replicated only once per cell cycle (McGarry and Kirschner, 1998). APC was ultimately much too big for one lab. It had many important functions outside of mitosis and is the locus for intricate regulation and is still very actively studied.

In one decade of work, the beating tell-tale heart of the cell cycle's autonomous oscillator was universally accepted, and the insights that came from the Hartwell metabolic pathway of the cell cycle and Paul Nurse's dissection of the regulation of the mitotic kinase were integrated into a detailed and universal model with a core oscillator and many feedbacks. The endogenous oscillator could explain all the basic properties of the cell cycle in eukaryotic cells. Before that clarification, there was some real discomfort. Was there one cell cycle model for frogs that looked like a clock and another that looked like a cascading row of dominoes for budding yeast? Could these processes be so different because they originated separately? Or were they created once and just simply diverged massively after 500 million years of evolution? Or were we failing to grasp their fundamental similarity? I remember one particular encounter with Lee Hartwell after I had finished a talk at a meeting. Lee said (as I remember it), "Marc, it is strange that the cell cycle of the frog egg should be so different from yeast. I would have expected that they would be conserved." But then he said he had been thinking, "maybe the basic structure of the cell cycle is more like the frog than we thought, and the appearance of a contingent linear pathway is due to *checkpoints* that restrain it when it is unsafe to proceed." It was a brilliant insight and the key to much wonderful biology that Lee contributed on checkpoints. In large part, thanks to him, we understand the answer. The frog egg has an easy life, provisioned with all its needs. It functions without growth at all for more than a dozen divisions. By contrast, the yeast cell has a hard scrabble existence; it cannot count on its mother provisioning it with everything it needs for the future. Like most cells, it has to duplicate everything, including components of the cell cycle oscillator. If it runs out of something, it needs to wait to make it. Today we know that metazoan cell cycles are replete with feedbacks on the autonomous kinase oscillator and APC; when these feedbacks fail and the cell tries to divide, there is permanent damage. From the perspective of cells in our bodies, both yeast cells and frog eggs are bizarre. But it is precisely their strange properties that made them so valuable for revealing the common thread that ties them together and to every eukaryotic organism.

There have been a lot of wonderful people who had worked on and are still working on the cell cycle in many different systems: human cells in culture and surf clam eggs, syncytial slime modes and human stem cells, starfish oocytes, Drosophila embryos, and nematode eggs. Each contributed in its own way to our understanding of the common features of the biology of proliferation. There are still many unanswered questions, particularly with regard to growth and cell cycle. Biased by their chosen tools or organisms, different scientists looked at the same fundamental mechanisms from different angles and saw things that others had not seen. This was not scientific redundancy; it was science efficiency at its best. The work of all these people left us not only with a better understanding of common processes in cell division but also with an appreciation of how evolution crafts cellular chemistry and maintains and adapts even the most ancient, the most complicated, and the most deeply embedded systems.

About 10 years after the cell cycle came together, I ran into a beginning graduate student in Boston and we started to talk, and I asked him what he was interested in studying and he said he was fascinated by the cytoskeleton and the cell cycle. My ears perked up. I asked him how he wanted to approach such complex systems and he said confidently that he wanted to study them in yeast, which did not surprise me and in fact seemed reasonable. But I then asked him what attracted him to yeast to study these systems and he looked very surprised and said, "that is where all the important discoveries were made." I am never bitter about the ignorance of young students (I have a separate scale for professors). But his answer caused my attention to jump to a very different time and place, when I first played a flickering 16-mm black and white movie and saw a frog egg with no nucleus mysteriously pulsating away to the beat of the cell division cycle; that vision comforted me and I suddenly felt grateful for what I had been able to experience in science.

I thank my students, postdocs, and assistants who through their dedication, ingenuity, and hard work helped peel away the mystery of cell division. I also thank the many scientists, young and old, from many labs, from many countries, and from many experimental systems who helped create this field in what seemed like a day. Their generosity and their keen competition kept things moving and we all were able to avoid traps along the way. I wish I could say enough about John Gerhart, but suffice it to say that I am grateful that our

scientific lives have been so intertwined for almost 50 years. Finally, I thank Randy King and Andrew Murray for reading this reverie of mine and pointing out errors. The errors that are left are all mine. These days there is a lot of talk about science in the news, its economic value, its objectivity, its grounding in facts. But that description omits how kind and vital a community it can be. I wish we could share that reality, so that the world can understand.

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