YEAST AND CANCER

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by

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My research career has been motivated by a desire to understand cancer. Each time I have identified an intriguing aspect of the cancer problem, I have found that it could be approached more effectively in the simpler eukaryotic cell, *Saccharomyces cerevisiae*, than the human cell. Each time the yeast cell has revealed some of its secrets. I will relate four vignettes involving studies on the genetics of cell division, the control of genome fidelity, therapeutics for cancer and the role of natural genetic variation in disease susceptibility. In the first two instances, yeast has told us something that is relevant to mankind. For the other two, it is too soon to tell.

When I was finishing my graduate studies and thinking about what area of science to pick for my postdoctoral work, I wanted to study a field that was still a mystery and would offer ample opportunity for a career. I chose to study the control of growth and proliferation in relation to cancer cells with Renato Dulbecco and Marguerite Vogt. Even in plastic petri dishes, cancer cells grew when normal cells did not and I became thoroughly engrossed with the problem. However, while in Renato's laboratory I had not been able to establish an experimental system that I thought could lead to fundamental insights. When I set up my own laboratory at the University of California at Irvine I was not sure what to work on.

I had applied for and received a grant to work on the control of DNA synthesis in mammalian cells. However, after much pondering and an influential conversation with Dan Wulff, I decided to work on a model system that permitted genetic analysis. I had been imprinted as an undergraduate with the insights that Bob Edgar and Bill Wood had attained on the pathway of viral morphogenesis using genetics. The processes of the cell cycle, chromosome replication and segregation, seemed like morphogenetic problems of the same nature. Yeast was the obvious choice because it grew as single cells, an important property for studies of cell division. Moreover, Don Williamson had shown that *S. cerevisiae* had a eukaryotic cell cycle with G1, S, G2 and M periods (1) and C.F. Robinow had demonstrated the presence of an intranuclear spindle (2). These were important facts since yeast has, at various times, been accused of not being a proper eukaryote. There was even a time when people thought that yeast lacked DNA.

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GENES THAT CONTROL CELL DIVISION

Since cell division was an essential process, I set out isolating temperature-sensitive mutants that could grow at room temperature but not at 36C. We isolated about a thousand mutants and characterized each for protein, RNA, and DNA synthesis, cell division, and cell morphology following a shift from the permissive to the restrictive temperature. Although most of the mutants had unremarkable phenotypes, a significant number behaved as if defective in a specific one of these processes (3).

I thought it important to demonstrate that we could identify the defective protein in some mutants. Calvin McLaughlin (Fig. 1) who was an expert in protein synthesis generously agreed to collaborate and we began studying the protein synthesis mutants. We did identify mutations in genes that coded for two aminoacyl-tRNA synthetases and another mutant defective in the initiation of protein synthesis (4).

CDC genes. Three years into the project, I moved to the Genetics department at the University of Washington. I have had a number of very bright undergraduates in my lab over the years and one, Brian Reid (Fig. 2), set us on the path to the cell cycle. Although we had a few mutants that behaved like DNA synthesis or cell division mutants from the original survey, we didn't have any clever ideas about how to analyze them. Brian, through a very amusing bit of serendipity that I have described elsewhere (5), discovered how useful photomicroscopy was for analyzing the *S. cerevisiae* cell cycle. This was because the cell forms a small bud on its surface at the beginning of a cell cycle and the bud grows in size as the cycle progresses. Thus one can easily tell where a cell is in the cycle merely from its morphology. Mutants with specific defects in the cell cycle were recognized because the asynchronous population of cells grown at the permissive temperature became synchronously arrested in the cell cycle at the restrictive temperature (Fig.3).

Figure 1. Calvin McLaughlin.

Figure 2. Brian Reid.
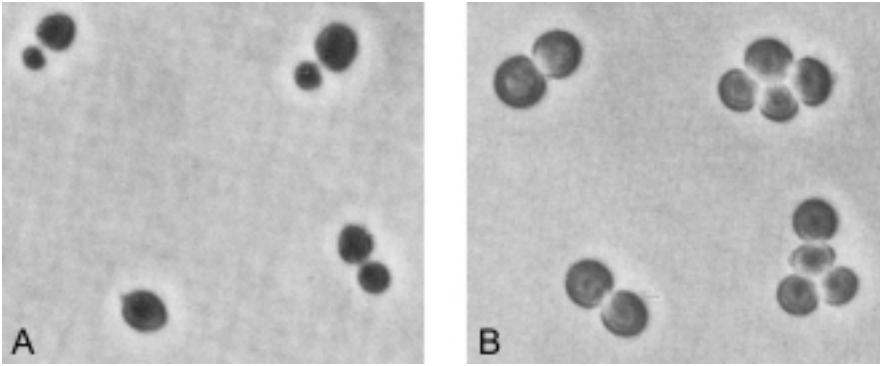
Figure 3. Time-lapse photomicroscopy of a *cdc* mutant cells growing at the permissive temperature (A) and several hours after a shift to the restrictive temperature (B).

When we realized this, we were able to find hundreds of cell cycle mutants in a few months. C.F. Robinow agreed to visit the lab and teach us how to stain the nuclei of cells, and Joe Culotti (Fig. 4), a new graduate student, was able to show that the mutants that arrested with a uniform cell morphology also arrested with a uniform nuclear morphology. At this point we were excited that we had some very interesting mutants (Fig. 5) and we were confident that they would reveal new insights into the cell cycle (6).

Figure 4. Joe Culotti.

Pathways. We ordered the mutants in a number of ways. First, by how far they traversed the cycle before arresting. We analyzed the following cell cycle events: budding, DNA synthesis, nuclear division cytokinesis and cell division. The event that stopped first after a shift from the permissive to the restrictive temperature was considered the primary defect. After the primary defect, other cell cycle events would occur or not depending upon the particular mutant. Eventually, the cell would arrest development and generate a terminal phenotype, depending on which events occurred and which did not. Mutants were found with primary defects in each of the cell cycle events that we monitored. The phenotypes of the mutants suggested a relatively simple pathway of dependent events leading to cell division. The first event,
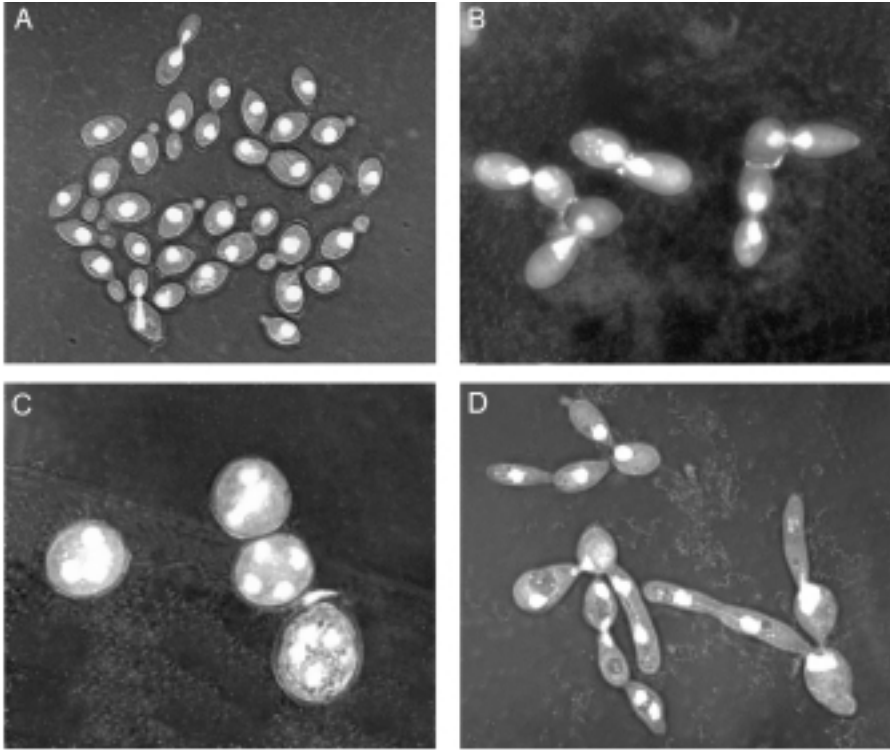
Figure 5. Normal cells and *cdc* mutant cells several hours after incubation at the restrictive temperature. (A) wild type, (B) *cdc8* (C) *cdc24* (D) *cdc10.*

controlled by the *CDC28* gene, was required for initiating two pathways, one of which led to budding, nuclear migration, cytokinesis and cell division (Fig. 6). The second led to DNA replication, nuclear division and joined the first prior to cytokinesis and cell division. We tested our conclusions by constructing all possible double mutants and, indeed, the phenotypes of double mutants was precisely that expected for the pathway (7).

It is interesting, in hindsight, that the pathway of dependent events was not the least surprising to me. Although the results could have been very different, the pathway we found was sensible with late events requiring the completion of earlier events in the cycle. This is just the way that bacteriophage morphogenesis was organized.

I thought we might learn more about the mutants by looking at them at higher resolution in the electron microscope. When I approached Breck Byers about this, he immediately responded that there was only one structure worth looking at and that was the spindle and its poles. He and Loretta Goetsch examined the spindle and were able to resolve additional events of spindle pole duplication, separation of the two poles, and elongation of the spindle (Fig. 7). This analysis resolved three mutants, all required for the initiation of DNA synthesis into successive steps involving duplication of the spindle pole body (*CDC28*), separation of the poles (*CDC4*) and the initiation of DNA synthesis (*CDC7*) (8).
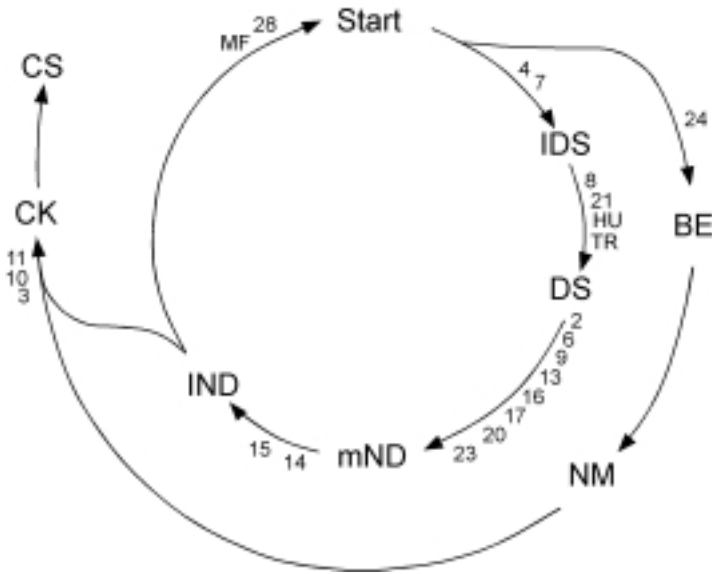
Figure 6. A pathway of gene controlled events in the *S. cerevisiae* cell cycle. Numbers refer to *cdc* genes. Abbreviations are: iDS, initiation of DNA synthesis, DS, DNA synthesis, mND, medial nuclear division; lND, late nuclear division; BE, bud emergence; NM, nuclear migration; CK, cytokinesis; CS cell separation; MF mating factor. Reprinted from ref 7 with permission.
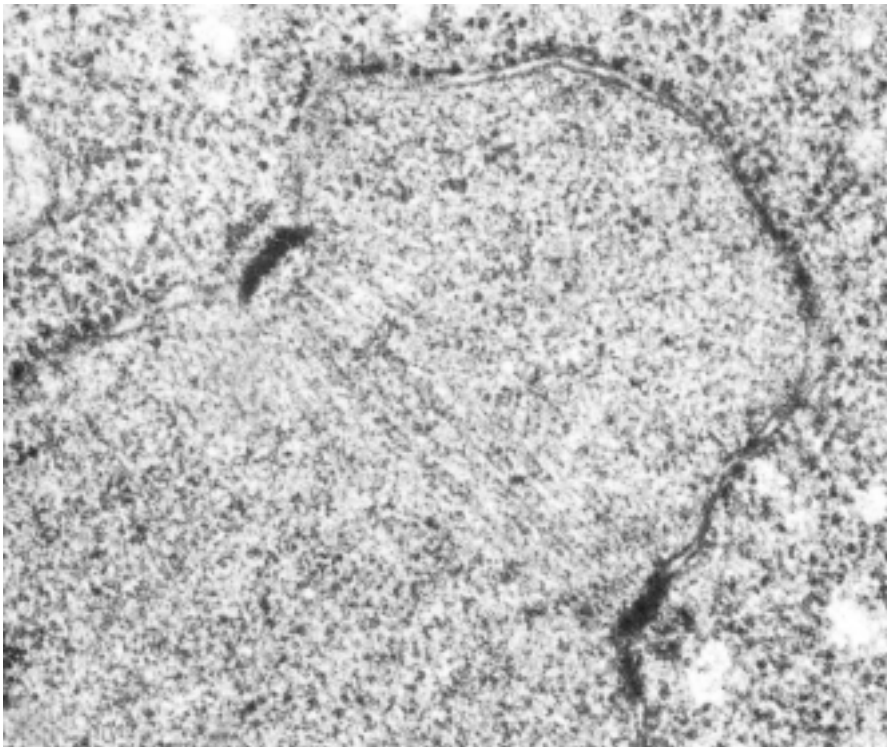

Figure 7. Electron microscopic image of the yeast nucleus revealing the electron dense spindle pole bodies imbedded in the nuclear membrane with microtubules emanating from them. Reprinted from ref 8 with permission.

The methods that we had used to order mutants into pathways utilized the phenotypes of single and double mutants. Lynna Hereford and Joe Culotti thought of a method we called "reciprocal shifts" that utilized two conditional agents that arrested the cell cycle independently. Jarvik and Botstein (9) independently introduced the same rationale to study bacteriophage morphogenesis. Lynna applied this method to mating factor (see below) and the *cdc* mutants showing that mating factor arrested cells at the *CDC28* step prior to the *CDC4* and *CDC7* steps (10). These results were in agreement with the spindle pole phenotypes determined by Byers & Goetsch.

Mating and cell division. Wolfgang Duntze and Tom Maney had discovered the presence of a mating pheromone made by *Matα* cells and Duntze had found that it inhibited DNA synthesis of *Mata* cells (11). We collaborated with them to analyze the cell cycle response to mating pheromone and found that it arrested cells at the *CDC28* step (12). Over the next few years, we investigated the relationship between the mating pathway and the division pathway. I found that when asynchronous cultures of *Mata* and *Matα* cells were mixed, they arrested one another in G1, and at the time of cell fusion, both cells were at the G1 stage (13; Fig. 8). John Pringle and Linda Wilkenson found evidence for a *Mata* pheromone that arrested *Matα* cells at the *CDC28* step (14). These experiments demonstrated that cell division was integrated with cell mating because both haploid cell types produced pheromones that arrested the other cell at the beginning of the cell cycle.

Breck Byers studied mating cells by electron microscopy and found that nuclear fusion in the zygote occurred by fusion of the two unduplicated spindle pole bodies resulting in a single nucleus with a single large spindle pole body (8). This assures that the newly formed diploid nucleus begins the cell cycle in G1 at the unduplicated spindle pole body stage. Brian Reid challenged cells to mate at other stages of the cell cycle by first arresting them with different cell cycle mutations. He found that only cells arrested at the *CDC28* controlled step were capable of mating (15).
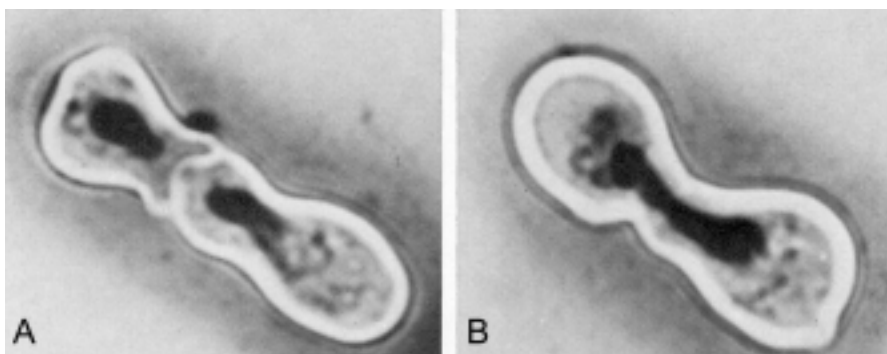
Figure 8. Stained cells revealing the earliest stage of cell fusion during mating with two unbudded, G1 cells undergoing cell fusion (A) followed by nuclear fusion (B). Reprinted from ref 13 with permission.

Growth and division. John Pringle (Fig. 9) became interested in the regulation of growth in the cell cycle. Williamson and Scopes had found that stationary cultures of yeast cells were unbudded (16). John Pringle and Gerry Johnston followed up on this and showed that growth is integrated with the division cycle prior to budding (17), once again at the *CDC28* step. They found that cells were able to complete the cell cycle after abrupt nitrogen starvation in the absence of any net growth, but did not start new cell cycles (Fig. 10). However, if division was interrupted with a cell cycle mutation, the growth of the cell continued unabated for several hours.

Yeast monitored other essential nutrients prior to budding as well. Michael Unger used a series of mutations in the sulfur assimilation pathway to ask whether an intermediate in sulfur assimilation acted as a signal to arrest cells. The idea was that interruption of the pathway after the signal would fail to produce arrest as unbudded
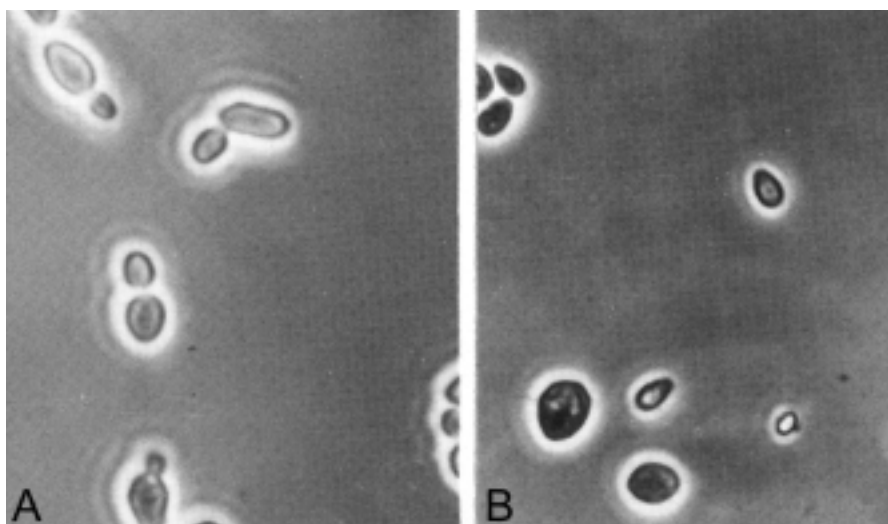

Figure 9. John Pringle.

Figure 10. Growing (A) and nitrogen starved (B) yeast cells illustrating that starved cells complete cycles without growth and generate very tiny cells from new buds; all cells end up arrested in G1 as unbudded cells. Reprinted from ref 17 with permission.

cells. He found that limitation for sulfur assimilation at any step, including the charging of methionyl-tRNA resulted in arrest at the *CDC28* step, suggesting that the cell was monitoring protein synthesis or the accumulation of protein (18).

Start. The *CDC28* step was the first step in the cell cycle. From it branched the two pathways of gene activity leading to the cell surface events, budding and cytokinesis, and the nuclear events, spindle pole duplication, separation and elongation, and DNA replication and nuclear division. It was also the step where growth was integrated with division. *CDC28* is not executed until the cell reaches a specific size but once the *CDC28* event was completed the cell could finish the rest of the cycle without appreciable growth. *CDC28* was also the step at which mating was coordinated with the cell cycle. Both pheromones arrested cells at a position, interdependent with the *CDC28* step so that when the two cells fused in G1 they were synchronized at the unduplicated spindle pole body stage. Thus *CDC28* was of central importance. We called the event that was performed by *CDC28* "Start" (Fig. 11).

Given the fundamental importance of the *CDC28* gene, it is amusing how close we came to missing it in our mutant screen. We obtained only one allele in the original mutant screen and that allele was present in a mutant strain with another temperature-sensitive mutation that limited growth at the high temperature. The double mutant was a clear cell cycle mutant. However, when the second mutation was crossed away from the *cdc28* allele, the strain grew extensively and became very odd shaped after prolonged incubation at the restrictive temperature. It is quite possible that we would have missed it all together without the second mutation in the background. As with most areas of science, the cell cycle is replete with serendipity. Paul Nurse discovered the very important wee mutants at the wrong end of a size gradient and Tim Hunt discovered cyclin while looking for something quite different.

Cyclin Dependent Kinase. My initial work on temperature-sensitive mutants had been motivated by an interest in cancer with the hope of learning about the genes that controlled cell division. The genes we discovered, especially *CDC28* seemed like they might eventually shed some light on why cancer cells divided. When Steve Reed (Fig. 12) joined the lab as a postdoc, he decided to
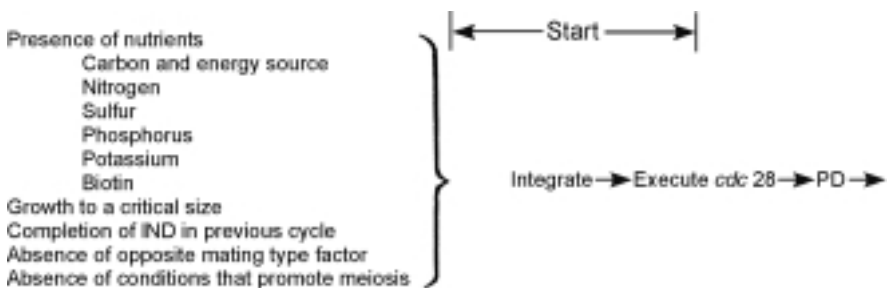

Figure 11. Summary of the events that are integrated at "Start". Reprinted from ref 49 with permission.

Figure 12. Steve Reed.

focus on the *CDC28* gene, setting up selections that uncovered new alleles as well as new genes that arrested at the same step. In 1980 he and Kim Nasmyth used recently developed techniques to clone the *CDC28* gene (19) and after Steve set up his own laboratory, he found that it had protein kinase activity (20). The ultimate unification of the cell cycle field with the discoveries relating *CDC28* of *S. cerevisiae, cdc2* of *S. pombe*, and MPF of Xenopus embryos to cyclin dependent kinases centered largely in the laboratory of Paul Nurse with important contributions by many others and has been elegantly described recently by Kim Nasmyth (21).

Expectations fulfilled. I find it quite interesting philosophically that two different groups approached the cell cycle with different expectations and each found exactly what they were looking for. At first, the results looked very different, perhaps because we were imposing our expectations. I approached the study of the cell cycle with the paradigm of bacteriophage morphogenesis in mind. Indeed what we found looked a lot like phage morphogenesis – a series of gene controlled, dependent events leading to progressive stages in division. Paul Nurse, on the other hand, was most interested in rate limiting steps. He found a rate-limiting step at mitosis controlled by the *cdc2* gene of *S. pombe* (22). From this point of view, the most important gene of fission yeast, *cdc2*, seemed very different than what we viewed as the most important gene in budding yeast, *CDC28*, because the former acted in G2 while the latter acted in G1. Once it became clear that the two genes could substitute for one another (23), the apparent differences were resolved. In fact, both proteins acted in each organism at G1 and G2. Under rapid growth conditions the G1 activation of *CDC28* in *S. cerevisiae* is dependent on cell growth while in *S. pombe* it is the G2 activation of *cdc2* that is dependent on growth.

GENOME FIDELITY

Cancer cells not only divide when they shouldn't but they also reproduce with far poorer fidelity than normal cells. Chromosome aberrations and chromosome losses are very common in cancer cells. This loss of fidelity seemed central to cancer because it would permit the cancer cells to evolve more

quickly. We thought we might be able to learn more about how chromosome fidelity is maintained in the normal cell cycle by studying the fidelity of chromosome transmission in yeast cells.

Essential machinery. We found that normal yeast cells lose a chromosome or undergo mitotic recombination at rates of about one event in 10^5 cells. We wondered whether limitation of any of the essential cell cycle functions would alter that fidelity. An undergraduate, David Smith and I studied chromosome loss, recombination and mutation in temperature-sensitive cell cycle mutants when they were grown at their maximum permissive temperature. We found that most mutants had greatly elevated rates of chromosome loss, recombination or mutation (Fig. 13), implying that defects in these functions could be important in the fidelity of cell division (24).

Other essential components were also important for fidelity. Doug Koshland examined the role of centromere structure and dicentrics on chromosome fidelity (25); Meeks-Wagner defined the importance of correct ratios of histones (26); and Megan Brown studied a new gene involved in centromere function, MIF2 (27).
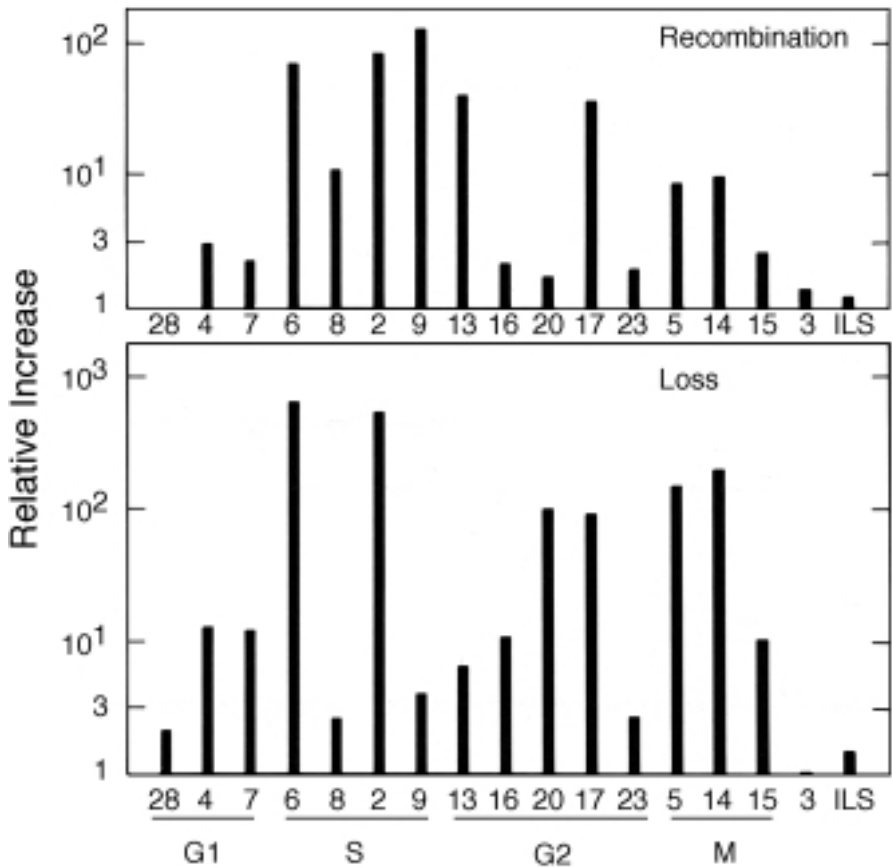

Figure 13. Rates of mitotic recombination and chromosome loss in *cdc* mutants grown at their maximum permissive temperature. Reproduced from ref 24 with permission.

These results suggested that mutations in essential components of the cell cycle machinery might be responsible for the genetic instability of tumor cells in addition to the known role of DNA repair defects in certain forms of cancer susceptibility (e.g. DNA excision repair in skin cancer and mismatch repair in colon cancer).


Figure 14. Ted Weinert.

DNA damage checkpoint. Fortunately, my interest in genomic instability coincided with Ted Weinert's (Fig. 14) interest in studying the regulation of cell division. He thought it likely that all of our cell cycle mutants were identifying genes that contributed to the machinery of cell division and was interested in studying something that was more clearly an example of cell cycle regulation. I had remembered noticing that yeast cells became arrested synchronously in the cell cycle by radiation and mutagens and he began looking at radiation sensitive mutants to see if any were altered for their cell cycle response. He quickly found that some radiation sensitive mutants failed to arrest the cell cycle in response to radiation (28). He demonstrated that deletion of the *RAD9* gene eliminated the regulation of the cell cycle by radiation, demonstrating a regulatory role for this gene (Fig. 15) and discovered a number of additional genes involved in the DNA damage checkpoint (29).

Ted's discovery led to an appreciation of the role of checkpoints in the fidelity of chromosome transmission. The *rad9* mutant exhibited a 20 fold increase in the rate of chromosome loss in the absence of any extrinsic DNA damage. An explanation for the increase in chromosome loss in checkpoint defective cells was provided by Ted's discovery that the same mutations that rendered cells insensitive to arrest of mitosis by radiation also rendered cells insensitive to arrest by defects in DNA replication (29; Fig. 16). This suggested that intrinsic errors in DNA replication occurred stochastically in rare cells of the population and that the checkpoint function was needed in those cells to assure correct repair of the damage. The same checkpoint genes were later found by Mandy Paulovitch to control the rate of replication over damaged DNA (30) and by Siede, Friedberg and Friedberg to control the rate at which cells enter S phase when they experience damage in G1 (31).

The role of the DNA damage checkpoint in assuring genomic fidelity was further elucidated by Daivd Toczyski when he was a postdoc in the lab. San-
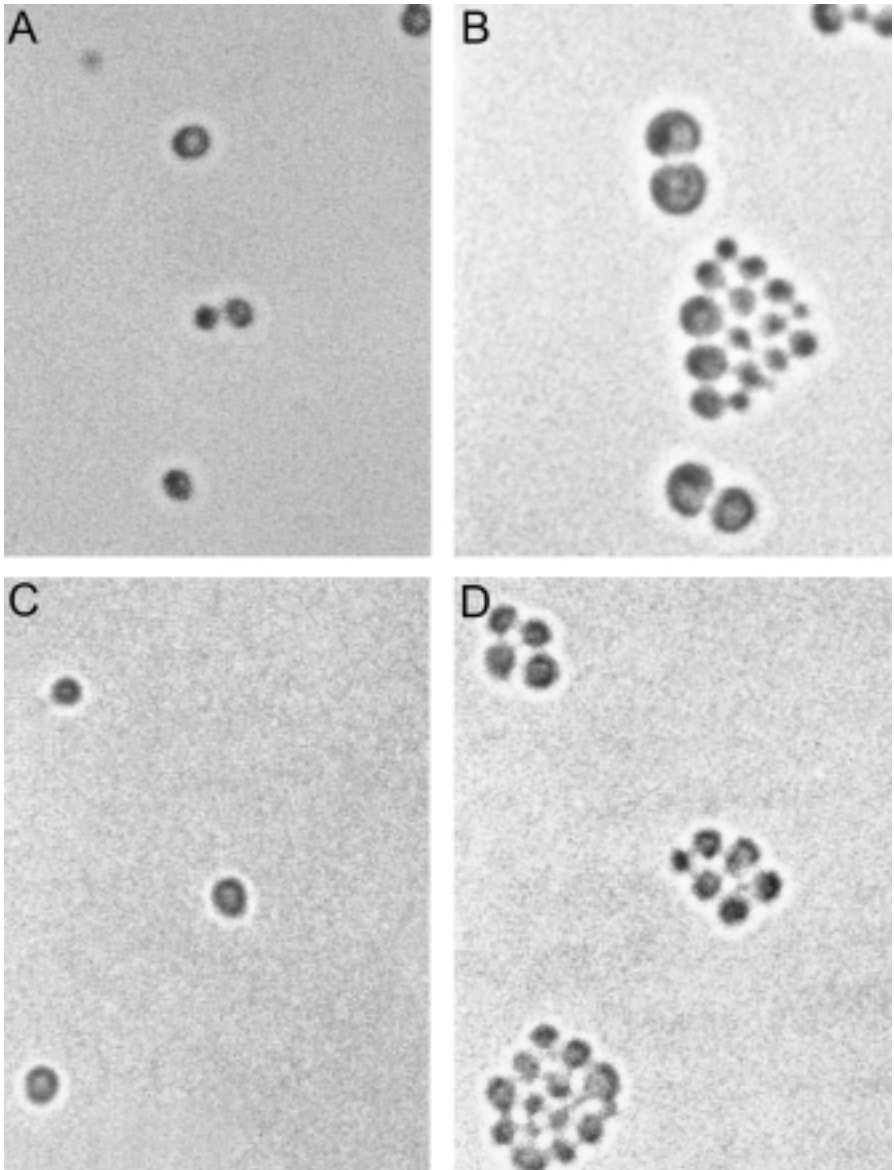
Figure 15. Time-lapse photomicroscopy of cells irradiated with X-rays. Wild type cells at the time of irradiation (A) and several hours later (B). Note that the originally unbudded G1 (A) cells have remained arrested as large budded cells (B) while the budded G2 cell (A) has resumed cell division (B). G1 haploid cells are very inefficient at repairing double strand breaks because of the lack of a template for homologous recombinational repair. *rad9* mutants cells at the time of irradiation (C) and several hours later (D). Note that the G1 unbudded *rad9* cells (C) do not arrest division but continue dividing producing dead microcolonies (D).

dell and Zakian had demonstrated, very elegantly, that cells which contain a single double strand break arrest at mitosis for a number of hours and then begin dividing again even if they cannot repair the break (32). This result indicated that the checkpoint pathway has an intrinsic adaptation component.
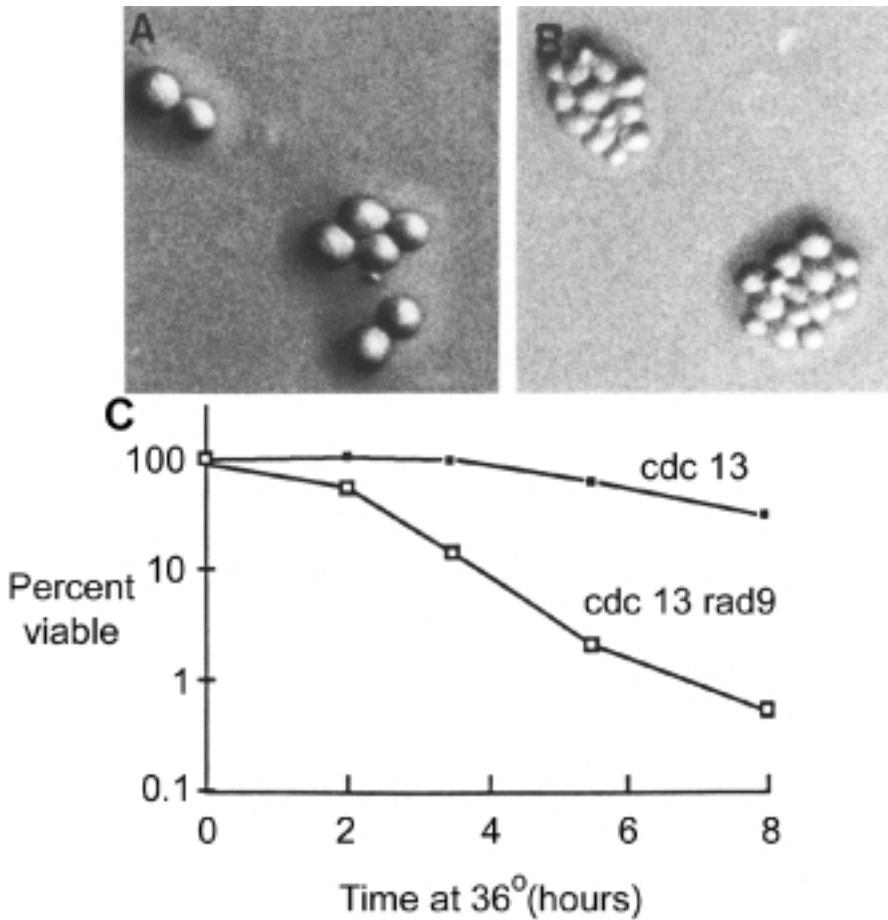
Figure 16. Cells defective in telomere replication fail to arrest cell division at the restrictive temperature. *cdc13 RAD9* cells incubated at the restrictive temperature for several hours have arrested cell division and retain viability (A) but *cdc13 rad9* cells failed to arrest and die (B). Reproduced from ref 29 with permission.

David discovered mutants defective in adaptation of the DNA damage checkpoint and showed that wild type cells actually transmit more damage to offspring than adaptation defective cells (33). The p53 tumor suppressor gene (34) acts as a cell cycle checkpoint gene and its function is also regulated by adaptation. Several other cell cycle checkpoints have been discovered. One controls mitosis if chromosomes are not attached to the mitotic spindle (35). A recent review of the DNA damage checkpoint has appeared (36).

Paradox resolved. After the discovery of cyclin dependant kinases, there remained an important paradox in our understanding of the cell cycle. By 1988 it was clear that the CDK played a controlling role in the cell cycle of frogs as well as yeast. Gerhart, Wu and Kirschner had demonstrated that the maturation promoting activity (CDK) of Xenopus eggs displayed a periodic activation at the onset of mitosis (37). Murray, Solomon and and Kirschner had

shown that accumulation of the cyclin mRNA was the limiting component for activating mitosis (38). Thus it seemed that the Xenopus cell cycle was driven by periodic activation of the CDK through cyclin synthesis. Moreover, this CDK "clock" was autonomous from the events of the cell cycle. Inhibition of DNA replication does not block mitosis during the earliest divisions of the egg.

How could we reconcile this picture with that derived from the two yeasts? The yeast cell cycle did not behave as though it were being run by a clock undergoing periodic activation. Instead, it had the appearance of a series of dependent events where each event provided the substrate or activated the subsequent event. Growth was the activator of the CDK each cycle and there was no need for a clock. To phrase the problem more precisely, if periodic activation of the CDK was driving cell cycle events, why did the cell cycle stop in yeast when the machinery for DNA replication or mitosis was defective? Why wouldn't the clock keep ticking and activate mitosis in the absence of DNA replication or cytokinesis in the absence of mitosis? The discovery of mutants that no longer arrested division in response to radiation provided the answer – a series of regulatory signaling pathways, checkpoints, that keep the cell informed of each event's progress. If an event stops, checkpoint signaling arrests the clock. The frog embryo is different because the checkpoint only becomes active after about the twelfth embryonic division (39).

A unified view. The genetic control of cell division provided two important lessons that have been repeated over and over in molecular, cellular and developmental biology. The first is the conservation of proteins and their functions throughout evolution. This was not a surprising conclusion because all living organisms share a common ancestor. However, we did not know how conserved the machinery would be. We did not know that a newly sequenced gene from nearly any organism would be seen to fit into a family of proteins like kinases, myosins, or anthranylate synthetases. Indeed, the molecular conservation of many of the proteins involved in the cell cycle and in cell cycle checkpoints is not very evident across the prokaryotic / eukaryotic boundary but within eukaryotes the conservation is striking. A second, and of course related issue is how easily biology uses the same parts to make different things. Here again, the cell cycle is a dramatic example. Of the three organisms most studied, Xenopus, fission yeast and budding yeast there appeared to be little in common. The frog embryonic cell cycle was controlled by a clock, the fission yeast by a rate controlling step in G2 and the budding yeast by a gene that initiated spindle pole duplication in G1. Yet all three activities turned out to encode the same cyclin dependent protein kinase, and the differences were only superficial.

CANCER THERAPEUTICS

The genetic instability of cancer cells are due to genetic defects that affect the cell cycle machinery, DNA repair, or cell cycle checkpoints. Examples of the latter two are well known. Since we know a lot about the biochemistry of each

of these processes it has been possible to define the genetic changes that lead to loss of fidelity in some cancers and it should be possible in many cancers. These defects should create a vulnerability for the cancer cell relative to the normal cell that could provide a powerful therapeutic advantage if the appropriate vulnerability were targeted for therapeutic intervention.

This brings me to a third application of the yeast model to cancer research. A new adventure was in the making when I had the good fortune to be invited to give a seminar at Massachusetts General Hospital at about the time that Stephen Friend was exploring his interest in drug discovery. Stephen had spent some time investigating the drug discovery programs at some of the major pharmaceutical companies. Mark Groudine, Jim Roberts, and Bob Eisenman had gotten me interested in the potential for drug discovery in the area of cell cycle checkpoints. Stephen got interested in this idea as well and was recruited to the FHCRC to head up a program in molecular pharmacology. Stephen and I set up a drug discovery program that was originally supported by Merck and the NCI to use yeast genetics to discover and validate cancer targets.

Our goal was to identify drugs and drug targets that would present a therapeutic advantage. That is, where the cancer cells would be more sensitive to the drug or to inhibition of the target than the normal cells. The idea was to construct yeast cells that contained mutations characteristic of specific tumors (altered in mismatch repair, cyclin, activated telomerase, etc.). The mutant and normal cells were then screened for drugs that killed the mutant yeast more effectively than a wild type yeast (40). The results have been encouraging. A test of 23 commonly used chemotherapeutic agents revealed that some had a high "therapeutic advantage" (41). Cis-platin preferentially kills cells with defects in post-replication repair genes, *rad6* and *rad18* (Fig. 17); the nucleotide analogue, cytarabine is preferential for an *sgs1* mutant, a homologue of the Bloom's syndrome and Werner's Syndrome helicases; camptothecin, a topoisomerase I inhibitor, and idarubicin and mitoxantone, two topoisomerase II inhibitors, are specific for cells with defects in double strand break repair, *rad50, rad51* and *rad52*. Other agents were selective for a broader range of DNA damage repair mutants and some agents were nonspecific.

This approach identifies drugs with therapeutic advantage. We were also interested in identifying protein targets which if inhibited by drugs would provide therapeutic advantage. These would be "validated" targets. Yeast lends itself to this goal as well. There are methods for identifying mutations in an unknown gene that are synthetic-lethal with mutations in a second known gene (42). So, we could start with a mutation characteristic of certain types of cancer and identify mutations in other genes that would be synthetic-lethal with the first. These genes would be potential drug targets. Targets were identified that would kill mutants defective in the DNA damage checkpoint. These targets were enzymes of deoxynucleotide biosynthesis and components of the DNA replication apparatus (Eric Foss and Patrick Paddison, unpublished).
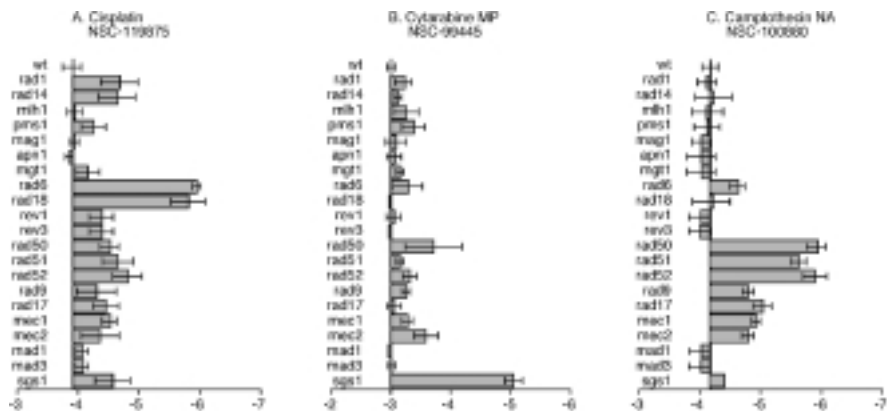
Figure 17. Sensitivities of different DNA repair and checkpoint mutants to common chemotherapeutic agents. (A) Cisplatin, (B) Cytarabine, (C) Camptothecin. The horizontal axis is a log scale. Reproduced from 41 with permission.

NATURAL GENETIC VARIATION

I am currently interested in the individual differences in susceptibility to cancer. How does the genetic variation that exists in the human population manifest in differences in disease susceptibility? Will we be able to assess disease predisposition for most people at an early age so they can choose life styles or frequent screening that will lead to better health? Will there be an individualized medicine?

The current preoccupation in molecular genetics is in the use of inbred organisms as models of human disease. When a gene is assigned a function in a mouse we reason that it is likely to play a similar role in the human. Yet the model systems that we study are genetically homogeneous except for the single gene perturbation that we study, whereas the human population is genetically outbred such that all individuals are very different (except for identical twins). It is clear that this genetic individuality has a very important role in the transformation of genotype into phenotype because the same mutation in different inbred strains of mice or yeast often have different phenotypes. Are we missing something by studying inbred model organisms?

I seem to have extremely good luck in finding colleagues who point the way. Just as I was considering these issues, I had the good fortune of meeting Stan Leibler who taught me about the robustness of biological circuits (43). I have come to think that this concept of robustness is critical to understanding the phenotypic consequences of natural genetic variation. Because of buffering, some genetic variation in protein activity or amount will have no phenotypic consequence.

There are several seminal contributions to our understanding of robustness. Redundant pathways, feed back control, and certain metabolic pathways have this robust behavior. One is the insight of Kczar and Burns to the robustness of metabolic pathways (44). They demonstrated that the flux of me-

tabolic pathways (in which the enzymes followed Michaelis-Menton kinetics and none of the enzymes are saturated for their substrates) would be relatively insensitive to changes in the amount or activity of individual enzymes in the pathway. Stan Leibler and his colleagues have come to the same conclusion for the more complicated bacterial chemotaxis behavioral response because of its intrinsic feed-back regulation (45). An investigation of the genetic circuitry that controls Drosophila segmentation by vonDassow, Meir, Munroe and Odell has come to the surprising conclusion that robustness may be a property of almost any circuit with a significant complexity (46).

Since this buffering capacity is a product of the circuit and its components there will be natural genetic variants that compromise the buffering capacity itself. The consequence will be that many polymorphisms in a population will present no phenotypic consequence in the most prevalent genotypes. However, in rare genotypes that happen to compromise the buffering capacity for the particular polymorphism in question, a phenotype may result. If this reasoning is correct, then many disease susceptibilities will not be assignable to single polymorphisms but only to combinations.

How can we identify alleles that interact to produce phenotypes in combination that neither produce alone? Here again, yeast is an ideal organism in which to investigate this issue at its most fundamental level. As described above, there are methods in yeast for identifying synthetic-lethal interactions or synthetic-phenotypes (42).

For my purpose, which is to understand the genetic basis of phenotypic variation in an outbred population, it is important to understand the number of synthetic relationships that each gene is subject to. Obviously, the more interactions that can create synthetic phenotypes, the greater the genetic complexity potentially underlying a phenotype. Recent work in yeast from Tong, *et al.* (47) and unpublished work by John Hartman in my own laboratory suggest that genes interact with 20 or more other genes in the genome to create synthetic phenotypes. Or, to put it another way, variations in the activity of any single protein are buffered by interactions with as many as 20 other proteins in the genome.

It is clear that the prospects for genetic complexity underlying disease phenotypes in outbred populations is very great. So great, in fact, that if even a small percentage of the potential genetic variation were present in the population, then nearly all phenotypes would be the result of highly complex genetic interactions and most individuals with the same phenotype would have different combinations of alleles generating that phenotype. Attempts to identify natural polymorphisms that account for the differences in cancer susceptibility between two inbred mouse strains often does uncover great complexity (48). What is not clear, is how much functional variation is actually present in the human population. This depends on the history of the population, its size, its age, etc. For the human population, we know that about 1 nucleotide in 600 is polymorphic but we do not have any idea how many of these polymorphisms effect function or expression of gene products.

In order to learn more about the principles underlying the genetic com-

plexity of human phenotypes we can hope to learn again from yeast. Important questions are the number and identity of genes that can interact synergistically to generate a phenotype, the patterns in which they interact, and the fraction of natural polymorphisms that have functional consequences. An investigation of these questions in yeast might help identify the set of candidate genes that could be associated with a particular human disease.

We still have a lot to learn from yeast and other model organisms about the nature of human disease.

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