YEAST AS TOOL IN CANCER RESEARCH
Yeast as Tool in Cancer Research

Edited by

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# TABLE OF CONTENTS

Foreword........................................................................................................ix  
List of Contributors......................................................................................xi  
Introduction..................................................................................................xv  

Chapter 1  
FROM DNA REPLICATION TO GENOME INSTABILITY  
IN *SCHIZOSACCHAROMYCES POMBE*: PATHWAYS TO CANCER.................................1  
Julie M. Bailis and Susan L. Forsburg  

Chapter 2  
DISSECTING LAYERS OF MITOTIC REGULATION  
ESSENTIAL FOR MAINTAINING GENOMIC STABILITY.................................37  
Jennifer S. Searle and Yolanda Sanchez  

Chapter 3  
YEAST AS A TOOL IN CANCER RESEARCH:  
NUCLEAR TRAFFICKING..............................................................................75  
Anita H. Corbett and Adam C. Berger  

Chapter 4  
STUDIES OF PROTEIN FARNESYLATION IN YEAST.................................101  
Nitika Thapar and Fuyuhiko Tamanoi  

Chapter 5  
FROM BREAD TO BEDSIDE: WHAT BUDDING YEAST HAS TAUGHT US ABOUT THE IMMORTALIZATION OF CANCER CELLS.................................123  
Soma S. R. Banik and Christopher M. Counter
# Table of Contents

Chapter 6  
HSP90 CO-CHAPERONES IN *SACCHAROMYCES CEREVISIAE*  
Marija Tesic and Richard F. Gaber

Chapter 7  
YEAST AS A MODEL SYSTEM FOR STUDYING CELL CYCLE CHECKPOINTS  
Carmela Palermo and Nancy C. Walworth

Chapter 8  
METABOLISM AND FUNCTION OF SPHINGOLIPIDS IN *SACCHAROMYCES CEREVISIAE*: RELEVANCE TO CANCER RESEARCH  
L. Ashely Cowart, Yusuf A. Hannun and Lina M. Obeid

Chapter 9  
EXPLORING AND RESTORING THE p53 PATHWAY USING THE p53 DISSOCIATOR ASSAY IN YEAST  
Rainer K. Brachmann

Chapter 10  
FUNCTIONAL ANALYSIS OF THE HUMAN p53 TUMOR SUPPRESSOR AND ITS MUTANTS USING YEAST  
Alberto Inga, Francesca Storici and Michael A. Resnick

Chapter 11  
ABC TRANSPORTERS IN YEAST – DRUG RESISTANCE AND STRESS RESPONSE IN A NUTSHELL  
Karl Kuchler and Christoph Schüller

Chapter 12  
THE FHCRC/NCI YEAST ANTICANCER DRUG SCREEN  
Susan L. Holbeck and Julian Simon

Chapter 13  
YEAST AS A MODEL TO STUDY THE IMMUNOSUPPRESSIVE AND CHEMOTHERAPEUTIC DRUG RAPAMYCIN  
John R. Rohde, Sara A. Zurita-Martinez and Maria E. Cardenas

Chapter 14  
USE OF YEAST AS A MODEL SYSTEM FOR IDENTIFYING AND STUDYING ANTICANCER DRUGS  
Jun O. Liu and Julian A. Simon
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Authors</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>GENETIC ANALYSIS OF CISPLATIN RESISTANCE IN YEAST AND MAMMALS</td>
<td>Seiko Ishida and Ira Herskowitz</td>
<td>393</td>
</tr>
<tr>
<td>16</td>
<td>USING YEAST TOOLS TO DISSECT THE ACTION OF ANTICANCER DRUGS: MECHANISMS OF ENZYME INHIBITION AND CELL KILLING BY AGENTS TARGETING DNA TOPOISOMERASES</td>
<td>Anna T. Rogojina, Zhengsheng Li, Karin C. Nitiss and John L. Nitiss</td>
<td>409</td>
</tr>
</tbody>
</table>

Index........................................................................................................................................................................429
Yeast has proved to be the most useful single-celled organism for studying the fundamental aspects of cell biology. Resources are now available for yeast that greatly simplify and empower new investigations, like the presence of strains with each gene deleted, each protein tagged and databases on protein–protein interactions, gene regulation, and subcellular protein location. A powerful combination of genetics, cell biology, and biochemistry employed by thousands of yeast researchers has unraveled the complexities of numerous cellular processes from mitosis to secretion and even uncovered new insights into prion diseases and the role of prions in normal biology. These insights have proven, time and again, to foretell the roles of proteins and pathways in human cells.

The collection of articles in this volume explores the use of yeast in pathway analysis and drug discovery. Yeast has, of course, supplied mankind’s most ubiquitous drug for thousands of years. In one aspect, the role of yeast in drug discovery is much like the role of yeast in other areas of biology. Yeast offers the power of genetics and a repertoire of resources available in no other organism. Using yeast in the study of drug targets and metabolism can help to make a science of what has been largely an empirical activity. A science of drug discovery would permit rigorous answers to important questions. What is the target of the drug? Is there more than one target and what are the relative affinities? What is the physiological consequence of inactivating a particular protein? Which drug in a panel is the most specific? How many ways can a cell mutate to resistance? What is the consequence of inhibiting two proteins? Which proteins in the cell if inhibited would produce a desired physiological outcome? Are all the proteins in a pathway equivalent targets? Can one identify drugs that alter the location or interactions of proteins without affecting their activity? Each of these questions can be rigorously answered in yeast but not in most other...
systems. Questions like these have rarely been answered in the field of drug discovery.

A more challenging question is: Can yeast be made more applicable for the discovery of drugs against human targets? While many human drugs are active in yeast many are not. The lack of effect in yeast can be due to the fact that yeast does not have the targets at all – e.g., cell surface hormone receptors, or because the orthologous protein in yeast is sufficiently different, or that yeast cells have redundant proteins and inhibition of one is masked by the second. However, even if drugs against human targets are active in yeast, the yeast orthologue is likely to be different enough to preclude optimization of drug identification in yeast. One way to solve this problem is by substitution of human orthologues for yeast ones. One could even substitute human transport proteins and drug-metabolizing proteins to further optimize the yeast system.

Moreover, our ultimate interest in drugs is to alter physiology, which is the product not of single proteins but of pathways and networks of proteins acting in concert. The most effective use of yeast would probably result from substituting entire pathways of yeast proteins with their human counterparts. With the aid of reporters that quantitatively reveal the activity of the pathway at different points one could enter a new era of drug discovery that interrogates modulation of the pathway at different sites. Finally, we should think about using the same approach for pathways that do not normally exist in yeast – for example pathways that synthesize hormones. By constructing the pathway in yeast \textit{de novo} with appropriate reporters one could screen for drugs that modulate hormone synthesis and easily localize the target in the pathway. This sounds like fun. I suspect the dough has only begun to rise on what yeast has to offer in the arena of drug discovery.
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INTRODUCTION

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In 1970, Lee Hartwell reported a series of genetic experiments showing that progression through the cell cycle in yeast was amenable to genetic analysis. At about the same time, several investigators, including Michael Resnick and Brian Cox identified the first yeast mutants that were shown to be defective in DNA repair processes. Walt Fangman and his co-workers were characterizing the basics of yeast chromosomes and yeast DNA replication (even though cytogenetics was not practical, and because yeast lack thymidine kinase, pulse labeling of DNA was not possible). Gerry Fink was identifying the many ways a eukaryote regulates gene expression, while Fred Sherman carried out studies on cytochrome c that illuminated translation (and much else). Other investigators were becoming convinced that yeast could shed light on many fundamental processes that were not accessible in multicellular eukaryotes. Since many investigators committed to using yeast as an experimental system, there was also considerable efforts to increase the scope of yeast genetics by developing new genetic tools, which became an effort to develop molecular, biochemical, and cell biological tools. The important tools developed in yeast are too numerous to mention (although the discovery of gene replacement by homologous recombination surely requires note). Contemporary biologists, even those studying “large” eukaryotes, continue to learn from yeast systems.

Despite the impressive roster of accomplishments in basic biology obtained using yeast as a model, there are areas of importance of cancer research where yeast has not been extensively utilized. In addition, many investigators and clinicians working in many areas of cancer research tend not to think of yeast as being relevant to their areas of interest. In some, cases, yeast researchers have not made the appropriate effort to communicate their results to the cancer research community. The goal of this book is to highlight the contributions that yeast systems have made to in a variety of
Introduction

areas of cancer research. Accordingly, this volume is intentionally directed more to workers outside the "yeast world" and toward investigators interested in cancer. We have requested the authors to highlight areas where yeast-based systems have made contributions not readily accessible with other experimental systems, and to try to communicate clearly to workers who may not be familiar with yeast.

This book is broadly organized into three sections. The first section, including Chapters 1 through 8 highlight areas of biology that are particularly relevant to cancer research. These include studies of DNA metabolism (Chapters 1, 2, and 7), protein localization and trafficking (Chapters 3, 4, and 6), and cell immortalization (Chapter 5). Chapter 8, a discussion of sphingolipids, is relevant both to the biology, and potentially, the development of novel cancer treatments.

The second section, Chapters 9 and 10 describe how yeast can be used to study human p53. These chapters highlight the ability to learn about the function of human oncoproteins using yeast.

The third section is broadly concerned with studying anticancer drugs in yeast. Some of the chapters discuss concerns broadly relevant to drug action (Chapters 11 and 14), while the actions of specific anticancer drugs, such as rapamycins, platinum compounds, and topoisomerase inhibitors are explored in Chapters 13, 15, and 16. Finally, Chapter 12 describes one broad effort to use yeast as a tool for drug discovery.

There are many other areas of interest not included in this volume where yeast systems have made important contributions to cancer research. These areas include important methodologies such as yeast two hybrid, areas of basic biology such as the study of yeast Ras proteins and yeast kinases, and areas of great relevance to anticancer drugs, such as yeast systems of DNA repair. While we hope to include such topics in future volumes, we also felt that there were other superb sources already available for topics such as a general introduction to yeast.

This book would not have been possible without the efforts of Peggy Vandiveer in the Word Processing Center at St. Jude Children’s Hospital. Peggy carefully formatted all of the chapters and cheerfully and quickly handled a huge amount of work. Thanks are also due to Jeffrey Berk and Aman Seth in the Nitiss laboratory, who carefully checked all of the chapters and caught many things that might have slipped through. Support for the generation of this book was provided to JLN by the American Lebanese Syrian Associated Charities (ALSAC).
Chapter 1

FROM DNA REPLICATION TO GENOME INSTABILITY IN SCHIZOSACCHAROMYCES POMBE: PATHWAYS TO CANCER

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1 INTRODUCTION

The genetic integrity of cells depends on the complete, accurate replication of each genome cell cycle. Cells are particularly susceptible to genetic changes during the DNA synthesis (S) phase of the cell cycle, because only one complete copy of the DNA template exists, and potentially damaging breaks and unwinding occur as part of the replication process. Genome instability may result in deletion or amplification of genetic information within a chromosome, translocation of part of a chromosome to another chromosome, or gain or loss of whole chromosomes. These changes, in turn, can have important consequences for chromatin structure and gene expression. In wild-type cells, DNA replication is tightly regulated and involves multiple mechanisms that prevent amplification or loss of genetic information. In addition, internal controls such as checkpoints delay or arrest replication if active replication forks are blocked. Cancer cells, in contrast, are characterized by uncontrolled proliferation and chromosome instability.

The fission yeast Schizosaccharomyces pombe provides an outstanding model for studies of replication and chromosome dynamics, with replication origins and centromeres that are similar to those of metazoans [46, 205]. This review will focus on DNA replication in S. pombe and its role in maintenance of genome integrity. We will consider the choice and
organization of origins of DNA replication, the proteins that assemble at origins and those that are responsible for actual DNA synthesis. We will also describe regulatory mechanisms that promote origin firing, control the timing of the replication program, and limit replication to once per cell cycle. Then, we will discuss the cellular response to blocks to DNA replication. Finally, we will evaluate how studies of DNA replication in fission yeast and other model organisms have demonstrated relevance to cancer biology, concentrating on those pathways that, when disrupted, lead to genome instability and progression toward cancer.

1.1 DNA replication origins and the origin recognition complex

The fission yeast genome, like that of other eukaryotes, is divided among multiple linear chromosomes each requiring multiple origins of replication. DNA synthesis must therefore be coordinated both between the different chromosomes and within each individual chromosome. The identification of specific fragments of the genome that could replicate autonomously on plasmids provided the first step toward identifying *S. pombe* origins of replication. The autonomously replicating sequence (ARS) elements in *S. pombe* that have been described thus far are at least 500 bp in size and correspond to intergenic regions of the genome [24, 53, 114, 203]. Interestingly, centromeric regions of *S. pombe* chromosomes appear to be enriched for DNA fragments with ars activity [170]. In some chromosomal regions, two or more origins are clustered close together [40, 147]. Replication initiates from discrete, defined sites within each origin region [24, 39, 40, 53, 147, 203].

*S. pombe* replication origins preferentially lie in adenine–thymine (A–T) rich regions of the genome where the local sequence of As and Ts is asymmetric [210]. Although short consensus sequences similar to that of the *S. cerevisiae* ARS element have been identified within some of the *S. pombe* origins, these are not essential for origin activity [114]. Fine structure examination of individual origins has revealed multiple short stretches of DNA where replication initiates as well as adjacent accessory sequences that promote origin function [29, 39, 85]. Thus, the organization of *S. pombe* replication origins appears to be modular, with redundant, dispersed, and degenerate elements contributing to efficient activity.

Different replication origins display distinct firing efficiencies [147]. It is not known whether the choice of origins is regulated or stochastic. Within those regions of the chromosome where origins are clustered, there may be a hierarchy of preferential origin usage determined at least in part by local enhancer sequences [84]. In the *S. pombe* genome, the number of potential
replication origins has been estimated at 1 per 20 kb [114] to 1 per 55 kb [203]. The recent publication of the *S. pombe* genome sequence [205] should facilitate genome-wide identification of replication origins as has recently been described for budding yeast [153, 206], as well as provide information about origin usage under different growth conditions.

Replication initiation at origins requires the association of multiple conserved proteins with the origin region [41, 80]. As in other eukaryotes, the *S. pombe* origin recognition complex (ORC) binds to the origin DNA and marks it as a potential site of replication initiation [27, 28, 179]. ORC is composed of six related proteins (ORC1–6), each one of which is essential and conserved in eukaryotic organisms [13]. In some organisms, several of the ORC subunits may contact the origin DNA [13]. In *S. pombe*, ORC binding to the origin DNA is mediated by the Orp4 subunit, which contains multiple A–T hooks [27, 28, 90]. This motif, which is not present in the Orc4 subunit of other eukaryotes, is thought to promote protein binding within the DNA minor groove in regions that are A–T rich [27].

Unexpectedly, *S. pombe* ORC can bind to multiple specific sites within a single replication origin [28, 179]. These sites correspond to the regions of the origin where replication initiates as determined by two-dimensional gel analysis [179]. Thus, the position of the ORC complex within the origin may determine the location and direction of the replication machinery. It is not known whether *S. pombe* ORC also associates with nonorigin DNA, as has been described for other eukaryotes and which may reflect the additional role of ORC in heterochromatic silencing [13, 206].

The majority of ORC protein in *S. pombe* associates with chromatin, including replication origins, throughout the cell cycle [107, 146]. The Orp2 (Orc2) subunit, originally identified as a cyclin-dependent kinase (CDK) binding protein, contains consensus sites for CDK phosphorylation [95]. Orp2 becomes phosphorylated in a CDK-dependent manner during mitosis, and is dephosphorylated during G1/S of the cell cycle [107, 197]. It remains to be determined whether other ORC subunits also become modified during the cell cycle.

## 2 THE PREREPLICATIVE COMPLEX AND LICENSING OF ORIGINS

The preparation for S phase begins as early as mitosis (M phase) or G1, when additional proteins required for DNA replication assemble sequentially at replication origins to form the prereplicative complex (preRC). A series of additional steps is required to convert the preRC into an initiation complex that actively synthesizes DNA. While the proteins and general pathways
involved in this process are conserved in eukaryotes, many of the details and order of their functions are still incomplete. Figure 1 provides a model of our current view of the assembly and activation of proteins at replication origins.

![Figure 1: Model of assembly and activation of the preRC based on information from multiple systems. Cdc18 and Cdt1 bind to ORC at the origin and facilitate the loading of the MCM protein complex. The preRC is activated by the actions of the CDK and Hsk1/Dfp1 kinases ("P" indicates phosphorylation). This promotes the association of other replication factors with the complex localized to the origin, and leads to initiation of DNA synthesis. Many of the relevant substrates of Hsk1 and CDK have yet to be identified.]

The *S. pombe* Cdc18 (Cdc6 in other eukaryotes) and Cdt1 proteins bind to origin sites marked by ORC [55, 95, 141]. ORC and Cdc18 physically interact [28], as do Cdc18 and Cdt1 [141]. ORC, Cdc18, and Cdt1 together recruit the minichromosome maintenance (MCM) proteins to the complex assembling at origins [79, 146]. Genetic interactions between the MCM proteins and Cdc18, and the MCM proteins and ORC, have been demonstrated in *S. pombe* [49, 55, 101, 102]. Although each of the preRC components is essential for DNA replication, the different proteins carry out distinct functions in assembly and activation of replication origins.

The Cdc18/Cdc6 and Cdt1 proteins are thought to act as a “licensing factor” that is a critical determinant of the onset of DNA replication [81, 109, 142]. Expression of *S. pombe* Cdc18 and Cdt1, which is controlled by the Cdc10 transcription factor [65, 81], is restricted to the G1/S window of the cell cycle [81, 141]. In addition, the Cdc18 protein is regulated by phosphorylation, which targets Cdc18 for ubiquitin-mediated proteolysis [68, 69]. It is not known whether *S. pombe* Cdt1 protein levels are also regulated. In *Xenopus*, Cdt1 activity is inhibited by association with another protein, geminin [16]; however, a geminin homolog has not been described in yeast. The cell cycle-regulated activity of Cdc18 and Cdt1 both promote
Chapter 1: Replication in Fission Yeast

preRC assembly and prevents its reassembly until the next cell cycle [17, 80].

The MCM complex is not only a preRC component, but also a compelling candidate for a replicative helicase [41, 80, 98, 148]. The complex is composed of six homologous subunits (MCM2–7) with similarity to a large family of ATPases [91]. Each subunit is conserved in other eukaryotes, and each is essential for replication and cell viability [148, 193]. Mutation of individual *S. pombe* MCM genes results in defects in replication, irreversible S-phase arrest and delocalization of the entire MCM complex from the nucleus [34, 49, 101, 149]. The *mcm* mutant arrest occurs with the bulk of replication completed and requires the DNA damage checkpoint, suggesting that the chromosomes are damaged in *mcm* mutant cells [101, 102, 108, 123, 178].

Interestingly, although the MCM proteins are estimated to be at least tenfold more abundant than the predicted number of origins, reducing the amount of a single MCM protein results in chromosome instability and defects in the completion of S phase [49, 102]. Replication can still initiate with low levels of MCM protein, suggesting that the amount of MCM protein required to initiate DNA replication is much less than that needed to complete S phase [102]. Genetic interactions between *S. pombe* MCM genes and factors involved in the elongation step of DNA replication suggest that MCM complex function is required throughout S phase [49, 102]. This is consistent with more direct experiments in *S. cerevisiae*, where specific degradation of one of the six MCM subunits during S phase blocks further DNA replication [94, 103]. The current model suggests that a heterohexameric MCM complex with all six MCM subunits is present at replication origins, and then travels with the replication fork [78, 148, 193].

Although all six *S. pombe* MCM subunits interact [1], distinct MCM subcomplexes have been identified in vitro and in vivo, suggesting that individual MCM proteins have different relative affinities for each other [96, 162, 163]. The Mcm4, 6, and 7 proteins are thought to form a “core” complex that is tightly associated [96, 163]. Mcm2 associates with this core through interactions with Mcm4 [163]. The Mcm3 and 5 proteins form a dimer that is also loosely associated with the MCM core proteins [162], probably through interaction with Mcm7 [101]. Similar subcomplexes of MCM proteins have also been described in other eukaryotes [67, 160]. The core complex of Mcm4, 6, and 7, but not the heterohexameric complex of Mcm2, 3, 4, 5, 6, and 7, demonstrates weak helicase activity in vitro in *S. pombe* [96] and in human cells [67]. Curiously, point mutations of conserved residues in each Mcm protein that are predicted to inhibit ATPase or helicase activity display different effects in vivo in both *S. pombe* [49, 52] and *S. cerevisiae* [160] depending on the subunit mutated. This suggests division of labor
amongst the MCM subunits, and may explain why six related proteins are required for MCM activity.

Although MCM nuclear localization is regulated in budding yeast, MCM proteins localize to the nucleus constitutively throughout the cell cycle in fission yeast and in metazoans [149, 193]. In these organisms, the association of the MCM proteins with chromatin varies: MCM proteins localize to chromatin, including replication origins, in late M phase and dissociate during S phase [79, 146]. Thus, regulation of MCM chromatin binding is one mechanism of control of MCM complex function. Part of this regulation is provided by the Cdc18 and Cdt1 loading factors, which limit MCM binding to M/G1 of the cell cycle [80]. In some organisms, Mcm4 is also regulated by CDK-dependent phosphorylation. In *Xenopus*, this promotes Mcm4 dissociation from chromatin [62, 152]; in *S. cerevisiae*, Mcm4 phosphorylation leads to its exclusion from the nucleus [140]. However, CDK consensus site mutants of *S. pombe* Mcm4 do not display obvious phenotypes in vivo [52].

3 REPLICATION INITIATION REQUIRES TWO DISTINCT PROTEIN KINASE ACTIVITIES

Although several of the proteins required for DNA replication are assembled at replication origins prior to the start of S phase, origin firing and initiation of DNA synthesis requires the activities of two protein kinase complexes, the CDK [17] and DDK (Dbf4-dependent) kinases [73, 110, 161]. While some substrates of these protein kinases have been identified, it is clear that our understanding of their role is still incomplete. In fission yeast, there is a single CDK, *cdc2*+, that functions as the major regulator of cell cycle transitions including S phase onset [127]. Importantly, the ability of *S. pombe* Cdc2 to functionally complement cross-species [11, 97] suggests that the principles of function and regulation of the CDKs are conserved in eukaryotes.

*S. pombe* Cdc2 associates with different cyclins in different stages of the cell cycle. The G1/S phase transition is promoted by the assembly of Cdc2 with the B-type cyclin Cig2 [45, 125]. In mitosis, Cdc2 associates with the Cdc13 cyclin [127]. Fission yeast CDK activity varies through the cell cycle, and this global regulation controls the dependency of S phase on completion of mitosis and prevents multiple rounds of replication within a single cell cycle [21, 31, 126].
Figure 2. Control mechanisms that prevent rereplication. CDK activity is regulated so that the preRC can only assemble once each cell cycle. CDK phosphorylates Drc1, which promotes association with Rad4/Cut5 and replication initiation. CDK phosphorylation of Cdc18 and Orc2 prevents origins from refiring.

Remarkably, the CDK kinase complex plays both positive and negative roles in S phase progression (Figure 2). Replication initiation is positively activated by CDK-mediated phosphorylation of Drc1, which results in the association of Drc1 with Rad4/Cut5 [143]. Rad4/Cut5 is essential for the initiation of DNA synthesis and also acts in the DNA damage checkpoint [44, 156, 157]. Although the molecular role of Rad4/Cut5 is unclear, the interaction between Rad4 and Drc1 may promote the association of the DNA polymerases α and ε with origins [143]. The S. pombe homologs of Rad4/Cut5 in S. cerevisiae (Dpb11) and in human cells (TopBP1) interact with DNA polymerase as part of their replication function [112, 182]. Each of these proteins contains BRCT motifs, which are also found in the human DNA repair gene XRCC1 and the BRCA1 tumor suppressor [19].

CDK activity negatively regulates DNA replication by preventing origins from refiring in a single cell cycle [17, 80]. CDK-mediated phosphorylation of Cdc18 and its subsequent degradation prevent reassembly of the preRC until the next cell cycle, because recruitment of preRC factors such as the MCM proteins depends on Cdc18 [68]. Overproduction of Cdc18 or expression of a nonphosphorylated version of Cdc18 induces re-replication, presumably by resetting origins to the G1 state [80]. Mutation of the CDK phosphorylation sites in Orp2 also allows re-replication [197], although the mechanism by which CDK-mediated phosphorylation of Orp2 prevents re-replication is not clear [95, 107, 197]. Unlike Cdc18, Orp2 appears to remain associated with the ORC complex at the origin [107]; it is not known whether phosphorylation of Orp2 changes its affinity for other origin-associated proteins.

Manipulation of the Cdc2 protein kinase itself can result in re-replication. Cells that lack mitotically active Cdc2 [21] or the mitotic cyclin Cdc13 [61] re-replicate DNA, as do cells overproducing the CDK inhibitor
Rum1 [126]. These observations demonstrated that increased levels of CDK kinase activity after the G1/S transition are required to prevent reinitiation of DNA replication within a single cell cycle. Re-replication appears to mimic a normal S phase because it requires all of the proteins that function in DNA synthesis in a normal S phase [172]. This suggests that re-replication results solely from a disruption of the order of the cell cycle.

CDK kinase activity is not the only control exerted over the onset of S phase. Initiation of DNA replication also requires the activity of the Cdc7 protein kinase [161]. Hsk1, the *S. pombe* homolog of Cdc7 [23, 111] associates with a subunit, Dfp1 (Dbf4 in other eukaryotes), which is required for Hsk1 kinase activity toward its substrates [23, 180]. Although the levels of Hsk1 protein appear to be constant in different phases of the cell cycle, Dfp1 levels and the associated Hsk1-dependent protein kinase activity are specifically upregulated during G1/S [22]. The Mcm2 protein is a substrate of Hsk1/Dfp1 in vitro [22] and in vivo [181]. However, the biological effect of Mcm2 phosphorylation remains to be determined. It is also possible that Hsk1 has additional, as yet unidentified, substrates in vivo; in *S. cerevisiae*, DNA polymerase α and another replication factor, Cdc45 (the homolog of *S. pombe* Sna41), are targets of Cdc7 [82, 145, 201].

*S. pombe* Hsk1 has three apparent roles in the cell. First, Hsk1/Dfp1 may activate individual origins of replication by phosphorylating components of the preRC, as has been described for Cdc7 in *S. cerevisiae* [20, 37]. This role for Hsk1 may also involve control of the temporal order of origin firing, since the kinase is a potential target of the replication checkpoint [22, 171, 181]. Second, Hsk1 is involved in the recovery from replication blocks such as hydroxyurea [171]. Third, Hsk1 may influence the establishment of sisterchromatid cohesion during S phase [8, 171, 181]. The potential mechanisms of these activities are discussed below.

### 4 ADDITIONAL REPLICATION FACTORS

Once the preRC is formed and activated by the CDK and Cdc7 protein kinases, additional components of the replication machinery become associated with the complex, which becomes the active replication fork. Many of these proteins are essential for viability and conserved in all eukaryotes, including Cdc23/Mcm10, Sna41/Cdc45, the single-strand DNA binding protein replication protein A (RPA), DNA polymerase α DNA polymerase δ DNA polymerase ε DNA, replication factor C (RFC), and proliferating cell nuclear antigen (PCNA) [198].

*S. pombe* cdc23 mutants, like the corresponding mutants in other eukaryotes, display defects in replication and demonstrate genetic
interactions with other replication mutants [7, 77, 100]. A mutant of the 
*S. cerevisiae* homolog of *cdc23*+, MCM10, can be complemented by
*S. pombe* *cdc23*+, suggesting conservation of gene function [7]. *S. cerevisiae*
MCM10 appears necessary to recruit the MCM complex to chromatin [66],
and also functions in replication elongation [116]. In both *S. cerevisiae* and
*S. pombe*, Cdc23 associates with chromatin throughout the cell cycle [66, 101]. In *Xenopus*, MCM10 is dispensable for MCM complex binding to
chromatin but is required to localize Cdc45/Sna41 [204].

Sna41/Cdc45 appears to be required both for replication initiation and
eelongation. The association of Sna41 with chromatin requires the Slr3
protein, which has been described in both the budding and fission yeasts [74, 136]. Sna41/Cdc45 is required to load other replication factors such as RPA,
DNA polymerase α, DNA polymerase ε, and PCNA [182]; in *S. pombe*,
Sna41 [124, 194] has been shown to interact with DNA polymerase α [195] and
recruit it to the MCM protein complex at replication origins [195]. A
role for Sna41/Cdc45 in origin DNA unwinding has been suggested from
experiments in *Xenopus* [199] and in *S. cerevisiae* [74]. In *S. cerevisiae*,
Cdc45 function has been shown to be required throughout S phase [189].

Many of the genes involved in DNA replication in *S. pombe* are
homologous to those described in other eukaryotes, and the enzymology of
the gene products is conserved [198]. RPA is needed to recruit the DNA
polymerases α, δ, and ε to replication origins [198]. Interestingly, DNA
polymerase α may have an additional function beyond replication initiation
and DNA synthesis (see section VIII). The eukaryotic DNA polymerase
processivity factor PCNA becomes associated with the replication fork
through the actions of the clamp loader, RFC [198]. *S. pombe* PCNA also
interacts with DNA polymerase δ [155] and promotes its processivity [6].
Components of *S. pombe* RFC and primase are involved in the replication
checkpoint as well as in DNA synthesis [56, 164].

Several proteins involved in lagging strand metabolism, such as DNA
ligase [72], the Dna2 helicase [54, 75], and the homolog of the FEN1
endonuclease, Rad2 [132] have also been characterized in *S. pombe*. The
dna2 mutant can be suppressed by overproduction of DNA polymerase δ,
DNA ligase or Rad2, suggesting that Dna2 has a central function in Okazaki
fragment maturation [75]. Mutants of a novel *S. pombe* replication factor,
cdc24+, are suppressed by overproduction of Dna2, as well as by
overproduction of PCNA or RFC [54, 184]. *S. pombe* *cdc24*+ has no known
sequence homolog in other systems [54]. Mutants of *cdc24*+ are defective in
the completion of S phase and display chromosome breakage, which is
not typical of replication mutants [54]. Cdc24 thus may play a role in
maintenance of chromosome integrity during S phase, perhaps through a function in Okazaki fragment maturation.

5 TIMING OF REPLICA TION ORIGIN FIRING

Coordination of multiple replication origins on multiple chromosomes is essential to maintain stability of the genome. In wild-type cells, there is a temporal program of origin firing: some origins fire early in S phase, others are active in the middle of S phase, and still other replication origins do not initiate synthesis until late in S phase. In general, heterochromatic regions are late-replicating in most organisms [208]. However, a recent analysis of replication origins in *S. pombe* [86] suggests that a centromeric origin in *S. pombe* replicates early in S phase. In contrast, origins located within other regions of heterochromatin, in particular the telomere and the rDNA, replicate late in S phase. Although most [14, 16] of the origins analyzed in this study [86] replicate early in S phase, it remains to be determined whether these are representative of other *S. pombe* origins.

Control of the timing of origin firing has been investigated using the drug hydroxyurea (HU) to block cells in S phase. HU treatment causes an array of lesions in the cell, including inhibition of the enzyme ribonucleotide reductase, which depletes the nucleotide pools and prevents further DNA replication [80, 106, 158, 188]. For the few origins that have been analyzed in *S. pombe*, HU treatment prevents the activity of normally late-firing origins, but does not restrain early origin firing [86]. This mechanism is checkpoint-dependent [86], as both early and late origins are active in mutants lacking the checkpoint kinases Rad3 (a homolog of human ATM) or Cds1 (the equivalent of human CDS1/CHK2 and budding yeast Rad53). Checkpoint-dependent inhibition of late origin firing was previously described in budding yeast [158, 166], and the conservation of replication timing control between these divergent yeasts suggests that a similar mechanism should also operate in human cells.

6 RESPONSE TO REPLICA TION BLOCKS

During S phase progression, active replication forks may encounter blocks to further synthesis. In *S. pombe*, HU-induced arrest has been most often used to study the effects of blocks to replication. Cells treated with HU undergo checkpoint-mediated arrest in S phase that depends on Cdc2 and six “checkpoint rad” proteins [25]. Wild-type cells can recover from HU treatment, resulting in restart of DNA replication, the completion of S phase
and subsequent progress through the cell cycle. This suggests that replication fork structure is preserved during S phase arrest caused by HU treatment.

The checkpoint kinase Cds1 may play a role in stabilization of stalled replication forks. *S. pombe* *cds1* mutants arrest in HU, but are defective in recovery from the arrest [104]. The HU-induced arrest of *cds1* cells results from the activation of the damage checkpoint kinase Chk1, suggesting that Cds1 is needed to prevent chromosomal damage in the presence of HU [104]. Consistent with this hypothesis, aberrant replication structures are observed in *S. pombe* *cds1* and *rad3* mutants treated with HU [86]. Loss of replication fork integrity has also been observed in *rad53* mutants of *S. cerevisiae*, suggesting a conserved mechanism of damage tolerance [106, 188].

*S. cerevisiae* Rad53 is phosphorylated in response to S phase damage [150, 188] and phosphorylates components of the replication machinery to restrain its progression [150]. Similarly, *S. pombe* Cds1 becomes phosphorylated in response to HU treatment [104]. This in turn leads to Cds1-dependent phosphorylation of both Hsk1 and its activator Dfp1, suggesting that Hsk1 and Dfp1 are targets of the cellular response to replication blocks [22, 171]. These phosphorylation events may serve to regulate the interaction of Hsk1/Dfp1 with proteins localized to replication origins and to prevent the activation of origins under conditions unfavorable to the cell [70, 110].

Although *S. pombe* Hsk1 and Dfp1 have an essential role in activation of replication origins, Cds1 is dispensable during a normal S phase [104, 129]. Recently, an S-phase specific upstream activator of Cds1, Mrc1, was identified [3, 185]; both Mrc1 and Cds1 are nonessential for cell viability [3, 129]. Future work should determine how the cell senses blocks to replication and how this information is directed to Mrc1 and Cds1.

Recovery from HU-induced S phase arrest also requires Rqh1, the fission yeast homolog of the *Escherichia coli* RecQ helicase [175]. The wild-type function of Rqh1 during S phase is not known but may involve preventing inappropriate recombination events when replication forks are stalled [175]. Mitotic recombination is elevated in *rqh1* mutants that are treated with HU [131, 175]. Mutants of *rqh1* also display synthetic genetic interactions with components of the replication machinery [131, 171]. In *S. cerevisiae*, the Rqh1 homolog *SGS1* localizes with the Rad53 kinase and promotes its phosphorylation in response to HU [51]. However, *SGS1* mutants are hyperrecombinant even in the absence of HU, suggesting there may be some differences in the function between *SGS1* and *Rqh1* [51].
Several replication mutants display increased levels of recombination, suggesting a link between these processes. Certain alleles of *S. pombe* DNA polymerase α, DNA ligase, and rad2+ have mutator phenotypes [105]. The increase in mutation frequency in these mutants suggests that the corresponding wild-type proteins prevent genome changes and rearrangements, which may result from recombination during S phase. Recombination is elevated in *mcm* mutant cells that have been arrested in S phase [102]. In addition, *S. pombe rad2* mutants are synthetically lethal in combination with mutants of *rad50*, *rhp51*, or *rhp54* (the *S. pombe* homologs of *RAD50*, *RAD51*, and *RAD54*), suggesting that recombination functions become essential when Okazaki fragment metabolism is compromised [58, 130, 132]. The association of impaired replication function with increased recombination has also been described in *S. cerevisiae* [36, 117] and prokaryotes [119], suggesting this is a general feature of S phase.

Conversely, certain recombination mutants display S phase defects. In the *S. pombe rad50* mutant, S phase is delayed relative to wild type and the cells are sensitive to HU [58]. In vertebrate cells, inactivation of the recombination proteins Rad51 [173] or Mre11 [32] leads to DNA strand breaks and cell lethality. These and other observations have led to the suggestion that recombination proteins are normal components of S-phase progression in eukaryotes that protect genome integrity [133, 135]. Thus, replication fork stalls and starts may occur as part of normal S phase in eukaryotes, as has been described in prokaryotes [33, 119].

There are several possible consequences of a stalled replication fork, which may depend on its cause. Ideally, fork structure is protected and its components remain assembled during the arrest (Figure 3). However, the fork may lose structural integrity if this protection fails, resulting in its collapse and the generation of DNA breaks; these breaks are likely to be lethal to the cell if they are not repaired [33, 119].

Recombination is one mechanism that can reestablish a replication fork from a DNA break [33, 120]. Although recombination-dependent replication has been best characterized in prokaryotes, there is evidence that a similar process operates in eukaryotes. In *S. cerevisiae*, break-induced replication (BIR) can replicate hundreds of kilobases of DNA starting from a chromosomal break [92]. In *S. pombe*, cells lacking telomerase can replicate telomere sequences, presumably by a recombinational mechanism [137]. Importantly, replication mediated by recombination is predicted to be independent of replication origins and origin proteins. Thus, there may be mechanistic links between recombination and replication throughout S phase.
in *S. pombe* and other eukaryotes, which are likely to be significant for the maintenance of overall genome stability.

![Diagram of replication restart](image)

Figure 3. Model for replication restart (based on 33,119). When cells are treated with HU, replication forks stall. If the structure of the fork can be maintained through the arrest, then the fork may resume synthesis once HU is removed from the media. If the fork structure cannot be maintained, the fork may collapse, generating DNA double-strand breaks. Recombination is one mechanism that may repair DNA breaks and reestablish stalled replication forks.

## 8 COORDINATION OF S PHASE EVENTS WITH MITOSIS

The events of S phase are closely linked with the later events of the cell cycle. As described above, maintenance of the order of the cell cycle and alternation of S phase with mitosis preserves the genome integrity. In addition, chromosomal processes such as sister-chromatid cohesion and silencing are regulated coordinately with the replication of the DNA. Intriguingly, the replication fork is the one structure that contacts all of the DNA, once, in a single cell cycle. This positions the replication machinery in a unique state to monitor and modify any region of the genome during S phase.

Cohesion between newly replicated sister chromatids holds them together from S phase until their separation during mitosis. This arrangement is essential for the proper attachment of kinetochores to the microtubule spindles and correct segregation of the chromatids during mitosis [139]. The
close apposition of sister chromatids throughout G2 and M phase likely also facilitates repair of damaged DNA off the homologous template [169]. Cohesion is mediated by a conserved complex of proteins first identified in *S. cerevisiae*, the cohesins [57, 118]. Homologs of the cohesins also exist in fission yeast [15, 187, 192] and have provided important insights as to how cohesion assembly is regulated during S phase.

The fission yeast Eso1 protein is critical for the activation of cohesion function, although it is not required for the assembly of cohesin proteins with chromatin [186]. Interestingly, Eso1 appears to be a fusion of two budding yeast proteins: the cohesin activator Eco1 and the DNA damage bypass polymerase Rad30. This homology suggests a direct link between cohesion and DNA damage repair.

In fission yeast, cohesion sites along the chromosome have been identified and are particularly concentrated at the repeat regions of centromeres [14, 192]. Like human centromeres, *S. pombe* centromeres are relatively large (compared to *S. cerevisiae* centromeres) and contain heterochromatin. Two recent studies indicate that the heterochromatin protein Swi6 (the equivalent of mammalian HP1) is specifically required for the efficient assembly of cohesion proteins to the centromere [14, 144]. Cohesion association with chromatin arms occurs independently of Swi6.

Unexpectedly, the Hsk1 kinase also has a role in sister-chromatid cohesion [171, 181]. Like Swi6, Hsk1 specifically influences the association of the cohesin complex with centromeres [8]. Swi6 physically interacts with both Hsk1 and its subunit Dfp1, suggesting direct recruitment of cohesin to centromeres by these replication proteins [8]. These findings suggest a role for Hsk1 and Dfp1 in sister-chromatid cohesion that may be separable from their role in replication initiation.

Replication proteins also affect heterochromatin function. Within regions of heterochromatin, including centromeres, most genes are normally silenced. At least in budding yeast, silencing involves progression through S phase, although perhaps not fork passage per se [50, 88, 99, 121]. As noted above, eukaryotic ORC proteins are required for gene silencing and localize to heterochromatin regions in addition to replication origins [13]. In *S. cerevisiae*, the PCNA [209] [43], RFC, and Cdc45 proteins also contribute to silencing [43]. In *S. pombe*, mutants of DNA polymerase α display defects in silencing [138]. DNA polymerase α interacts with Swi6 and is required for its proper localization to heterochromatin [2, 138] suggesting that disruption in silencing can be attributed to defective heterochromatin structure. *hsk1* mutants also display a defect in silencing, even though the Swi6 protein is localized to heterochromatin [8]. This suggests that heterochromatin function depends on the assembly of other proteins with Swi6; whether this requires a
passing replication fork or other aspects of S-phase progression remains to be determined.

9 DNA REPLICATION IN FISSION YEAST

MEIOSIS

Meiosis is a specialized cell cycle that generates recombinant, haploid progeny cells from a diploid cell. The meiotic cell cycle differs from the vegetative cell cycle in two outstanding respects. First, the S phase that occurs prior to meiosis (premeiotic S) is followed by two successive rounds of chromosome segregation, rather than alternating between S phase and mitosis. Second, the first meiotic division is reductional, resulting in the maintenance of cohesion between sister chromatids but the separation and segregation of homologous chromosomes. The preparation for this modified cell division cycle involves lengthy interaction between homologous chromosomes during the prophase stage of meiosis I but is likely to initiate as early as premeiotic S phase.

Premeiotic S phase is longer than S phase in vegetative cells in most organisms [26]. The cause of this difference is unclear, since experiments in budding yeast suggest that the same replication origins are active in vegetative and meiotic cells [30]. However, other experiments in S. cerevisiae suggest that meiosis-specific chromosomal factors required during prophase might assemble during premitotic STET [26].

Recent studies in S. pombe have addressed the question of whether the replication machinery that functions during premeiotic S phase is the same as that utilized in the vegetative cell cycle [48, 103, 128]. S. pombe proteins required for the actual synthesis of the DNA in vegetative cells, such as DNA polymerase α and ribonucleotide reductase, also are essential for premeiotic S phase [48]. In contrast, mutants defective in initiation of DNA replication, such as the mcm mutants and cdc18, display different phenotypes in meiosis and mitosis. In mitosis, conditional alleles of these mutants allow bulk DNA replication but cause cells to arrest in late S phase [49, 81, 102]. In contrast, in similar conditions these mutants can proceed through the meiotic divisions and sporulate [48]. With more extreme conditions, these mutants delay replication and the subsequent meiotic divisions [103, 128]. This may reflect a quantitative difference: meiotic cells may tolerate a lower amount of certain replication proteins than that needed during the vegetative cell cycle. It is also possible that other meiotic factors, perhaps recombination proteins, can contribute to premeiotic replication.

The S. pombe MCM protein complex is associated with chromatin during premeiotic S phase [103], consistent with the hypothesis that these proteins
function in premeiotic DNA replication. However, MCM proteins are not localized to chromatin in between the meiotic divisions, when an additional round of DNA replication is suppressed [103].

Similar to the vegetative cell cycle, the meiotic cell cycle is subject to checkpoint controls. Fission yeast cells that have been induced to enter meiosis block the cell cycle when treated with HU [48, 128]. As in the vegetative cell cycle, HU-induced arrest during meiosis is likely to be checkpoint-dependent. Future work should resolve the components of this response, which are likely to be essential for the viability of gametes.

Importantly, premeiotic S phase is closely coupled to the downstream events of meiosis such as recombination [47]. This has been best demonstrated in budding yeast, where blocks to premeiotic DNA synthesis prevent meiotic recombination and changes in the timing of premeiotic replication result in corresponding changes in the timing of initiation of meiotic recombination [18]. The molecular mechanism by which this occurs is still unclear [47].

10 REPLICATION: A CONSERVED PROCESS

Most, if not all, of the proteins involved or implicated in DNA replication in *S. pombe* have homologs in other eukaryotes including human cells. Analysis of these proteins in *S. pombe* and other model organisms has greatly facilitated the identification and characterization of the human counterparts. However, many of the details of regulation of the replication process differ between yeast and human cells, which may reflect internal differences in cell cycle control between the different organisms as well as the additional complexity required in multicellular organisms.

*S. pombe* cells maintain genome stability and order of the cell cycle through multiple and overlapping pathways. In particular, the process of DNA replication itself is tightly regulated so that the genome is duplicated once, entirely and accurately, in each cell cycle. Mechanisms are also in place to restrain passage into mitosis until replication is complete. It is possible that additional mechanisms, as yet uncovered, also act to maintain the integrity of the genetic material. *S. pombe* cells that have lost this control capability may re-replicate DNA, lose chromosomes, and die. In comparison, deregulation of S-phase control in human cells may result in genome instability and contribute to cancer progression.