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DEFINING RESPONSE PATHWAYS OF BUDDING YEAST CHECKPOINT GENES

by

Richard Donald Gardner

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STATEMENT BY AUTHOR

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Dedication

This dissertation is dedicated to:

My wonderful parents (Mom and Dad),

My favorite sister Rosanne,

and especially,

My loving wife, Naomi.
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ABSTRACT

Cell cycle events are ordered correctly; mitosis follows DNA replication. To ensure correct order, cells employ checkpoints that delay the cycle when DNA replication, repair, or spindle assembly have not been completed. In this dissertation, I have focused on the DNA damage checkpoint, which arrests the cell in G₂ in response to DNA damage (the G₂/M checkpoint). I have studied the roles of several checkpoint genes in the budding yeast *Saccharomyces cerevisiae* involved in the response to DNA damage, focusing on a key gene called *MEC1*. I have tested genetically where in several pathways checkpoint genes act:

**G₂/M checkpoint pathway:** I found that after DNA damage, *MEC1* signals G₂/M cell cycle arrest using two pathways, one involving *RAD53* and *DUN1*, and the other involving *PDS1*. Both pathways must be functional for full checkpoint arrest; either pathway acting alone produces only a partial arrest. I speculate why there are two pathways for arrest.

**TEL1:** I also tested the roles of *TEL1*, a putative *MEC1* homolog. I showed that *TEL1* has no normal checkpoint function. However, when overexpressed, *TEL1* produces a constitutive G₂ delay, independent of DNA damage, a delay that requires the *PDS1* pathway. This constitutive delay is responsible for the suppression by *TEL1* of the UV sensitivity of *mec1* mutants.
overexpressed, *TEL1* also restores damage-inducible transcription to *mec1* cells. I discuss *TEL1*'s possible roles in checkpoint mediated responses.

**Essential function pathway:** Previous results showed that *MEC1* and *RAD53* are also required for the transcriptional induction of repair genes and for an essential function. The nature of their essential function(s) remains unknown. My results, from a complex series of genetic tests, suggest that *MEC1* and *RAD53* share the same essential function, and that this function may in fact be related to the transcriptional function. I speculate on the nature of the essential function.

I also present evidence that *MEC1* and *RAD53* may have a role in DNA replication.

My results have led to refined models of pathways leading to checkpoint arrest, damage-inducible transcription, and an essential function(s).
CHAPTER 1: BACKGROUND

INTRODUCTION

The cell cycle and its controls are both fascinating and complicated. Precise regulation of the levels and activities of many proteins are required for proper cell cycle control. An understanding of such complicated phenomena is greatly benefited by genetic analysis, to which budding yeast is well suited.

Are studies of the yeast cell cycle relevant to the mammalian cell cycle? Many of the cell cycle control mechanisms in budding yeast and mammals are similar, having been at least partly conserved through evolution, even though budding yeast and mammals diverged long ago. For instance, the cyclin dependent kinases are conserved in yeast and mammals. The similarity of cell cycle controls in yeast and mammalian cells indicates that studies in yeast are useful for understanding mammalian cells. For example, my studies have been concerned with checkpoints in the yeast cell cycle — mechanisms which delay the cycle when triggered by DNA damage or incomplete DNA replication (for review see 52). If checkpoint delay does not occur, the cell may die or accumulate mutations (155).

The similarity of yeast cell cycle controls to their human counterparts suggests that studies in yeast may enhance our understanding of human cells and human cancers. Many human cancers have checkpoint defects which allow
mutations to occur and accumulate: Defects in the p53 checkpoint gene result in many human cancers (115), and defects in the human homolog of another yeast checkpoint gene, *BUB1*, have been implicated in most colon cancers (13). A human homolog of the yeast checkpoint gene *MEC1*, called ATM, results in ataxia telangiectasia and disposition to cancer when mutated (129). Thus, lack of checkpoint control allows the development of mutations and genomic instability. Progress in curing and preventing cancer may depend in part on our understanding of the cell cycle and cell cycle genes.

Yeast provides a powerful model organism to study the cell cycle, offering many experimental advantages over mammalian systems. Yeast is especially amenable to both genetic and molecular biological studies. Many techniques which cannot be performed in mammalian cells are easily done in yeast. For example, deleting specific yeast genes is a straightforward process, and yeast strains with multiple mutations are readily obtained through genetic crosses. In some cases yeast cell cycle controls contain fewer redundancies than do mammalian cells, making study of the cell cycle in yeast more straightforward. Yeast, for example, contains one cyclin dependent kinase, while mammalian cells contain several. Because of budding yeast's advantages as a model organism, it is not surprising that studies of this organism have led to many advances in the cell cycle field.
This chapter provides brief background information to yeast nomenclature, the yeast cell cycle in general, and to the concept of checkpoints and specific checkpoint genes in budding yeast. These topics are a prelude to studies of the $MEC1$ gene and its homologs, and to the events mediated by checkpoint genes after DNA damage, the focus of my dissertation.

**YEAST NOMENCLATURE**

In this work, I use standard nomenclature (inherently somewhat confusing) to describe genes, alleles, and proteins of budding yeast and occasionally of fission yeast. In budding yeast, wild-type genes or dominant alleles are written in capital italics, e.g. $MEC1$. Recessive alleles are written in small italics, e.g. $mec1$, to indicate all recessive mutations of $MEC1$; specific recessive alleles are written with a hyphen, such as $mec1-1$. The protein encoded by the gene is written in non-italic, with only the first letter capitalized, such as Mec1, and a final p is often (though not always) included to emphasize that a protein is referred to (Mec1p).

In the fission yeast *Schizosaccharomyces pombe*, by contrast, wild-type genes are written in small italic letters followed by a + sign, such as $rad3^+$. Recessive alleles are written without the + sign, and alleles are designated when appropriate ($rad3-1$). Proteins are written in lower case non-italicized letters ($rad3$).

Unfortunately the confusing differences in nomenclature (which arose historically) are not longer reconcilable.
YEAST CELL CYCLE

The eukaryotic cell cycle is divided into four phases: G_1, S, G_2, and M. G_1 (for Gap) is a growth control phase in which cells commit to another cycle after specific nutrient and size conditions have been met. In the S (synthesis) phase, the genome is replicated. G_2 is another control phase in which the cell cycle can be delayed if DNA damage has occurred, and in the M (mitosis) phase, the duplicated chromosomes segregate.

There are variations on this cell cycle theme. For example, early embryonic cells lack G phases (reviewed in 95), and many mature mammalian cells exit the cycle and become terminally differentiated in a state known as G_0. Yeast cells grown in liquid media until saturation eventually deplete the nutrient supply and also enter a G_0, or stationary phase. The meiotic cycle is also different than the normal mitotic cycle: it includes two rounds of cell division but only one round of DNA replication. DNA breaks that occur as a normal part of meiosis trigger a cell cycle delay, just as DNA damage triggers a G_2 delay in the mitotic cycle, as discussed above.

A particular decision point in G_1 has been designated as "START". As the cell passes this point it commits to undergo a complete cycle (95). Cells must normally achieve a minimum size and have completed any necessary DNA repair before passing START. Soon after passing START, DNA replication begins and
the yeast cell starts budding. The G₂ phase provides another opportunity for the cell to delay its cycle in response to DNA damage. After mitosis, when the nucleus is divided between the mother and daughter, cytokinesis occurs and the bud separates from the mother cell (See Figure 1-1).

Figure 1-1. The budding yeast cell cycle. Bud sizes and nuclear division at each stage are shown. A schematic representation of the chromosome cycle shows a single chromosome in G₁, a replication bubble in S phase, the sister chromatids in G₂, and their separation in M. The figure also shows that cells can reversibly enter a G₀ state when nutritional conditions are poor.

**CDC Genes**

A large set of proteins is required to regulate the cycle, and to ensure that the many steps are performed in the correct order. Many cell cycle proteins are required at specific times for the cell to correctly complete its cycle. If one of these proteins is inactivated, the cell cycle is often arrested at a discrete point.
For example, DNA polymerase delta is required during S phase to complete replication, and its inactivation leads to arrest of the cell cycle in S phase. Mutants in the budding yeast genes encoding proteins required at specific times of the cell cycle are called *cdc* mutants (for cell division cycle). They were first discovered in a large screen by Hartwell (51), and subsequently discovered in fission yeast as well. I have used certain *cdc* mutations, especially *cdc13*, both to arrest the cell cycle and to generate DNA damage.

**Cyclin Dependent Kinases**

A substantial portion of cell cycle regulation occurs through the phosphorylation/dephosphorylation and/or degradation of regulator proteins (reviewed in 26). A key regulator in cells from all organisms is a protein kinase called the cyclin dependent kinase (CDK). This kinase is a heterodimer, and consists of a kinase subunit and a cyclin subunit. Activity of this kinase regulates the transition from START to S phase and from G2 to M. The kinase itself is regulated in part by the cyclin subunit. Cyclins are proteins that are synthesized and degraded in a stage-specific manner (for a general review of cyclins see 113; an excellent article that summarizes the role of yeast cyclins is 132). At specific times during the cell cycle, different cyclins are synthesized, form heterodimers with the CDK, then are degraded as a new type of cyclin appears (Figure 1-2). (For a review of the role of protein degradation in cell cycle progression, see 26).
The cyclin dependent kinase, along with its cyclin partners, has been referred to as the "engine" that drives the cell cycle (96). The primary importance of CDKs and cyclins has long been recognized; for instance, the complex of a CDK and the M-phase producing cyclin was initially named MPF, for Mitosis or Maturation Promoting Factor, in 1971 (91) (see also 57, 77). Subsequently, CDK/cyclin complexes have been identified in all eukaryotic cells.

Although regulation of CDK/cyclin activity is important in checkpoint mechanisms in many organisms, our understanding of the roles of CDK/cyclins has not led to a full understanding of checkpoint arrest in budding yeast after DNA damage.

The budding yeast cyclin dependent kinase is encoded by \textit{CDC28} (\textit{cdc2} in fission yeast). Because Cdc28 controls the G\textsubscript{1}/S and G\textsubscript{2}/M transitions, temperature sensitive mutations in \textit{CDC28} result in cell cycle arrest in G\textsubscript{1} and G\textsubscript{2}. Inactivation of Cdc28 is also necessary for the cell to exit mitosis. CDKs are regulated in many ways (92): 1) by association with cyclins as discussed above, 2) by phosphorylation/dephosphorylation, and 3) by CKIs (cyclin dependent kinase inhibitors). Some of these modes of CDK regulation are relevant to checkpoint regulation in several cell types.
Figure 1-2. Cyclin synthesis. Different cyclins are synthesized at different parts of the cell cycle to control specific events, such as the G1/S transition or the G2/M transition, and then are degraded. Although transcript levels are shown, protein levels show a similar pattern. This figure is based on (132).

DAMAGE CAUSES DELAYS BY CHECKPOINT CONTROLS

Cell viability requires that the steps of the cell cycle are performed in proper order and with proper timing. For instance, mitosis must follow DNA replication.

It is thought that the essential cell cycle controls, including the cyclin dependent kinase "engine" described previously, are usually sufficient to promote the proper steps at the proper time. Under normal circumstances, one might imagine that the kinetics of activation of the cyclin dependent kinase engine ensures that DNA replication and mitosis are timed so that they occur in the correct order — the cell cycle is "hard wired". Perhaps, for example, an S-phase promoting complex, "SPF" (the CDK plus an S-phase cyclin), is always formed before the mitosis promoting complex MPF. This would ensure that DNA replication precedes mitosis.
However, DNA damage, inhibition of DNA replication, or microtubule poisons could potentially cause lethal havoc on the cell cycle. It is essential for the cell to delay its cycle until the repair, induced by such disruption, has been completed (for a review, see 109). After DNA damage, there are conceivably two mechanisms to ensure proper order and timing (52): 1) A late step in the cell cycle is impossible to execute until the previous steps have taken place, because the previous steps provide the “substrate” for the succeeding steps. Under this hypothesis, for example, a cell could not physically complete mitosis if replication is not completed (or if DNA damage exists), because completely replicated chromosomes are a necessary substrate for mitosis. In this case, after damage the cell would be forced to delay its cycle until generation of intact chromosomes. 2) Alternatively, there may be specific control mechanisms, or “checkpoints”, that monitor the progress of the cell cycle and prevent later events, such as mitosis, until earlier events, such as replication or damage repair, have been completed. By this model, a defect in these checkpoint mechanisms would allow continuation of the cell cycle even when chromosomes are not intact.

Work from several laboratories, starting with studies by Weinert and Hartwell (52, 158), has shown that many mechanisms of the second type (checkpoints) govern the order of many cell cycle events. Most of this evidence was obtained genetically: yeast mutants were identified in which later events occurred before earlier ones had been satisfactorily completed (often resulting in
lethality). For example, wild-type (WT) yeast cells that have been exposed to X-rays arrest their cell cycle in G2. However, rad9 cells exposed to X-rays (which break DNA strands) fail to arrest their cell cycle in G2, and mitosis occurs before the broken DNA is repaired. The WT forms of these mutant genes are called checkpoint genes. They are required to prevent late events until early events are complete. For example, RAD9 is required to prevent mitosis (a late event) until DNA repair (an early event) is complete.

Checkpoint genes have now been found in *Saccharomyces cerevisiae* (budding yeast), *Schizosaccharomyces pombe* (fission yeast), other fungi, and importantly, in higher eukaryotes. For instance, the mammalian gene encoding p53, a tumor suppressor gene, has checkpoint function. It is required to arrest the cell cycle in G1 after DNA damage. The lack of p53 checkpoint function contributes to cancer (see for example 58, 115). The human ATM gene also has checkpoint activity (6, 125).

**CHECKPOINT RESPONSES**

A major focus of this dissertation is checkpoint delay in G2 after damage, but checkpoint proteins also act in many other stages of the budding yeast mitotic cell cycle to cause both cell cycle delays and altered gene expression (reviewed in 32, 154; See Table 1-1 for specific references). Genes have been identified that are required for the following responses:
DNA damage and replication checkpoint responses

1) The G_{1}/S checkpoint delays the cell in G_{1} before START after DNA damage. This is a minor checkpoint in budding yeast; the G_{2}/M checkpoint is the major damage-induced delay. (In mammalian cells, however, the predominant arrest point after DNA damage is at the G_{1}/S checkpoint.)

2) The intra-S checkpoint slows down replication when DNA has been damaged.

3) The S/M phase checkpoint prevents mitosis until the completion of DNA replication.

4) The G_{2}/M checkpoint delays mitosis when the DNA has been damaged.

5) The mid-anaphase checkpoint arrests the cell in anaphase apparently after DNA damage.

6) Checkpoints are also involved in the meiotic cell cycle, to delay the cycle in response to the DNA breaks which are a normal part of meiotic recombination. Many of the same checkpoint genes required for mitotic checkpoints are active in meiosis (84).

Other checkpoint responses

1) An M phase checkpoint arrests the cell cycle in mitosis in cases of microtubule disruption ("spindle assembly checkpoint").
2) The cell morphogenesis checkpoint delays mitosis if budding has been inhibited ("budding checkpoint").

Since this work focuses on the DNA damage and replication checkpoints, the following discussion does not address the M phase, cell morphogenesis, or meiotic checkpoints further.

**Damage-inducible transcription response**

Another response mediated by checkpoint genes to DNA damage is the transcriptional induction of certain repair genes (124). For example, one damage-inducible gene is \( RNR3 \), which encodes subunits of ribonucleotide reductase, an enzyme involved in dNTP production (33, 34), and which I discuss in Chapters 4 and 6. Some other induced repair genes are \( RNR1 \) and 2, which also encode subunits of ribonucleotide reductase (33, 34); \( RAD54 \) (23) whose product is involved in recombinational repair; \( POL1 \) (63), which encodes DNA polymerase \( \alpha \); \( RAD2 \) (120), which is involved in excision repair; \( UBI4 \) (150), involved in protein degradation; and \( CDC9 \) (112), which encodes DNA ligase.

Since certain checkpoint genes are required for damage-inducible transcription, one might imagine that checkpoint arrest is activated by induced transcription of cell cycle inhibitory genes. However, damage-induced transcription is probably not required for the \( G_2/M \) checkpoint to function. The evidence for this is that adding the protein synthesis inhibitor cycloheximide to \( G_2 \) cells does not inhibit damage-induced checkpoint arrest (157). Because protein
synthesis is not required for G\textsubscript{2}/M checkpoint function, transcription probably is not required as well. In contrast, in mammalian systems, transcription is required for checkpoint arrest, at least at the G\textsubscript{1} checkpoint. For example, p53, the well known mammalian checkpoint protein, causes arrest via transcription of p21, a CKI (111). It is possible that other yeast checkpoints, in addition to the G\textsubscript{2} checkpoint, also function independently of induced gene expression, but since continuation of the cell cycle at these other points also requires translation, the appropriate experiments cannot be done.

**Essential function**

Most checkpoint genes are required only after DNA damage or other cellular insults. However, some checkpoint genes are essential for viability even in the absence of damage or other cellular insults. The nature of the essential functions of these genes has been enigmatic.

My studies concern the essential functions of two genes, \textit{MEC1} and \textit{RAD53}. The nature of the essential function of \textit{MEC1} and \textit{RAD53} is not understood, but may involve maintaining proper dNTP levels, as discussed in Chapter 5.

**IDENTIFICATION OF CHECKPOINT GENES**

Checkpoint genes are involved in mediating many responses after damage, as discussed above. They are most often identified when mutant cells fail to
arrest their cycle after damage. Identification of checkpoint genes is often facilitated because mutations in many of these genes result in a lethal phenotype when cells are exposed to DNA damaging agents or inhibitors of DNA synthesis. When DNA damage occurs, checkpoint controls cause the wild-type (WT) cell to delay entry into mitosis until the cell has had time to repair the damage. Similarly, when DNA replication is inhibited, checkpoint controls cause wild-type cells to delay entry into mitosis until replication has been completed. In checkpoint defective cells, death often occurs when the cell attempts mitosis prior to the completion of synthesis or the repair of damaged DNA. Therefore, checkpoint mutants are usually extra-sensitive to DNA damaging agents (especially if they have an associated repair function), and this provides an easy way to perform a primary screen for new mutants. In a secondary screen, candidate checkpoint mutants are identified because they do not arrest after DNA damage.

In the laboratory, there are several ways to damage yeast DNA. Damage may be induced by UV, X-rays, or MMS (an alkylating agent), or it can be induced by defects in DNA metabolism. This can be achieved by incubating the cells at the restrictive temperature of temperature-sensitive alleles of certain genes such as CDC9 (encodes DNA ligase, 62) or CDC13 (involved in telomere metabolism, 40). The lack of CDC9 or CDC13 activity results in DNA damage and, consequently, in a G$_2$/M checkpoint arrest. Inhibition of DNA replication is usually done by treatment with hydroxyurea (HU), which inhibits the enzyme
ribonucleotide reductase (responsible for the production of the dNTPs needed for replication (2, 31). This results in a stalled replication fork, and consequently, in S/M arrest. Alternatively, thymidylate kinase, which is encoded by \textit{CDC8} (64, 133) and is needed for the production of dTTP (and therefore essential to replication), can be eliminated by incubating \textit{cdc8}^{ts} cells at their restrictive temperature. The lack of dTTP results in a stalled replication fork.

Many of the budding yeast checkpoint genes identified to date are listed in Table 1-1. (This table is intended primarily for reference use.)
Table 1-1. Checkpoint responses and roles of budding yeast checkpoint genes. This table is intended for reference purposes and is current as of July 1998. Genes listed in brackets have lesser or less-clear roles. References are shown in parenthesis.

<table>
<thead>
<tr>
<th>G1/S</th>
<th>intra-S</th>
<th>S/M</th>
<th>G2/M cell morphogenesis</th>
<th>mid-anaphase</th>
<th>M (spindle)</th>
<th>Damage-inducible transcription</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEC1 (138)</td>
<td>MEC1 (108)</td>
<td>MEC1 (159)</td>
<td>RAD9 (157, 158)</td>
<td>SWE1 (136)</td>
<td>RAD9 (166)</td>
<td>MPS1 (160)</td>
</tr>
<tr>
<td>RAD53 (3)</td>
<td>RAD53 (108)</td>
<td>RAD53 (159)</td>
<td>RAD17 (156)</td>
<td>CDC28 (76, 136)</td>
<td>MAD1 (77)</td>
<td>RAD53 (3)</td>
</tr>
<tr>
<td>RAD9 (140)</td>
<td>[RAD9] (110)</td>
<td>POL2 (101)</td>
<td>RAD24 (159)</td>
<td>MAD2 (77)</td>
<td>DUN1 (173)</td>
<td></td>
</tr>
<tr>
<td>RAD24 (139)</td>
<td>[RAD17] (110)</td>
<td>DPB11 (5)</td>
<td>MEC1 (159)</td>
<td>MAD3 (77)</td>
<td>SWI6 (137)</td>
<td></td>
</tr>
<tr>
<td>[MEC3] (79)</td>
<td>[RAD24] (110)</td>
<td>RFC5 (147)</td>
<td>MEC3 (159)</td>
<td>BUB1 (57)</td>
<td>RFC5 (146)</td>
<td></td>
</tr>
<tr>
<td>DDC1 (81)</td>
<td>MEC3 (79)</td>
<td>RAD53 (159)</td>
<td>BUB2 (57)</td>
<td>RAD9 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWI6 (137)</td>
<td>DDC1 (81)</td>
<td>PDS1 (165)</td>
<td>BUB3 (57)</td>
<td>POL2 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RFA1 (80)</td>
<td>RFC5 (146)</td>
<td>DDC1 (81)</td>
<td>PDS1 (165)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[PRI1] (90)</td>
<td>RFA1 (80)</td>
<td>[RFC5] (146)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRI1 (90)</td>
<td></td>
<td>CDC28 (78)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DUN1 (this study and 107)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*rfc5 cells are sensitive to DNA damaging agents, but no G2/M checkpoint role has been shown directly (146).
BUDDING YEAST G₂/M CHECKPOINT GENES

Most of my checkpoint studies have been concerned with the G₂/M DNA damage checkpoint. So far, ten checkpoint genes that control the G₂/M transition have been identified in budding yeast (Table 1-2). Until my work (Chapter 3) and that of Pati et al. (107), DUN₁, a gene required for damage inducible transcription, was thought not to have checkpoint activity (173). All of these G₂/M checkpoint genes have been cloned. "MEC" indicates Mitotic Entry Checkpoint; "RAD" indicates genes which are radiation sensitive; "PDS" indicates Premature Dissociation of Sisters, "DUN" means Damage Uninducible, and "DDC" means DNA Damage Checkpoint.

The biochemical activity of many of these genes is known (see Table 1-2; this table is intended primarily for reference).

Many of my studies have involved two of these genes, MEC₁ and RAD53, the only G₂/M genes which are also required for the S/M checkpoint. Also, unlike most of the others, MEC₁ and RAD53 are essential; their function(s) is required even when there is no DNA damage or inhibition of S phase (T. Weinert personal communication and 99, 172). Table 1-2 summarizes some of the functions of the G₂/M checkpoint genes.
Table 1-2. DNA damage checkpoint genes of budding yeast and their putative molecular roles. All have G₂/M checkpoint function (references under each gene). This table is intended primarily for reference.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Possible role in G₂/M arrest*</th>
<th>S/M checkpoint function</th>
<th>Essential?</th>
<th>Other functions</th>
<th>Biochemical Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEC1</td>
<td>upstream signal transduction</td>
<td>yes (159)</td>
<td>yes (66, Weinert unpublished, 99)</td>
<td>damage-inducible transcription (70)</td>
<td>putative PI and protein kinase</td>
</tr>
<tr>
<td>MEC2/ RADS3</td>
<td>signal transduction</td>
<td>yes (159)</td>
<td>yes (172)</td>
<td>damage-inducible transcription (3)</td>
<td>protein kinase (172)</td>
</tr>
<tr>
<td>MEC3</td>
<td>Processes damage (87)</td>
<td>no (159)</td>
<td>no</td>
<td>damage-inducible transcription (70)</td>
<td>?</td>
</tr>
<tr>
<td>RAD9</td>
<td>Processes damage (87)</td>
<td>no (159)</td>
<td>no (157)</td>
<td>damage-inducible transcription (1, 70)</td>
<td>?</td>
</tr>
<tr>
<td>RAD17</td>
<td>Processes damage (87)</td>
<td>no (159)</td>
<td>no</td>
<td>damage-inducible transcription (70)</td>
<td>putative exonuclease (87)</td>
</tr>
<tr>
<td>RAD24</td>
<td>Processes damage (87)</td>
<td>no (159)</td>
<td>no</td>
<td>damage-inducible transcription (70)</td>
<td>limited replication factor C homology</td>
</tr>
<tr>
<td>PDS1</td>
<td>Downstream target of APC (165)</td>
<td>no (165)</td>
<td>essential at restrictive temperature (164)</td>
<td>spindle checkpoint (164, 165)</td>
<td>?</td>
</tr>
<tr>
<td>DUN1</td>
<td>signal transduction (this work)</td>
<td>not tested</td>
<td>no</td>
<td>damage-inducible transcription (173)</td>
<td>protein kinase (173)</td>
</tr>
<tr>
<td>DDC1</td>
<td>not known</td>
<td>no (81)</td>
<td>no (81)</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>CDC28</td>
<td>a target (but not the target) (4, 78, 144)</td>
<td>?</td>
<td>yes (51)</td>
<td>controls cell cycle (73, 118)</td>
<td>protein kinase (117)</td>
</tr>
</tbody>
</table>

* Not necessarily comprehensive.
HOW CHECKPOINT GENES MAY ACT IN THE G₂/M RESPONSE PATHWAY

A checkpoint pathway can be viewed as a signal transduction system with three main components (85): a molecule(s) capable of sensing a signal such as DNA damage or incomplete replication (sensor); a target molecule(s) that acts to arrest the cell cycle (effector); and a signal transducing molecule(s) that relays the message from the sensor to the effector. (Of course, it is possible that a single molecule could perform two or all three of these functions.) Order of function of checkpoint genes and the identification of the pathways these genes operate on are a major focus of this research. Figure 1-3 shows a working model of yeast checkpoint genes involved in sensing damage, signal transduction, and in effecting cell cycle arrest at the G₂/M checkpoint. This model, which serves as useful background information for much of the work in this dissertation, especially for Chapter 3, is based on our current understanding, the evidence for which is given in the following sections.
Sensors of DNA damage in budding yeast

Data from our laboratory (87) suggest a model in which at least four G₂/M checkpoint proteins (Rad9, Rad17, Rad24, and Mec3) are involved in processing a DNA lesion after damage, possibly to provide a substrate which signal transduction proteins, such as Mec1p, could recognize (see Figure 1-4). Rad17p is a putative exonuclease. In vivo experiments, in which the amount of ssDNA near a lesion is quantitated, suggest that Rad17 might cause resection (exonucleolytic digestion) of the DNA at the site of damage, perhaps aided by Rad24p and Mec3p. (Although the roles of Rad24 and Mec3 are unknown, they are in the same epistasis group as Rad17, and are thus likely involved in the
same pathway (86).) Rad9p seems to be required to limit the amount of resection, according to in vivo experiments (resection, measured by the amount of single stranded DNA at the lesion, is increased in cells lacking Rad9p) (87).

It is plausible that several checkpoint gene products, including Rad9, Rad17, Rad24, and Mec3, are also involved in the repair of damaged DNA. It has been suggested that the act of repair itself may signal checkpoint arrest response, or that repair substrates, such as ssDNA, are required for detection by checkpoint proteins (87, 104).

After replication blocks (such as that caused by hydroxyurea), S/M checkpoint arrest occurs in a Rad9, 17, 24, and Mec3 independent manner. However, S-phase specific checkpoint genes, such as *Pole*, are required for arrest (101). It may be that after a replication block, the same type of lesion processing that occurs after DNA damage is not needed to provide a substrate for the signal transducing proteins to recognize. Instead, the stalled replication fork itself, modified by S-phase specific checkpoint proteins, may form an appropriate substrate.
Signal transducers of DNA damage in budding yeast

Yeast checkpoint proteins that are thought to transduce the arrest signal are the protein kinases Rad53 and Dun1, and the putative protein kinase Mec1.

There are several lines of evidence that suggest that Mec1 is likely to be the upstream signaler, with Rad53 and Dun1 acting downstream. First, Rad53 is phosphorylated after DNA damage in a MEC1 dependent manner (127, 148).

The simplest way to explain this is to place MEC1 upstream of RAD53. Second,
although all three proteins are required for damage-inducible transcription, *MEC1* is required for induction of a larger set of transcriptionally regulated proteins than is *RAD53* or *DUN1* (70). This again suggests that Mec1p may act upstream of Rad53p and Dun1p. Third, as I show by genetic epistasis analysis in Chapter 3, *MEC1* is likely to be upstream of two subpathways leading to checkpoint arrest, one of which involves *RAD53* and *DUN1*. (The other involves *PDS1*, as shown in Figure 1-4.) Fourth, *MEC1* is homologous to DNA-PK, a mammalian protein kinase activated by DNA breaks (discussed in more detail below). This suggests that *MEC1* might function at the site of damage rather than downstream. The roles of Mec1 in arrest, and the roles of arrest pathways downstream of Mec1, are discussed in Chapter 3.

**INTEGRATING CHECKPOINT PATHWAYS INTO THE RESPONSES**

Less is known about the targets of checkpoint arrest in budding yeast than about the upstream components of arrest pathways, the sensors and signal transducers. Although my research has focused on the G₂/M checkpoint, I discuss briefly here our current understanding of the targets of the G₁/S, intra-S, and S/M checkpoints. Perhaps knowledge of the targets of these checkpoints may aid our understanding of the target of the G₂/M checkpoint.
The target of the G₁/S checkpoint

For the G₁/S checkpoint, recent evidence shows that at least one mechanism for arrest involves the Cln cyclins. These are cyclins which promote the G₁/S transition, and are encoded by CLN1 and CLN2. After damage, their transcription is decreased, thus slowing the transition through START. This decrease in transcription is dependent on RAD53, which is thought to inhibit the SWI4/SWI6 complex, upon which transcription of CLN1 and CLN2 depend (137).

In mammalian systems, cyclin-dependent kinases have also been shown to act as targets for the G₁/S checkpoint. DNA damage results in activation of a CKI called p21, which then inactivates the cyclin dependent kinase, resulting in G₁ arrest (28). Therefore, G₁/S arrest involves transcription and involves the CDK in both budding yeast and mammals, yet details of the arrest mechanisms differ.

The target of the Intra-S checkpoint

For the Intra-S checkpoint, DNA primase may be involved. Evidence for primase's role in the intra-S checkpoint comes from a dominant primase mutation, which prevents activation of the intra-S checkpoint (90). Primase's activity is required for both initiation of replication and elongation, and is downregulated by checkpoint proteins such as Mec1p and Rad53p.
The target of the S/M checkpoint

Nothing is known about the target of the S/M checkpoint in budding yeast. The S/M checkpoint could share target(s) with the G_2/M checkpoint, since in both cases mitosis is inhibited (although it is not known if it is the same stage of mitosis that is inhibited in both cases). However, *PDS1*, a putative target gene required for the G_2/M checkpoint (see below) is not involved in the S/M checkpoint (165).

In other organisms, such as fission yeast and Aspergillus, the CDK cdc2 is the target of the S/M checkpoint, as well as the target of the G_2/M checkpoint (35, 168). Further, it has been reported that in cell-free extracts derived from *Xenopus* eggs, coupling of S phase and mitosis also occurs via phosphorylation/dephosphorylation of p34^*cdcc2* (72, 143). Therefore, at least in some organisms, p34^*cdcc2* is important for checkpoint arrest, in addition to its normal role of driving the cell cycle engine.

Cyclins have also been implicated as targets for the S/M checkpoint in other organisms: ablation of cyclin A in *Xenopus* extracts resulted in the loss of dependence of mitosis on DNA replication (152). Both the CDK and cyclin components of the CDK/cyclin complex may therefore be important for the S/M checkpoint.
THE ENIGMA OF CDK/CYCLIN AS A TARGET OF THE G₂/M CHECKPOINT

What is the target of G₂/M checkpoint arrest in budding yeast? One theory is that it is p34cdc2 (encoded by CDC28 in budding yeast), the cyclin-dependent kinase component of MPF. p34cdc2 as a target of checkpoint control has been extensively characterized in fission yeast. In that organism, cdc2 activity is known to be regulated by at least three proteins: the kinases wee1 and mik1 (82), and the phosphatase cdc25 (126). (Homologs of these genes are found in budding yeast and higher eukaryotes.) The fission yeast kinase cdc2 is active (that is, it promotes mitosis) when bound to cyclin B and dephosphorylated on tyrosine 15, and inhibited when phosphorylated on tyr 15. Experiments involving the kinase wee1 and phosphatase cdc25 have shown that fission yeast checkpoints work by regulating the phosphorylation state of cdc2 (See Figure 1-5.) (35, 119, 123). After damage, wee1 kinase activity is maintained, while cdc25 phosphatase activity is inhibited, thus maintaining the p34cdc2 phosphorylated state. As these observations predict, a cdc2 allele that cannot be phosphorylated cannot cause delay after DNA damage (45).

It appears that p34cdc2/MPF may be a target of checkpoint genes in many organisms, including Aspergillus (169) and humans (61). It is also possible that Drosophila checkpoints also act through cdc2 (7, 14, 29, 30), although conclusive evidence is not yet available.
In budding yeast there seems to be no role of CDK phosphorylation in checkpoint arrest. Researchers from two different laboratories have found that, while Cdc28 is phosphorylated on equivalent tyrosine residues as in fission yeast, mutations that prevent this phosphorylation by changing the tyr (and other potentially phosphorylatable amino acids) to another amino acid do not lead to inactivation of checkpoint arrest. There are no other known amino acids in Cdc28 that would inhibit Cdc28 activity when they are phosphorylated, as does the fission yeast tyrosine 15. This suggests that budding yeast checkpoint genes may not act through Cdc28, or, if so, not through Cdc28 phosphorylation (4, 144). This was a surprising result in light of the fact that there is so much conservation between fission and budding yeast cell cycle systems. For example, there are budding yeast homologs of wee1 and cdc25. More recent observations deepen the enigma of the checkpoint role of Cdc28: A recent report discusses a new
allele of $CDC28$ in which checkpoint arrest is compromised (78). This suggests that Cdc28 may be involved in budding yeast G$_2$/M checkpoint control by mechanisms different than those used by $S.~pombe~cdci2^-$. The role of Cdc28 in arrest is yet to be determined.

**Other possible targets of G$_2$/M arrest**

Another potential target of G$_2$/M arrest in budding yeast is the other component of the Cdc28/cyclin complex, the cyclins. Cyclins have also been implicated as targets for G$_2$/M checkpoint systems in other organisms: The levels of cyclin B mRNA and cyclin B protein have been shown to decrease in HeLa cells upon exposure to ionizing radiation (97). This in turn delays mitosis (65). Recently, a DNA damage-induced cell cycle delay was described in budding yeast in which the delay is attributed to an increase in the stability of the Clb5 cyclin protein. Clb5 is a G$_1$ cyclin, and its increase in stability is thought to prevent mitosis by keeping the cell in a G$_1$-like state. However, this checkpoint system may be completely independent of other described DNA damage checkpoints, since a key checkpoint gene, $MEC1$, is not required for the arrest (41).

Another possible mode of action for budding yeast G$_2$/M checkpoint gene products is activation of Cyclin Dependent Kinase Inhibitors (CKIs) (98). CKIs are involved in mammalian checkpoints (For review see 111). For example, p21, a CKI, inhibits a CDK and thereby activates cell cycle arrest (28). While CKIs have
been found in budding yeast (see for example 131), there is no direct evidence yet of their role in checkpoint controls.

In budding yeast there may be more than one target for checkpoint arrest. Perhaps one checkpoint mechanism, acting through Cdc28, arrests the cells in G2, and another mechanism, inhibiting the Anaphase Promoting Complex (which degrades cyclins and other substrates so that the cell can exit mitosis and enter a new cell cycle), arrests the cells in metaphase. This idea is discussed in Chapter 3. Figure 1-6 summarizes current understandings or hypotheses about the targets of the G1/S, G2/M, intra-S, and S/M checkpoints.
After DNA damage (top), the G1/S checkpoint functions by reducing the transcription of Cln1 and 2, which are required for S-phase entry. The G2/M targets are less well defined, but may include two arrest points: one at G2, mediated by Cdc28, and one at metaphase (M), which works via the APC pathway ("A" stands for Anaphase). After DNA damage during S-phase (bottom), primase is inactivated which causes a slowing of replication. Also, S-phase specific checkpoint genes prevent entry into mitosis when DNA replication has been inhibited (indicated in the figure by the presence of a stalled replication fork). Cdc28 is a possible, but unproven, target.
**MEC1**

The checkpoint gene I have studied most intensively is *MEC1*. *MEC1* is a large gene (with an open reading frame of 7104 bases encoding a protein of 2368 amino acids) with many functions. Its checkpoint functions include the G1/S (138), intra-S (108), S/M, and G2/M (159) checkpoints; it is required for damage-inducible transcription of several classes of genes (70); and it has an essential function (T. Weinert personal communication and 66, 99). *MEC1* is also a meiotic checkpoint gene; it is required for arrest in a *dmc1* mutant, in which meiotic double strand breaks persist (84), and there is some evidence it may have additional role(s) in meiosis (10). It is not surprising, in light of *MEC1*‘s many functions, that cells with mutant alleles of *mec1* are very sensitive to DNA damage caused by agents such as MMS, X-rays, and UV, and are also sensitive to DNA damage caused by a lack of *CDC 8,9, or 13* activity. Mutant alleles of *mec1* are also sensitive to inhibition of DNA replication by HU.

Several alleles of *MEC1* were first isolated based on their lethal phenotype in a *cdc13* background at *cdc13*‘s restrictive temperature (159). Later, *MEC1* was also isolated by several other groups: it was identified as *ESR1* on the basis of MMS sensitivity (66), identified as an essential gene during the yeast genome project (99), identified as *SAD3* by HU sensitivity and S/M checkpoint deficiency (3), and most recently it was found in a synthetic lethal screen of a DNA primase mutant (81).
**MEC1 is essential**

Work from several laboratories has shown that *MEC1* has an essential function (T. Weinert unpublished data and 66, 99). In each case, *MEC1* was found to be essential because meiotic segregants that were *mec1A* failed to grow. *MEC1*’s essential function has not been defined, and determining the nature of the essential function is one objective of this research.

There are two general ideas that might explain the nature of *MEC1*’s essential function: 1) *MEC1*’s essential function may be to ensure that in each cell cycle, replication is complete before mitosis begins. This function would be similar to the S/M checkpoint function. 2) *MEC1*’s essential function may not be related to the S/M checkpoint function, but might involve some other cell cycle function. See Chapter 5 for further discussion on this topic.

An analysis of suppressors of *MEC1*’s essential function may shed light on the nature of that function. *MEC1*’s essential function is easily suppressed. The strongest allele, *mec1-1*, grows well even though it has no checkpoint or transcription activity. It was shown by researchers in other laboratories (and confirmed by myself and others in our laboratory) that *mec1-1* is likely to be a null allele and that growth of *mec1-1* strains depends on the existence of a spontaneous suppressor (110, 121). This suppressor has been termed *sml1*, for suppressor of *mec1* lethality (110). *sml1* has no independent phenotype and behaves recessively. One theory for how *sml1* suppresses *mec1* is discussed in Chapter 5 and in Appendix A.
Separation of \textit{MEC1}'s functions

Our understanding of the different response pathways in which Mec1 functions may be aided by learning whether \textit{MEC1}'s functions are separable (see Figure 1-7). If \textit{MEC1}'s functions are separable, this may indicate that there are different substrates, targets, or downstream components for each function. The existence of opposite-acting alleles of \textit{mec1}, such as alleles proficient for G$_2$/M arrest but deficient for S/M arrest, and deficient for G$_2$/M arrest, but proficient for S/M arrest, would show that the functions of \textit{MEC1} are genetically separable. Work in progress by others in the Weinert laboratory is focused on finding opposite-acting alleles, and there is now reason for cautious optimism that such alleles exist.

The existence of suppressors such as \textit{sml1} which suppress only the essential but not checkpoint functions suggests that these functions may be genetically separable. The caveat in this case, however, is that the checkpoint function(s) and essential functions may be the same activity; the checkpoint function(s) simply may require more suppressor activity than the essential function.
Figure 1-7. Models demonstrating genetically separable and unseparable MEC1 functions. 

a, genetically separable functions model predicts the possibility of alleles proficient in only certain functions. b, genetically unseparable functions model predicts that mutations in mec1 will affect all functions. The functions shown are examples, and not meant to be comprehensive.

**MEC1 AND PI KINASES**

*MEC1* contains a putative phosphatidyl inositol (PI) and protein kinase domain at its 3' end. (PI kinases are defined by what position of the inositol ring they phosphorylate. PI3 kinases phosphorylate the position 3 of the inositol ring.)

*MEC1's* kinase domain is shared with several other genes in diverse organisms, such as the gene encoding mammalian p110 (53), the mammalian DNA-PK, ATM, and ATR genes (see below); budding yeast *VPS34* (130), *TOR1* and *TOR2* (74), *PIK1* (39), *FAB1* (163), *STT4* (37, 170), *TEL1* (see below); *Drosophila mei-41* (see below); and fission yeast *rad3* (see below). Figure 1-8 shows the overall
structures of some of these genes. (See Figure 2-2 on page 67 for a partial sequence alignment of the kinase domain of several PI kinase genes.) So far, ATM, ATR, rad3*, and mei-41 have been shown to have checkpoint activity. All of these kinases have protein kinase activity.

The best characterized PI kinase domains are in PI3 kinases. Perhaps for this reason, the literature is replete with reference to MEC1's (and its homologues') PI3 kinase domains. However, it now seems likely that Mec1 may have protein kinase activity, but not PI kinase activity (see below). Although Mec1 has not been shown so far to have either PI or protein kinase activity, some other genes with PI kinase domains (such as PIK1, a PI-4 kinase, and p110 and VSP34, which are both 3-kinases) do have both PI and protein kinase activity (see (39), (53), and (130) respectively).

It is thought that, among the members of the kinase family, the homologous regions in the kinase domain identify the catalytic sites, but not the substrate binding sites, which are less well conserved (71).
Figure 1-8. Structures of some genes in the PI kinase family. The gray boxes represent highly conserved regions; the first gray box represents the kinase domain, and the second gray box represents an undefined sequence at the carboxy termini.

**MEC1 HOMOLOGUES**

**DNA-PK as a precedent for kinase activity stimulated by damaged DNA**

In mammalian cells, a protein kinase called DNA-PK is activated by DNA breaks of various types, including nicks and single stranded gaps (44, 93). DNA-PK operates as a heterotrimer with a DNA binding subunit called KU (which is a heterodimer). So far, DNA-PK has not been shown to have any PI kinase activity, just protein kinase activity. Because of the homology between DNA-PK and MEC1, there is speculation that Mec1 also probably does not have any PI kinase activity (59), just protein kinase activity.

DNA-PK provides an excellent precedent for activity stimulated by DNA breaks. It is thought that Mec1's putative protein kinase activity may also be stimulated by DNA breaks. A KU-homolog has been identified in yeast, but it apparently has no checkpoint phenotype, based on unpublished work in our laboratory. Human DNA-PK may not have DNA-damage checkpoint activity.
because after cells defective in DNA-PK were exposed to several different kinds of DNA damaging agents, their arrest response was indistinguishable from WT cells. The caveat in this experiment is that the DNA-PK alleles used were not nulls (116).

**ATM**

Recently, the human gene responsible for the disease Ataxia telangiectasia (called ATM) has been cloned and sequenced (129). It shares the PI kinase motif of MEC1 (with high homology) and its homologues, and protein kinase activity has been shown (67). Outside of the kinase domain, Mec1 and ATM show only slight similarity. ATM− cells are checkpoint deficient (6, 125, see also 162). ATM patients also develop cancer at high rates (129, see also 161), which is likely due to the lack of checkpoint activity.

**rad3**

The MEC1 homolog in the fission yeast *Schizosaccharomyces pombe* is called rad3*. rad3* shares similar checkpoint functions with MEC1, but it is not essential. So far, the reasons for this difference in the essential function are unknown. rad3’s kinase domain is required for rad3’s functions (9). Protein kinase activity, but not PI kinase activity, has been shown. MEC1 and rad3* have not been shown to complement each other (9, see also 60, 134).
ATR

ATR is a human gene; its name refers to Ataxia and rad related. It was identified as a human homolog of *S. pombe* rad3', and cloned by degenerate PCR (9). The ATR protein is more closely related to rad3 than it is to ATM, and its function in humans is still unclear. Like ATM and rad3, ATR has protein kinase activity (67). ATR overexpression in budding yeast can complement UV sensitivity of a *mec1* allele called *esr1-1*, although it cannot complement HU sensitivity. Nor can it complement UV or HU sensitivity of *rad3* mutants in fission yeast (9). A kinase inactive allele of ATR confers checkpoint and radiation sensitivity defects when overexpressed in human cells (20).

*mei-41*

*mei-41* is a *Drosophila* checkpoint gene. Mutations in *mei-41* result in lack of G$_2$/M checkpoint arrest, sensitivity to ionizing radiation, and high levels of chromosome instability (49).

*TEL1*

*TEL1* is a budding yeast gene with little homology to *MEC1* outside of the kinase domain. However, it shares with *MEC1* a highly conserved protein/PI kinase domain. One of *TEL1*'s functions is to ensure proper telomere size (83). In *tel1Δ* cells, telomeres are shorter than those of WT cells, but do not continue to shorten and *tel1Δ* cells do not senesce. *TEL1* has no checkpoint function, but its
overexpression can restore the essential function of mec1 cells as well as partial UV and HU viability (46, 94). I discuss TEL1 in greater detail in Chapter 4.

**ADAPTATION TO CHECKPOINT ARREST**

It is conceivable that while some of the checkpoint genes that have been discussed in this chapter are required for the initiation of arrest, others may be required not for the initiation of arrest, but rather for the maintenance of arrest. One possible role for checkpoint genes in the maintenance of arrest is to block the process of adaptation. Adaptation is defined as follows: If DNA is damaged irreparably, the cell will delay for several hours, but the cell often eventually proceeds to M phase with the unrepaired damage. Adaptation is this eventual release from checkpoint arrest, even in the presence of irreparable DNA damage. Adaptation seems to be genetically controlled (149). In Chapter 3, I test the role of adaptation controls in maintenance of checkpoint arrest.

Wild-type cells can grow in the continued presence of hydroxyurea (HU), a DNA synthesis inhibitor, because after checkpoint arrest they are able to complete DNA synthesis by an unknown mechanism. This process is also referred to as adaptation. However, it should be noted that these two uses of the term "adaptation" do not imply a common mechanism; although adaptation after DNA damage is lethal, adaptation to HU is not. Adaptation to HU probably
involves an increase in ribonucleotide reductase levels, which overcomes the inhibitory effect of HU.
CHAPTER 2. CREATION OF SPECIAL ALLELES OF MEC1

INTRODUCTION.

This chapter describes three new alleles of MEC1 which I have created, and briefly describes how they were used to study MEC1 functions. Questions I asked included: a) Is MEC1 essential? b) What responses require MEC1's kinase domain? c) What can I learn about MEC1 from a study of a conditional (temperature sensitive) allele?

RESULTS

Table 2-1 summarizes the questions being asked and the general methods used. These are presented in detail in the individual sections.
IS MEC1 ESSENTIAL?

Previous evidence had indicated that MEC1 was essential, or at least nearly so (66, Weinert unpublished, 99). A partial deletion of mec1 called mec1Δ-1

<table>
<thead>
<tr>
<th>Question</th>
<th>Experiment</th>
<th>Results</th>
<th>Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Is MEC1 essential?</td>
<td>create mec1Δ — is it viable?</td>
<td>dead*</td>
<td>MEC1 is essential</td>
</tr>
<tr>
<td>What function(s) require the kinase domain?</td>
<td>create kinase inactive allele - does it complement MEC1 functions?</td>
<td>no complementation of S/M or G2/M checkpoint, transcription, or essential responses</td>
<td>these responses require kinase function</td>
</tr>
<tr>
<td>What does a mec1Δ allele reveal about MEC1's functions?</td>
<td>isolate mec1Δ — what happens when Mec1 activity is lost?</td>
<td>mec1 phenotypes: checkpoint arrested cells fail to maintain arrest spontaneous suppressors arise telomere length is unaffected</td>
<td>MEC1 is required continuously for arrest MEC1's essential function is easily suppressible Essential function of MEC1 is not telomere maintenance Phenotypes ascribed to mec1 are due to mec1, not suppressors</td>
</tr>
</tbody>
</table>

* Depending on strain background (see text)
which contained 475 bp of the original 7104 bp ORF, had resulted in dead
spores or small colonies when *mec1Δ-1/+* cells were sporulated and dissected.
However, the deletion still left larger than desirable sections of open reading
frame, which may have accounted for the small *mec1Δ* colonies. This left the
question of whether *MEC1* is truly essential.

**Phenotypes of *mec1Δ* cells are slightly different depending on strain
background**
To ask if *MEC1* is essential, I constructed a new plasmid, *mec1Δ-2*,
designed to delete more of the *MEC1* open reading frame. This deleted all but
the first 104 and last 127 bases (34 and 41 amino acids, respectively, of the 2368
total amino acids), and replaces the *MEC1* gene with the *URA3* gene, allowing
one to follow the *mec1* deletion by the Ura" phenotype. This plasmid was
integrated into a WT diploid strain in the A364a genetic background, and the
diploid strain was then sporulated and tetrads dissected. After two days it
appeared that viability segregated 2:2, which would indicate that *MEC1* is
required for viability, but some poorly growing spores grew up after
approximately another 3 days, producing small colonies (Figure 2-1); the large
and small colonies were confirmed to be Mec" and *mec1Δ*, respectively, by their
Ura" and Ura" phenotypes. Cells from the small Ura" colonies were hydroxyurea
sensitive (similar to viable *mec1-1* cells). When cells from the small Ura" colonies
were replated, resulting colony sizes varied widely and had low cell viabilities
(about 10-30%). One explanation for the existence of these small meclΔ colonies, as well as the widely varying colony sizes they produce upon replating, is that the small colonies were the result of the generation of spontaneous suppressors. The growth of these small colonies presumably allows the occurrence of additional spontaneous suppressors. It is unlikely that the meclΔ colonies resulted from residual MEC1 function, given the small amount of MEC1 open reading frame present, although I cannot rule out residual Mec1 function from the MEC1+/Δ diploid. meclΔ spores that do not result in macrocolonies produce from 1 to about 50 cells. Therefore, meclΔ cells in the A364a strain background do grow, albeit very poorly.

In contrast, in the W303 strain background, colonies resulting from meclΔ spores rarely if ever occur. This suggests either that the essential function is absolutely required in the W303 strain background, while it is only very important in the A364a background, or that spontaneous suppressors arise at a higher frequency in A364a than in W303 cells. (Spontaneous suppressors of MEC1’s essential function do arise in cells of the W303 background under different conditions.) Figure 2-1 shows the results of sporulating and dissecting mecl+/Δ2 cells from each of these strain backgrounds. The conclusion, therefore, is that MEC1 is essential, or very important for cell growth. Subsequent to these studies, two other laboratories reported that MEC1 is essential (66, 99). Neither reported that meclΔ spores can produce colonies as I have seen in the A364a
background (Kato and Ogawa (66) did not report which strain background they used. Nasr et al. (99) used the W303 background).

Figure 2-1. Sporulation and dissection of a MEC1+/Δ strain gives different results in two different genetic backgrounds. Left, When sporulated and dissected, a MEC1+/Δ-2 strain in the W303 background (RGY60) reveals 2:2 viability after 5 days at 23°C. Right, sporulation and dissection of a strain of the same genotype in the A364a background (RGY6) shows the small mec1Δ-2 colonies growing after 4 days at 23°C.

THE KINASE DOMAIN IS REQUIRED FOR ALL FUNCTIONS OF MEC1 TESTED.

Over its entire length, Mec1 has low homology to known (or putative) phosphatidylinositol (PI) and protein kinases, but at its carboxy region Mec1 has stretches of sequence very highly conserved with PI/protein kinases. This domain includes the ATP binding and catalytic sites. (Protein kinase activity has been reported from studies of three MEC1 orthologs, rad3+ from fission yeast and ATM and ATR from human cells (9, 67), but not yet for Mec1p.) What is the biological significance of Mec1's putative kinase domain? It was conceivable that Mec1 kinase activity could be required for certain of Mec1's functions but not for others. For example, it may be required only for checkpoint function but not the essential function, or vice versa. (There is precedent for kinases that still retain function even when their kinase activity is abolished. Two examples of protein
kinases in yeast that have function(s) in addition to the kinase function are Fus3 and Kss1. Mutations in these proteins which abolish kinase activity do not abolish their other activities (89).)

In order to address the question of which function(s) are dependent on the Mec1 kinase domain, I constructed a site-specific mutant allele of MEC1. (See Figure 2-2; see methods #4 on page 213 for details of construction.) By sequence comparison, it was possible to identify specific amino acids in Mec1 corresponding to specific amino acids in known kinase domains, having known roles in ATP binding and catalysis. One of these, an asparagine, is conserved in all PI kinases, and is part of the "catalytic loop" region, involved in ATP binding and catalysis (48, 71). This asparagine had been replaced with a lysine both in the budding yeast protein VPS34 (130), and in the fission yeast protein rad3 (a Mec1 homolog) (9) resulting in loss of function of both mutant genes. Therefore I made a similar change in Mec1, changing asn (amino acid #2229) to lys. Identification of the mutant allele was facilitated because the mutation also created a new restriction site. The region in which the mutation was made was sequenced to confirm the presence of the mutation, as well as to confirm the absence of other mutations.
**mec1N2229K mutation**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
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</thead>
<tbody>
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<td>MAAMGHILGL</td>
<td>GDRHCE NIL</td>
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<td>VCITYILGLV</td>
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<td>-----YILG-</td>
<td>GDRH---N</td>
</tr>
</tbody>
</table>

**pkinase conserved residues**

- D-K- NL- - - - - DFG

---

**Figure 2-2. MEC1 has a protein/PI kinase motif.** Comparison of MEC1 sequence with sequences of other members of the PI/protein kinase family shows that MEC1 is a PI and protein kinase by sequence (only part of the kinase domain sequence is shown). Bold residues indicate complete/near complete conservation. In consensus and protein kinase sequences, nonbold residues indicate a high degree of, but not complete, conservation. Sequence line-up modeled after (74); protein kinase sequence based on (48). The location of the mec1 N2229K mutation is indicated.

A plasmid with the mutated mec1 gene, hereafter called mec1N2229K, was introduced into a MEC1+/Δ diploid strain and into two mec1 haploid strains (one strain contained a temperature sensitive allele of mec1, and the other strain contained the genomic suppressor sml1, which restores viability, but not other MEC1 functions, to mec1 cells). The mec1N2229K allele was then tested for complementation as shown in Table 2-2 and in Figure 2-3. The mec1N2229K allele failed to complement all responses for the mec1 alleles tested. Even a sensitive kinetic assay of G2/M delay in a strain containing the mec1N2229K allele shows no detectable G2/M delay. I conclude that the kinase domain is required for MEC1’s essential function, transcription function, and S/M and G2/M checkpoint functions. (It seems likely that Mec1’s kinase domain is also required for other checkpoints, such as the G1/S and intra-S checkpoints.) Several
proteins have now been shown to become phosphorylated in vivo in a \textit{MEC1}-dependent manner after DNA damage (RPA, Rad53, and Pds1 -see 12, 21, 127). The importance of protein phosphorylation dependent upon \textit{MEC1} would account for failure of the \textit{mec1N2229K} kinase-inactive allele to complement any defects in \textit{mec1} mutants (Figure 2-3).

\begin{table}
\centering
\caption{The \textit{mec1N2229K} allele does not complement HU sensitivity or temperature sensitivity of \textit{mec1} strains. These were scored as growth or no growth on plates (+ or -). \textit{mec1}[^{15}], RGY2; \textit{mec1-1sml1}, TWY177.}
\begin{tabular}{|l|c|c|c|}
\hline
 & + vector & + p\textit{MEC1} & + p\textit{mec1N2229K} \\
\hline
Growth of \textit{mec1-1sml1} cells on 2M HU plate & - & + & - \\
\hline
Growth of \textit{mec1}[^{15}] cells on plates at 37°C & - & + & - \\
\hline
\end{tabular}
\end{table}
Figure 2-3. The mec1N2229K allele is deficient for all tested functions. Comparison of the suppression of several mec1 defects by pMEC1, vector alone, or pmeclN2229K shows that pmeclN2229K fails to suppress these defects. In each case, the WT response was normalized to 100% response. The bars represent % WT response. (In some cases the pMEC1 control was not done, so the response of WT cells is shown.) Damage-inducible transcription was measured as the amount of RNR3 transcript on a Northern blot (normalized to a control URA3 transcript) after 4 hr exposure to .01% MMS. S/M or G2/M arrest bars show the percent arrested cells at the indicated times of HU exposure or cdc13 damage. GAL NK refers to overexpression of the NK allele under control of the GAL promoter. Spore viability was measured by sporulation and dissection of a MEC1+/Δ strain containing either pMEC1, vector, or pmeclN2229K. pMEC1 may have produced more viable mec1Δ spores but plasmid loss may have occurred during sporulation. Viability after a transient HU exposure was measured by plating the cells after they had been exposed to HU, and determining the percentage of microcolonies that were alive (methods #3). mec1cdc13, TWY432; mec1+/Δ diploid, RGY6; WT, RGY4.
Possible Caveat to the Conclusion that Mec1’s kinase domain is required for all Mec1 responses

One caveat to the conclusion that Mec1’s kinase domain is required for all Mec1 responses is that the kinase-inactive allele may simply be destabilized due to the N2229K mutation, and perhaps be degraded. Western blotting shows, however, that at least some full length Mec1N2229K protein is present in cells expressing the \textit{mec1N2229K} allele (Figure 2-4).

One might hypothesize that the \textit{mec1N2229K} protein can still interact with other checkpoint proteins that WT Mec1 interacts with. If so, this would suggest that the \textit{mec1N2229K} allele might be dominant negative, because the \textit{mec1N2229K} allele might interfere in Mec1p function. Evidence that \textit{mec1N2229K} is dominant negative would suggest \textit{mec1N2229K} is not simply degraded. However, galactose inducible overexpression of the \textit{mec1N2229K} allele in a WT background did not result in a Mec\(^{-}\) phenotype; cells were HU and UV resistant and still arrested at the G\(_2\)/M checkpoint in a \textit{cdc13} test (data not shown and see Figure 3-3 (page 87) for a description of the \textit{cdc13} test). Therefore, the \textit{mec1N2229K} allele is not dominant negative by these criteria.
Figure 2-4. Mec1N2229K protein of the correct size can be detected on a Western Blot. This blot shows both immunoprecipitated (IP) Mec1 and Mec1N2229K protein, and protein from total cell lysates. The same number of cells was used in each total protein lane and the same number of cells was used in each IP lane. In all cases, the proteins were LexA fusions, and were immunoprecipitated and probed with the LexA antibody (courtesy J. Little). They were visualized by using a secondary antibody conjugated to peroxidase in conjunction with the ECL detection reagent. (See methods #9, 10, 13, on pages 216, 217, and 219.)

CREATION AND CHARACTERIZATION OF THE mec1ts ALLELE

Conditional alleles are useful tools in studying gene function. They are especially useful when the gene of interest is essential, as is the case with MEC1. A typical type of conditional allele is the temperature-sensitive (ts) allele. I created ts alleles of mec1 by hydroxylamine mutagenesis of WT MEC1 present on a plasmid (see methods #6 on page 214). The plasmid-linked mec1ts alleles
were identified by the complementation of the HU sensitivity of a genomic \textit{mec1-1} strain at 23°C but not at 37°C. The 28 plasmid-linked \textit{mec1}^{is} alleles I obtained were compared for HU sensitivity at different temperatures. Overall, they all behaved similarly: they were HU resistant at 23°C and HU sensitive at 30-37°C. The strongest \textit{mec1}^{is} allele was selected for further study and integrated into the yeast genome. After integration, this \textit{mec1}^{is} allele was genetically mapped to the \textit{MEC1} locus. (Progeny from the sporulation and tetrad dissection of a \textit{mec1-1/mec1}^{is} diploid strain produced no WT [HU-resistant at 37°C] segregants in 46 tetrads dissected. If \textit{mec1}^{is} and \textit{med-l} were not linked, one-quarter of the meiotic progeny would be expected to be WT [HU-resistant at all temperatures].) The integrated \textit{mec1}^{is} cells grow well on rich media and HU at 23°C and 30°C; hydroxyurea sensitivity on plates occurs at or above 32°C, and poor growth occurs on rich media from about 34°C. Figure 2-5 shows that \textit{mec1}^{is} is still functional at 23°C with respect to HU/UV sensitivity, but loses these functions at 36°C.

The \textit{mec1}^{is} allele was useful for asking whether Mec1p is required to act continuously in G\textsubscript{2} to maintain G\textsubscript{2} arrest (this chapter); for the generation of suppressors of \textit{mec1}'s essential function (Chapter 5); for showing that \textit{mec1-1}'s phenotypes are due to mutation(s) at the \textit{MEC1} locus and not due to \textit{sml1} (this chapter); for asking whether ensuring proper telomere length might be \textit{Mec1}'s essential function (Chapter 5); for looking for synthetic lethality between alleles of
MEC1 and PIK1 (Appendix 2), and for testing MEC1's role in RNR1 transcription (Chapter 5).

![Graph showing UV sensitivity of mecV allele at different temperatures.](image)

**Figure 2-5.** Characterization of UV and HU sensitivity of the mec1ts allele at different temperatures. **a**, mec1ts cells (RGY2) have the same UV sensitivity as WT cells (RGY4) at 23°C, are slightly more sensitive at 30°C, and are as sensitive as mec1-1 cells (TWY177) at 36°C. Cells were incubated at each temperature for three hours both before and after UV exposure, then outgrown at 23°C. Viability was scored as the percentage of microcolonies that were growing. **b**, (following page) mec1ts cells have the same HU sensitivity as WT cells at 23°C, and over time at 37°C eventually show the same sensitivity as mec1-1 cells.
Figure 2-5 continued. Legend on previous page.

DNA damage-dependent roles of MEC1 are unaffected by a suppressor of the essential function.

Subsequent to the isolation of the mec1-1 allele (159), Paulovich et al. (110) and Rothstein (121) discovered that the mec1-1 strain contains an unlinked suppressor called sml1 that is required for viability of mec1-1 mutants. The presence of the suppressor mutation sml in our mec1-1 strains required that we determine whether the previously assigned roles of MEC1 are due to mutations in MEC1 and not to mutations in the suppressor sml1. I used my mec1ts allele to compare the phenotypes of strains containing the mec1ts mutation shifted to the restrictive temperature to the phenotypes of mec1-1 sml1 strains. For all phenotypes assayed, I found that mec1ts mutants have quantitatively similar
mutant phenotypes as \textit{mec1-1 sml1} strains. The phenotypes include UV sensitivity, sensitivity to hydroxyurea, G2/M arrest after DNA damage and transcriptional induction of \textit{RNR3}, a damage-inducible transcript (Figure 2-6). (The HU-sensitivity does shows a phenotypic lag in \textit{mec1}\textsuperscript{ts} strains, which may be in part due to kinetics of inactivation of the \textit{mec1}\textsuperscript{ts} allele; see experiments in Figure 2-7 in the following section). I conclude that the roles ascribed to \textit{MEC1} from studies of the \textit{mec1-1 sml1} strains are due to \textit{MEC1} gene function. It has been previously shown by similar criteria that the meiotic roles of \textit{MEC1} inferred from analysis of \textit{mec1-1 sml1} strains are due to the \textit{MEC1} gene (84).
Figure 2-6. Comparison of mec1-1sml1, mec1\(^{as}\), and mec1\(^{as}\) sml1 phenotypes. Bars represent percent WT response, where WT is normalized to 100% response. All assays were done at mec1\(^{as}\) restrictive temperature (36°C or 37°C). N.D., not done. For viability assays, cells were treated as indicated, plated, and the percentage of viable microcolonies was determined (see methods #2, page 212.). For cdc13 or UV delay experiments, the bar represents the percentage of cells delayed in medial nuclear division (the G2/M arrest point) (see methods #11 and 12, page 217 and 218.) Transcriptional induction was measured by quantitating the RNR3 signal on a Northern blot and normalizing it to the signal from a control probe (see methods #13, page 219). Growth on .1M HU plates (not shown in the figure) was also determined and scored qualitatively as growth or no growth. While WT strains grow on HU plates, none of the mec1 strains shown in the figure do. mec1-1sml1, TWY308; mec1\(^{as}\), RGY2; and mec1\(^{as}\) sml1 (RGY7)
**MEC1 is required to act continuously in G2 for G2 arrest.**

Results presented in this dissertation and published elsewhere (70, 94, 159) indicate that *MEC1* is a central regulator for many cell responses to DNA damage. A simple model of *MEC1* function postulates that as long as DNA damage persists, *MEC1* will signal responses. An alternative model postulates that *MEC1* may need only initiate the responses, and once initiated other mechanisms maintain the responses until damage has been repaired. To distinguish between these models, I made use of the conditional *mec1*<sup>ts</sup> allele and determined whether *MEC1* is needed to both establish and to maintain arrest at the G2/M checkpoint. To generate DNA damage I made use of conditional expression of the site-specific endonuclease HO which introduces a single double strand break at the *MAT* locus (see methods #7 and 8, page 215 and 216). I prevented repair of the double strand break by introducing a *rad54* mutation that eliminates double strand break repair. Arrest after an HO-induced DSB is profound in cells unable to repair the break (8 and Weinert lab unpublished results). I used this form of DNA damage and tested if *MEC1* is required to establish and to maintain arrest. Induction of HO endonuclease in a *mec1*<sup>ts</sup> *rad54* strain grown at the permissive temperature resulted in a quantitative arrest of cells at the G2/M checkpoint (Figure 2-7a). To test if *MEC1* function was required to maintain arrest, I shifted arrested cells to the restrictive temperature of 36°C. *mec1*<sup>ts</sup> cells resumed mitosis 2-4 hours after shift to the restrictive temperature while *mec1*<sup>ts</sup> mutant cells maintained at 23°C, or Mec<sup>+</sup>
cells at either high or low temperatures, remained arrested (Figure 2-7b). I conclude that in the presence of damaged DNA, Mec1p function is required continuously to both establish and maintain the arrest response.

Figure 2-7. Kinetics of inactivation of the G2 checkpoint activity of mec1ts. a, experimental protocol (see also methods #8, page 216). It took about six hours after the addition of galactose (which promoted induction of the HO endonuclease, a source of DNA damage) for 80% of the cells to become arrested at the G2/M checkpoint. b, MEC1 is required to maintain G2 checkpoint arrest. The graph shows the actual percent of cells (not normalized) arrested at the G2/M checkpoint ("medial nuclear division"). Average of duplicates is plotted; bars indicate values of each duplicate. Where bars are not shown, they are smaller than the plot symbol. Mec+, TWY185; mec1ts, RGY5.
CHAPTER 3: A DNA DAMAGE CHECKPOINT GENE NETWORK AND THE ROLES OF MEC1 ACTING ON PARALLEL PDS1 AND RAD53/DUN1-DEPENDENT PATHWAYS

FOREWORD TO CHAPTER 3

This chapter is an adapted and expanded version of a portion of a manuscript being prepared for publication. I performed all of the experiments described here, except as follows: The UV kill curve experiments (Figure 3-11), were done by C. Putnam (although I provided some of the strains); and although the Western blots (Figure 3-10) were mine, the Western blotting experiments as a whole were a joint venture between myself and T. Weinert.

INTRODUCTION

After DNA damage, all eukaryotic cells respond by altering cell cycle progression and gene expression — responses that serve to both maintain cell viability and genomic stability. After DNA damage, delays occur in many stages of the mitotic cycle as well as the meiotic cell cycle, and increases in transcription of many repair genes occur as well (For reviews, see 32, 105, 109). Cell cycle controls called checkpoints regulate all of these damage-inducible responses; the cell cycle delays apparently increase the available time to repair the damage before the ensuing cell cycle events, and transcriptional-induction of repair genes
serves to increase the cells' capacity for repair (1, 3, 52, 70, 100). Unrestrained cell cycle progression of damaged cells can have either of two deleterious consequences, cell death or genomic instability. Studies of mammalian p53 and ATM genes suggest that checkpoint controls maintain genomic stability and therefore prevent genetic rearrangements that lead to cancer (50).

Checkpoint controls have been genetically dissected in several organisms, including budding and fission yeasts, flies, filamentous fungi and mammalian cells (32, 49, 169). Studies of these organisms show that the physiological responses to DNA damage are highly conserved, and the underlying molecular mechanisms appear at least in part to be conserved as well (reviewed in 154). Checkpoint controls regulate delays in G1 (the G1/S checkpoint), in G2 (G2/M checkpoint), and regulate two delay responses during S phase (slowed replication and inhibition of mitosis — the S phase progression and S/M checkpoints, respectively). Checkpoint controls also mediate a delay during meiotic recombination (84) that shares some but not all features of the mitotic DNA damage checkpoints.

A current model for how checkpoint gene dependent responses are triggered by DNA damage involves three types of gene products that are hypothesized to recognize damage, transduce signals, and mediate arrest, respectively. This model is at present supported mostly by genetic studies, with
as yet little direct biochemical evidence. After DNA damage, proteins that may act directly on damage include Rad9, Rad24, Rad17, Mec3, and Ddc1 (81, 87). That the proteins encoded by these genes may act directly on DNA damage derives from in vivo studies that suggest they mediate degradation of dsDNA to ssDNA (87). *RAD17* encodes a protein with sequence similarity to *REC1*, a bona fide 3'-5' exonuclease from *U. maydis*; therefore the in vivo degradation observed may be a direct consequence of checkpoint protein function.

The proteins that act on damaged DNA regulate the activities of protein kinases that signal arrest. The signal transducers include two types of protein kinases. *MEC1* encodes a putative PI-3 kinase, with homologs *rad3* in fission yeast, and ATM and ATR in mammalian cells (for reviews see 54, 171). The budding yeast *MEC1* gene is involved in all checkpoint responses and the damage-inducible transcription response, whereas most other checkpoint genes have more specialized functions (i.e. *RAD9* does not act in the S/M checkpoint (159) and its role in slowing of replication is less dramatic than that of *MEC1* (110)). The *MEC1* homologs in other organisms appear to mediate a similar but not identical spectrum of responses (9, 49).

Two conventional protein kinases, encoded by *RAD53* and *DUN1*, are also involved in checkpoint pathways. *RAD53* in particular appears to act in most if not all *MEC1*-dependent pathways, an issue addressed further in this report. *RAD53* appears to act downstream of *MEC1* because phosphorylation of
Rad53p after damage requires an intact MEC1 gene (127, 148). Similarly, DUN1 is hypothesized to act downstream of RAD53 in transcriptional induction because Dun1p is phosphorylated after damage, and phosphorylation requires RAD53 (3). Clues to epistasis can be deduced from such patterns of phosphorylation, though a caveat to epistasis comes from the potential for feedback by downstream kinases on upstream regulators. The kinase Fus3 phosphorylates the upstream protein Ste7 in pheromone response, for example (174). In addition, the role of phosphorylation in the DNA damage pathways is unclear, and at least in one case phosphorylation does not seem to correlate with arrest (Pati et al., in press).

Finally, the downstream targets of checkpoint pathways in budding yeast remain largely elusive. An exception is the G₁/S delay which occurs by inhibiting CLN cyclin transcription through regulation of Swi4 and Swi6 transcription factors (137). The target of the G₂/M checkpoint, studied here, appears to involve at least PDS1, a gene required for both spindle assembly and DNA damage checkpoints (164, 165).

Details on the mechanisms of activation of specific response pathways are now emerging. A very general model for activation is provided from biochemical studies of a related mammalian PI-3 kinase called DNA-PK (44, 93). DNA-PK (which has no reported role in cell cycle control, 116) is activated indirectly by damage; the DNA-PK catalytic subunit associates with two proteins (called KU) that bind DNA damage directly and thereafter activate the catalytic subunit. In an
analogous manner, in budding yeast Mec1p may serve as the catalytic subunit activated by binding to proteins, perhaps those encoded by RAD24 and RAD9 classes of genes, that bind damage directly. The activated Mec1p would then activate, by phosphorylation, Rad53 and Dun1p, which themselves activate response pathways by phosphorylation.

The goal in this chapter is to test the order and role of genes acting in the G2/M response pathway regulated by MEC1. I performed epistasis tests, using both inactivating alleles as well as activating alleles (where available) in checkpoint genes. In general my results verify general features of the current checkpoint pathway described above; that RAD9 and RAD24 classes of genes act upstream of MEC1, RAD53, and PDS1, for example. I provide two important modifications to the current checkpoint pathways in budding yeast. First, to mediate G2/M arrest MEC1 appears to act upstream of parallel pathways, one involving RAD53 and a second involving PDS1. Each pathway contributes about equally to the G2/M arrest response. Cohen-Fix and Koshland recently came to a similar conclusion from a different approach (21). Second, DUN1 and RAD53 act in a single pathway with very similar roles in arrest and transcription (though from UV survival curves RAD53 and DUN1 appear to make unique contributions to DNA repair). I speculate on the significance of parallel pathways of arrest. Discussion of the parallel pathway model is continued in Chapter 4.
RESULTS PART A: TESTS OF LOSS OF FUNCTION MUTANTS

Epistasis tests of checkpoint gene functions
My principal aim here was to understand the pathway(s) by which MEC1 regulates G2/M cell cycle arrest. (Pathway(s) by which MEC1 regulates damage-inducible transcription, and the pathway leading to MEC1’s essential function are discussed in Chapters 4 and 5.) To determine order of gene function I manipulated appropriate gene functions and performed quantitative assays of the G2/M checkpoint arrest response. Cell cycle arrest in G2/M is assayed in mutants with a temperature-sensitive cdc13 allele; at the restrictive temperature cdc13 defective haploid cells show a robust arrest with 2C DNA content (87). I started with a preliminary model (Figure 3-1) which I tested using genetic epistasis techniques.

The model I derived from the following studies is shown in Figure 3-2 (presented here to explain experimental rationale).
Figure 3-1. Preliminary epistasis model. In this model, G_{2}/M checkpoint arrest is completely dependent on MEC1, RAD53, and PDS1. DUN1 is required for transcriptional induction after damage but is not involved in checkpoint arrest. M/A stands for metaphase/anaphase.

Figure 3-2. Current Model of checkpoint arrest pathways. This model summarizes our current understanding of genetic pathways leading to G_{2}/M checkpoint arrest. The arrest signal acts on two downstream pathways, both of which are required for complete arrest. M/A stands for metaphase/anaphase.
Roles of Checkpoint Genes in $G_2$/$M$ arrest

The key to understanding the pathways involved in $G_2$/$M$ arrest was the use of a kinetic and quantitative assay of arrest. My primary assay for the $G_2$/$M$ checkpoint uses $cdc13$ strains (additional assays of arrest are described subsequently). $CDC13$ encodes a gene product that when defective leads to accumulation of ssDNA near chromosome ends (40). The ssDNA apparently contributes to a robust and prolonged cell cycle arrest (87, 156). I analyzed cell cycle arrest in cells with DNA damage as they proceeded synchronously through one cell cycle. Briefly, cells were synchronized at the permissive temperature for $cdc13$ ($23^\circ$C) in the $G_1$ stage by treatment with alpha factor (Figure 3-3). $G_1$ cells were released from arrest by washing out the alpha factor and shifted to the restrictive temperature ($36^\circ$C) for $cdc13$ to allow generation of DNA damage (See methods #11 on page 217). Cells treated in this way either arrest at the $G_2$/$M$ stage (in checkpoint proficient cells) or proceed to subsequent stages of cell division (in checkpoint deficient cells). I included a second mutation in these strains, $cdc15$, to prevent cells that proceed past $G_2$/$M$ from entering the next cell cycle. Mutations in $cdc15$ cause cells to arrest in telophase as large budded cells with a divided nucleus, after the DNA damage $G_2$/$M$ checkpoint. Hence cells lacking $G_2$/$M$ checkpoint function have a distinctive morphology (binucleate) (Figure 3-3) easily distinguishable from checkpoint arrested cells (mononucleate). The combination of $cdc13$ and $cdc15$ mutations results in an assay very sensitive to changes in gene function. I determined the progression of
cells through the cell cycle by examining cell and nuclear morphology in ethanol
fixed and DNA-dye (DAPI)-stained cells (see methods # 11) at times after cells
exit from $G_1$. Extent of delay is determined from the percent of cells arrested in
medial nuclear division (generally 100 cells were scored from each timepoint).

![Figure 3-3. Assay for G$_1$/M checkpoint arrest.]

**MEC1** and **RAD9** are required for complete arrest, but **RAD53** is only
partially required for arrest.

Using the $cdc13$-based assay, I evaluated the roles in cell cycle arrest of
genes apparently involved in detecting DNA damage (**RAD9**) and in signaling
(**MEC1, RAD53, DUN1**). By this assay, Mec$^-$ cells showed a robust arrest in
which $>80\%$ of cells arrest 2 hours after release from $G_1$, and remain arrested for
more than 4 hours. $rad9$ cells showed no detectable arrest compared to $CDC13^+$
(undamaged) cells, as reported previously ((87); Figure 3-4). **mec1** mutants are
also completely defective for arrest (Figure 3-4). (The **mec1** strain shown in
Figure 3-4 is **mec1-1sml1**: I show in Chapter 2 that except for inviability, **mec1**
phenotypes are not affected by the **sml1** suppressor, which confers viability to
**mec1** cells.)
Figure 3-4. *mec1* and *rad9* cells do not delay after *cdc13* damage. **a**, **G**$_2$/M checkpoint delay of WT (DLY408), *mec1* (DLY557), and *rad9* (DLY409) cells after *cdc13* damage. Cells were released from **G**$_2$ arrest before induction of *cdc13* damage. WT cells delay in medial nuclear division in response to damage, while *mec1* and *rad9* cells with *cdc13* damage do not show any more delay than WT cells without damage (*CDC13^+*, DLY418). **b**, percent of cells in part (a) that had proceeded through medial nuclear division. This panel shows that the cells that did not arrest in **G**$_2$ were in fact cycling. In both panels, averages of duplicates are shown. Error bars in these experiments (standard deviations) are smaller than the plot symbols. All strains contain the *cdc15* mutation.
I then examined the role of RAD53, a protein kinase. My initial model (Figure 3-1) and other published models (see for example 3, 25, 100, 127, 148) predict that Rad53 is completely required for G₂/M checkpoint arrest. I was surprised therefore, to find that, rad53-11 cells did arrest for about two hours, after which many arrested cells proceeded through mitosis (see Figure 3-5a). I asked if this partial delay might result from the hypomorphic nature of the rad53-11 mutation (that rad53-11 is not a null allele is inferred because rad53 nulls are inviable and rad53-11 mutants are viable. See 68, 159, 172). Alternatively, this mitotic delay may have been due to slow growth of rad53-11 cells. I therefore generated a rad53A strain that was kept alive by a high copy plasmid encoding the RNR1 gene (24, 127). (RNR1 overexpression has no obvious phenotype in WT cells.) The rad53ApRNR1 cells, like rad53-11 cells, also showed a partial arrest similar to that of rad53-11 (Figure 3-5 b, c).

I then considered whether the mitotic delay in rad53Δ mutant cells was due to some aspect of cell physiology (i.e. poor growth or a delay in S phase of rad53Δ cells caused by the pRNR1 suppressor plasmid per se), or if the delay was indeed the consequence of a specific DNA damage checkpoint signal. To distinguish between these possibilities, I reasoned that any DNA damage-specific delay would require intact checkpoint genes that act upstream of RAD53, like RAD9 or RAD24 (both rad9 and rad24Δ strains show a complete arrest defect (87 and data not shown)).
I therefore analyzed the response of a rad53Δrad24ΔpRNR1 strain and found that the partial delay present in rad53Δ pRNR1 strains was eliminated by the rad24 mutation (Figure 3-5 b, c). I conclude that the delay seen in rad53Δ mutants is indeed due to signaling by a checkpoint pathway.
Figure 3-5. *rad53* cells show G1/M checkpoint delay after cdc13 damage. Cells were released from G1 arrest before induction of cdc13 damage. Averages of duplicates were plotted; error bars represent value of each duplicate. Where bars do not appear, they are smaller than the plot symbol. a, an accumulation of medial nuclear division cells indicate that *rad53-11* cells (DLY554) delay after cdc13 damage. b, similarly, *rad53* cells (RGY81) show mitotic delay after cdc13 damage. This delay is dependent on *rad24*, (*rad53-rad24cdc13*, RGY91) and therefore is checkpoint delay. c, the percentages of post mitotic cells from the strains in part (b) show that the cells that do not arrest at medial nuclear division are indeed cycling.
I also considered whether RAD53's partial role in arrest may in some way be peculiar to the type of DNA damage in cdc13-defective cells. I therefore analyzed cells containing one unrepairable double strand break (from the HO-endonuclease, see methods #7 on page 215 for details), and found similar results; rad9 and mec1 mutants are completely arrest-defective while rad53Δ mutants again show a partial arrest defect (Figure 3-6. The background delay seen in rad9 and mec1 mutants is likely to be due to slow growth resulting from a non-optimal carbon source, or a slight asynchrony of cells entering the cell cycle after release from the alpha factor block). I conclude that the mechanism of arrest after DNA damage proceeds completely through RAD9 and RAD24, completely through MEC1, but may involve both RAD53 and another parallel pathway. This hypothesis is tested below.
Figure 3-6. *dun1* and *rad53* cells show partial checkpoint delay after double stranded break DNA damage. Cells were arrested in G1, with alpha factor and simultaneously HO damage was induced by addition of galactose (the HO endonuclease was under control of the GAL promoter.) After induction of an HO double-stranded break, WT cells (TY54) arrest in response to the DNA damage; *rad9* (RGY133) and *mec1* (RGY131) cells do not. As is the case after *cdc13* damage, described in this and the following section, *dun1* (RGY135) and *rad53* (RGY132) cells arrest partially. Arrest levels for WT, *dun1* and *rad53* strains are not as high as after *cdc13* damage because of incomplete efficiency (about 70%) of HO induction or cutting (see methods #7, page 215). Averages of duplicates were plotted; error bars represent value of each duplicate. Where bars do not appear, they are smaller than the plot symbol.

**DUN1 and RAD53 act in the same pathway for G1/M arrest**

*DUN1* is a protein kinase initially reported to act specifically in transcriptional induction of repair genes (173). In testing roles of genes shown in the checkpoint pathways in Figure 3-1, I reevaluated the role of *DUN1* in arrest and found that *dun1* mutants also show a partial arrest defect very similar to that of *rad53* mutants (Figure 3-7a ). In addition, in cells with an irreparable double
strand break, dun1 mutants also showed a similar partial arrest defect (Figure 3-6). Recently, dun1 mutants were reported independently to have an arrest defect after UV irradiation (107).

DUN1 and RAD53 genes therefore appear to have similar roles in providing for part of the G2/M arrest. Both genes encode protein kinases with similar motifs outside of the kinase domains (see Discussion), so the two genes may have overlapping functions. I therefore tested whether the partial arrest in each single mutant may be due to residual arrest activity provided by the other intact gene. If so, a dun1 rad53 double mutant would be completely arrest defective. To test this hypothesis, I generated rad53 dun1 double mutants and tested cell cycle arrest. (During strain construction I discovered that rad53-11 dun1Δ double mutants are inviable, but viability was restored with high copy RNR1 plasmid, enabling the experiment to be done.) I found that a cdc13 rad53Δ dun1Δ pRNRI strain shows a partial arrest similar to that of either single mutant (e.g. rad53Δ cdc13 pRNRI) (Figure 3-7b). The largest component of this partial delay was due to checkpoint signaling because I showed that most of the partial delay of a cdc13 rad53 dun1 strain does require an intact RAD24 gene (Figure 3-7b. Again, a slight background delay is evident; this may be due to a slightly asynchronous release from G1 arrest and is not considered further). I conclude that RAD53 and DUN1 act in single pathway in G2/M arrest. The basis for partial
arrest phenotypes of *rad53* and *dun1* single mutants may still be explained by
identification of another separate pathway.
Figure 3-7. DUN1 is a checkpoint gene which acts in the RAD53 G2/M arrest pathway. In both panels, cells were released from G1 arrest and cdc13 damage was induced. Averages of duplicates were plotted; error bars represent value of each duplicate. Where bars do not appear, they are smaller than the plot symbol. a, dun1Δ (RGY201 or RGY86, not shown) cells fail to maintain arrest after cdc13 damage, and thus DUN1 is a checkpoint gene. b, the rad53Δ dun1Δ double mutant (RGY102) shows a mitotic delay similar to either the rad53Δ or dun1Δ single mutants after cdc13 damage. (Compare the rad53Δ response in Figure 3-5b.) This delay depends on RAD24 (rad53Δ dun1Δ rad24Δ strain, RGY105), and thus is G2/M checkpoint delay. Because the double mutant behaves as either single mutants, RAD53 and DUN1 are in the same pathway for G2/M arrest.
The role of *PDS1* in the parallel pathway of arrest.

*PDS1* was previously identified as a gene required for arrest at the spindle assembly checkpoint and the DNA damage checkpoint. After DNA damage, Yamamoto et al. reported that *pds1* mutants appeared to exhibit a partial arrest after X-irradiation (e.g. they found that arrest kinetics of *pds1* mutants, judged from bud morphology, appeared distinct from the arrest kinetics of *rad9* mutants (165)). The nature of the residual delay in *pds1* mutants after damage was unknown. I have analyzed quantitatively the arrest of *pds1* cells (Figure 3-8).

Quantitation of arrest is critical, and is complicated in *pds1* mutants because at 36°C they have an additional temperature-sensitive defect in progression through mitosis (164). I therefore performed the DNA damage arrest assays at 30°C (instead of at 36°C), as the lower temperature is more permissive for *PDS1*'s essential function yet still restrictive for *cdc13*. (At 30°C, *pds1 CDC13* mutants, without DNA damage, still show some mitotic delay. The residual delay at 30°C persists in *pds1 CDC13* *rad9* mutants and is therefore DNA damage and checkpoint gene-independent; Figure 3-8b).

Following generation of DNA damage in *cdc13* mutants grown at 30°C, *pds1 cdc13* mutants exhibit a substantial mitotic delay (Figure 3-8a). This additional *cdc13*-dependent delay, above that seen in *pds1 CDC13* cells, is due to checkpoint signaling because that additional component of the delay does require an intact *RAD9* gene (e.g. no additional delay in *pds1 cdc13 rad9*; Figure
3-8a). I conclude that \textit{PDS1} contributes to G\textsubscript{2}/M arrest, but that another pathway may account for the partial delay.
Figure 3-8. *pds1Δ* cells show G2/M checkpoint delay. Parts a and b represent results from one experiment, but are shown on separate plots for clarity. For each strain, G1 cells were released from arrest before induction of *cdc13* damage. a, *pds1Δ cdc13* cells (CPY189) show a mitotic delay after *cdc13* damage at 30°C. A substantial amount of this delay is checkpoint dependent because the delay in a *pds1Δ rad9 cdc13* strain (DLY677) is much lower. (*RAD9* is completely required for G2/M checkpoint arrest.) The background delay in the *pds1Δ rad9 cdc13* strain is due to the effect of *pds1Δ* mutation at semi-restrictive temperatures, and is eliminated in the *rad9cdc13* (*PDS1*) strain (DLY409). b, additional controls show that WT *cdc13* cells (DLY408) show checkpoint arrest at 30°C, and that in *pds1Δ* cells without DNA damage (*CDC13*) (CPY207) there is still background delay due to the *pds1Δ* mutation. 30°C was used as the *cdc13* restrictive temperature instead of 36°C because *pds1Δ* strains are temperature sensitive and exhibit less background delay at the lower temperature.

Averages of duplicates were plotted; error bars represent value of each duplicate. Where bars do not appear, they are smaller than the plot symbol.
G2/M checkpoint arrest is eliminated in pds1rad53 cells

Because each of the RAD53 (and DUN1) and PDS1 pathways account for only part of the arrest response, I determined if these two sets of genes act in the same or in parallel pathways to contribute to G2/M arrest. I generated strains that were defective for pds1 and either rad53 or dun1, and analyzed the cell cycle kinetics as before. I found that the checkpoint dependent cell cycle arrest is completely eliminated in rad53Δpds1Δ pRNRIceWs, in rad53-11pds1Δ cells, and in dun1Δpds1Δ cells (Figure 3-9). In each case, the level of arrest of the double mutant (pds1rad53 or pds1dun1) after damage is nearly exactly the same as the level of arrest due to the background delay of pds1Δ mutants. These results are incorporated into the model shown in Figure 3-2 in which MEC1 mediates G2/M arrest by acting separately through both the RAD53/DUN1 pathway and the PDS1 pathway. The roles of both pathways in yet other MEC1-dependent events are considered below.
Figure 3-9. *pds1*rad53 and *pds1*dun1 strains do not show checkpoint delay after *cdc13* damage. Following Page. In each case, cells were released from G1 arrest before the induction of *cdc13* damage. For clarity, the *pds1*CDC13*+* controls (CPY201) were placed on separate plots to the right of each figure they accompany. These controls show the level of background delay due to the *pds1*Δ mutation alone, without DNA damage. Averages of duplicates were plotted; error bars represent value of each duplicate. Where bars do not appear, they are smaller than the plot symbol. a and b, *rad53-11*cdc13 (DLY554) and *pds1*Δ*cdc13* (CPY189) strains show mitotic delay after damage, which is substantially reduced in the *pds1*Δ*rad53-11*cdc13 double checkpoint mutant (CPY238). This level of reduction represents the amount of mitotic delay due to the checkpoint. The level background delay of a *pds1*Δ strain without damage (b) is nearly identical to the level of delay of the double mutant in part (a) with damage. c and d, *rad53Δcdc13 pRNR1* (RGY167) and *pds1Δcdc13* strains (CPY189) show mitotic delay after damage, which is substantially reduced in the *pds1Δrad53Δcdc13 pRNR1* double checkpoint mutant (RGY169). e and f, *dun1Δcdc13* (RGY86) and *pds1Δcdc13* (CPY189) strains show mitotic delay after damage, which is substantially reduced in the *pds1Δdun1Δcdc13* double checkpoint mutant (CPY215). The level background delay of a *pds1*Δ strain without damage (f) is nearly identical to the level of delay of the double mutant in part (e) with damage.
% medial nuclear division

- Graphs showing data with labels for time and CDC13.
Phosphorylation of Pds1p

Recently Cohen-Fix and Koshland showed that Pds1p becomes phosphorylated after damage, and phosphorylation requires an intact MEC1 gene, but does not require RAD53 (21). Rad53p phosphorylation dependent on MEC1 was shown previously as well (127, 148). The role of phosphorylation of Rad53p or Pds1p in arrest remains, however, unknown. The model in Figure 3-2 predicts that the phosphorylation of Pds1 after damage should not require RAD53 or DUN1. Indeed, as mentioned above, recent experiments (21) have shown that RAD53 is not required for Pds1 phosphorylation after damage. However, since RAD53 and DUN1 share several functions and may be partially redundant, we thought it important to determine if phosphorylation of Pds1 depends on either RAD53 or DUN1. To test this, we used a rad53-1 dun1Δ double mutant (kept alive by pRNR1), and examined the level of phosphorylation of Pds1 with or without cdc13 damage. While the effect is usually very slight (and sometimes Pds1 phosphorylation is not seen after damage even in the WT control), it appears that damage induced phosphorylation still occurs in the dun1rad53 strain (Figure 3-10 shows the clearest data I obtained). I conclude that the dun1rad53 pathway does not seem to be required for Pds1 phosphorylation after damage.
Figure 3-10. Rad53 and Dun1 are not required for Pds1 phosphorylation after damage. a, Western blot shows that phosphorylation of Pds1p in WT (RYG206) and rad53dun1 (RYG205) strains increases slightly after cdc13 damage (30°C) as compared to no damage (23°C). Identification of bands was determined by comparing to a no Pds1HA control (not shown). The phosphorylation signal does not seem to increase in mec1 cells after damage (RYG225, not shown) but mec1 results were less conclusive (blots were hard to interpret). b, quantitation of signals (Stratagene Eagle Eye Densitometer). The phosphorylation increase after damage is slight and not always reproducible. Shown in this figure are the best representative data. See methods #13, page 219.
UV resistance mediated by checkpoint pathways

The MEC1-dependent pathways contribute to survival of DNA damage by at least two mechanisms: increased gene expression of DNA repair proteins and cell cycle delay. We have tested the contributions to UV survival from both elements of the checkpoint pathways by comprehensive analysis of UV sensitivity of checkpoint mutants defective for G2/M arrest. (Figure 3-11). We found that *pds1* mutants are the least UV sensitive, consistent with its specific role in partial cell cycle arrest. *dun1* and *rad53* have greater sensitivity than do *pds1* mutants, concordant with their roles in transcriptional induction and in partial arrest. Eliminating both G2/M arrest pathways, in *pds1 dun1* or *pds1 rad53* mutants, increases UV sensitivity beyond defects in either pathway, as would be expected if each contributes to UV resistance independently. (Note that *rad53* mutants are more UV sensitive than are *dun1* mutants in our strains (A364a and W303 backgrounds). The basis for *RAD53*’s greater contribution to UV survival than *DUN1* is unknown, though it may be due to *RAD53*’s greater role in the S/M checkpoint; *rad53* mutants are very HU sensitive while *dun1* mutants are less so (unpublished)). *dun1 rad53* double mutant cells are more UV sensitive than either single mutant, suggesting different roles for the two genes at additional levels of control beyond G2/M arrest (fig. 3-19). Finally, the UV sensitivity of *mec1smi1* or *mec1ΔpRNR1* mutants (pRNR1 was used to suppress the essential function of *MEC1*) is still greater than UV sensitivity in *pds1 rad53*
mutants and even in *pds1 dun1 rad53* mutants. This suggests that *MEC1* has at least one additional role in tolerance to DNA damage that is *RAD53*, *DUN1* and *PDS1*-independent. The nature of that role is unknown, though transcriptional induction of *MEC1* and *RAD53* themselves requires *MEC1* but not *RAD53* (70); the relevance of this or other mechanisms to explain *MEC1*'s control of damage tolerance remain to be tested.
Figure 3-11. UV survival curves of checkpoint deficient strains. 

(a) comparisons of double mutants of \(pds1\), \(dun1\) or \(rad53\)-11. (Double mutants of \(dun1\) and \(rad53\) are nonviable). 
(b) In order to compare strains carrying deletions of the essential genes \(MEC1\) and \(RAD53\), as well as the double mutant \(dun1\Delta rad53\), those strains were transformed with a high copy \(RNR1\) plasmid to maintain viability. 
For reasons not clear, strains in which \(RNR1\) expression was not required for survival exhibited a high rate of plasmid loss, especially so when \(PDS1\) was deleted. Consequently, this analysis was limited to the strains requiring \(RNR1\) for viability. 
(c) Rank order of UV sensitivity, compiled from the UV survival curves presented in (a) and (b), as well as many similar experiments (all of which gave consistent results). Note that the sensitivity of the \(mec1\) strain (shown in part a) or of the \(mec1\Delta\) strain (b) is considerably greater than that of strains carrying any combination of \(dun1\), \(rad53\) and \(pds1\) mutations.

Strains used in part a: WT, DLY 408; \(pds1\), CPY 189; \(dun1\), RGY86; \(rad53\)-11, DLY554; \(pds1\Delta dun1\), CPY215; \(pds1\Delta rad53\), CPY238; \(mec1\)-1sml1, DLY557. Strains used in part b: WT, RGY241; \(rad53\), RGY244; \(pds1\Delta rad53\), RGY246; \(dun1\Delta rad53\), RGY247; \(dun1\Delta pds1\Delta rad53\), RGY248; \(mec1\), RGY240. (Additional strains containing \(pRNR1\) used in other experiments which gave results consistent to the ones shown were: \(dun1\), RGY242; \(dun1\Delta pds1\), RGY245; \(pds1\), RGY243)
RESULTS PART B: TESTS OF GAIN OF FUNCTION MUTANTS

Tests of overexpression of *DUN1*, *RAD53* and *PDS1* on G\textsubscript{2}/M arrest

The model presented in Figure 3-2 predicts that active alleles of genes downstream from *MEC1* may cause a G\textsubscript{2}/M delay independent of upstream genes. In further tests of this model, I found, as reported previously, that overexpression of either *RAD53* (68, 148) or of a non-degradable allele of *PDS1* (*PDS1*\textsubscript{mdb}) (22) caused the cells to delay in medial nuclear division (Figure 3-12). I found that the delay by *RAD53* overexpression was both *PDS1*- and *MEC1*-independent, and that the delay caused by *PDS1*\textsubscript{mdb} was both *RAD53*, *DUN1* and *MEC1*-independent (Figure 3-12). (*DUN1* overexpression did not cause a detectable cell cycle delay; data not shown.) These results are consistent with the model in which *RAD53* and *PDS1* act in separate pathways and act downstream of *RAD9*, *MEC1*, and DNA damage. The interpretations from these epistasis experiments carry an important caveat, however: I cannot show that the delays are indeed due to effects on the checkpoint pathways. (In studies discussed in the following chapter, I show that delays caused by overexpression of *TEL1* are *PDS1*-dependent, and therefore do occur by activation of a checkpoint pathway.) Delays caused by overexpression of *RAD53* and of *PDS1*\textsubscript{mdb} may provide genetic tests of roles of candidate genes that may act downstream.
Figure 3-12. RAD53 or PDS1 overexpression induce delay in strains of many genotypes. RAD53 or PDS1mdb were overexpressed under a GAL promoter. In the RAD53 experiments, cells were arrested in alpha factor and released. The highest percentage of medial nuclear division cells over a several hour period is shown, for cells either overexpressing RAD53 or containing an empty vector. Delay caused by RAD53 overexpression was PDS1-independent. Averages of duplicates were plotted; error bars represent value of each duplicate.

In the PDS1mdb experiments, cells were arrested with either alpha factor or HU (for technical reasons), and the highest percentage of medial nuclear division cells over a several hour period is shown. All the cells contained the PDS1mdb construct, but the cells were either induced with galactose or were grown in raffinose, in which case the GAL promoter was not activated. Delay caused by PDS1 overexpression was RAD53-independent. The PDS1mdb experiments were not done in duplicate. WT, DLY62; mec1, DLY258; dun1, RGY68 and 69; tel1, RGY88 and a derivative made by integrating PDS1mdb; pds1, CPY201; rad53, DLY259.
RESULTS PART C: TESTS OF ALTERNATIVE HYPOTHESES
THAT MAY EXPLAIN THE PHENOTYPES OF rad53, dun1, AND pds1 MUTANTS

RAD53, DUN1, and PDS1 do not promote maintenance of checkpoint arrest through CDC5

The model presented in Figure 3-2 (page 85) accounts for the observations presented so far in this chapter, and specifically accounts for those observations showing that pds1, dun1, and rad53 mutant cells show a transient cell cycle delay after damage (Figures 3-5,6,7,8 on pages 91, 93, 96, and 99). The model postulates two pathways leading to arrest, either of which acting alone can produce partial arrest. I considered alternative explanations for RAD53s, DUN1's, and PDS1's role in G2/M checkpoint arrest; arrest may be established by one pathway and maintained by the pathway. In fact, there is evidence for separate controls in yeast required to establish and to maintain arrest. The evidence for separate mechanisms comes from the following observation: Yeast cells with damage arrest but, even when the damage persists, those cells eventually resume cell cycle progression (128, 149, Weinert lab unpublished). Resumption of cell cycle progression when damage persists is called adaptation. (Apparently, normal cells, even with continuous DNA damage, eventually exhibit a "defect" in maintaining arrest and adapt after about 10 hours.) Toczyski and Hartwell identified a yeast mutation, called cdc5-ad, in which mutant cells with damage fail to adapt after 10 hours; rather they remain arrested indefinitely.
I therefore considered whether in normal cells, the \textit{RAD53 (DUN1)} or \textit{PDS1} pathways might contribute to arrest during the four hour arrest by inhibiting adaptation. By this model, for example, \textit{rad53} mutant cells would establish arrest by the \textit{PDS1} pathway, but then fail to maintain arrest because adaptation, normally inhibited by \textit{RAD53}, is not inhibited. If either the \textit{RAD53} or \textit{PDS1} pathways normally inhibit adaptation, then that "adaptation regulatory" pathway would not be required to maintain arrest in \textit{cdc5-ad} mutants (where adaptation does not occur). Figure 3-13 illustrates this model. I found, however, that \textit{pds1 cdc5-ad, dun1 cdc5-ad} and \textit{rad53 cdc5-ad} mutants all show only a partial arrest phenotype in a \textit{cdc13} background (Figure 3-14). The simplest explanation for these findings is that \textit{RAD53, DUN1, and PDS1} do not act by inhibiting adaptation. Although I cannot rule out that \textit{RAD53, DUN1, and PDS1} could be inhibiting adaptation by a Cdc5-independent mechanism, the checkpoint arrest phenotypes of these genes are most easily explained by the model in Figure 3-2. The possible roles of parallel arrest pathways is addressed further in the Discussion.
Figure 3-13. Model of hypothetical role of Pds1, Dun1, and/or Rad53 in maintaining G2/M checkpoint arrest by inhibiting Cdc5. By this hypothesis, Pds1, Dun1, and/or Rad53 are involved in inhibiting Cdc5 activity, which itself inhibits checkpoint arrest. In this way these proteins would be required for maintenance, but not initiation, of checkpoint arrest.
Figure 3-14. Dun1, Pds1, and Rad53 are not involved in maintenance of arrest by inhibiting Cdc5. Cells were released from G1 arrest followed by induction of cdc13 damage. Averages of duplicates were plotted; error bars represent value of each duplicate. Where bars do not appear, they are smaller than the plot symbol. cdc13 damage induced delay is not increased by a cdc5-ad mutation in pds1, rad53, and dun1 cells. dun1, RGY86; dun1cdc5-ad, RGY125; pds1, RGY177; pds1cdc5-ad, RGY200; rad53, DLY380; rad53cdc5-ad, RGY123.
The *CDC28AF* mutation does not eliminate G$_2$/M checkpoint arrest in combination with mutations in *RAD53* or *PDS1*.

I next considered whether either of two pathways may normally act on the Cyclin Dependent Kinase (CDK) p34\textsuperscript{Cdc2/Cdc28} kinase to achieve arrest. In fission yeast and higher eukaryotes, checkpoint proteins promote arrest by inhibiting CDK activity by phosphorylating (or preventing dephosphorylation of) specific CDK residues (35, 119, 123); however, the following evidence indicates that in budding yeast, checkpoint controls do not seem to achieve arrest by phosphorylating the corresponding residues of p34\textsuperscript{Cdc28}: Amon et al. (4) and Sorger and Murray (144) found that strains carrying *CDC28AF*, in which the phosphorylated amino acids have been mutated to non-phosphorylatable residues, still arrest after DNA damage. I thought it plausible that an overlapping role for inhibitory phosphorylation of Cdc28 in arrest might be revealed in *pds1* or *rad53* mutants. I found, however, that *CDC28AFpds1* and *CDC28AFrad53-11* mutant cells retained a brief delay as in *pds1* and *rad53* checkpoint mutant strains (Figure 3-15). Therefore, a role for Cdc28 phosphorylation after DNA damage is still unclear.
Figure 3-15. **CDC28AF mutations do not decrease checkpoint delay.** rad53 (a) and pds1 (b) strains' partial checkpoint delay after cdc13 damage does not depend on phosphorylation of Cdc28 residues thr18 and tyr19. The experiments were done the same as those described before (cells were released from arrest followed by induction of cdc13 damage), except the CDC28AFpds1 experiment (b) was started from HU arrested cells instead of alpha factor arrested cells. (This was done simply because one of the strains was not available with the mating type a, which is required for arrest by alpha factor.) Averages of duplicates were plotted; error bars represent value of each duplicate. Where bars do not appear, they are smaller than the plot symbol. rad53cdc13CDC28+, RGY114; rad53cdc13CDC28AF, RGY113; cdc13CDC28AF, RGY116; pds1cdc13CDC28AF, RGY178; pds1cdc13, RGY177; pds1Cdc*, RGY143.
DISCUSSION

Mec1-mediates responses through a checkpoint gene network

The principal goal of the work presented in this chapter was to analyze checkpoint pathways mediated by MEC1 leading to damage-inducible cell cycle arrest. The principal findings are summarized in the model in Figure 3-2 (page 85), which is a substantial revision of previous models (3, 107). Most importantly, I found that MEC1 mediates G2/M arrest by acting through two parallel pathways. One pathway contains the RAD53 and DUN1 encoded protein kinases, and the second pathway contains the PDS1 gene product. Each pathway makes roughly equal contributions to the G2/M arrest response. This conclusion comes from data that show that neither the rad53 pds1 nor the dun1 pds1 double mutant strains show checkpoint arrest after cdc13 damage (Figure 3-9, page 101). The model in Figure 3-2 is supported by 1) the UV sensitivity data in Figure 3-11 (page 107, discussed below); 2) data showing that overexpression of either RAD53 or PDS1 delay the cell cycle, independently of genes thought to act upstream (Figure 3-12, page 109); and 3) by experiments presented in the following chapter in which TEL1 is overexpressed.

Recently, Cohen-Fix and Koshland (21) developed a similar model of two pathways based on a different and less direct experimental strategy. They characterized the phosphorylation of Pds1, and found that Pds1p becomes
phosphorylated after damage, and that phosphorylation required *MEC1* but did not require *RAD53*. They also reported that transcriptional regulation required *RAD53* but not *PDS1*, and therefore suggested a model similar to that shown in Figure 3-2. However, they did not address the role of *DUN1*. Our preliminary assays suggest that Pds1 phosphorylation is *DUN1* and *RAD53* independent, consistent with the model (tested in *rad53dun1* double mutants, Figure 3-10, page 104).

The UV sensitivity of mutants in specific genes in each pathway is consistent with current understanding of their relative contribution (Figure 3-11, page 107). For example, mutating both arrest pathways (e.g. *pds1 dun1* double mutant) led to greater UV sensitivity than mutating either arrest pathway alone (e.g. *pds1* and *dun1* single mutants). The UV sensitivity profiles also indicate that the *RAD53* and *MEC1* genes contribute to UV survival by mechanisms in addition to G2/M arrest and transcriptional induction. For example, both *mec1* and *rad53* single mutants are more UV sensitive than *dun1 pds1* double mutants. *mec1* strains in particular have the greatest UV sensitivity, greater than even a *rad53 dun1 pds1* triple mutant that eliminates the major responses evaluated here, the G2/M delay and transcriptional regulation. Therefore *MEC1* must have additional role(s) that contribute to UV repair. DNA damage survival curves, as shown in Figure 3-11, will be useful in evaluating other *MEC1*-mediated response pathways. Additional pathways likely include the G1 checkpoint (in which *RAD53*
also acts (3), damage-induced delays during S phase (108), additional roles in transcriptional regulation (70), and perhaps more direct roles in DNA repair.

**Possible mechanisms underlying two pathways leading to G₂/M arrest.**

In considering why two pathways mediate the mitotic arrest response, I considered, and eliminated, one model in which one pathway might establish arrest while the second inhibits adaptation (the ability of cells to recover from arrest even when damage persists).

I consider here two other models to explain parallel pathways of arrest. First, both pathways may converge on a common regulator to achieve optimal regulation. For example, one pathway may activate an inhibitory activity and the other pathway inhibit an activating pathway (Figure 3-16). In fission yeast, for example, complete arrest in G₂/M seems to require chk1, which may regulate two such pathways (102, 153): chk1 may activate the wee1 kinase which maintains an inhibitory phosphorylation on cdc2, and chk1 may inhibit the cdc25 phosphatase which normally activates cdc2 by dephosphorylation (38, 103, and see Chapter 1). By analogy in budding yeast the RAD53 and PDS1 pathways may also converge on one common regulator. The identity of that regulator is unknown, but it certainly does not appear to involve p34^{Cdc28} phosphorylation (4, 144). A role for CDC28 in G₂/M arrest has been recently identified (78), though where CDC28 might fit into the checkpoint pathway in Figure 3-2 remains unclear. See Figure 3-15 (page 115).
Figure 3-16. A model for G2/M arrest involving two pathways analogous to the fission yeast system.
We consider a second, more unusual model to explain parallel pathways of arrest. In this model RAD53 and PDS1 pathways inhibit two consecutive and independent steps in mitosis; RAD53 (and DUN1) may inhibit a G2 to metaphase transition, and PDS1 may inhibit a metaphase to anaphase transition (Figure 3-17). This model rests on several observations that suggest to us that damaged yeast cells may arrest in either a G2-like or a metaphase-like state. That yeast cells with damage may arrest in an interphase-like state seem plausible for the following reasons: arrested cells contain at least some phosphorylated Cdc28, a biochemical marker of interphase (4, 144), and at least some damage-arrested cells do not have fully condensed chromosomes (47). That yeast cells can also arrest in metaphase, at the metaphase to anaphase transition, comes from the following observations: First, benomyl-treated yeast cells, when irradiated, show a robust RAD9-dependent arrest (158). The benomyl-arrest state appears to be metaphase-like because chromosomes are condensed (relative to interphase cells; (47)) and Cdc28, the Cdc2 homolog in budding yeast, is completely dephosphorylated relative to interphase cells. Chromosome condensation and dephosphorylation of Cdc2 are both phenotypes of metaphase in other eukaryotic cells. Second, Pds1p appears to be an inhibitor of the metaphase to anaphase transition, as argued from other observations (164). Third, one report
suggests that cells may experience a \textit{RAD9}-dependent delay even as cells attempt anaphase (166).

I speculate, therefore, that normal cells after damage may be able to arrest in either $G_2$ or in metaphase. Arrest in two states may explain the existence of two pathways to arrest. (That neither pathway is apparently sufficient for arrest suggests an added complexity, and an equilibrium between those states.) There is evidence of mammalian cells moving from a mitotic-like state to interphase after DNA damage. Carlson found that after irradiation, cells in prometaphase (having partially condensed chromosomes) will return to interphase before again progressing through mitosis (discussed in 16, 17). The two stage arrest hypothesis in budding yeast awaits experiments that will determine if, for example, a damaged metaphase cell may return to a $G_2$-like state.
Figure 3-17. A model for G₂/M checkpoint arrest involving two pathways and two arrest points. In this model, the kinases Rad53 and Dun1 promote arrest at G₂, while Pds1p promotes arrest at metaphase. The role of Cdc28 is speculative.

The roles of RAD53 and DUN1

Earlier studies on the protein kinases RAD53 and DUN1 demonstrated their roles in responses to DNA damage (3, 173). Subsequent studies continue to refine and redefine their roles. Recently Pati (107) et al. reported a defect in dun1 mutants in cell cycle delay after UV irradiation, and here I report an arrest defect in dun1 mutants after two other types of damage (in cdc13-defective cells and in cells with a double strand DNA break). I also found that RAD53 and DUN1 act in one pathway contributing to G₂/M arrest (See Figure 3-7 b, page 96). The basis
for their action in a single pathway remains unknown. Curiously, \textit{RAD53} and \textit{DUN1} encode protein kinases related in sequence outside of the kinase domains; both contain an FHA domain (Fork head associated domain), also found in the fission yeast \textit{RAD53} homolog called \textit{cds1} \cite{55}. The role of the FHA domain, as yet unknown, may provide a clue to how these two related protein kinases collaborate in regulating many responses (other responses are discussed in the following chapters). Each protein kinase may have unique substrate targets, each contributing independently to one response, because \textit{rad53 dun1} double mutants show synthetic lethality, as well as greater UV sensitivity than either single mutant (Figure 3-11, page 107). Cell cycle arrest may require that both protein kinases phosphorylate unique substrates, each of which is required for function of the arrest pathway.
CHAPTER 4: TESTS OF THE ROLES OF TEL1 IN MEC1-MEDIATED RESPONSES

FOREWORD TO CHAPTER 4

Chapter 4 is an expanded version of part of a manuscript being prepared for publication. I performed all the experiments discussed here, which the exception of the chromosome recombination/loss assay (Figure 4-9), which was done by L. Shanks.

INTRODUCTION

After DNA damage, budding yeast cells exhibit several responses including checkpoint arrest and damage-inducible transcription (for review, see Chapter 1 and 32, 109). It is thought that checkpoint arrest allows the cell more time to repair the DNA damage, and that this repair is facilitated in part by damage-inducible transcription of repair genes. Checkpoint arrest at multiple checkpoints, including the G2/M and S/M checkpoints (which are discussed in this chapter), as well as damage-inducible transcription of a large set of repair genes, requires a key gene called MEC1 (see references under the MEC1 heading of Chapter 1 and see 154). In addition to its roles in responding to DNA damage, MEC1 is essential (Chapter 2 and 66, 99). (Although the nature of MEC1's essential function is not known, some ideas are discussed in Chapter 5). MEC1 encodes a putative Phosphatidylinositol (PI)/protein kinase; while kinase activity has not yet
been shown, the kinase domain is required for MEC1’s checkpoint, damage-inducible transcription, and essential functions (see Chapter 2).

TEL1 was initially identified as a gene that when mutated caused shortening of telomeres (46, 83). In contrast to the continually shortening telomeres in strains containing mutations in certain other genes required for proper telomere length (such as the EST genes (75) and TLC1 (142)), telomeres in tel1Δ strains are shorter than WT telomeres but do not continue to shorten. tel1Δ strains are viable and grow well (46, 83). The TEL1 gene has sequence similarity to MEC1, especially in the highly conserved kinase domain at the 3’ end of each gene. When overexpressed, TEL1 suppresses several defects in mec1 mutant cells: TEL1 overexpression partially restores viability after radiation or hydroxyurea (HU) exposure (94); restores damage-induced phosphorylation of Rad53p in mec1 mutants (127); and restores viability to mec1Δ cells (94). In addition, while viability of both mec1Δsml1 cells and tel1Δ cells is high, mec1Δsml1tel1 strains grow extremely poorly, suggesting that MEC1 and TEL1 share an overlapping role in an essential function. Taken together, all of these results suggest that MEC1 and TEL1 may share some cellular functions, and that TEL1 may act in checkpoint pathways.

The goal of this chapter is to understand TEL1’s role, if any, in the G2/M checkpoint response, in damage-inducible transcription, and to understand by what pathway(s) overexpressed TEL1 can restore viability to mec1Δ cells, and
can suppress the sensitivity of mec1 mutants to DNA damaging agents and to HU.

I show that TEL1 does not act normally in the G2/M checkpoint pathway, yet when overexpressed, it can activate several responses by genetically defined checkpoint pathways. The pathways activated by TEL1 confirm several aspects presented in a checkpoint pathway model presented in Chapter 3. I speculate on the significance of possible cellular roles of TEL1.
RESULTS

**TEL1 is not required for the G₂/M checkpoint after cdc13 damage**

The results summarized above suggested that *MEC1* and *TEL1* may share some cellular functions, and that *TEL1* might act in checkpoint pathways. To test for roles of *TEL1*, I first analyzed a *tel1Δ* strain and found that mutants still arrest completely after DNA damage (Figure 4-1). Furthermore, since *mec1* mutants are completely defective for arrest (Figure 3-4, page 88), and since *tel1Δ* cells still arrest after damage, the entire mitotic delay is accounted for by *MEC1* with no apparent requirement for a second signaling pathway parallel to *MEC1*. 
Figure 4-1. *tel1Δ* cells are G2/M checkpoint proficient. *tel1Δ* (RGY103) cells were released from G1 arrest before induction of *cdc13* damage. They maintain mitotic arrest for several hours and are thus G2/M checkpoint proficient by this assay (note that release from G1 in this experiment was slow; however, this does not alter the conclusion that the *tel1Δ* cells are checkpoint proficient). Compare with *mec1cdc13* and WT*cdc13* cells, in Figure 3-4 on page 88, and with *rad53cdc13* and *dun1cdc13* cells in Figures 3-5 and 3-7, on pages 91 and 96, noting different time scales. *tel1Δ* (RGY 234) cells were also tested by *cdc13* plate assay and were again found G2/M checkpoint proficient. data not shown.

**TEL1 overexpression causes a G2/M cell cycle delay**

I next examined the role of overexpressed *TEL1* in the cell cycle. To our knowledge, how *TEL1* overexpression suppresses *mec1* mutant phenotypes is unknown, as is whether *TEL1* indeed activates bona fide checkpoint pathways to achieve suppression of *mec1* damage sensitivity. To evaluate the role of overexpression of *TEL1* on checkpoint pathways, I first tested the effect of overexpression on restoration of G2/M arrest in *mec1* mutants after DNA
damage. *TEL1* overexpression (achieved by introducing into the cell a single copy centromere plasmid containing the *TEL1* gene under the control of its own promoter, hereafter designated "pTEL1"), did restore a partial arrest to *mec1* mutants (Figure 4-2). I determined by FACS analysis (not shown) that this arrest occurs in G2 (see methods #14 and 15 on page 220). I tested whether the partial arrest occurs by a bona fide checkpoint pathway by testing if upstream and downstream acting checkpoint genes are required for the partial arrest. Surprisingly, pTEL1 caused a partial arrest response in *cdc13 rad9* cells, and even in *CDC13* cells that presumably do not have any DNA damage (Figure 4-3 and 4-4). Therefore, pTEL1 appears to constitutively activate an arrest pathway independent of DNA damage and independent of *MEC1* and *RAD9* gene functions. (In many of my cell cycle experiments, including those just described, strains that carry a plasmid show a higher level of background delay than do strains that do not carry a plasmid. Compare with Figure 3-4a, page 88, for example. This plasmid-induced background delay seems to be increased at higher temperatures, and has been reported by others (T. Weinert, personal communication). The cause of this background delay is unknown but does not affect my conclusions.)
Figure 4-2. pTEL1 causes a delay in mec1cdc13 cells after cdc13 damage. Cells (DLY557) were released from G1 arrest followed by induction of cdc13 damage. a, pTEL1 (single copy TEL1 plasmid) produces a mitotic delay above that of vector alone. b, the percentage of post-M cells from the same experiment. Fewer of the cells containing pTEL1 have completed a cell cycle, since they delay in G2. Averages of duplicates were plotted; error bars represent value of each duplicate. Where bars do not appear, they are smaller than the plot symbol.
Figure 4-3. pTEL1 causes a delay in rad9cdc13 cells after cdc13 damage. Cells (DLY409) were released from G1 arrest followed by induction of cdc13 damage. a, pTEL1 (single copy TEL1 plasmid) produces a mitotic delay above that of vector alone. b, the percent of post-M cells from the same experiment. Fewer of the cells containing pTEL1 have completed a cell cycle, since they delay in G2. Averages of duplicates were plotted; error bars represent value of each duplicate. Where bars do not appear, they are smaller than the plot symbol.
Figure 4-4. pTEL1 causes constitutive cell cycle delay. Checkpoint proficient cells (DLY418) were released from G1 arrest and were not subject to DNA damage. pTEL1 (single copy TEL1 plasmid) produces a mitotic delay above that of vector alone. Averages of duplicates were plotted; error bars represent value of each duplicate. Where bars do not appear, they are smaller than the plot symbol.

To determine if pTEL1 is in fact causing arrest by acting on a bona fide checkpoint pathway, I tested if the partial arrest required either the RAD53 (and DUN1) or PDS1 pathways. I introduced pTEL1 into appropriate strains (i.e. CDC13\* pds1 or CDC13\* rad53) and tested for cell cycle delay. Strikingly, I found that the pTEL1-dependent delay is entirely dependent on PDS1 (the delay is eliminated in a pTEL1 pds1 strain, Figure 4-5d) and entirely independent of RAD53 and DUN1 (Figure 4-5 a-c). These results indicate that the pTEL1-
dependent arrest does operate through a bonafide checkpoint pathway, and only through the \textit{PDS1}-dependent pathway.

Figure 4-5. \textit{pTEL1} delay is dependent on \textit{PDS1}, but independent of \textit{DUN1} and \textit{RAD53}. Cells were released from G\textsubscript{1} arrest and not subjected to DNA damage. Averages of duplicates were plotted; error bars represent value of each duplicate. Where bars do not appear, they are smaller than the plot symbol. \textbf{a}, \textit{pTEL1} delay is greater than background delay (vector alone) in \textit{dun1\Delta} cells (RGY68). \textbf{b}, \textit{pTEL1} delay is greater than background delay (vector alone) in \textit{rad53ApRNR1} cells (RGY108). \textbf{c}, \textit{pTEL1} delay is greater than background delay (vector alone) in \textit{rad53-11} cells (DLY259). This experiment was done at 36°C because the \textit{rad53-11} allele is temperature sensitive and behaves more like a null allele at high temperatures. \textbf{d}, \textit{pTEL1} delay and background delay (vector alone) are indistinguishable in \textit{pds1\Delta} cells (CPY201).
The delay induced by \( pTEL1 \) allows us to make several genetic inferences with respect to the model of checkpoint pathways in Figure 3-2 (page 85). First, the finding that the \( pTEL1 \)-dependent delay requires only one of the two pathways supports the existence of independent \( RAD53 \)- and \( PDS1 \)-dependent parallel pathways. Second, the finding that the \( pTEL1 \)-dependent delay does not require \( RAD9 \) suggests that Rad9p is acting upstream of PI3/protein kinases in the checkpoint pathways. Both of these inferences are concordant with the checkpoint pathway model in Figure 3-2 (page 85).

\( pTEL1 \) is a centromere-containing (single copy) plasmid. Therefore, cells containing \( pTEL1 \) have only one extra copy of \( TEL1 \) per cell. I was surprised that even a single extra copy of \( TEL1 \) could cause a cell cycle delay. I wondered if the plasmid-borne copy of \( TEL1 \) was indeed simply a second copy of the gene, or whether the plasmid-borne copy of \( TEL1 \) was altered in some way. I reasoned that if the plasmid-borne copy of \( TEL1 \) was expressed like the genomic copy, then \( pTEL1 \) should not cause a delay in cells deleted for the genomic copy of \( TEL1 \). Indeed, \( pTEL1 \) did not cause a delay in four separate transformants of \( tel1\Delta \) cells (Figure 4-6); therefore, by this genetic assay, a single extra dose of the \( TEL1 \) gene can cause \( G_2/M \) delay.
Figure 4-6. pTEL1 constitutive arrest requires only a single extra copy dose of TEL1. In a tel1Δ strain (RGY88) synchronously proceeding through the cell cycle at 30°C, one plasmid copy of TEL1 does not cause constitutive arrest over background levels. (Compare with results from Figure 4-4 which shows a pTEL1 delay in TEL1* cells.) For pvec, the mean of duplicates was graphed; for pTEL1, the mean of quadruplicates was graphed. Bars indicate standard deviation; where bars do not appear, they are smaller than the plot symbol.

The delay by pTEL1 suggests that even mild overexpression of TEL1 results in a constitutive arrest signal that cannot be activated further by DNA damage (because delay was not increased in cdc13 defective cells, Figures 4-2 and 4-3.) All the previous pTEL1 delay results are summarized in Figure 4-7.
Figure 4-7. **pTEL1 induced delay is not significantly altered by cdc13 damage.** This figure summarizes pTEL1 arrest data previously presented in Figures 4-2 to 4-6. Bars show maximum levels of G2 delay with either vector or pTEL1 in specified strains (in all cases the maximum delay occurred at the same time point for both pTEL1 and vector-containing strains, except for the rad53A strain. In that case, the highest delay level of the vector-containing cells occurred at 75 minutes because, as Figure 4-5b shows, they were caught as many of them were passing through mitosis, as opposed to delaying in mitosis. Therefore the bar corresponding to the vector delay in rad53A shows the delay at 105 minutes, which was when pTEL1 showed maximum delay.) Although in some cases delay in the vector control strains is high, results are reproducible when pvector and pTEL1 are tested in the same experiment. In all cases, the net delay (pTEL1 delay minus background vector delay) was about 20%, and was independent of cdc13 damage. Error bars show the actual levels of each of 2 duplicates. vector, pRS416; pTEL1, cenURA3TEL1.
The only type of damage tested thus far for activation of the TEL1-dependent delay in mec1sm11 mutants was in cdc13 cells that have ssDNA specific for chromosome ends. I therefore have also tested whether pTEL1 might mediate an arrest response after a different type and location of damage: a double strand break at the MAT locus that is not near a telomere. I analyzed the delay of pTEL1 in mec1 mutants expressing the HO endonuclease (a checkpoint deficiency such as a mec1 mutation in the strain is necessary; otherwise, nearly all the cells will arrest in response to damage, masking any effect by pTEL1), and found no increase in pTEL1-mediated arrest above that found generated by the constitutive delay (compare the level of pTEL1 delay above the delay in the pvector control after double stranded break damage in Figure 4-8, next page, to the level of pTEL1 delay above background after no damage in Figure 4-4, page 132). Therefore, pTEL1 cannot respond to either of two types of damage to cause G2/M arrest.

I also tested whether overexpression of pTEL1 can mediate arrest in response to MMS-induced damage. This seemed plausible because pTEL1 does restore transcriptional induction of RNR3 in mec1 cells after MMS-induced damage (see below). The results from studies of cell cycle arrest are not interpretable, however, because even checkpoint mutant cells (including mec1 or rad9) and WT cells arrest as single nucleate large budded cells after MMS exposure (110 and my unpublished data). The basis for this checkpoint gene-
independent delay in MMS is unknown. (It could be related to a novel DNA damage checkpoint that operates independently of other known checkpoints (possible precedent for this in 41), or that many MMS treated cells simply die with the large budded, mononucleate morphology, giving the appearance of G₂ arrest.)

**GAL HO rad54mec1 + . . .**

![Graph showing % medial nuclear division over time for pTEL1 and pvec](image)

Figure 4-8. *pTEL1* cannot restore G₂/M damage-induced checkpoint arrest to *mec1* cells after double strand break damage. This graph shows that *pTEL1* (in triplicate) does not restore G₂/M checkpoint arrest in *mec1* cells (RGY137) experiencing a double stranded GALHO break, but only produces the level of constitutive delay above that of pvec seen without damage (compare levels of delay with those in Figure 4-4, page 132. In fact, in this case the constitutive delay levels may be even lower than those in Figure 4-4.) The *rad54* mutation prevents double stranded break repair, thus prolonging the HO-induced damage. Standard deviation error bars are smaller than the plot symbols.

I conclude that *pTEL1* causes a constitutive delay by the *PDS1*-dependent pathway, and that *pTEL1* cannot respond to DNA damage to increase the arrest
response. The basis for the constitutive delay by TEL1 is unknown. I favor the view that the constitutive delay is due to constitutive signaling when TEL1 is expressed inappropriately. One alternative explanation for the delay is that overexpression of TEL1 actually causes DNA damage which then activates a novel checkpoint pathway not involving RAD9 nor MEC1. While this hypothesis cannot be conclusively ruled out, it seems unlikely for the following reason: DNA damage is probably not induced in cells overexpressing pTEL1 because mitotic recombination (an indicator of DNA damage) is not elevated in strains containing pTEL1 compared to vector alone (Figure 4-9). Although it is possible that not all types of damage may result in an increase in mitotic recombination, this is the most unambiguous indicator of damage. The simplest explanation is that pTEL1 does not cause DNA damage. Why pTEL1 activates the PDS1-dependent pathway is addressed further in the Discussion.
pTEL1 does not increase mitotic loss or recombination. This is representative of three separate experiments in which chromosome loss/recombination is measured in a chromosome 7 disome strain (TWY120) containing either single (cen) or high copy (2u) TEL1, versus vector. Controls show that after HU exposure (which causes DNA damage), mitotic loss is increased. See methods #16, page 221, for details.

pTEL1 suppresses mec1’s defect in damage-inducible transcription, and suppression requires DUN1 and RAD53 but not PDS1

Evaluation of the effects of TEL1 overexpression reveals that overexpression of TEL1 (pTEL1) restored other responses to mec1 mutants as well. I found that pTEL1 restores damage-inducible transcription of RNR3 in mec1-1sml1 (Figure 4-10a) or in mec1Δ (data not shown) to WT levels. In these experiments, in cells treated with MMS, pTEL1 apparently restores only inducible RNR3 transcription; the transcriptional induction is not constitutive like the effect of pTEL1 on cell cycle delay. I tested if restoration by pTEL1 of transcription occurs by checkpoint pathways, and if so which pathways. I found that RAD53 and DUN1, but not PDS1, are required for pTEL1 to restore damage-inducible
transcription to mec1 mutants (Figure 4-10 b-c). This result is also incorporated in the model of checkpoint pathways, diagrammed in Figure 4-15 in the Discussion. (Since both RAD53 and DUN1 are required for damage inducible transcription — see references in Chapter 1 — and are required for pTEL1 to restore damage induced transcription to mec1 mutants, one might predict that DUN1 or RAD53 overexpression would also restore induced transcription to mec1 mutants. However, overexpression of RAD53 and DUN1 do not restore induced transcription of RNR3 to mec1 mutants, data not shown).

I also show that neither PDS1 nor TEL1 are required for RNR3 induction after damage (Figure 4-10c). (That PDS1 is not required was also shown by Cohen-Fix and Koshland (21).)
Figure 4-10. pTEL1's restoration of damage-inducible transcription in mec1 strains. a, pTEL1 restores RNR3 induction after 0.01% MMS damage in mec1 mutants (TWY177, A364a background; same results in DLY258, W303 background). b, pTEL1 induces RNR3 in mec1 (TWY177) but does not induce RNR3 in mec1dun1 strains (RGY27). "cen" refers to a single copy plasmid, and "2u" refers to a high copy plasmid. TEL1 on either plasmid suppresses mec1's damage-inducible transcription defect equally well. c, (next page) summary of relative RNR3 induction in various strains: WT, DLY62 and RGY4; mec1, TWY177 and DLY258; mec1dun1, RGY27; rad53, DLY259; pds1, RGY85; mec1pds1TEL1, RGY217; tel1Δ, RGY53. Quantitations were done by dividing the RNR3 signal by the loading control (URA3 or ACT1). Results from different experiments were combined on the bar graph by normalizing values to that of the positive control in each experiment.
pTEL1’s suppression of mec1’s essential function also requires DUN1 and RAD53 but not PDS1

Finally, I have evaluated pTEL1’s restoration of MEC1’s essential function (as demonstrated originally by Morrow et al. (94)). From an extensive segregational analysis (see methods #17, on page 222) I found that RAD53 and DUN1, but not PDS1, are required for pTEL1 to restore cell viability to mec1Δ mutants. (See Figure 4-11. For example, from sporulation of a MEC1+/Δ
DUN1+/Δ diploid strain containing pTEL1. mec1ΔDUN1+ spores containing pTEL1 were viable, but mec1Δdun1Δ spores were not viable, indicating that DUN1 is required for pTEL1 to restore viability to mec1Δ cells.) (See methods #17 for further experimental details).

pTEL1 therefore acts through the RAD53 and DUN1 pathway to suppress both transcriptional and essential function defects in mec1 mutants. The essential function of MEC1 remains unknown, though it does appear to be related to transcription and to TEL1's function (also unknown).

Several additional genetic observations strengthen the inference that the normal role of TEL1 may be related to MEC1's essential role. I found that the normal gene dosage of TEL1 is required for both pDUN1 and pRNR1 to effectively suppress mec1 mutant inviability (e.g. pRNR1 efficiently suppresses mec1 inviability in a TEL1+ strain and but does not efficiently suppress mec1 inviability in a tel1' strain; Figure 4-11d). The identification of the essential cell function apparently shared by TEL1 and MEC1 remains to be determined.
Figure 4-11. pTEL1's (and other genes') requirements for restoration of mec1's essential function. Starting diploid strains are shown in the heading of each graph. Plasmids, shown along the top of each graph, were introduced into the diploid strains which were then sporulated. Genes contained on plasmids are expressed under control of their own promoters, except for RAD53, which is expressed by the GAL promoter. Tetrads were either dissected or spores were analyzed randomly. Horizontal lines indicate expected number of segregants (one fourth of the total in each case) of each genotype if suppression was complete. a, pTEL1 suppression of mec1Δ requires DUN1. RGY51 and 139. b, pTEL1 suppression of mec1Δ requires RAD53. RGY58. c and d, next page.
mec1+/Δ pds1+/Δ. pTEL1 can restore essential function in mec1 cells in the absence of PDS1.

Figure 4-11 continued. c, pTEL1 does not require to PDS1 to restore essential function in mec1 cells. RGY87. Note that pds1 cells are sick by themselves and thus appear at lower than otherwise expected frequencies. d, suppression of the mec1 essential function by plasmid suppressors is poor in a tel1Δ background (RGY73). Only GALRAD53 suppresses.
pTEL1 does not restore S/M checkpoint function to mec1 cells

The S/M checkpoint prevents entry into mitosis until DNA replication has been completed. In WT cells, inhibition of DNA replication results in S/M checkpoint arrest which can be monitored by lack of both spindle elongation and nuclear division. By contrast, in checkpoint deficient cells, inhibition of DNA replication does not result in S/M arrest; the lack of S/M arrest results in both spindle elongation and nuclear division.

Although I had shown that the pTEL1-induced constitutive arrest was not increased by DNA damage, it remained possible that pTEL1 might suppress mec1's S/M checkpoint defect, or increase the level of constitutive delay in response to hydroxyurea (HU), which inhibits DNA replication. Indeed, Morrow et al. have shown that TEL1 overexpression partially restores viability to mec1 cells after HU exposure; one possible mechanism for this would be suppression of mec1's S/M checkpoint defect. One reason to hypothesize that HU treatment might provoke a cell cycle arrest response by pTEL1 while other kinds of DNA damage (such as cdc13 or double stranded-break damage) do not, is that the S/M checkpoint appears to act by a different mechanism than the G2/M checkpoint. For example, the S/M checkpoint does not require several checkpoint proteins that are required for the G2/M checkpoint, including Rad9, Rad24, and Pds1. I therefore compared the S/M checkpoint proficiency in WT cells and in mec1-1sml1 cells carrying either vector or pTEL1. I synchronized the
cells in G₁, then released them into hydroxyurea-containing medium, and inferred by fluorescent staining (see methods #18, on page 223) of the spindles if the cells remained arrested in S-phase (short spindles), or attempted mitosis (long/aberrant spindles). As Figure 4-12 shows, pTEL1 did not suppress the S/M checkpoint defect of mec1-1 cells. I conclude that restoration of S/M checkpoint function cannot explain pTEL1’s restoration of viability to HU-treated mec1 cells. (This assay was not precise enough to measure a constitutive delay by pTEL1).

Figure 4-12. TEL1 overexpression does not suppress the S/M checkpoint defect of mec1 cells. In this experiment, G₁ synchronized cells were released from G₁ arrest and then treated with 0.1M hydroxyurea. After treatment with hydroxyurea, WT cells (DLY62) arrest with short spindles, while mec1 cells (DLY258) carrying either pTEL1 or vector attempt mitosis, producing long and aberrant spindles. Shown is the % abnormal spindles at selected times in hydroxyurea-containing media. (See methods #18, page 223.)
Mechanism of suppression by pTEL1 of UV and HU sensitivity in mec1 cells

I considered explanations for pTEL1’s restoration of viability to mec1 cells after exposure to UV or HU (94). Having previously ruled out a restoration of the S/M or damage-induced G₂/M checkpoint functions, I considered whether suppression might be due to either the constitutive G₂/M delay caused by pTEL1, the restoration of damage-inducible transcription, or both. I tested these hypotheses by measuring UV and HU viability in mec1 strains that were also pds¹⁻ (in which no pTEL1 delay occurs), or dun¹⁻ (in which pTEL1 cannot restore transcriptional induction). I found that in mec1 cells pTEL1’s restoration of UV resistance depends on PDS1, (because restoration of resistance does not occur in a mec1pds1 pTEL1 strain), and does not depend on DUN1 (Figure 4-13). The simplest conclusion is that pTEL1’s constitutive G₂/M delay appears to partially restore UV resistance to mec1 mutants. This conclusion is strengthened by the observation that in a different strain background (A364a), pTEL1’s restoration of viability to UV-treated mec1 cells is very weak or nonexistent; pTEL1’s constitutive delay is smaller as well (data from 2 experiments, not shown).

The mechanism of restoration of viability of mec1 cells after HU exposure is more complex; neither DUN1 nor PDS1 are required because restoration of HU resistance occurred even in mec1dun1pds1 pTEL1 mutant cells (Figure 4-14). The mechanism of restoration by pTEL1 of viability to mec1 mutants after HU exposure must therefore be unrelated to the G₂/M delay or to damage-inducible
transcription, and may perhaps involve a response related to DNA replication itself. It may involve *RAD53*, but this has not been evaluated further.
Figure 4-13. *pTEL1* requires *PDS1* for restoration of viability to *mec1* cells after UV exposure. After UV exposure, *pTEL1* increases viability in *mec1* (DLY258) (a) and *mec1* *dun1* (RGY181) (b) strains, but not a *mec1* *pds1* strain (RGY179) (c). Viability of microcolonies was scored (see methods #2, page 212). Averages of duplicates were plotted; error bars represent value of each duplicate. Where bars do not appear, they are smaller than the plot symbol. Strain background, W303.
Test of Pds1 phosphorylation by TEL1 overexpression

Recently Cohen-Fix and Koshland showed that Pds1p becomes phosphorylated after damage, and phosphorylation requires an intact MEC1 gene, but does not require RAD53 (21). Rad53p phosphorylation dependent on MEC1 or overexpression of TEL1 was shown previously as well (127, 148). The role of phosphorylation of Rad53p or Pds1p in arrest remains, however, unknown.

Because pTEL1 causes a PDS1-dependent delay, it was conceivable that in cells containing pTEL1, Pds1 would be constitutively phosphorylated. I could
not, however, distinguish differences on Western blots in the levels of phosphorylated Pds1p in cells overexpressing *TEL1* compared to cells with vector alone (see methods #13, page 219). This was not unexpected, since the p*TEL1* delay is small (about 20% of synchronized cells; see Figure 4-7, page 136). Therefore, while I cannot rule out that p*TEL1* causes constitutive phosphorylation of Pds1, the mechanism by which p*TEL1* mediates a *PDS1*-dependent delay remains to be determined.
DISCUSSION

**Overexpression of TEL1; ectopic activation of the checkpoint pathway?**

I have evaluated the possible roles of TEL1 in several MEC1-dependent responses and find no role for TEL1, in its normal gene dosage, in MEC1-dependent checkpoint pathways, including G2/M arrest, S/M arrest, and transcriptional induction of repair genes. TEL1 does have some role in cell viability and tolerance of DNA damage because, for example, mec1tel1sml1 mutants have very poor growth and greater damage sensitivity than mec1sml1 mutants (94 and Chapter 5). The nature of TEL1's normal roles, as well as the essential function of MEC1, remain obscure.

TEL1 overexpression (by pTEL1) did provide important and surprising results relevant to understanding checkpoint pathways. In particular, overexpression of TEL1 confirms the parallel pathway model presented in Chapter 3 (see Figure 3-2 on page 85) and identifies which responses each pathway regulates. Specifically, TEL1 overexpression produced a partial arrest that was dependent on PDS1 but not RAD53 or DUN1. It also restored both essential and transcription functions to mec1 cells in a manner that was dependent on RAD53 and DUN1 but not on PDS1 (Figures 4-5, 10, 11, on pages 133, 142, 145).

In addition, the delay by pTEL1, because it appears to act constitutively, provides additional genetic inferences. Because the pTEL1-mediated delay is
independent of both DNA damage and RAD9 (Figures 4-3, 7 on pages 131 and 136), and because TEL1 and MEC1 are homologous, by inference Mec1p may also act downstream of DNA damage and downstream of gene products involved in recognition of DNA damage (e.g. Rad9). (A more convincing demonstration that Mec1p acts downstream of Rad9p would require showing that a constitutively active allele of MEC1 still causes a cell cycle delay in a rad9 mutant strain. A constitutively active allele of MEC1, however, is not yet available.)

Figure 4-15 summarizes the role of pTEL1 on each of the two pathways.

I also uncovered one mechanism of pTEL1-mediated suppression: increase in UV resistance of mec1 mutants by pTEL1 was due to the constitutive PDS1 dependent G2/M delay (Figure 4-13, page 151).

Recently, pTEL1 was found to restore transcriptional induction to an RFC mutant defective in some checkpoint responses (146). Earlier studies showed that pTEL1 causes damage-inducible phosphorylation of Rad53p (127), which I infer does not lead to arrest since pTEL1 does not confer damage-inducible arrest responses.

Two possible explanations for the role of TEL1 in checkpoint pathways are as follows. First, overexpression of TEL1 may aberrantly activate checkpoint pathways by crosstalk, as seen in some MAPK cascades (see 167 for example). By this model overexpression of TEL1 mimics certain aspects of MEC1 function
but only when overexpressed, and it clearly is not being similarly regulated. A
second explanation, not mutually exclusive with the crosstalk model, is these
phenotypes reveal a normal role for TEL1 in joining some aspect of chromosome
metabolism to cell cycle control. TEL1 does not appear to simply join
conventional DNA damage to cell cycle controls, because, for example, I see no
restoration of an inducible cell cycle delay by pTEL1 in mec1 mutants with
specific forms of damage (in cdc13-defective cells and in cells with a specific
double stranded break; Figures 4-7, 8, on pages 136 and 138). An obvious
aspect of chromosome metabolism to consider is telomere synthesis because
tel1 mutants have shorter telomeres, revealing a defect in telomere synthesis.
Many mutants have a similar shorter telomere phenotype, including mre11, sir2,
sir3, and sir4 (106). Other yeast mutants have a yet more dramatic telomere
defect, including rad50, est1, est2, and tlc1 (69, 75, 142). Because pTEL1
causes a checkpoint-mediated delay (through PDS1), and because tel1 mutants
have shorter telomeres, I speculate that TEL1 may normally impose a cell cycle
delay until telomere synthesis is complete. By this model, telomere synthesis
may require a cell cycle delay to be completed, a delay mediated by TEL1 acting
on part of the MEC1-dependent pathway involving PDS1. Overexpression of
TEL1 may reveal that role. This hypothesis remains to be tested.
Overexpressed TEL1 acts in different ways on each of two different checkpoint pathways. This is an adapted version of the model shown in the previous chapter, in Figure 3-2 (page 85). Normal pathways are shown in thick arrows, and pathways resulting from TEL1 overexpression in thin arrows. Normally, the DNA damage signal requires RAD9, RAD24, MEC1, RAD53, DUN1, and PDS1 for complete G2/M arrest. Also, normally MEC1, RAD53, and DUN1 are required for transcriptional induction and an essential function (MEC1 and RAD53 are essential, and probably share the same essential function as will be discussed in Chapter 5. DUN1, though not essential, can act on the essential function pathway because rad53-11 and dun1Δ alleles are synthetically lethal (Chapter 3). Note that the essential function does not require any genes upstream of MEC1.) The figure shows that TEL1, when overexpressed, acts through the PDS1 pathway to cause a constitutive cell cycle delay, and that it acts through the RAD53/DUN1 pathway to restore both Transcriptional Induction and the Essential Function to mec1Δ cells. pTEL1's constitutive delay could possibly be related to a normal telomere checkpoint role for TEL1.
CHAPTER 5: MEC1 AND RAD53 SHARE AN ESSENTIAL FUNCTION

INTRODUCTION

After DNA damage, WT cells arrest at checkpoints, presumably in order to allow time to complete DNA repair (32, 109). The genes required for this arrest are called checkpoint genes. In budding yeast, several checkpoint genes have been identified (see Chapter 1 for review). Normally, many of these genes are not required unless DNA damage or blocks to DNA replication have occurred. Some of these genes, though, have another function which is essential to normal viability. For instance, pol ε is essential, because in addition to its checkpoint role, it has a required polymerase function (101).

MEC1 (Weinert lab unpublished, Chapter 2, and 66, 99) and RAD53 (172) are also essential. However, the nature of the essential function(s) of MEC1 and RAD53 has been enigmatic. MEC1 and RAD53 are both required for several checkpoint responses (3, 108, 138), including the S/M checkpoint response (3, 159) and damage-inducible transcription (3, 70). Is the essential function of MEC1 or RAD53 related to either of these functions, or to an unidentified function?
Is the essential function of *MEC1* and *RAD53* related to the S/M checkpoint response?

One possible reason why *MEC1* and *RAD53* are essential has to do with their S/M checkpoint function. This function inhibits entry into mitosis if DNA replication is incomplete. Perhaps in most or all cycles, the cells' tendency is to proceed through mitosis before the completion of DNA replication, and this lethal event is prevented by a constitutive S/M checkpoint function of *MEC1* and *RAD53*. By this hypothesis, in the absence of either of these genes, cells die because they proceed through mitosis with unreplicated DNA.

Alternatively, the essential function of *MEC1* and *RAD53* may be completely unrelated to their checkpoint function. There is precedent for genes or proteins having two unrelated functions — for an extreme example, the yeast CAP (for Cyclase-Associated Protein) protein's amino and carboxy termini have completely different functions. The amino terminus responds to RAS, and the C terminus responds to nutrient conditions (42).

Is the essential function of *MEC1* and *RAD53* related to the transcriptional induction response?

Another response that *MEC1* and *RAD53* share is induced transcription of repair genes after damage (3, 70). Among the genes transcriptionally induced after damage is *RNR3*, a gene involved in dNTP synthesis. Its induction after DNA damage or replication inhibition requires both *MEC1* and *RAD53* (3, 70). It has previously been shown that the yeast G2/M checkpoint functions in the
absence of protein synthesis (157); it seems likely that other checkpoints, such as the S/M checkpoint, also do not require protein synthesis (although the experiments to test this cannot be done due to technical reasons). This suggests that the checkpoint and transcription responses may be separable. It is conceivable that the essential function may be related, not to the S/M checkpoint response as discussed above, but instead to the transcription response. Previous results from our laboratory suggest that this may be the case. Both the essential function (94) and damage-induced $RNR3$ transcription (Chapter 4) can be restored by overexpression of $TEL1$ in a $mec1$ mutant. In both cases (essential and transcription functions), this restoration requires $RAD53$ and $DUN1$, two genes that are also normally required for damage-inducible transcription (Chapter 4). Furthermore, overexpression of either $RAD53$ (127) or $DUN1$ (99) themselves also restores viability in a $mec1$ mutant. Taken together, these results suggest that restoration of the transcription function correlates with restoration of the essential function.

Restoration of the essential functions of both $MEC1$ and $RAD53$ can also be accomplished by $RNR1$ overexpression (24, 127). $RNR1$ is the essential gene which encodes the large subunit of ribonucleotide reductase, which is required for the production of dNTPs. (It is functionally interchangeable with $RNR3$ (34), although instead of being strictly damage-inducible as is $RNR3$, $RNR1$'s transcript is cell cycle regulated, peaking at the beginning of S phase.) One
simple explanation, then, for the essential functions of both *MEC1* and *RAD53* is that they are required for the constitutive expression of *RNR1*, and therefore are indirectly required for DNA replication.

**The essential function of MEC1 is suppressed by sml1**

A genomic suppressor called *sml1* can suppress the essential function defect of *mec1* cells (110); while the mechanism of suppression is not fully understood, it has been shown recently that Sml1p and Rnr1p physically interact (121). This again suggests involvement of *RNR1* in *MEC1*'s essential function. Efforts at understanding the function of *SML1* may help us to understand the essential function of *MEC1*.

*RNR1* overexpression and the *sml1* mutation do not restore transcriptional induction to *mec1* cells as does p*TEL1*. This suggests that *mec1*’s essential function is either related to *RNR1* transcription itself, as mentioned above, or that the essential function is not related to transcription at all.

The goal of the work in this chapter is to gain a better understanding of the essential functions of *MEC1* and *RAD53*, as well as how these functions are suppressed. In Chapter 4, I discussed overexpressed genes (*TEL1*, *RAD53*, and *DUN1*) that suppress *mec1*’s essential function. I continue here with an analysis of spontaneous suppressors (such as *sml1*), and other genomic suppressors of the essential function of *MEC1*. I have used a conditional allele of *mec1* to ask
questions about the essential function, as well as to show that functions ascribed to \textit{MEC1} are indeed functions of \textit{MEC1} and not of \textit{sml1}.

I present evidence that \textit{MEC1} and \textit{RAD53} share the same essential function, and that this function is not directly related to the S/M checkpoint. I discuss and eliminate three hypothesis as to \textit{MEC1}'s essential function: that it is involved in telomere length maintenance, that it is required for yeast origin of replication (ARS) firing, and that it is involved in maintaining proper \textit{RNR1} transcript levels. The results I present may contribute to our understanding of the roles of \textit{MEC1} and \textit{RAD53}. 
RESULTS

Suppressors of MEC1's essential function arise frequently

The isolation of MEC1 mutants and characterization of the strongest allele, mec1-1, was reported by Weinert et al. (159). From analysis of mec1-1 strains they showed that MEC1 is involved in cell cycle arrests after a block to DNA replication and after DNA damage (S/M and G₂/M checkpoints), and that MEC1 is involved in transcriptional induction of DNA repair genes (70). MEC1 has also been shown to be essential (Weinert lab unpublished, Chapter 2 herein, 66, 99). Subsequently Paulovich et al. (110) and Rothstein (121) found that the mec1-1 strains used in the above studies contain an unlinked suppressor (sml1) required for viability of mec1-1 mutants. I have confirmed their observations by meiotic segregation analysis; mec1-1/+ SML1+/sml1 yields near the expected frequency of inviable cells in tetratype tetrads that I can infer are mec1-1, and mec1-1/+ sml1/sml1 strains show high spore viability (26 of 28 tetrads have 4:0 viability). (The lethality of mec1-1SML1- was originally missed, in part, due to the high frequency of unlinked suppressors, reported later in this Chapter.) I considered that sml1 or other spontaneous suppressors might provide insight into the nature of MEC1's essential function.

I asked if spontaneous suppressors could also arise in mec1Δ strains, and if so, if these suppressors could restore other functions in addition to the essential function. (See Chapter 2 and methods #6, page 214, for information related to
the creation and characterization of the $mec^{1s}$ allele.) I reasoned that suppression of more than one function by the same suppressor mutation may indicate the functions are related. I observed that when $mec^{1s}$ cells are plated at 37°C, the cells die slowly while continuing to divide, producing microcolonies of varying size, averaging about 100 cells. After about 3 days spontaneous macrocolonies form (at a rate of about $3 \times 10^{-3}$ cells or 1 in 300 cells plated); these cells when replated at 37°C are as viable as $MEC^{1+}$ cells.

This high rate of suppression prompted me to ask if each suppressed strain results from mutations in the same gene. To test this, I mated a $MATa$ $mec^{1s}$ strain carrying a suppressor mutation to five different $MAT\alpha$ $mec^{1s}$ strains, each carrying independent suppressor mutations. By analysis of meiotic progeny, I concluded that the suppressor mutation in the $MATa$ strain (denoted $sup^{1}$ - see below) wasn't linked to any of the 5 suppressor mutations from the alpha strains. (In each case, I analyzed four tetrads, and observed that 3 to 5 meiotic segregants from the four tetrads had the nonsuppressed $mec^{1s}$ phenotype in each strain. This is the expected result for unlinked genes. If the different suppressor mutations were in the same gene, all the segregants would have the suppressed phenotype.) This indicates that perhaps many different suppressor mutations (at least two) can restore the essential function to $mec^{1s}$ cells at the restrictive temperature. Furthermore, all of the above-mentioned strains of $mec^{1s}$ carrying suppressor mutations are HU sensitive at 37°C, indicating that the
suppressors do not affect responses to HU, such as the S/M checkpoint function. The mec1<sup>ts</sup> sup<sub>1</sub> strain is also UV sensitive at 37°C. (The other strains were not tested for UV sensitivity.) These findings suggest that the suppression may be specific for the essential function, and therefore the essential and checkpoint functions may be separable.

**Analysis of sup<sub>1</sub>/sml<sub>1</sub>**

I analyzed the mec1<sup>ts</sup> sup<sub>1</sub> strain further in an attempt to understand how MEC1's essential function is suppressed. sup<sub>1</sub> was shown to occupy a single locus because I obtained 2:2 segregation for growth at 37°C upon sporulation of a mec1<sup>ts</sup>/mec1<sup>ts</sup> sup<sub>1</sub>/+ diploid. SUP1 is not linked to MEC1, because meiotic segregation from a mec1<sup>ts</sup>/+ sup<sub>1</sub>/+ diploid resulted in some mec1<sup>ts</sup> segregants lacking the sup<sub>1</sub> phenotype. Furthermore, by mating mec1<sup>ts</sup> sup<sub>1</sub> to a mec1<sup>-1</sup>sml<sub>1</sub> strain and analyzing the meiotic progeny, I showed that the mec1<sup>ts</sup> suppressor mutation sup<sub>1</sub> was allelic to sml<sub>1</sub>, and that sup<sub>1</sub> confers the sml<sub>1</sub> suppressor phenotype on mec1<sup>-1</sup> cells. I asked which general type of suppressor sml<sub>1</sub>/sup<sub>1</sub> was — some suppressors act by changing protein-protein interactions; others, called bypass suppressors, simply eliminate the need for the protein they suppress. sml<sub>1</sub>/sup<sub>1</sub> is a bypass suppressor because it suppresses the essential function in a mec1<sup>-</sup> strain. (It does not, however, suppress the checkpoint or transcription functions of mec1<sup>-</sup>, data not shown.) Although the location of the mutation(s) in the mec1<sup>-1</sup> allele aren't known, its phenotypes,
compared to those of $mec1\Delta$, and the observation that $sml1$ is a bypass suppressor, indicate that $mec1^{-1}$ is likely to be a null or very near null mutation.

The finding that $sml1$ could restore viability to $mec1\Delta$ cells suggested that $sml1$ should also restore viability to $mec1^{ts}$ cells at their restrictive temperature. In testing whether $sml1$ could suppress the essential function in $mec1^{ts}$ cells, I found that among the meiotic progeny resulting from sporulating a $mec1^{-1}/mec1^{ts}$ $sml1/+\$ strain, half of the $mec1^{ts}$ segregants are still temperature sensitive for growth on hydroxyurea (HU) plates, but grow well on rich media at 36°C. I infer that these segregants are $mec1^{ts}sml1$. This indicates that the essential function has been restored by the $sml1$ mutation, but that other function(s) associated with HU sensitivity, such as the S/M checkpoint function, or a possible DNA replication function, have not been restored. This again supports the idea that $MEC1$'s essential function is genetically separable from its other functions.

In addition to these results, I was interested to know if $sml1$ and $sup1$ had the same phenotype; although I showed that $SML1$ and $SUP1$ are the same gene, $sml1$ and $sup1$ may have represented alleles of different strengths. Figure 5-1 shows that $mec1^{-1}sml1$, $mec1\Delta sml1$, and $mec1\Delta sup1$ strains have similar phenotypes. It is likely therefore that $sup1$ and $sml1$ are similar alleles.
Figure 5-1. *mec1* cells have similar phenotypes in either a *sup1* or *sml1* background. a, after HU exposure; b, after UV exposure. In parts a and b, lines with error bars show the average of duplicates; bar shows value of each duplicate. Lines without error bars indicate a strain not tested in duplicate; c (next page) RNR3 transcription after 4 hours of .01% MMS treatment, and S/M checkpoint arrest (% arrest after 4 hours HU treatment) of *mec1* cells are similar in *sup1* or *sml1* backgrounds. Bars show % response normalized to WT. Growth on an HU plate was also examined but was not included on the graph because it was scored qualitatively instead of quantitatively; Mec* cells grow but *mec1-1sml1* and *mec1Δsup1* cells fail to grow. Mec*, TWY397; *mec1-1sml1*, TWY308; *mec1Δsml1*, RGY55; *mec1Δsup1*, RGY9.
In attempting to elucidate the role of sml1/sup1, I have found that these suppressor mutations are semi-dominant for growth at 37°C (qualitatively, the growth of strains follows this order from best to worst: Mec* / mec1^ss / mec1^ss sup1/sup1 > mec1^ss / mec1^ss sup1/+ > mec1^ss / mec1^ss SUP1/SUP1).

sml1 in a Mec^- background has no discernible phenotype, such as altered cell cycle distribution nor detectable HU or UV sensitivity (my data and (110)), and its DNA sequence is not informative (121). However, there are recent unpublished observations that suggest that the Sm1 and Rnr1 proteins interact, and that Wt SML1 may act to inhibit dNTP synthesis (121).
mec1ts cells die slowly at their restrictive temperature
   I considered that the mec1ts allele may be useful for learning more about

MEC1's essential function. (See Chapter 2 for information about the isolation and
characterization of the mec1ts allele.) Specifically, does death in mec1ts cells at
the restrictive temperature require cell cycling, and do the cells die at a uniform
place in the cell cycle? I found that when a liquid culture of midlog mec1ts cells is
shifted to the restrictive temperature (36°C or 37°C), the cells continued to divide
for several hours, even as they died. Death occurred slowly; in one experiment it
took about 24 hours for nearly all the cells (>95%) to die (not shown).
Furthermore, by analyzing the budding and nuclear morphology of the dead or
dying cells, I could determine that these cells represented all phases of the cell
cycle (see Table 5-1). Reasons for the slow loss of viability and lack of uniform
death point could include: 1) The essential function may not be cell cycle related.
2) It may be that the mec1ts allele isn't fully inactive at 36°C. But by data in
Chapter 2, (Figure 2-7, page 78) this allele becomes inactivated by 3-4 hours at
this temperature, at least for the G2/M checkpoint function. 3) There may be a
phenotypic lag of cell cycling after death. This is consistent with an essential
function of promoting production of some essential but relatively stable RNA,
metabolite, or protein. Upon inactivation of Mec1p, enough of this RNA,
metabolite, or protein remains to ensure viability for several cell cycles.
**MEC1 and TEL1 may share an essential function**

Morrow et al. have shown (94) and I confirmed that mec1-sml1tel1 cells have very poor viability. (I also showed in Chapter 4 that while the viability of mec1ΔSML1+ cells can be restored by overexpression of DUN1 (99), or RNR1 (24, 127), that this restoration of viability requires a TEL1+ background). These results indicate that even though TEL1 itself is not essential, MEC1 and TEL1 may share an essential function. In fact, mec1ts tel1 cells go through fewer divisions and die faster at the restrictive temperature than do mec1ts TEL1+ cells, (Figure 5-2, and from examination of overnight growth on YEPD plates at 37°C), and accumulate many fewer spontaneous suppressors (data not shown).

Table 5-1. Cell cycle distribution of mec1ts and mec1ts tel1Δ cells after 15 or 24 hours at 36°C. These data are from the same experiment shown in Figure 5-2. 100 cells were scored for each sample as unbudded, small budded, large budded with one nucleus (medial nuclear division), or large budded with two nuclei (post anaphase).

<table>
<thead>
<tr>
<th></th>
<th>Unbudded</th>
<th>Small budded</th>
<th>Medial nuclear division</th>
<th>Post anaphase</th>
</tr>
</thead>
<tbody>
<tr>
<td>mec1ts 15 hr</td>
<td>36</td>
<td>26</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>mec1ts tel1 15 hr</td>
<td>46</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>mec1ts 24 hr</td>
<td>49</td>
<td>19</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>mec1ts tel1 24 hr</td>
<td>47</td>
<td>14</td>
<td>18</td>
<td>22</td>
</tr>
</tbody>
</table>
Figure 5-2. Death of mec1ts and mec1ts tel1 cells at the restrictive temperature. a, growth rate of cells over a 24 hour timecourse. mec1ts (RGY224) and mec1ts tel1 (RGY229) cells increase in number more slowly presumably due to cell death. Mec+Tel+, DLY62. b, death rate of the cells. Cells at 23°C of all strains grow at the same rate and retain high viability (not shown). Death was measured by plating the cells and scoring the number of viable microcolonies the following day. In both panels, averages of duplicates were plotted; error bars represent value of each duplicate. Where bars do not appear, they are smaller than the plot symbol.
Cell cycling is required for slow loss of viability in mec1Δ cells

If the function of mec1Δ were not cell cycle related at all, this would predict that death could occur even in noncycling cells at the restrictive temperature. I attempted to test the role of cycling in two ways, taking advantage of the faster death rate of mec1Δ tel1Δ cells over mec1Δ cells. First, I arrested mec1Δ tel1Δ cells at the permissive temperature in G, with alpha factor, then shifted the cells to the restrictive temperature. Then over time I tested the viability of the cells. This experiment required keeping the cells arrested for several hours, since they retain viability for several hours at the restrictive temperature. Long alpha-factor arrest was possible because of the bar1 mutation I introduced into the strains. (The BAR1 gene encodes a protease which cleaves alpha factor (88). The bar1 mutation vastly increases the sensitivity of cells to alpha factor and decreases their ability to adapt to it (19, 145).) I found that even WT cells arrested in G, by alpha factor for several hours gradually begin to lose viability. Cycling mec1Δ tel1Δ cells lost viability faster than noncycling mec1Δ tel1Δ cells (Figure 5-3).

As a second method of testing the role of cycling, I obtained noncycling cells simply by starting with a late-log instead of early mid log culture. In late log cultures, where cells only divide one or two more times before entering stationary phase, the cells retain viability over many hours at their restrictive temperature (Figure 5-3). I obtained similar results using the mec1Δ TEL1Δ strain (data not shown). Taken together, these results suggest that cycling of the cells is required for death after the loss of MEC1 and TEL1 function. (I only tested
mec1Δ cells arrested in stationary phase and G₁ phase. It is possible that mec1Δ cells arrested at other points in the cycle, such as in S or G₂ phase, may lose viability at the restrictive temperature. These experiments were not done due to technical difficulties associated with them.) I conclude that if the phenotypic lag hypothesis (discussed above) is correct, the requirement for cycling suggests that the essential protein, metabolite, or RNA is cell cycle related or is required at specific times during the cell cycle (or at least is not required in the G₁ or G₀ phases).

Figure 5-3. mec1Δtel1Δ cells at their restrictive temperature require cell cycling for death. Noncycling or reduced-cycling cells (either alpha factor arrested, or in late log/stationary phase) do not die. Although the data shown here is for mec1Δ tel1Δ cells (RGY229), the same findings apply to mec1Δ cells (RGY2, not shown). Mec+Tel+, DLY62. N.D., not done.
Other Genomic suppressors of \textit{MEC1}'s essential function

As noted above, meiotic segregants from a \textit{mec1-1/+/sml1/+} diploid show a low spore viability, consistent with segregation of \textit{sml1} away from \textit{mec1-1}. However, in meiotic segregation of a \textit{mec1-1/+/sml1/+} diploid in a \textit{cdc13} background, we observed higher viability (e.g. some \textit{cdc13mec1-1SML1'} colonies were viable). I considered that mutations in other genes might have the same effect as mutations at the \textit{cdc13} locus, and, if so, that such mutations might be informative with regards to the essential function of \textit{MEC1}. To ask if other \textit{cdc} mutations could confer viability on \textit{mec1\^A}, I deleted one copy of \textit{MEC1} from homozygous \textit{cdc} diploid strains (\textit{cdc/cdc \textit{mec1\^A/MEC1}'}). Upon sporulation and dissection, I observed that several tetrads showed 4:0 viability (usually 2 large and 2 small colonies) in homozygous \textit{cdc7, 13, and 28} strains, but not in a homozygous \textit{cdc15} strain (Figure 5-4). Colonies were counted at 2-3 days before \textit{mec1\^A} colonies started appearing in the WT control plate (see Chapter 2 for a discussion of \textit{mec1\^A} cell growth in the A364a strain background). I also tested suppression of \textit{MEC1}'s essential function in a homozygous \textit{cln1cln2} strain (151); this produced the most dramatic results, because nearly all the tetrads were 4:0 for viability and all the colonies in a tetrad were often the same size (Figure 5-5c). However, these \textit{mec1\^Acln1cln2} cells (identified by an auxotrophic marker of the \textit{mec1} deletion locus) were still HU sensitive, HU arrest defective, and defective for transcriptional induction of \textit{RNR3} after MMS damage,
indicating that not only were they bona fide mec1Δ cells (based on these mec1 phenotypes), but that the suppression was probably specific to the essential function (data not shown).

**Figure 5-4. Mutations in CDC and CLN genes suppress MECT's essential function.** Strains homozygous for the indicated mutations and heterozygous for MEC1 were sporulated and dissected. Shown are the number of tetrads producing 1, 2, 3, or 4 spores. cdc15 mutations do not suppress MEC1's essential function. These experiments were repeated with similar results. Homozygous strains are as follows: WT, RGY 6; cdc7, RGY41; cdc13, RGY43; cdc28, RGY47; cdc15, RGY45; cln1,2, RGY48.

I reasoned that one possible mechanism of suppression was the slow growth in the early portion of the cell cycle caused by a lack of Cln1 and Cln2 proteins. To ask if slow growth in general could suppress the inviability of mec1Δ cells (in a Cln' Cdc' background) I dissected MEC1+/Δ tetrads on 3ug/ml nocodazole plates (which delays the G2/M progression), but this did not increase viability. I concluded that the best suppressors of mec1 are generally in genes
that act early in the cell cycle. Slowing down the later part of the cell cycle by a cdc15 mutation or by a low concentration of nocodazole did not suppress MEC1's essential function. Suppression of mec1Δ by cdc13 (from sporulation of a cdc13/cdc13 MEC1*/mec1Δ diploid) was an enigmatic result in this regard, in that CDC13 causes delay in late G2/M (although an earlier function cannot be ruled out). Although I do not know how these mutations restore viability to mec1 cells, I hypothesize that by slowing down the early part of the cell cycle, they allow more time for the cell to complete the function for which MEC1 is normally required.

**MEC1 and RAD53 probably share the same essential function**

In the course of evaluating the nature of MEC1's essential function, I became interested in the essential function of RAD53 — specifically whether it shared the same essential function as MEC1. Several previous results had indicated that Mec1p may act upstream of Rad53p, interact with it, and share functions with it: First, Rad53 becomes phosphorylated in a Mec1 dependent manner after DNA damage (127, 148). Second, while both MEC1 and RAD53 are required for damage-inducible transcription, data suggest that MEC1 is required for transcription of a greater number of genes than is RAD53, consistent with Mec1p acting upstream of Rad53 (70). Third, overexpression of RAD53 suppresses the essential function of mec1 (127). Fourth, both genes are required
for checkpoint arrest (159), and the model I present in Chapter 3 (Figure 3-2, page 85) predicts that Rad53 acts in an arrest pathway downstream of Mec1.

Fifth, both mec1Δ and rad53Δ cells can be suppressed by overexpression of RNR1 (24, 127). These results led me to hypothesize that MEC1 and RAD53 share the same essential function. This hypothesis predicts that other suppressors of MEC1’s essential function are also likely to suppress RAD53’s essential function. Therefore, I tested the effect of sml1 and cln1cln2 mutations and overexpression of TEL1 and DUN1 on rad53Δ cells. (Overexpression of TEL1 or DUN1 suppresses mec1’s essential function (94, 99 and see Chapter 4)). sml1, and a cln1cln2 double mutation (Figure 5-5), and pTEL1 and pDUN1 (my data, not shown) all restore viability to rad53Δ cells. pTEL1 and pDUN1 suppress inviability of rad53 cells qualitatively about as well as they suppress inviability of mec1 cells; however, as Figure 5-5 shows, sml1, and the cln1 cln2 double mutation do not suppress inviability of rad53 cells as effectively as they suppress inviability of mec1 cells. This supports the idea that MEC1 and RAD53 share the same essential function, but that these genes act in different parts of the pathway (see model in Figure A-1 in Appendix A, page 236). sml1 even suppresses a mec1rad53 double mutant strain, as well as rad53dun1, mec1dun1, and rad53tel1 double mutant strains (not shown), but suppression is relatively poor in all cases where the strain contains a rad53 mutation. Growth of strains was as follows, from best to worst (Table 5-2):
<table>
<thead>
<tr>
<th>strain</th>
<th>plate viability</th>
<th>growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>mec1sml1 (DLY258)</td>
<td>about 95%</td>
<td>healthy looking microcolonies</td>
</tr>
<tr>
<td>mec1dun1sml1 (RGY181)</td>
<td>85%</td>
<td>healthy looking microcolonies</td>
</tr>
<tr>
<td>rad53Δsml1 (RGY140)</td>
<td>73%</td>
<td>microcolonies nearly as good as above</td>
</tr>
<tr>
<td>rad53Δtel1Δsml1 (RGY183)</td>
<td>about 75%</td>
<td>same as rad53Δsml1</td>
</tr>
<tr>
<td>mec1rad53sml1 (RGY190)</td>
<td>60%</td>
<td>smaller microcolonies than RGY140</td>
</tr>
<tr>
<td>rad53Δdun1Δsml1 (RGY173)</td>
<td>41% - 65%</td>
<td>poor looking microcolonies, slow to form macrocolonies</td>
</tr>
<tr>
<td>mec1tel1Δsml1 (RGY39)</td>
<td>39%</td>
<td>poorest looking microcolonies, slowest to form macrocolonies</td>
</tr>
</tbody>
</table>
Sporulation and dissection of...  

_MEC1^+/Δ_ strains

- a
- c
- e
- g

_RADIUS3^+/Δ_ strains

- b
- d
- f

homozygous _cln1cln2_ background

homozygous _smil_ background

Figure 5-5. _smil_ and _cln1,2_ genomic suppressors of _MEC1's_ and _RAD53's_ essential function. a and b, sporulation and dissection of a _MEC1^+/Δ_ strain (RGY60) and a _RAD53^+/Δ_ strain (RGY75), respectively, reveals 2:2 viability. c and d, same as a and b, but in a homozygous _cln1,2_ background (RGY48 and 197, respectively), lethality of _mec1Δ_ and _rad53Δ_ cells is suppressed. e and f, same as a and b but in a homozygous _smil_ background (RGY151 and RGY152 respectively). Suppressed _mec1Δ_ cells produce colonies of the same size as WT colonies, while suppressed _rad53Δ_ cells produce smaller than WT-size colonies. g, sporulation and dissection of a _RAD53^+/Δ_ _MEC1^+/Δ_ _smil1/smil1_ strain (RGY186). Half of the small colonies are _mec1Δrad53Δ_ double mutant strains.
Since \textit{RAD53} overexpression can restore viability to \textit{mec1A} cells (127 and Chapter 4), I also tested if the reverse were true: can \textit{MEC1} overexpression restore viability to \textit{rad53A} cells? \textit{MEC1} overexpression did not suppress \textit{rad53} cells (not shown), which may indicate that \textit{MEC1} lies upstream of \textit{RAD53} on a pathway leading to the essential function, as well as lying upstream of \textit{RAD53} on other pathways leading to other functions discussed above and in Chapters 3 and 4. (See, for example, Figure 4-15 on page 157.)

\textbf{pTEL1 suppression of \textit{RAD53}’s essential function requires \textit{DUN1}}

I showed in Chapter 4 that \textit{pTEL1}’s suppression of \textit{MEC1}’s essential function requires both \textit{RAD53} and \textit{DUN1}. This finding led to a model where \textit{pTEL1} acts upstream of \textit{RAD53} and \textit{DUN1} on a pathway leading to the essential function (see a diagram of the model in Figure A1 of Appendix A, page 236). By this model, \textit{pTEL1} acts through \textit{DUN1} to suppress \textit{RAD53}’s essential function, and therefore \textit{pTEL1} should not restore viability to a \textit{rad53dun1} double mutant strain.

To test this model, I examined the numbers of meiotic segregants of different genotypes resulting from sporulation of \textit{RAD53+/Δ DUN1+/Δ} diploid strain, containing either \textit{pTEL1}, \textit{pvector}, or \textit{pRNR1} (as a positive control, since I had shown in Chapter 3 that \textit{pRNR1} suppresses inviability of the \textit{rad53dun1} double mutant). Figure 5-6 shows that while \textit{pRNR1} restores viability to both
rad53\textsuperscript{DUN1+} and rad53\textsuperscript{dun1} cells, \textit{pTEL1} restores viability only to rad53\textsuperscript{DUN1+} cells. Therefore, \textit{pTEL1} acts through \textit{DUN1} in order to suppress \textit{RAD53}'s essential function.

**Figure 5-6.** \textit{pTEL1} suppression of \textit{rad53\textsuperscript{A}} requires \textit{DUN1}. \textit{RAD53\textsuperscript{+}/\textit{ADUN1\textsuperscript{+}/\textit{ADUN1}} strains, containing one of the indicated plasmids, were sporulated and the spores were analyzed randomly. Horizontal lines indicate expected number of segregants (one fourth of the total in each case) of each genotype if suppression were complete. The graph shows that while \textit{pTEL1} can restore viability to \textit{rad53\textsuperscript{A}} strains, it cannot restore viability to \textit{rad53\textsuperscript{A}/\textit{ADUN1}} strains. As a control, \textit{pRNR1} restores viability to both \textit{rad53\textsuperscript{A}} and \textit{rad53\textsuperscript{A}/\textit{ADUN1}} strains. Both \textit{TEL1} and \textit{RNR1} were expressed on high copy plasmids under their own promoters. \textit{RAD53\textsuperscript{+}/\textit{ADUN1\textsuperscript{+}/\textit{ADUN1}} pTEL1, RGY 238; RAD53\textsuperscript{+}/\textit{ADUN1\textsuperscript{+}/\textit{ADUN1}} pRNR1, RGY 237; RAD53\textsuperscript{+}/\textit{ADUN1\textsuperscript{+}/\textit{ADUN1}} pvec, RGY 239.**

\textbf{MEC1 is not required for telomere maintenance}

In considering the essential function of \textit{MEC1}, I asked if knowledge of \textit{TEL1}'s functions could help elucidate the essential function of \textit{MEC1}. There are at least three reasons to believe that studies of \textit{TEL1} may aid in our understanding of the essential function of \textit{MEC1}: 1) \textit{TEL1} overexpression suppresses several functions of \textit{mec1} (94 and Chapter 4); 2) \textit{TEL1} and \textit{MEC1}
have a high degree of homology in their kinase domains; 3) \textit{mec1tel1sml1} (or \textit{mec1tel1} cells kept alive by \textit{pDUN1} or \textit{pRNR1}) cells have very low viability (94), so their essential functions may overlap as discussed earlier. \textit{TEL1} is not essential, and its only known function is to regulate telomere length (83). \textit{tel1} mutants have short telomeres; these do not progressively shorten and cause cell senescence or death as do some other telomere mutants, and \textit{tel1Δ} cells are not visibly sicker than WT cells. However, I thought it was reasonable to suspect, based on the homology of \textit{MEC1} and \textit{TEL1}, that \textit{MEC1} may also be involved in telomere regulation — specifically, that the essential function of \textit{MEC1} may be to stabilize telomere length. By this hypothesis, \textit{mec1} cells die due to progressive shortening of telomeres in each generation. This could explain why it takes several generations for \textit{mec1ts} cells to die. Previously, Greenwell et al. noted that \textit{mec1-1sml1} cells do not have shortened telomeres, in contrast to the shortened telomeres of \textit{tel1} cells (46). However, I reasoned that since a \textit{mec1-1sml1} strain has an intact essential function due to \textit{sml1}, it would be a less than ideal strain to test whether \textit{MEC1}'s essential function may be telomere maintenance. In a 24 hour timecourse experiment, at the \textit{mec1ts} restrictive temperature (37°C), WT cells and \textit{mec1ts} cells retain their telomere length, although \textit{mec1ts} cells lose their viability (Figure 5-7). Therefore, \textit{MEC1}'s essential function is not telomere-length related. This does not rule out some other type of telomere-related metabolism as \textit{MEC1}'s essential function.
Figure 5-7. Telomere length is unaffected in mec1" cells at their restrictive temperature. 

a. Southern blot (see methods #13, page 219, including Table 7-1) showing telomere sizes (arrow). Lanes 1 and 2, WT (DLY62) and tel1 (RYG53) controls. Note tel1 cells have shorter telomeres. Other lanes, timecourse at 37°C shows that WT and mec1" (RYG2) cells have telomeres of the same size. The difference in upper bands is probably due to the different strain background. 

b. Viability of mec1" cells decreases during the experiment, indicating that the Mec1 protein is in fact becoming inactivated. The decrease in viability in the WT control is probably due to an overheating accident that occurred at the 12 hour timepoint.
RNR1 transcript levels are not significantly different in mec1 strains

Based on observations made by others that RNR1 overexpression restores viability to mec1Δ and rad53Δ cells (24, 127), and the suggestion from my data in Chapter 4 that the essential function and the damage-inducible transcription function are related, I hypothesized that MEC1's essential function may be to ensure adequate transcription of the essential gene RNR1 (The "RNR1 hypothesis"). In addition, MEC1 (hereafter "MEC1" will also indicate RAD53) may help promote transcription of other important genes, also likely involved in DNA replication. Promoting transcription of other genes, however, would be a lesser role for MEC1, since overexpressed RNR1 by itself can restore viability to mec1Δ cells. (The RNR1 hypothesis postulates that the reason mec1 cells kept alive by RNR1 overexpression do not grow as well as WT cells is because of the deficient levels of other transcripts MEC1 regulates.) Restoration of viability to mec1 cells by DUN1, RAD53, or TEL1 overexpression would occur because of increased RNR1 transcription. An argument against the simple hypothesis that MEC1's essential function is to simply promote RNR1 transcription is that RNR1 overexpression does not suppress mec1tel1 cells (Chapter 4). It still could be, however, that TEL1 shares responsibility with MEC1 for the production of yet another essential or near essential transcript as discussed above. It is possible that RNR1 overexpression suppresses mec1 cells, not because it promotes dNTP synthesis (its normal function), but that in high amounts Rnr1 can perform other functions. One such other function may be the reduction of cellular levels of
oxidative stress, as the MEC1 homolog ATM has been speculated to have such activity (122).

To test the RNR1 hypothesis, I measured relative RNR1 transcript levels in these strains: WT, mec1-1sml1, and mec1Δ cells suppressed by pDUN1, pTEL1, or pRAD53. The data shows that mec1-1sml1 may have slightly less RNR1 transcript than WT, and that the level is increased upon overexpression of DUN1, TEL1, or RAD53 (Figure 5-8 a, b). Since these mec1 strains all had suppressed essential functions, I also measured RNR1 transcript levels in a mec1ts tel1Δ strain. In asynchronous mec1ts tel1Δ cells incubated at 36°C for 6 hours (Figure 5-8c) or eight hours (not shown), RNR1 levels are not significantly different from those of WT cells, or of mec1ts tel1Δ cells incubated at 23°C. Although the cells were cycling, they were grown to late-log phase prior to performing the experiment and kept in late-log phase during the experiment so that the mec1ts tel1Δ cells incubated at 36°C would not lose viability too rapidly. (Viability of the mec1ts tel1 cells decreased from near 100% at the beginning of the experiment, to about 50% after 6 hours at 36°C, to about 20% after 8 hours at 36°C. WT cells retained their viability.)

I conclude that RNR1 transcript levels are not decreased upon loss of MEC1 function, and therefore that MEC1's essential function is not involved in promoting RNR1 transcription.
Figure 5-8. *RNR1* levels in asynchronous cells. a, comparison of several strains of two different backgrounds shows that although *RNR1* transcript levels do not vary widely, *DUN1* and *TEL1* overexpression increase *RNR1* transcript levels. The presence or absence of *smi1* does not affect *RNR1* transcript levels. Strains were grown in duplicate; averages are shown, and error bars indicate the level of each duplicate. Asterisks (and lack of error bars) indicate a strain not measured in duplicate. b, in another experiment (A364a background), *RNR1* transcript levels in *mec1* cells were somewhat lower in comparison to *RNR1* transcript levels in WT cells. Strains used are shown below bars. Part c, next page.
**mec1 cells do not appear to be defective in ARS firing**

One possible explanation for the slow death of *mec1Δ* cells at their restrictive temperature, which takes into account *MEC1's* possible replication role, is chromosome loss or damage caused by lack of ARS firing. (ARS stands for Autonomously Replicating Sequence, and is an origin of replication in yeast chromosomes. Chromosomes that are not replicated become lost, since one of the daughter cells will lack that chromosome after mitosis.)

D. Koshland has developed an assay for loss of minichromosomes (centromere-containing plasmids, hereafter called plasmids) from failure of
replication (56). This assay employs a centromere containing minichromosome (plasmid), which carries the selectable markers LEU2 (used simply as a standard auxotrophic selectable marker when introducing the plasmid into yeast) and ADE3. Introducing the plasmid into an ade2ade3 strain (which is white) causes it to become ade2 ADE3* which produces a red color. Loss of the plasmid in turn results in a reversion to white. Plasmid loss is measured by plating the cells on nonselective media and examining the color of individual colonies. Entirely white colonies arise from cells that contained no plasmid when plated. Colonies that arise from cells containing the plasmid are red, or are red in the center and red/white sectored elsewhere (indicating plasmid loss subsequent to plating the individual cell). There are two versions of the plasmid: one version contains only 1 ARS sequence, and the other version contains seven ARS sequences. Koshland has shown that mutations which cause defects in ARS firing result in minichromosome loss, and adding additional ARSs to the plasmid increases the chance that an ARS will be fired, and therefore that the plasmid is replicated, thus preventing plasmid loss (56).

I used this assay to ask if cells lacking MEC1 function are defective in ARS firing. To do this, I measured plasmid loss in mec1Δ cells versus WT cells at mec1Δ's permissive and restrictive temperatures. If mec1Δ cells do not fire ARSs correctly at the restrictive temperature, the one-ARS plasmid may be lost at a higher rate than the rate in either mec1Δ cells at the permissive temperature, or
in Mec⁺ cells at either temperature. Furthermore, the extra ARSs of the multi-ARS plasmid may prevent or at least decrease plasmid loss in mec1ˢ at the restrictive temperature.

I measured plasmid loss rates of WT and mec1ˢ cells after 8 and 12 hours at either 23°C or 37°C by comparing the numbers of red-centered and white colonies produced by plating the cells at 23°C. Although as expected the mec1ˢ cells lost viability compared to the WT cells (Figure 5-9 b), there was little difference in plasmid retention rates between WT and mec1ˢ cells, containing either the 1 or 7-ARS plasmids, at either 23°C and 36°C (Figure 5-9a). This suggests that mec1 cells do not have a defect in ARS firing per se, although other replication defects are not ruled out.

The colonies produced by mec1ˢ cells that had been incubated at 37°C quickly lost their plasmids (either the 1- or 7-ARS versions) and therefore were red only in a small area in the center, whereas colonies produced by WT cells (which had been incubated at either temperature) or by mec1ˢ cells that had been incubated at 23°C lost their plasmids much more slowly, indicated by colonies with very little white around the edges (see schematic in Figure 5-9c). Although the significance of this observation is not entirely clear, it could be due to the formation of dicentric chromosomes in mec1ˢ cells at 37°C. This is consistent with reports from others, who have observed high levels of mitotic recombination in mec1 strains (66).
Figure 5-9. mec1 cells do not appear to be deficient for ARS firing. Wt and mec1<sup>ts</sup> cells containing plasmids with 1 or 7 ARS sequences were incubated at 23°C or 37°C for the indicated times, then plated at 23°C. Plasmid retention was measured by measuring the % red colonies that resulted (see text for details.) a, plasmid retention in WT (RGY64) and mec1<sup>ts</sup> (RGY65) cells at the permissive and restrictive temperature of mec1<sup>ts</sup>. Plasmid loss rates are not significantly different, and are not affected by the presence of additional ARS sequences. b, viability of cells from (a), showing that the Mec1 protein was indeed inactivated by the mec1<sup>ts</sup> cells' loss of viability. c, schematic diagram of colonies plated (see text for details of the experiment). The black in the center represent cells that have not lost the plasmid, while the white portion represents cells having lost the plasmid. mec1<sup>ts</sup> cells that had been incubated at their restrictive temperature produced colonies like the one shown on the left (rapid plasmid loss). All other colonies resembled the one on the right (slow plasmid loss).
DISCUSSION

The essential function of MEC1 and RAD53

The main conclusion of this chapter are that MEC1 and RAD53 probably share the same essential function. This conclusion comes primarily from data showing that inviability of both rad53 and mec1 strains is suppressed in the same ways: by sml1, cdc and cln mutations (Figure 5-5 on page 179), and by overexpression of TEL1, DUN1, and RNR1; and because RAD53 overexpression suppresses inviability of mec1 mutants. Although the identity of the essential function and role(s) of MEC1 and RAD53 in replication are still unclear, various experiments have either ruled out certain possibilities or have helped to acquire information relevant for future models. Whether or not the essential function is the same as a putative replication function (see next chapter) is not yet known.

It seems likely that MEC1’s essential function is related to its transcriptional function. The reasons for this conclusion are 1) data presented in Chapter 4 showed that suppression of either function by TEL1 overexpression acts on the same pathway, and 2) A role for MEC1 in transcribing an essential gene, (which I hypothesize would happen indirectly, via RAD53) rather than a more direct essential role, is consistent with the slow death of mec1ts cells at the restrictive temperature. Among other possibilities, this transcribed gene could be a), a gene involved in DNA replication, b) RAD53 itself, or c) some important gene involved in oxidative stress response. The oxidative stress concept is based on recent
reports about the *MEC1* homolog ATM (122) and postulates that *MEC1*’s essential function is to either lower the amount of oxidative damage that occurs in the cell or to aid in repair of this damage. Mec1p could perform this function either directly, or indirectly by increasing the levels of other proteins involved in the oxidative stress response. If *MEC1*’s essential function relates to the oxidative stress response, why does *RNR1* overexpression suppress the essential function? One would have to postulate that Rnr1p, in addition to its specific role in dNTP production, either has another specific role in the oxidative stress response or can act nonspecifically in this response and probably on other pathways as well.

I also tested several other likely scenarios for *MEC1*’s essential function. While I cannot rule out a role for some type of telomere maintenance as a function of *MEC1*, I did show that telomere length is maintained even after loss of *MEC1* function (Figure 5-7, page 183). In fact, it may be that a role in telomere maintenance could be uncovered in the *mec1ts tel1* strain. A role in firing of replication origins (ARSs) might have explained both *mec1*’s essential and replication functions, and even implied that they were the same function. However, I did not find evidence for a role in ARS firing (Figure 5-9, page 190). This does not rule out a role for *MEC1* in DNA replication. A likely explanation for the plasmid loss in *mec1ts* cells after their shift back to the permissive temperature (Figure 5-9 part c) is the formation of dicentric plasmids.
Another possibility for MEC1's essential function was an essential role in \( RNR1 \) production. Overexpression of \( \text{DUN1, TEL1 or RAD53} \), which restore viability to \( \text{mec1} \) cells, also increased the levels of \( RNR1 \) transcript (Figure 5-8a, b, page 186). However, the \( RNR1 \) transcript levels in \( \text{mec1-1sml1} \) strains or a \( \text{mec1}^s \; \text{tel1}^\Delta \) strain were not significantly lower than those of WT cells. Very recent results suggest that the Sml1 and Rnr1 proteins can interact (121). This suggests that the essential function of \( \text{MEC1 and RAD53} \) may be regulation of Rnr1 at the protein level, rather than at the level of transcription. In Appendix A, I present a model of pathways leading to the essential function.
CHAPTER 6: MEC1 AND RAD53 MAY SHARE A DNA REPLICATION FUNCTION

FOREWORD TO CHAPTER 6

I performed all of the experiments in this chapter, with the exception of the DNA replication experiments involving viability assays, FACS and pulse-field gels from HU-treated cells (Figures 6-1, 2). These were first done by T. Weinert, and were repeated by M. Nejad and myself working in collaboration.

INTRODUCTION

MEC1 and RAD53 cells are sensitive to inhibition of replication by hydroxyurea

Both mec1 (159) and rad53 (3) cells are very sensitive to the drug hydroxyurea (HU). HU inhibits DNA replication by inhibiting the enzyme ribonucleotide reductase, which is responsible for dNTP production. Low dNTP levels presumably stall the replication forks. There may be several reasons for the HU sensitivity of mec1 and rad53 mutants. One reason is that mec1 and rad53 cells are S/M checkpoint deficient; they cannot restrain mitosis in the event that DNA replication is inhibited. Entry into mitosis with unreplicated DNA is presumably lethal.

Another possible reason for the HU sensitivity of mec1 and rad53 cells, discussed in this chapter, is their lack of damage-inducible transcription function. Normally, WT cells increase transcription of RNR3 (which encodes a component
of ribonucleotide reductase) upon HU exposure; this presumably is important in the adaptation process (in which WT cells can grow in the presence of HU after experiencing an initial delay), and may increase the fidelity of replication as well.

I show here that the reason(s) for HU sensitivity of mec1 and rad53 mutants is probably not related to the S/M checkpoint or damage-inducible transcription functions, but rather are probably due to a DNA replication function shared by MEC1 and RAD53.
RESULTS

**MEC1 may be involved in DNA replication**

Most checkpoint-mutant cells are sensitive to DNA damage and/or inhibition of DNA replication. Although it is common to associate sensitivity to damage or to inhibition of replication with progression through a checkpoint with damaged or unreplicated DNA, there may be additional causes for this sensitivity. For example, Lydall et al. have shown that rad9 cells are more sensitive to cdc13 damage than are rad24 cells, although each mutant has the same checkpoint defect. The increased sensitivity of rad9 cells appears due to the inability of rad9 cells to limit exonucleolytic digestion of DNA around the damaged area. Therefore, in addition to a checkpoint function, RAD9 probably also has a direct role in DNA metabolism. Based on precedents such as these, I tested if mec1 cells may be sensitive to inhibition of DNA replication for reasons not directly related to the lack of S/M checkpoint function.

If HU-treated mec1 cells die because they enter mitosis with unreplicated DNA, one might predict that these cells could be rescued by removing the HU and inhibiting mitosis for a period long enough to allow completion of DNA replication. Alternatively, if mec1 cells are sensitive to HU for reasons besides their lack of S/M checkpoint function, an artificial delay of mitosis might not rescue them. To distinguish between these two hypothesis, we performed the following experiment (see methods #19 on page 224 for detailed protocol): We
released G₁-synchronized WT and \textit{mec1-1sml1} cells into media containing HU; the HU was removed after 40 minutes. (Since the HU pulse lasted only 40 minutes, the cells did not reach the G₂/M boundary or attempt mitosis by the time of HU removal.) A sample of the cells was then removed and plated on agar, to test their potential to form viable colonies, and the rest of the cells were allowed to grow for another 150 minutes in media containing nocodazole (which delays mitosis), and viability was again assayed. We asked if the nocodazole-induced delay would restore viability to the \textit{mec1} cells. FACS analysis of the cells shows that HU-pulsed \textit{mec1-1} cells do eventually complete DNA synthesis, albeit at a slower rate than WT cells (Figure 6-1b). (Both strains replicate their DNA with the same kinetics in the absence of HU, data not shown.) However, the viability of both the HU-pulsed plus nocodazole treated and HU-pulsed \textit{mec1} cells was low, (although the nocodazole treated cell viability was consistently slightly higher than the untreated, Figure 6-1a). These results suggested that while a small part of the inviability of HU treated \textit{mec1} cells is due to progression through mitosis with unreplicated DNA, most of the inviability must be due to other reasons.

I have confirmed others' observations that \textit{TEL1} overexpression, which causes a slight constitutive G₂ delay in \textit{mec1} cells but does not restore the S/M checkpoint (see Chapter 4), also increases \textit{mec1} cells' viability after HU exposure (94). In Chapter 4, I reported that the p\textit{TEL1}-induced restoration of viability in HU treated \textit{mec1} cells cannot be due to the constitutive G₂ delay
caused by TEL1 overexpression, because the restoration of viability also occurred in a mec1pds1dun1 cells, and the pTEL1-induced delay is PDS1 dependent. The observation that the pTEL1 restoration of mec1's HU viability does not require a G2/M delay is consistent with the above finding that a nocodazole induced G2/M delay does not play a large role in restoring viability. (The mechanism by which pTEL1 restores viability to mec1 cells after HU exposure remains unknown.)
Figure 6-1. Replication and viability defects of mec1 cells after transient HU exposure. a, viability of WT (DLY62) and mec1-1 (DLY258) strains after HU exposure. HU-induced death of mec1 cells is not suppressible by causing a G2 delay. b, FACS profiles show that Mec' cells replicate their DNA faster after removal of HU than do mec1-1 cells. This profile is typical of several experiments. dun1 cells (RGY33) do not replicate their DNA more slowly (although it appears faster than WT in this case, it is generally the same). 1c and 2c markers are approximate. Times are after HU removal.
Lack of damage-inducible transcription does not account for HU sensitivity of mec1 cells

Another possible explanation for mec1 cells' death in HU is mec1's lack of damage-inducible transcription. Perhaps DNA replication in mec1 cells is compromised by the lack of dNTPs after HU exposure; since RNR3 (which is responsible for increased dNTP production after DNA damage) is not upregulated in mec1 cells, the DNA replication that occurs in the HU-pulsed cells may be of poor quality. As mentioned above, TEL1 overexpression increases the viability of HU-treated mec1 cells; in addition, I showed in Chapter 4 that TEL1 overexpression restores damage-inducible transcription to mec1 cells. This correlation could mean that the pTEL1 restoration of damage-inducible transcription (of RNR3, for example) in mec1 cells causes the increase in viability of mec1 mutants after HU exposure. However, as noted in Chapter 4, TEL1 overexpression still increases HU viability even in a mec1dun1pds1 triple mutant. I interpreted this to mean that the viability suppression is unlikely due to pTEL1's restoration of damage-inducible transcription (since DUN1 is required for transcriptional induction of RNR3, both normally and by pTEL1 in mec1 mutants (173), Chapter 4). Therefore, it is unlikely that mec1 cells die in HU because of their lack of damage-inducible transcription (with the caveat that damage-inducible transcription of another transcript(s), not RNR3, may be important, and that induction of this transcript(s) may be MEC1 but not DUN1 dependent).
As a more conclusive test of the role of damage-inducible transcription in HU viability, I performed an HU-pulse experiment similar to the one described in the previous section using dun1Δ cells, which, as noted above, are also defective for damage-inducible transcription of RNR3. If the cause of mec1-1 death after an HU pulse was poor replication in response to a lack of dNTPs, then dun1 cells should also show low viability after the same 40 minute HU treatment after G1 release. However, the viability of the dun1 cells was consistently high (about 80%), suggesting that the cause of mec1-1 death is not related to the lack of RNR3 upregulation. (Although a 40 minute HU pulse does not kill dun1 cells, I do see that, as reported (173), dun1 cells grow poorly on .1M HU plates, depending on strain background. [A364a dun1 cells do not grow, while W303 dun1 cells grow poorly.] This is expected since continued HU exposure would prevent dun1 cells from completing S-phase.) dun1 cells also do not replicate their DNA more slowly after an HU pulse, as mec1 cells do (Figure 6-1b).

Although mec1 cells are able to replicate their DNA (by FACS) after the HU pulse, the observations that the sensitivity of mec1 cells to HU seems not to be directly related to their lack of S/M checkpoint function nor to their lack of damage-inducible transcription suggested that MEC1 may have a more direct role in DNA replication. We therefore asked about the quality of the replicated DNA in the HU-pulse experiment. Using pulse-field chromosome gels, we showed that the quality of the DNA from the HU-pulsed mec1 cells is poor. This is
evident by less well defined/fuzzy chromosome bands from \textit{mec1} cells compared to those from WT cells after the 40 minute HU exposure and 150 minutes growth in nocodazole-containing media (Figure 6-2). Results here were generally consistent, although T. Weinert's gels showed a more severe effect than those done jointly by M. Nejad and myself.

While we do not yet have proof that \textit{MEC1} plays a direct role in DNA replication, these results are strongly suggestive of one. What that role is remains to be determined.
Figure 6-2. DNA replication in synchronized mec1 cells after transient HU exposure is poor. In this chromosome gel (where each band represents a separate chromosome), chromosomes from mec1-1sm1 (DLY258) or mec1Δsup1 (RGY37) cells exposed to HU are less well defined than those from WT (DLY62) cells exposed to HU, or to cells of either genotype not exposed to HU.

*RNR1* overexpression does not restore viability to mec1/rad53 cells after HU exposure.

Evidence that *mec1* may have a role in replication, coupled with the idea that *MEC1*’s essential function may involve production of dNTPs via *RNR1* (see...
Chapter 5), led me to question if \textit{MEC1}'s putative replication role is related to \textit{MEC1}'s essential function. Under this hypothesis, \textit{MEC1}'s essential function is to promote replication indirectly by causing synthesis of necessary replication components (e.g. \textit{RNR1}). \textit{mec1} cells die as the dNTP pool becomes depleted. The rate of \textit{mec1} cell death at 37°C is slow (see Figure 5-2b, page 171) perhaps because there is a small excess of dNTPs. This hypothesis also postulates that the \textit{sml1} mutation may suppress \textit{mec1} lethality by promoting dNTP production. But after an HU pulse, as described in the previous section, \textit{sml1} cannot restore the dNTPs in time, and so replication is slow and of poor quality.

Since \textit{RNR1} overexpression (by high copy plasmid-containing \textit{RNR1} expressed under its own promoter — called \textit{pRNR1}) had been shown to suppress \textit{mec1} and \textit{rad53}'s essential function, it seemed possible that \textit{pRNR1} could also restore viability after HU exposure, if \textit{mec1/rad53} cells die because of poor replication due to a lack of \textit{RNR3}. (Recall that Rnr1p and Rnr3p are both similar subunits of ribonucleotide reductase, and are interchangeable. \textit{RNR1} is transcribed normally, and \textit{RNR3} is transcriptionally induced after damage.) Although I had already shown that the HU-induced death of \textit{mec1} and \textit{rad53} cells was probably not due to a lack of extra \textit{RNR3} transcription, it was still reasonable to ask if \textit{RNR1} overexpression could restore viability after HU exposure. However, in a timecourse of synchronous cells that had just been
released from G₁ arrest into media containing HU, RNR1 overexpression in both rad53 and mec1 cells did not increase cell viability above that of cells containing vector alone (Figure 6-3). As before, arresting the cells in nocodazole (for 90 minutes this time) slightly increased viability. Since nocodazole treatment did not significantly increase the viability of the rad53 cells, it seems likely that, as for mec1 cells, the reason rad53 cells die after HU exposure is not due simply to premature mitosis before replication is completed, and that, like MEC1, RAD53 probably has a replication function. This remains to be more rigorously tested.

There are 2 explanations for the finding that pRNR1 cannot restore viability to HU treated mec1 or rad53 cells. First, it may be that the essential function of mec1/rad53 is not related to DNA replication, and so restoring or strengthening the essential function by RNR1 overexpression would not be expected to affect viability after HU exposure. Second, it may be that MEC1's and RAD53's essential function is indeed proper replication, and while RNR1 overexpression directly or indirectly can partially suppress the essential function, the situation after HU exposure is too severe for overexpression of RNR1 by itself to be able to restore viability. (Perhaps additional genes encoding other subunits of ribonucleotide reductase, or other replication proteins, would also have to be overexpressed.) Whether MEC1/RAD53's essential and putative replication functions are the same as each other is still an open question.
Figure 6-3. *RNR1* overexpression does not restore viability to *mec1* or *rad53* cells treated with HU. Viability after a short exposure to HU is slightly increased by a nocodazole-induced G2 delay in both *mec1−1sml1* (DLY258) and *rad53−11* (DLY259) cells. However, viability in either *mec1* or *rad53* cells is not increased by *RNR1* overexpression vs. vector alone. Viability was measured by plating cells and scoring the percent viable microcolonies the following day. Bars show average viability of duplicates; error bars indicate actual viability of each duplicate. Where error bars do not appear, they are too small to be graphed.
DISCUSSION

Replication function

Evidence that MEC1 may have a role in DNA replication is indirect but can be summarized as follows: 1) the HU sensitivity of mec1 cells is only suppressed slightly by delaying the onset of M phase (Figure 6-1a). Therefore, there must be another cause(s) for HU sensitivity besides premature entry into mitosis. 2) pTEL1 can suppress the HU sensitivity of mec1 cells, and this suppression does not come about through restoration of the S/M checkpoint (Chapter 4) or by pTEL1's constitutive delay or restoration of damage-inducible transcription (Chapter 4). In fact, a deletion of another gene responsible for damage-inducible transcription, DUN1, does not contribute to HU sensitivity after short HU pulses (this chapter). This again implies that there must be another cause for HU sensitivity in mec1 cells. 3) After an HU pulse, DNA replication in mec1 cells is slowed compared to that in WT cells (Figure 6-1b, page 199). Again, this slow replication is probably not due to the lack of transcriptional induction because dun1 cells do not replicate their DNA more slowly after an HU-pulse (Figure 6-1b). 4) After an HU pulse, replication quality in mec1 cells is poor as assayed by pulse-field chromosome gels, indicating a molecular deficiency in some aspect of DNA replication (Figure 6-2, page 203).

It seemed reasonable that if RNR1 overexpression restores viability to mec1Δ cells, it may also restore viability to HU-pulsed mec1 cells, since RNR1
encodes a subunit of ribonucleotide reductase, the enzyme targeted by HU. However, \textit{RNR1} did not restore viability to HU-pulsed \textit{mec1} cells (Figure 6-3, page 206). This could have been because after HU treatment, other subunits of ribonucleotide reductase were also lacking, or that HU sensitivity results from defects in a more direct replication function in a \textit{mec1} mutant.

After DNA damage, WT cells replicate their DNA more slowly due to the intra-S checkpoint (108). \textit{mec1} cells, which are intra-S checkpoint defective (108), do not slow down their rate of replication after damage, and therefore replicate their DNA faster than WT cells do after damage. Therefore it is puzzling that after an HU pulse, \textit{mec1} cells replicate their DNA more slowly than WT cells do. One possible explanation is that the slow replication is due to the lack of damage induced transcription of \textit{RNR3}; however, as mentioned above \textit{dun1} cells also lack this function and yet do not replicate more slowly in identical conditions.

There are two hypothesis that might explain \textit{MECT}'s (and \textit{RAD53}s) role in replication: 1) after HU exposure, Mec1p (or another protein activated by Mec1/Rad53) is required to keep the stalled replication fork from disassembling and/or provide for proper reassembly. Although the fork can reassemble after HU is removed, and replication can be completed, the quality is poor due to improper reassembly. 2) Perhaps Mec1 and Rad53, contrary to promoting the firing of ARSs as discussed in the previous chapter, instead prevent their firing. Santocanale and Diffley (cited in 11) have shown that \textit{RAD53} is required to
prevent late ARS firing after HU exposure (11). It could be that after HU depletes the dNTP pool in *mec1* or *rad53* cells, the stalled replication forks remain stable, but the unprevented firing of late origins in the absence of dNTPs renders these ARSs useless. Subsequently, when HU is removed and replication resumes, only the early-firing ARSs are functional and so replication progresses more slowly. In WT cells, however, the late-firing ARSs could still be used. Furthermore, perhaps DNA structures caused by the aborted firing of late origins contributes to the poor quality of DNA. Currently there is no reason to favor one of these hypothesis over the other.
WHAT WE HAVE LEARNED

A major conclusion from the work presented in this dissertation is that cell cycle checkpoint control in general, and functions of individual genes involved in this control, is not as simple as we once thought.

We have learned that the G2/M checkpoint pathway involves two subpathways, each of which can produce a partial arrest by itself. There are likely to be further complications that account for the synergistic activities of these two pathways in promoting full arrest when they work together.

We have learned that genes such as TEL1 and DUN1 have more complex roles than we first ascribed to them. For example, while TEL1 has no checkpoint function, its overexpression causes constitutive cell cycle delay by one pathway, and causes damage-induced transcription by another pathway. It isn’t known yet how TEL1 overexpression can restore viability to HU treated mec1 cells, but it appears to operate by yet another pathway. Furthermore, while tel1Δ cells are viable, TEL1 has overlapping roles with MEC1 in an essential function.

DUN1 has a similar role in checkpoint arrest as RAD53, yet dun1 mutants are less UV sensitive. While dun1Δ cells are viable, DUN1 apparently has overlapping roles with RAD53 in an essential function.
We have learned that the essential function of *MEC1* may be more complex than once imagined: *MEC1*'s essential function isn't likely to be a merely constitutive S/M delay to prevent premature mitosis. *MEC1* may have more roles than once imagined, such as a role in DNA replication.
CHAPTER 7: MATERIALS AND METHODS

1. Strains and media.
   All strains used are shown in Table 7-2 (at the end of the chapter). For hydroxyurea (HU) plates, HU was used at a final concentration of 100mM. For liquid viability assays, it was used at 200mM. MMS was used at .01%.

2. Cell viability and morphology.
   Cells in liquid were plated at 23°C, and the following day the percentage of live microcolonies was determined from scoring at least 100 microcolonies. When HU was present it was washed out 2x with H₂O before plating. MMS was inactivated by addition of 10% sodium thiosulfate to a final concentration of 1.2%. For scoring arrest phenotype, cells were fixed in 70% EtOH for 1 hour or more, washed 2x, stained with DAPI [4,6-Diamidino-2-phenylindole], briefly sonicated, and scored by a fluorescence microscope. At least 100 cells were classified as unbudded, small budded, large budded, or large budded with divided nucleus.

   Viability after UV exposure was measured by plating cells, allowing the plates to dry (to avoid the UV shielding effect of water), and exposing the cells to UV by a Stratalinker (Stratagene). Viability was scored either by counting the percent of live (growing) microcolonies the next day, or by counting the number of macrocolonies that grew (normalized to the number that grew when not exposed to UV).
Scoring growth on HU plates at 37°C presents special problems:
Sometimes even WT strains don’t grow well on HU at 37°C, especially on
selective media. When this was a problem, we incubated the plates for 1 day at
37°C, then shifted the plate to 30°C. The WT control strain then grew, but a
mec1-1 or mec1ts cell did not.

3. MEC1 deletion.
A knockout plasmid was created (Figure 7-1, at the end of the chapter) by
ligating the 819bp Sacll/BamH1 fragment (to which was added a polylinker
fragment to provide a Sac1 end) from the 3' end of the gene (containing the first
98 bp of the MEC1 ORF), and the 455 bp Sacll/Sacl fragment from the 5' end of
the gene (containing the last 124 bp of the ORF) to the vector backbone
PRS406, a URA3 integrating vector. This plasmid was linearized by cutting at
Xba1 (Xba1 is in the polylinker fragment mentioned above), and yeast cells were
transformed.

See plasmid map for construction of pRG10 (mec1 knockout plasmid).
pRG38 (mec1::LEU2) was made by subcloning the Pvull fragment of pRG10 into
PRS 405.

4. Site Directed Mutagenesis
The N2229K allele was constructed in a 1012bp fragment of MEC1 (Kpn1-
Sacll) by using the Clonetech kit according to the manufacturer’s directions. The
mutation was identified by the creation of a new Ssp1 site. The 1kb fragment was then ressected into PRS415MEC1 and pDL297 (GalLexAMec1), and the mutated portion (1012 bp) was sequenced to confirm the presence of the N2229K mutation and the absence of other mutations.

5. DNA sequencing
   DNA sequencing was done by standard procedures (dideoxy method).

6. Creation of the mec1ts allele
   A mec1ts strain was created by hydroxylamine mutagenesis of a plasmid-borne MEC1 (URA3 marked PRS416 MEC1). (For the hydroxylamine mutagenesis protocol, see below in this section.) The checkpoint temperature sensitive phenotype was selected for in mec1-1 transformants of the mutagenized MEC1, by examining growth on .1M HU at 23°C vs. 37°C. 1600 transformants were patched and replica plated onto -ura HU at 23°C and 37°C, as well as -ura at 37°C. About 30 transformants showed growth at 23°C but not 37°C on HU, but still grew on -ura media at 37°C (showing that the temperature sensitive phenotype was not linked to the URA3 gene). About 125 transformants were non-conditional mec1 mutants (HU sensitive at 23°C and 37°C). All temperature sensitive transformants showed similar death kinetics in .2M HU. One temperature sensitive allele was chosen for integration; it was excised from the plasmid and transplaced into mec1+Δ diploid, to replace the null allele, and the diploid was sporulated and dissected. The integrated temperature sensitive
allele, in addition to its checkpoint defect at 37°C, also exhibits poor growth at
37°C: microcolonies of a few to up to about 100 cells form; they are rough
looking rather than round, and grow more slowly (due in part to lethality). Growth
is good at 23°C and 30°C.

**Hydroxylamine mutagenesis:** I mixed 15 ul of plasmid (about 1mg/ml) and
85 ul mutagenesis buffer [2M NH₂OH, 4mM EDTA, 100 mM
tetrasodiumpyrophosphate, 200mM NaCl, to pH 7 with NaOH]. This was
incubated at 75°C for 1 hour, and purified on a G50 column which had been
equilibrated with STE by twice running the column with 100 STE. (STE is TE
buffer plus a final concentration of .1M NaCl.) I got 25% as many transformants
from the mutagenized plasmid as I did with mock-mutagenized plasmid (where
the mutagenesis buffer was substituted with water), as expected based on
previous work in our laboratory.

7. **Double stranded break experiments**
   These were done with GAL HO strains; strains were grown in raffinose
overnight, then induced with galactose for two hours, resulting in a double strand
break at the HO locus. In addition, these strains contained a rad54 mutation so
that the break could not be repaired, resulting in about a 10 hour arrest in
checkpoint proficient cells. Cell viability after galactose exposure was measured
as a way of determining efficiency of HO cutting. Since the break could not be
repaired, it should in all cases be lethal.
8. GAL HO mec1ts experiment

We made a GalHO rad54 mec1ts strain, in which the HO endonuclease is galactose inducible. These cells, and Mec+ GALHO rad54 controls, were grown in raffinose to midlog before galactose induction. After 4-6 hours at 23°C, 80% of the cells were arrested at the G2 checkpoint due to the HO break. The cultures were split and incubated at either 23°C or 37°C. At various times, cells were removed for ethanol fixation and DAPI staining. When split, the cultures were also plated at 23°C and 37°C as an added control that the cells were either failing to maintain arrest or remaining arrested. Low viability of cells (2.5% and 7.7% for Mec- and mec1ts, respectively) indicated that most of the cells received the double stranded break.

9. Immunoprecipitation of GALLex A- Mec1

Since the cells don’t prefer raffinose as a carbon source, the cells were grown in selective media containing dextrose to mid-log; the cells were then washed and resuspended raffinose/galactose containing media. 5 mls of cells were grown in selective media + raffinose (2%) and galactose (2%) to about 1x10^7/ml. The cells were pelleted, resuspended in a microfuge tube in 300 microliters (ul) cold lysis buffer [contains: 50mM Tris pH 7.5, 150 mM NaCl, 5mM EDTA, 1% Triton X-100, protease inhibitors: PMSF (to 1mM), Leupeptin, chymostatin, pepstatinA, aprotinin, soybean trypsin inhibitor, and antipain (all to1
microgram/ml), and put in ice. 150 ul cold acid-washed glass beads were added, and the mixture was vortexed for 3 minutes at 4°C. The tubes were spun at high speed for 10 minutes at 4°C, and the supernatant was transferred to a new tube. 1 ul crude LexA antibody was added and incubated on ice for 1 hour. Thirty ul Staph cells or protein A beads, (which have been resuspended in lysis buffer) were added, and incubated for 30 minutes in ice (staph cells) or 1 hour at 4°C rocking (protein A beads). This was spun down, aspirated, and washed 3x with 500 microliters lysis buffer. For Western blots, the beads were resuspended in 20 ul cracking buffer [1 ml contains: 100 ul glycerol; 100 ul of 20% SDS; 125 ul 1M Tris pH 6.8; 100 ul B-ME; 575 ul water; bromophenol blue], then incubated 3 minutes at 100°C, spun 15 seconds; the supernatant was loaded onto the gel.

10. Extraction of total cell protein
   500 ul of overnight yeast culture was pelleted in a microfuge, to which 150ul acid washed glass beads and 20 ul sample buffer [1ml contains: 120ul 1M Tris pH 6.8; 100 ul 20% SDS; 200 ul glycerol; and 450 ul water. Also, 100 ul Beta mercaptoethanol is added just before use.] was added. This was boiled for three minutes, vortexed for one minute, and 80ul additional sample buffer was added. 5ul was used to run on a polyacrylamide gel.

11. \textit{cdc13} and other cell cycle assays
   For plate assays, cells were grown to mid log overnight in appropriate media (YEPD unless selection of a plasmid required selective media). They were
briefly sonicated, plated, and incubated at 37°C for 4-6 hours. Microcolony size of 100 microcolonies was scored as 2, 3, 4, or at least 5-budded. Arrest at 2 or 4 buds indicates cdc13 arrest.

For liquid assays, cells were grown in YEPD (including those cells containing pRNR1 which required pRNR1 for their survival), alpha factor arrested at 23°C, washed 3 times to remove alpha factor, and shifted to 36°C in liquid media. At various times aliquots were taken, EtOH fixed, DAPI stained, and nuclear morphology determined by fluorescence microscopy. For pds1 strains, cells were shifted to 30°C, where cdc13 arrest still takes place but the lower temperature was used to avoid complications with the temperature sensitivity of pds1.

To measure delay caused by TEL1 or RAD53 overexpression in Cdc13 strains, cells were grown in appropriate selective media at 30°C (or 23°C in the case of temperature sensitive strains, such as pds1.) The pTEL1 in rad53-11 delay experiment was done at 36°C since the rad53-11 strain is temperature sensitive. This was done to remove as much RAD53 activity as possible.

12. G2/M UV checkpoint experiments
Mid log cells were arrested in nocodazole (10 ug/ml) for 2 hours (or until >80% G2 arrest) at the appropriate temperature. Cells were plated, (up to about 2X10^7 cells/plate), the plates allowed to dry, and exposed to UV (Stratalinker) at 40 J/m^2. The cells were washed off the plate, washed twice more to remove
nocodazole, and resuspended in growth media. Aliquots were taken every 15 minutes for 1 hour, and were EtOH fixed, DAPI stained, and nuclear morphology was determined (see Cell viability and morphology).

13. Northern, Southern and Western Blots

These were done by standard procedures. For Northerns, RNA was prepared by a glass bead disruption protocol, and the signal was quantitated on a phosphoimager using the IP lab gel program. The number of counts of the RNA being tested (such as RNR1 or RNR3) was divided by the number of counts from a loading control probe, either URA3 or ACT1.

Immunoprecipitation of Pds1HA: cells were disrupted with glass beads in lysis buffer (50mM Tris pH7.5, .1% NP40, 2mM EDTA, 20mM NaCl and 50mM NaF + protease inhibitors aprotinin, leupeptin, and pepstatin A), incubated 1 hr on ice with mouse monoclonal HA antibodies (supplied by Carol Gregario), incubated at 4°C with rocking with protein A sepharose, and washed 3x with lysis buffer. Western blots were visualized by a horseradish peroxidase secondary antibody and Pierce supersignal. Some Western blots were quantitated by scanning the autoradiogram with an Eagle Eye densitometer (Stratagene).
Table 7-1. Some probes used in this study. The following table lists probes used for experiments (as opposed to probes used simply to check if the construct was made correctly).

<table>
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<th>transcript size*</th>
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<tbody>
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<td>pBR 1600 (Weinert lab)</td>
<td>3kb</td>
</tr>
<tr>
<td>RNR1</td>
<td>2.7 kb Nde1/NotI</td>
<td>pBAD070 (S. Elledge, B. Desany)</td>
<td>3kb</td>
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<td>1.1 kb BamHI</td>
<td>pTW004 (Weinert lab)</td>
<td>.7 kb</td>
</tr>
<tr>
<td>ACT1</td>
<td>.6 kb BamHI</td>
<td>pKS:ACT1 (C.Dieckmann)</td>
<td>slightly larger than .7 kb</td>
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<tr>
<td>telomere</td>
<td>.7 kb HhaI</td>
<td>pYT14 (135) (supplied by P. Hieter)</td>
<td>about 1.5 kb from genomic DNA cut with Xho1</td>
</tr>
</tbody>
</table>

* or size of expected band on Southern blot in the case of the telomere probe.

14. Determination of G₂ arrest caused by TEL1 overexpression

DAPI staining confirmed 30-40% medial nuclear division in a synchronous culture. This could have been S or G₂ arrest. The lack of an S peak by FACS suggested that the cells must all be arrested in G₂ by pTEL1 (data not shown). Also, had the delay been in S phase, the cells overexpressing TEL1 might have reached G₂ later in the cell synchrony experiment; they did not. FACS analysis on an asynchronous culture would theoretically be predicted to show a higher G₂ peak in strains overexpressing TEL1, but since the arrest level was low in synchronous cultures, it would have been even lower in asynchronous cultures and therefore the difference between strains overexpressing TEL1 or not overexpressing TEL1 probably would not have been distinguishable.
The TEL1 overexpression delay phenotype was examined in two backgrounds: it is stronger in W303 than in A364a.

We have noticed that sometimes preparations of TEL1-containing plasmid contain defective TEL1. This may be due to rearrangements of the plasmid while in bacteria. Therefore we were careful to use plasmid preparations that we knew were contained an intact TEL1 gene. As a test for an intact TEL1 gene, we asked for TEL1-plasmid that restored UV viability to mec1 cells. (Another test I performed once showed that the pTEL1 could restore proper telomere length to tel1Δ cells, as assayed by Southern blot.)

15. Preparation of cells for FACS
   About 5-7 X 10^6 cells/sample were fixed overnight in 70% EtOH at 4°C, washed 3x, incubated at 37°C for 1 hour in RNAse solution (95 ul 1 mg/ml RNase plus 5 ul 1M Tris pH 8), washed 2x, incubated at 37°C for 30 minutes in pepsin solution (5 mg/ml pepsin in 55mM HCL), washed twice, washed in FACS wash (100mM Tris pH 7.5, 180mM NaCL, 70 mM MgCl2), and resuspended in 100 ul 50 ug/ml PI in FACS wash.

16. Chromosome loss/recombination experiment
   Cells were grown in complete media to midlog, and the culture was split; to half the culture HU was added to .2M. The cells were grown 4 hours, and plated for individual colonies on CAN (canavanine), CAN -ade, and complete plates, so that the number of cells plated on each plate would be the same. Recombination
rate was measured as the number of colonies on CAN-ade plates divided by the number on complete plates; chromosome loss was measured as the number of colonies on CAN plates divided by the number on complete plates.

17. Crosses testing requirement of various genes for essential function restoration

   Before sporulation, diploids were grown in selective media for the desired plasmid. After dissection, the colonies were patched onto the appropriate selective plate for the plasmid; only those colonies containing the plasmid were examined further. In some cases, random spore analysis was done to increase the numbers of plasmid containing colonies that could be analyzed. (Again, only colonies containing the plasmid of interest were examined further.) In all crosses, \( mec1 \Delta \) strains were kept alive by the overexpression of either \( DUN1, TEL1, MEC1, RNR1, \) or \( RAD53 \); there were no \( sml1 \)-type mutations involved. Genotypes of meiotic segregants were followed by auxotrophic markers.

   In the case of \( pds1 \) crosses, \( pds1 \) appears in far less than half of the spores (probably due to sickness of \( pds1 \) cells); furthermore, suppression of \( mec1 \) by plasmid suppressors is imperfect, and so the number of double mutants are often very low.
18. Antibody staining for microtubules and other proteins

The following protocol is adapted after (114). Mid log cells are fixed 1.5 - 2 hours by adding 2.5 ml cell culture to 2.5 ml fresh 10% formaldehyde [made by using 5g/50ml paraformaldehyde, plus drops of 1M NaOH, pH to neutral with 1M HCL]. Cells are washed 3x [40mM KPO4, .5mM MgCl2 pH6.5] and resuspended in .5ml solution B [solution B is made by mixing 1M K2HPO4 with 1M KH2PO4 to obtain a solution at pH 7.5. This is diluted with H2O to 100mM, and sorbitol is added to 1.2M]. [DAPI can be added to a final concentration of .2ug/ml] About 200 ul cells are added to an equal volume of ZB [1ml solution B + 10ul zymolyase, 5mg/ml. When zymolyase digestion is complete (about 30 minutes, can be monitored by susceptibility of cell to lysis by detergent or squashing), cells are washed 2x in solution B, and resuspended in about 1ml solution B. In the meantime, 10-well immunofluorescence slides are prepared by placing 10ul/well .1% polylysine, which is aspirated after about 10 seconds, and are air dried. Slides are then washed by vigorous shaking in a 50 ml tube with water for 10 minutes. After air drying, cells are placed on the well and the fluid is aspirated, and the slide is again air dried, is immersed in MeOH for six minutes at -20°C, and acetone for 30 seconds at -20°C. The slides are again air dried, and 10% fetal calf serum in solution F [solution F is made by adding 10 mg KH2PO4 to 90 ml H2O and titrating to pH 7.4 with .1N KOH; then diluting to 100 ml with H2O, adding .85g NaCl, .1g BSA, and .1g NaN3] is added to the wells and incubated
45 minutes. The FCS is washed 3x with solution F, 10ul primary antibodies (diluted in solution F) are added, incubated 1-2 hours at RT, washed 10X in solution F, and the secondary antibodies, also diluted in solution F, are added. After 1-2 hours, the wells are washed in solution F 10X, a drop of mounting media [made by dissolving 100mg p-phenylenediamine in 10mlPBS, adjusting to pH9, adding 90ml glycerol with stirring; and adding DAPI to a final concentration of .2ug/ml] added and the coverslip is sealed with clear nail polish. Slides are stored at -20°C. Antibody concentrations depend on the antibody being used.

19. 40 min HU pulse experiments

WT and mec1-1sml1 bar1 cells (or mec1 sup1bar1 or dun1 bar1 cells) in YEPD were released from alpha factor arrest (by washing out the alpha factor 3x) into .2M HU, which was then washed out (2x) after 40 minutes. A sample of the cells was then plated for viability, and the cells were allowed to grow for another 150 minutes in 10 ug/ml nocodazole, which prevents entry into the next cell cycle. During this 150 minutes, samples were removed for FACS analysis (see #15 FACS). At the end of that time, cells were collected for pulse-field gel electrophoresis (see #20 Pulse field gels).

20. Pulse field gels

Cells were grown to saturation, washed, and resuspended in a .2ml solution of SCE (1M sorbitol, .1M Sodium citrate, and .06MEDTA, pH7), 100mM B-
mercaptoethanol and 100 units/ul zymolase. They were then mixed with 2% low melting agarose, poured into a plug mold, and incubated at 36°C for 24 hours. The plug was suspended in a solution of .5MEDTA, 10mM Tris, 1% N-lauroylsarcosinate, and .5mg/ml proteinase K, and incubated at 50°C for another 24 hours. Finally the plugs were washed several times with .05MEDTA, placed into a 1% agarose gel, and run according to the manufacturer's directions in order to separate the chromosomes.

21. G$_s$/S checkpoint function of MEC1 experiment

I deleted the RAD16 gene from the WT strain, then crossed the resulting strain to a mec1-1 strain to obtain a mec1-1rad16 strain as well. rad16 checkpoint proficient strains delay longer after damage because they repair the damage less efficiently. Cells were arrested in G, with alpha factor, then pelleted, resuspended in a low volume, plated, exposed to 40 J/m$^2$ UV (UV Stratalinker) washed off the plates, washed twice more to remove alpha factor, and resuspended in rich media (YEPD) and allowed to grow. At 15 minute intervals, aliquots were taken, and fixed overnight in 70% ethanol. These were prepared for FACS (see FACS in methods #15) and taken to be analyzed. At the plating stage, I tried to avoid getting over 2X10$^7$ cells per plate, as others had noted that more than this number of cells can result in the top cells shielding the bottom ones from UV. The viability of the cells were checked after the experiment; in one experiment, the mec1-1rad16 cells had no discernible viability; the WT cells had
a 28% viability; these numbers indicate that the cells received an appropriate UV dosage.

22. Quantitative βgal assays
1.5 to 2ml late log phase cells were harvested and pelleted in a microfuge tube. The cells were washed in 1 ml water, and then resuspended in 100 ul of Z buffer [600 mM Na2HPO4, 400 mM NaH2PO4, 100 mM KCl, 10mM MgSO4] on ice. About 150ul acid washed glass beads were added, and the tube was vortexed 5 minutes, and spun for 5 minutes, all at 4°C. In a disposable 1 ml cuvette, 50 ul supernatant was added to 1 ml buffer Z containing .7mg/ml ONPG [this was diluted from a 10x stock in NaP, pH 7], and gently mixed. Cuvettes were incubated at 30°C until the appearance of a slight yellow color; then the reaction was stopped by adding and mixing .3ml of 1M Na2CO3. The time and OD420 were recorded. Protein levels in the sample were determined by mixing 5 ul extract with 1ml Bradford reagent (BioRad), and OD595 was determined after 5-60 minutes. Units were calculated as follows:

units = \( \frac{1000 \times \text{OD420 per ml of extract}}{\text{(mg/protein/ml extract) \times \text{time (minutes)}}} \)

For the amounts of extract used (50 ul for the enzyme assay, and 5ul for the protein assay), units = \( \frac{(5291 \times \text{OD420})}{\text{(minutes \times \text{OD595})}} \)
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<td>MATα/MATα cdc15/cdc15 MEC1/mec1Δ::URA3 ura3/ura3 his3/+ his7/+ leu2/+ trp1/+</td>
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<td>MATα/MATα cdcn1/cdc1 cln2/cln2 MEC1/mec1Δ::URA3 ura3/ura3 bar1/+ his2/his2 ade1/ade1 leu2: GaiCLN1::LEU2/leu2 + pgalCLN1.</td>
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<td>MATα/MATα mec1+/Δ::URA3 dun1+/Δ::HIS3 leu2/leu2 HIS7/HIS7 ura3/ura3 trp1/trp1</td>
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<td>RGY51</td>
<td>MATα/MATα mec1+/Δ::LEU2 dun1+/Δ::HIS3 leu2/leu2 HIS7/HIS7 ura3/ura3 trp1/trp1</td>
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<td>MATa mec1Δ::URA3 trp1 leu2 his3 HIS7+ 2u LEU2 TEL1</td>
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<td>MATa ade2 ura3 his3 leu2 trp1 tel1::URA3</td>
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<td>T. Petes</td>
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<td>MATα/MATα mec1+/Δ::LEU2 ade2-1/ade2-1, trp1-1/trp1-1, can1-100/can1-100, leu2-3,112/leu2-3,112, his3-11,15/his3-11,15 ura3/ura3, GAL+GAL+, psi+/psi+, ssd1-d2/ssd1-d2 MAT? MEC+ sml1 ura3 trp1 leu? his7?</td>
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<td>MATa bar1 ade2 ade3 ura3 leu2 his7 MEC+ CDC+</td>
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<td>his3/his3 ura3/ura3 trp1/trp1</td>
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<td>his3 + pRNR1 (URA3 marked)</td>
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<td>RGY102</td>
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<td>RGY127</td>
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<td>RGY155</td>
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<td>RGY156</td>
<td>= DLY567 + pRS416</td>
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<td>RGY157</td>
<td>= DLY418 + pRS416</td>
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<td>RGY158</td>
<td>= RGY108 + pRS416</td>
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<td>RGY159</td>
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<td>RGY162</td>
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<td>RGY163</td>
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<td>RGY166</td>
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<td>MATα cdc13 cdc15 ade2 trp1 leu2 his3 ura3 + pTW128 (cenPDS1HA LEU2)</td>
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<td>RGY229</td>
<td>MATa bar1 mec1::HIS3 tel1A::URA3 ura3 his3 ade2 trp1 leu2</td>
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<td>MATa/MATα dun1::HIS3+/rad53::LEU2/+ ura3/ura3 his3/ade2 ade2 trp1/trp1 leu2/leu2 + 2uTRP1RNR1</td>
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<td>MATa =DLY258 + pRNR1</td>
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<td>RGY246</td>
<td>MATα pds1::LEU2 rad53::TRP1 ura3 leu2 trp1 his3 ade2 cdc15 + p2uURA3RNR1</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>RGY247</td>
<td>MATα dun1::HIS3 rad53::TRP1 ura3 leu2 trp1 his3 ade2 + p2uURA3RNR1</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>RGY248</td>
<td>MATα dun1::HIS3 pds1::LEU2 rad53::TRP1 ura3 leu2 trp1 his3 ade2 cdc15 + p2uURA3RNR1</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>Strain</td>
<td>Genotype</td>
<td>Background</td>
<td>Source</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------------------------------------</td>
<td>------------</td>
<td>--------</td>
</tr>
<tr>
<td>RGY249</td>
<td>MATa/MATα dun1::HIS3+/rad53::LEU2+/ura3/ura3 his3/his3 ade2/ade2 trp1/trp1 leu2/leu2 + p2uTRP1 vector</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>DLY62</td>
<td>MATa, bar1::hisg ade2-1 trp1-1 can1-100, leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>DLY65</td>
<td>MATa, mec1 ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL+, psi+, ssd1-d2</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>DLY258</td>
<td>MATa, bar1::hisg, mec1-1 ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>DLY259</td>
<td>MATa, bar1::hisg, rad53-11 ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>DLY394</td>
<td>MATa cdc13-1 cdc15-2 ura3 trp1 his3 leu2</td>
<td>A364a</td>
<td></td>
</tr>
<tr>
<td>DLY408</td>
<td>MATa, bar1::HISG cdc13-1 cdc15-2 ade2-1 trp1-1, can1-100, leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>DLY409</td>
<td>MATa, bar1::HISG cdc13-1 cdc15-2 rad9::HIS3 ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2</td>
<td>W303</td>
<td></td>
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<tr>
<td>DLY418</td>
<td>MATa bar1::HISG cdc15-2 ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>DLY498</td>
<td>MATa, bar1::HISG cdc13-1 cdc15-2 ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>DLY554</td>
<td>MATa, bar1::hisg, rad53-11, cdc13-1 cdc15-2 ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL+, psi+, ssd1-d2</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>DLY557</td>
<td>MATa, bar1::hisg, mec1-1 cdc13-1 cdc15-2 ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>Strain</td>
<td>Genotype</td>
<td>Background</td>
<td>Source</td>
</tr>
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<td>--------</td>
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<tr>
<td>DLY677</td>
<td>MATα, bar1::HISG cdc13-1 cdc15-2 rad9::HIS3 pds1::LEU2 ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL+, psi+, ssd1-d2</td>
<td>W303</td>
<td>Lisa Hartwell Sandell lab</td>
</tr>
<tr>
<td>20-13-3</td>
<td>MATΔ ade2 ade3:GalHO leu2 his3 trp1 can1 chy2r ura3 lys5 cdc13-1 CDC5</td>
<td>Lisa Hartwell Sandell lab</td>
<td></td>
</tr>
<tr>
<td>11351-8</td>
<td>MATΔ ade2 ade3:GalHO leu2 his3 trp1 can1 chy2r ura3 lys5 cdc13-1 Cdc5-ad</td>
<td>A364a Koshland lab</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>MATa bar1 pds1::LEU2 ura3 trp1</td>
<td>TWY120</td>
<td></td>
</tr>
<tr>
<td>971-1A</td>
<td>MATa bar1 pds1::LEU2 ura3 trp1</td>
<td>A364a T. Formosa</td>
<td></td>
</tr>
<tr>
<td>TWY120</td>
<td>MATα can1 ura3 sap3 CAN1:hxk2/+ ly2/+ +/-aro2 cyh2/+ trp5/+ leu1/+ ade6/+ +/-ade3 (chromosome 7 disome)</td>
<td>A364a</td>
<td></td>
</tr>
<tr>
<td>TWY177</td>
<td>MATa mec1-1 sml1 leu2 trp1 his3 ura3</td>
<td>AAY101 4</td>
<td></td>
</tr>
<tr>
<td>TWY185</td>
<td>MATa ura3 leu2 his3 lys2 rad54LEU2 GALHO::URA3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TWY308</td>
<td>MATa mec1-1 ura3 trp1</td>
<td>A364a</td>
<td></td>
</tr>
<tr>
<td>TWY359</td>
<td>MATa cdc13-1 ura3 his3 his7 leu2 trp1</td>
<td>A364a</td>
<td></td>
</tr>
<tr>
<td>TWY432</td>
<td>MATa cdc13-1 mec1-1 ura3 his3 his7 leu2</td>
<td>A364a</td>
<td></td>
</tr>
<tr>
<td>TY54</td>
<td>MATa GALHO::URA3 rad54::LEU2 ura3 his2 ade2 trp1</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>CPY189</td>
<td>MATa bar1 cdc13 cdc15 pds1::LEU2</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>CPY201</td>
<td>MATa CDC13+ cdc15 pds1::LEU2 ura3 leu2 his3 trp1 ade2</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>CPY207</td>
<td>MATa CDC13+ cdc15 pds1::LEU2 rad9::HIS3 ura3 leu2 his3 ade2 trp1</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>CPY215</td>
<td>MATa bar1 cdc13 cdc15 pds1::LEU2 dun1::HIS3 ura3 leu2 his3 trp1 ade2</td>
<td>W303</td>
<td></td>
</tr>
<tr>
<td>CPY238</td>
<td>MATa bar1 cdc13 cdc15 pds1::LEU2 rad53-11 ura3 leu2 his3 trp1 ade2</td>
<td>W303</td>
<td></td>
</tr>
</tbody>
</table>
mec1 deletion plasmid

pRS 416 backbone

Sac2
ATG
BamH

63  784  882

Figure 7-1. Map of the mec1Δ2 plasmid. Cutting at Xba linearizes the plasmid for integration into the yeast genome.
APPENDIX A: A MODEL FOR THE ESSENTIAL FUNCTION PATHWAY

Figure A-1 presents a general model of the essential function based on several of my observations, as well as a recent finding by R. Rothstein that Sml1p and Rnr1p physically interact (121).

GeneA

Figure A-1. Model of the essential function pathways of MEC1 and RAD53. Essential genes are shown in bold. Major pathways are shown in bold, and minor pathways, including those used when genes are overexpressed, are shown as thin lines. Arrows do not necessarily indicate direct protein-protein interactions; intermediate proteins may be involved. Recent data suggests Rnr1p is regulated post-translationally; proteins x and y may be regulated at any level (including transcriptionally).

Features of the model are as follows:

1) The essential function is to promote the transcription and/or activity of RNR1 and certain other genes or gene products (gene x and gene y. Could one of these be Dbf4? [R. Sclafani, unpublished]).
2) Genes x and y are important to the cell, and their absence makes the cell sick, but they are not absolutely essential. Alternatively, they may be essential but are transcribed constitutively at a low level without checkpoint genes.

3) RNR1 and genes x and y may be important for DNA replication or for another process such as oxidative stress response.

4) The levels of activity of Rnr1p, and proteins x and y are important to the cell; a low level of gene product may result in viable but sick cells. Activity levels can be regulated at the transcription or post-translational levels. mecl and rad53 mutants are inviable because the activity levels of Rnr1p, x, and y are lowered below a threshold. Suppressors of mecl and rad53 act in various ways to increase these levels. Different combinations of mutations and suppressors may give the same results if they produce the same levels of Rnr1p, x and y activity.

5) Either Rad53p or overexpressed Dun1p can increase activity of Rnr1p, and the activity or transcription of x, and y, but normally the activation is mainly done by RAD53. Sml1 inhibits the action of Rnr1 unless Rnr1p is activated by Rad53p or Dun1p, which is the normal case.
6) In *sml1* cells, Rnr1, x and y can act constitutively at a basal level, even in the absence of upstream genes. This basal level barely ensures viability.

7) *cln1,2* suppresses *mec1* and *rad53* cells by slowing down the early part of the cycle, allowing more time for levels of essential proteins to reach a necessary threshold. (Alternatively, in the absence of *CLN1* and *CLN2*, another G1 cyclin dimerizes with Cdc28, giving it the specificity to promote activation of Rnr1, and genes x and y.)

8) Dun1 and Rad53 operate together, perhaps even activating each other.

9) After DNA damage, Mec1, Dun1, and Rad53 cooperate in activating an additional transcription factor that specifically expresses genes upregulated after damage. (Perhaps the signal from damaged DNA causes these proteins to change their substrate specificity.) This also applies to *TEL1*, which, when overexpressed, restores damage inducibility, but not constitutive levels, of *RNR3* overexpression (Chapter 4).
This model is consistent with the following findings:

Table A-1. Explanation of essential function model.

<table>
<thead>
<tr>
<th>Finding</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>suppression of mec1Δ</td>
<td>1) In the absence of MEC1, overexpression of RAD53 or DUN1 can suppress the essential function (99, 127) (and confirmed by me in Chapter 5).</td>
</tr>
<tr>
<td></td>
<td>2) In the absence of MEC1, TEL1 (acting through DUN1 and RAD53, Chapter 4) overexpression can suppress the essential function (94).</td>
</tr>
<tr>
<td></td>
<td>3) mec1-1sml1 cells grow well.</td>
</tr>
</tbody>
</table>

sml1 can suppress a mec1 mutant by removing a block to Rnr1's (and protein x and y's) activity. This basal level of activity can barely support viability, but a small added level of input from RAD53 (activated constitutively at low levels by DUN1 and TEL1 even in the absence of MEC1) can cause enough activity for good growth seen in mec1-1sml1 cells.
4) *mec1* and *rad53* cells can be suppressed by *RNR1* overexpression (24, 127) (and confirmed by me in Chapters 4,5). Suppression doesn’t result in WT growth, due to lack of expression of genes x and y, or to lack of complete activation of the Rnr1 protein.

5) *mec1 tel1 sml1* cells are alive but sick (94) (and confirmed by me in Chapter 4). Rnr1p now has only the basal activity allowed by the *sml1* mutation. (Rad53 and Dun1 are no longer activated by Mec1 and Tel1, and thus cannot efficiently activate Rrn1.)

6) p*RNR1* suppresses a *mec1tel1* strain extremely poorly (Chapters 4,5). Although *RNR1* is expressed, it lacks activation from upstream pathways, and its activity may be inhibited by Sml1. Furthermore, activity and/or transcription of genes x and y are lacking.

7) *RAD53* overexpression can restore viability to *mec1tel1* cells Chapter 4), although they are somewhat sick; Dun1 overexpression cannot. Rad53 could suppress better than Dun1 because it is more involved in the essential function, but it could also be due to the high GAL expression levels, whereas *DUN1* was only overexpressed by a high copy plasmid. Also, p*DUN1* might not suppress because normally Dun1 is constitutively modified by *TEL1*, and *DUN1* overexpression cannot make up for a lack of such modification.
<table>
<thead>
<tr>
<th>Finding</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>suppression of rad53Δ</strong></td>
<td>8) <em>sml1</em> suppresses <em>rad53</em> cells but poorly (Chapter 5).</td>
</tr>
<tr>
<td></td>
<td><em>sml1</em> can suppress a <em>rad53</em> mutant by removing the block to Rnl1’s activity; however, without <em>RAD53</em>, the cells grow more poorly. Endogenous <em>DUN1</em> isn’t enough for good growth.</td>
</tr>
<tr>
<td></td>
<td>9) <em>rad53Δtel1</em> cells can be suppressed by an <em>sml1</em> mutation (Chapter 5).</td>
</tr>
<tr>
<td></td>
<td><em>sml1</em> can suppress a <em>rad53</em> mutant by removing the block to Rnl1’s activity; however, without <em>RAD53</em>, the cells grow more poorly. Endogenous <em>DUN1</em> isn’t enough for good growth.</td>
</tr>
<tr>
<td></td>
<td>10) <em>MEC1</em> overexpression cannot suppress <em>rad53Δ</em> (Chapter 5).</td>
</tr>
<tr>
<td></td>
<td>The model predicts that this is because Mec1 acts upstream of Rad53.</td>
</tr>
<tr>
<td></td>
<td>11) <em>rad53Δ</em> cells can be suppressed by <em>pDUN1</em> (Chapter 5).</td>
</tr>
<tr>
<td></td>
<td>They share functions.</td>
</tr>
<tr>
<td></td>
<td>12) <em>rad53Δ</em> cells can be suppressed by <em>pTEL1</em>. This requires <em>DUN1</em> (Chapter 5).</td>
</tr>
<tr>
<td></td>
<td>Although <em>pTEL1</em> requires both Rad53 and Dun1 to suppress the essential function in <em>mec1</em> cells, in this situation the presence of Mec1 may allow <em>pTEL1</em> to restore viability.</td>
</tr>
<tr>
<td><strong>suppression of rad53-11 dun1Δ or rad53Δdun1</strong></td>
<td>13) The <em>rad53-11</em> and <em>dun1Δ</em> alleles are synthetically lethal (Chapter 3).</td>
</tr>
<tr>
<td></td>
<td>As the model shows, both Rad53 and Dun1 can activate Rnl1 et al. Dun1 may act both on Rnl1 directly (at least when overexpressed) and through <em>RAD53</em>. The <em>rad53-11</em> protein may have lowered activity, and without Dun1 activity, the <em>rad53-11</em> protein has no activity.</td>
</tr>
<tr>
<td>finding</td>
<td>explanation</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>14) rad53-11 <em>dun1Δ</em> cells or rad53Δ <em>dun1</em> cells can be suppressed by <em>RNR1</em> overexpression (Chapter 3).</td>
<td>However, these cells do not grow as well as WT cells because gene X and gene Y are also limiting, and because Rnr1p activity is blocked by Sm1.</td>
</tr>
<tr>
<td>15) rad53<em>dun1</em> cells can be suppressed by <em>sml1</em>, but growth is even more poor than that of <em>rad53sml1</em> cells Chapter 5).</td>
<td><em>sml1</em> can suppress a <em>rad53</em> mutant by removing the block to Rnr1’s activity; however, without <em>RAD53</em>, the cells grow more poorly, and even worse without <em>DUN1</em>. The reason why <em>rad53dun1sml1</em> cells grow better than <em>mec1tel1sml1</em> cells is not known.</td>
</tr>
</tbody>
</table>
APPENDIX B: OTHER PROJECTS UNDERTAKEN

During my graduate studies I undertook several additional projects besides those reported in the previous chapters. In this Appendix I present the rationales, experiments, and results, or lack thereof, for some of these projects. Although in general these projects didn't produce very useful results, they are reported here because they concern important questions about MEC1 function, and ways in which they were addressed.

IS Mec1 a PROTEIN OR PI KINASE, AND IF SO, WHICH TYPE(S)?

Introduction
PI kinases are classified as to which position of the inositol ring they phosphorylate — for example, PI-3 kinases and PI-4 kinases phosphorylate on the positions 3 and 4, respectively. Many proteins with the PI-kinase motif have been shown to have PI kinase activity, while it seems that other proteins with the same motif may not have PI kinase activity (see Chapter 1 for references). Since Mec1 has a putative phosphatidylinositol (PI)/protein kinase domain, I attempted in collaboration with others to show that Mec1p was a PI/protein kinase, and to discover which type of PI kinase.
By knowing which type of PI kinase *MEC1* encodes, biochemical model(s) for how Mec1 functions can be created. On the other hand, since different phosphorylated PIs behave differently and act in different biochemical pathways, if it isn't known what kind of kinase Mec1p is, or if Mec1p even is a PI kinase, developing models is much more speculative and potentially time wasting.

Any protein or PI kinase activity found in Mec1p could be useful for epistasis experiments to order *MEC1* relative to other checkpoint genes. For instance, if Mec1p is upstream on a checkpoint pathway, its kinase activity should be turned on after DNA damage occurs, regardless of the status of putative downstream proteins (such as Rad53). Also, a biochemical assay for Mec1 activity would allow in vitro tests, in which various DNA substrates (double stranded, single stranded, nicked, gapped, etc.) could be assayed for their ability to activate Mec1. This would help us understand the nature of the signal that activates Mec1 in vivo. And lastly, once Mec1's kinase activity is identified and assayable, it would be easier to identify Mec1's substrate(s).

Many scientists now doubt that Mec1 is a PI kinase (see, for example, 59). Although there is no evidence to show this, their doubts are based on 1) a lack of data that Mec1 is a PI kinase, and 2) failed attempts at finding PI kinase activity in DNA-PK, the human DNA-activated protein kinase which also shares a PI kinase motif. Showing that Mec1 is indeed a PI kinase would put these arguments to rest; and repeated unsuccessful attempts to show PI kinase
activity, while not disproving anything, would still support the argument that Mec1 is not a PI kinase.

**Results**

In order to test Mec1p for kinase activity, my first step was to obtain Mec1 protein; the second step (mostly done by collaborators in other laboratories) was to use the Mec1 protein in assays for kinase activity. PI kinases are identified by production of specific phosphorylated PI
tides, which are distinguished by how far they run on a Thin Layer Chromatography (TLC) plate, versus known standards. Protein kinases are identified by the production of phosphorylated proteins (substrates tested were casein, histone, and raytide), which run more slowly in acrylamide gels than their non phosphorylated counterparts.

To obtain Mec1 protein (step one), I showed that Mec1p can be immunoprecipitated (see methods #9, page 216). I used a GAL LexA Mec1 fusion protein (made by D. Lydall) to overexpress Mec1, and LexA antibodies (from J. Little) to immunoprecipitate it. I showed that the full size protein was present by Western blot; I also showed by Western blot that total cell extracts of cells containing LexAMEC1 and LexAmec1N2229K contained roughly similar amounts of protein of the same size — suggesting that the lack of complementation of the NK allele is not due to simple degradation (see Figure 2-2).

Collaboration was done with two other laboratories to assay for kinase
activity. On two occasions, I delivered Mec1 protein that I had prepared to the Powis lab at the University of Arizona Cancer Center. Personal in this laboratory tested for PI-3 and protein kinase activity, but did not find activity on either occasion. However, the PI-3 kinase test conditions used were disfavorable to other types of PI kinases — thus Powis lab personal were prevented from finding any PI-4 or PI-5 kinase activity, for example.

I also sent Mec1 protein, yeast cells, LexA antibody, and immunoprecipitation protocols to the Cantley Lab at Princeton. Cantley lab personal, in particular K. Wong, performed several assays, and only once obtained an (unrepeatable) result: possible PI-5 kinase activity. Since the Cantley lab personnel determined that larger amounts of protein were needed for their assays, I developed a scaled-up protocol for the glass bead method of protein extraction, but this still gave no positive results. Eventually I was too busy with more promising avenues of research to continue on this project, and our collaborators were too.

Conclusion
Any result in regards to Mec1's biochemical activity, including PI and protein kinase activity, is still conceivable. It still may be that MEC1 encodes a PI-5 kinase based on the one TLC result from the Cantley lab, as well as on sequence alignments with other PI kinases (15). However, it may be that no PI kinase activity was found because Mec1p is not a PI kinase in spite of its sequence, a
view that has widespread support.

**IS PIK1 A CHECKPOINT GENE?**

**Introduction**

Based on the idea that Mec1p was a PI kinase, as well as a checkpoint protein, I wondered if other PI kinases have checkpoint activity. In particular, if \( MEC1 \) encoded a PI-5 kinase, as preliminary data suggested (see above), its substrate likely would be PI-4-P. This molecule is produced in yeast by phosphorylating PI on the 4 position, which is done by a gene product called Pik1p(36, 39). I asked two questions about \( PIK1 \):

1) Is it a checkpoint gene, and
2) Based on the similarity of its kinase domain with \( MEC1 \)'s, are alleles of the two genes synthetically lethal?

**Results**

We obtained from Dr. Mike Hall three strains with similar phenotypes, but each containing different temperature sensitive alleles of \( pik1 \) (39). (The strains were \( pik1 \) nulls containing a plasmid borne copy of \( pik1^{ts} \). Temperature sensitive alleles were used in place of null alleles since \( PIKI \) is essential.) I was able to verify that the \( pik1^{ts} \) cells behaved as \( pik1 \) null cells after 3 and 7 hours at the \( pik1^{ts} \) restrictive temperature, by assaying the cytokinesis defect that occurs when Pik1p becomes inactivated (39). (There were about 40% large budded cells containing 2 nuclei at 37°C verses 20% or fewer of these cells at 23°C or in
the WT control at either temperature. Furthermore, cell division, as measured by counting the \textit{pik1}ts cells on a hemacytometer, ceased by 3 hours at 37°C, but continued normally at 23°C or in a \textit{PIK1}+ background at 37°C.)

In order to assay the G2/M checkpoint activity of the \textit{pik1} strains, I followed the progression of the cells through the checkpoint after \textit{cdc13} damage. This experiment necessitated the creation of a \textit{cdc13pik1} double mutant. (This mutant was created by a genetic cross. Although it was somewhat difficult to distinguish the double mutant because both single mutant and double mutant strains were temperature sensitive, it was possible to find the double mutant in a non-parental ditype tetrad, in which two meiotic products were not temperature sensitive [and therefore inferred to be WT] and the other two meiotic products were temperature sensitive [and therefore inferred to be double \textit{cdc13pik1} mutants]. It was also helpful that the \textit{pik1} alleles were marked auxotrophically: a \textit{pik1ADE+} allele that showed a \textit{cdc13}-like arrest was in fact \textit{cdc13} if the other \textit{pik1} segregant from the same tetrad did not show \textit{cdc13}-like arrest and exactly one of the two Pik* segregants showed \textit{cdc13} arrest.)

If the \textit{pik1} cells had been checkpoint defective, they would not have maintained \textit{cdc13} arrest. But after 10 hours at \textit{cdc13}’s restrictive temperature, the \textit{pik1cdc13} cells remained arrested, while the \textit{pik1Cdc}− cells continued to cycle. Due to their temperature sensitivity, the \textit{pik1Cdc}− cells did not cycle very much as compared to WT cells (an average of 8 buds were produced by the \textit{pik1}}
cells, vs. 20 in the WT cells). It was clear, however, that they cycled beyond the cdc13 arrest point (which arrests cells on agar plates at the two or four budded stage, depending on if the cells were one or two budded when plated.)

To ask if alleles of MEC1 and PIK1 were synthetically lethal, I made double pik1ts mec1ts mutants. Growth was compared at 23°C, 30°C, and 33°C for the mec1ts, pik1, and the double mutant cells. These are intermediate temperatures for both the mec1ts and pik1ts alleles, where growth should still occur but the cells are somewhat sick. If the genes are synthetically lethal, the permissive temperature of the double mutant may be lowered. Of my two double mutants strains (independent meiotic sergeants with identical genotypes), one grew well, at these temperatures and one did not, but growth of the mec1ts controls was equally ambiguous.

Conclusion

PIK1 by the cdc13 assay is not a G2/M checkpoint gene. This does not rule out, however, other checkpoint functions for PIK1.

While the results of the synthetic lethal test were somewhat ambiguous, there was no obvious synthetic lethality between the two genes.

Mec1 LOCALIZATION ATTEMPTS

Introduction

Based on MEC1’s checkpoint and DNA damage-inducible transcription
functions, it seemed likely that Mec1 protein would be found in the nucleus. However, the presence of its lipid kinase domain suggested that it might localize to a membrane of the cell, where PI is found. Since PI has been reported to be present in the nucleus (27), localization to the nuclear membrane seemed most likely. One interesting possibility is that after DNA damage, Mec1’s localization may change — from the nucleus generally to the nuclear membrane, for example. Although I had strong predictions about nuclear localization, the slight possibility of cytoplasmic or some other localization would be important to know about as it would cause us to rethink how MEC1 functions.

The most common way to localize a protein in the cell is by fluorescence. I tried two methods: immunofluorescence, and GFP fluorescence.

Localization by Immunofluorescence
I first attempted to localize Mec1p by immunofluorescence. The immunofluorescence technique works by targeting the protein of interest with a specific antibody in fixed cells. A secondary antibody which is conjugated to a fluorescent molecule such as FITC, is then added. The secondary antibody binds to the primary antibody. Thus the fluorescent label is present at the locations of the protein of interest. Under a fluorescent microscope these locations can be identified.

Since no antibodies to Mec1p were available, I used a GAL LexAMec1 fusion (made by David Lydall, Weinert lab). I had previously shown that this GAL
LexAMec1 fusion complements mec1's essential function, and that it is recognized by LexA antibodies (provided by the J. Little Lab) on a Western blot. It is important to note that the presence of the LexA epitope has been reported to not contain known localization sequences, so that I expected it not to alter Mec1's localization (43, 141).

I carried out the antibody staining procedure I had successfully performed before and since for microtubule staining (see methods #18, page 223). With the primary LexA antibody (produced in a rabbit) and the anti-rabbit FITC conjugated secondary antibody, I should have seen a green glow under the fluorescence microscope wherever Mec1 protein was. However, there were several technical problems. First, a signal was present in the negative control cells: Cells grown in dextrose or raffinose, in which the GAL LexA Mec1 should not be produced, glowed all over, as well as cells with no GAL LexA Mec1 or with no LexA antibody added. In subsequent attempts, the neck of the budded cells fluoresced, also nonspecifically.

I considered that these problems may be due to impure antibody, and I therefore attempted to purify the LexA antibody. I used a protocol in which crude antibody is passed over a sheet of nitrocellulose to which is fixed LexA protein. LexA antibody should bind to the nitrocellulose while any contaminating antibody should not bind. The LexA antibody is then eluted off. Therefore, the purification procedure produces two fractions: purified LexA antibody, and crude antibody
without the LexA antibody. Upon retesting, the original crude antibody and the crude minus LexA fraction continued to give the same nonspecific fluorescence at the neck of the bud. However, the pure antibody produced no fluorescence even though Western blotting showed that my purified antibody still bound to LexA well.

I also obtained from the J. Little lab some column-purified LexA antibody. While the cells stained well for microtubules, (a control showing that the cells themselves were good), the results with the column-purified LexA antibody were ambiguous. The control cells (no LexA antibody or no epitope) did not fluoresce, while in about 10% of the experimental cells, the entire cell was stained except for the nucleus. However, I didn't consider this a strong enough result to be trusted.

At this point, I switched to another epitope. I had made an HA-Mec1 fusion; however, it couldn't be recognized by HA antibody on a Western blot. (The HA antibody was good, based on a Western blot control.) Nevertheless I attempted immunofluorescence with this HA-Mec1 fusion, since it complemented the mec1 phenotypes. However, with or without the HA Mec1, the cells glowed all over, nonspecifically.

Localization using GFP

GFP (Green Fluorescent Protein) is derived from a jellyfish and glows green in response to UV light. Since its recent discovery as a useful tool for localization,
(18) it has become commonplace to create fusion proteins of GFP and a protein being studied. This allows localization of the protein in live cells. No fixation is needed. It also eliminates the need for antibodies and incubation time, as well as the need to permeabilize cells.

I obtained the GFP gene on a plasmid, and amplified it using PCR, cloned it into a vector in order to amplify it further, and created a GAL-GFP-\textit{MEC1} inframe fusion. This was transformed into yeast cells. It took two attempts to get a GAL-GFP-\textit{MEC1} construct that complemented \textit{mec1}. When yeast cells were transformed with GAL-GFP alone (no \textit{MEC1}), grown in galactose to induce GFP, and observed under the fluorescence microscope, the whole cell glowed, as expected. However, the GAL-GFP-\textit{MEC1} construct under the same conditions gave no signal.

\textbf{Conclusions}

While it would have been informative to confirm the intracellular location of Mec1, I was unable to do this using multiple approaches coupled with many controls and much time spent troubleshooting. Work is now underway by others in our laboratory to use a GSTMec1 fusion, along with appropriate primary and secondary antibodies, to localize Mec1p in meiotic cells, using a somewhat different protocol than the one I used.
IS *MEC1* A G\textsubscript{s}/S CHECKPOINT GENE?

**Introduction**

So far all budding yeast checkpoint genes that have G\textsubscript{2}/M checkpoint function that have been tested for the G\textsubscript{s}/S checkpoint function have this function (3, 79, 81, 138-140). Although in mammalian cells, the G\textsubscript{s}/S checkpoint is the predominant arrest point after DNA damage, yeast cells pause there only slightly, and arrest much longer at the G\textsubscript{2}/M checkpoint. This short pause is one reason it is difficult to measure G\textsubscript{s}/S checkpoint delay in yeast. Another reason is that, unlike the G\textsubscript{2}/M checkpoint which is easy to assay by simply looking at nuclear staining, the G\textsubscript{s}/S checkpoint has no cytological markers. Instead, one must analyze G\textsubscript{s} synchronized cells that have then been exposed to DNA damage (such as UV) and determine the rate of initiation of DNA replication. DNA replication is measured by FACS (Fluorescence Activated Cell Sorting). *MEC1* was known to have a G\textsubscript{2}/M checkpoint function, and I attempted to find out if it also had a G\textsubscript{s}/S checkpoint function. I predicted that while WT cells exposed to DNA damage would delay at the G\textsubscript{s}/S boundary, *mec1-1* cells would not.

**Results**

To ask if Mec1 had G\textsubscript{s}/S checkpoint activity, I attempted to compare two strains (WT and *mec1-1*), each with and without UV damage. The detailed protocols are found in the methods section (#21, page 225). Following is an
overview of the experimental protocols: I constructed rad16 versions of each strain because without the UV repair function provided by RAD16, cells delay longer after UV and thus the delay is easier to measure (70). Cells were arrested in G1 (using alpha factor), exposed to UV, released from G1 arrest by removal of alpha factor, and cell aliquots were taken at various times, before preparing the cells for FACS. The first attempt failed because the cells (even the irradiated ones) began cycling prematurely during the 45 minutes it took to remove the alpha factor. (This indicated that any G1/S delay must be shorter than 45 minutes.) To avoid this problem, in subsequent experiments I UV irradiated and removed alpha factor in one strain at a time, thus allowing measurement of shorter G1/S delays. As predicted, I was unable to see a delay for mec1-1 cells. However, WT cells, which should have delayed, did not. To make sure that the cells were in fact being exposed to UV, I measured their viability. As expected, it was somewhat lower for irradiated WT cells than for unirradiated cells, and was very low for irradiated mec1 cells. To further confirm that our UV source was working correctly, I performed kill curve experiments using our UV source and compared the results to experiments done using another UV source that was thought to work well, and found that our source worked correctly. These results indicated that the reason cells failed to delay was not due to poor UV exposure.

Another reason I may not have seen G1/S delay in WT cells in response to UV is that only cells of certain strain backgrounds may have the G1/S checkpoint,
and our A364A strain was not one of them. Therefore, I obtained from W. Siede the SX46A strains which he had used in his G,S checkpoint assays in his published papers, as well as his experimental protocol, which differed slightly from mine. However, he informed me that he had already shown that \textit{MEC1} was a G,\textquoteright\textquoteright S checkpoint gene, although it was not yet published. (It has since been published: (138).) Therefore, I discontinued the project.

**Conclusion**

Although I did not repeat the experiment using W. Siede's strains and methods, it still may be useful in future studies of the G,S checkpoint in yeast to realize that an appropriate strain background may be required. If different strain backgrounds do indeed behave differently with regards to this checkpoint, this information may be of some use in learning about the genetic control of this checkpoint.

**EXAMINATION OF OTHER ALLELES OF \textit{MEC1} FOR SEPARATION OF FUNCTION**

**Introduction**

\textit{MEC1} has several functions: an essential function, several checkpoint functions, damage-inducible transcription, and perhaps others. Are these functions genetically separable? Can we find or obtain alleles of \textit{MEC1} that are proficient in some functions but deficient in others? If so, this may indicate that
the functions are separable. However, this may also simply indicate that two functions may require the same *MEC1* biochemical activity, but one function requires more activity than the other. For example, an allele may be proficient in the \(G_2/M\) checkpoint response, but deficient in the transcriptional induction response because the transcriptional induction response may require more activity. The allele may be a simple hypomorph, with enough activity to perform one function but not another. The existence of two alleles, each proficient in the function the other is deficient for, would argue that the two functions are indeed separable. For example, the existence of both a \(G_2/M\) checkpoint proficient, transcriptional induction deficient allele, and a \(G_2/M\) checkpoint deficient, transcriptional induction proficient allele would rule out the hypothesis that two functions only require different levels of activity.

**Strategy**

In order to find alleles proficient in some function(s) but deficient in other(s) (e.g. \(G_2/M\) checkpoint proficient but S/M checkpoint deficient), I examined a collection of twelve *mec1* alleles that resulted from the original screen in which *MEC1* was first identified (159).

**Results**

I examined three *MEC1* responses in twelve *MEC1* alleles: the S/M checkpoint, the \(G_2/M\) checkpoint, and damage-inducible transcription. Qualitative
measures of HU and MMS sensitivity on agar plates (scored as not sensitive, sensitive, or intermediately sensitive) of these alleles had been published previously (159). (HU and MMS sensitivities often correlate with defects in the S/M and G₂/M checkpoints, respectively.) To obtain a quantitative measure of HU or MMS sensitivity in these alleles, I measured the percent viability of cells after 4 hours of growth in media containing HU or MMS. I also quantitated damage-inducible transcription using a quantitative LacZ assay after MMS damage (see methods #22, page 226). After multiple experimental trials, trends were clear but there was often substantial variation, especially in the transcription data. I was able to divide the alleles into classes. In general, alleles that were hypomorphic for one function were hypomorphic for all three. However, there were some that were more MMS sensitive than HU sensitive.

The original mec7-containing strains were all *cdc13* (because the *mec1* alleles were originally identified on the basis of sensitivity to *cdc13* damage). Since each strain was temperature sensitive due to the *cdc13* mutation, in order to ask if the *mec1* alleles had temperature sensitive defects, I obtained *CDC13*⁺ versions of several of the respective strains by crossing them to a *CDC13*⁺ strain. I directly examined the S/M and G₂/M checkpoint proficienties of several of the strains, by measuring S phase delay after HU treatment (in nuclear-stained cells) and delay after UV irradiation, respectively. Table A-2 shows the classes of alleles and questions that I asked about each class, and some experiments that
could be done.
<table>
<thead>
<tr>
<th>Class</th>
<th>Alleles</th>
<th>Criteria</th>
<th>Hypothesis/question</th>
<th>HU viab&lt;sup&gt;a&lt;/sup&gt;</th>
<th>S /M chkpt&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MMS viab&lt;sup&gt;g&lt;/sup&gt;</th>
<th>G&lt;sub&gt;j&lt;/sub&gt;/M UV chkpt</th>
<th>tx&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>WT</td>
<td>proficient in all functions</td>
<td></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>II</td>
<td>1-1</td>
<td>very low proficiency for all functions except essential function</td>
<td>&lt;1 n.d. 20</td>
<td>-</td>
<td>7</td>
<td>- n.d.</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>1-6</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1-4</td>
<td>very poor growth under normal conditions; phenotypes not tested further</td>
<td></td>
<td></td>
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<tr>
<td>IV</td>
<td>1-12</td>
<td>- slightly MMS sensitive</td>
<td>85 n.d. 52 n.d.</td>
<td>+/-</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>V</td>
<td>1-2</td>
<td>- UV, MMS, and HU resistant</td>
<td>Are these G&lt;sub&gt;j&lt;/sub&gt;/M checkpoint&lt;sup&gt;+&lt;/sup&gt; for UV/Xray damage and checkpoint&lt;sup&gt;-&lt;/sup&gt; for cdc13? Do they show damage-specific responses?</td>
<td>90 87 91 n.d. 83 n.d.</td>
<td>+ + + + n.d. + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-5</td>
<td>-lacks G&lt;sub&gt;j&lt;/sub&gt;/M checkpoint after cdc13 damage</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>1-9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>1-11</td>
<td>- HU/MMS sensitive on plates</td>
<td>Does this allele separate HU (and to a lesser extent) MMS viability from checkpoint status? Why is it sensitive to HU/MMS on plates but not 4 hr liquid exposure?</td>
<td>78 - 51 - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- only slightly HU and MMS sensitive during 4 hr exposure</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>- S/M and G&lt;sub&gt;j&lt;/sub&gt;/M checkpoint deficient</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>1-7</td>
<td>- MMS sensitive (+/-)</td>
<td>Do these alleles separate S/M and G&lt;sub&gt;j&lt;/sub&gt;/M checkpoints? (Based on MMS vs. HU sensitivity)</td>
<td>75 76 n.d. 47 n.d.</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-8</td>
<td>- HU resistant (+/-)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+/</td>
</tr>
</tbody>
</table>

<sup>a</sup>, numbers for HU and MMS viability are average percent viabilities for several experiments, in which viabilities were measured after 4 hours in liquid media containing MMS or HU. n.d., not done

<sup>b</sup>, S/M or G<sub>j</sub>/M arrest was measured scoring the % medial nuclear division of DAPI stained cells (synchronized in G<sub>j</sub> in the case of the G<sub>j</sub>/M checkpoint assay) exposed to HU or UV, respectively. n.d., not done; +, proficient; -, deficient; +/-, somewhat proficient

<sup>c</sup>, tx, damage-inducible transcription of RNR3 after MMS damage, measured by LacZ assay (see methods #22, page 226). Actual measurements from various experiments are not comparable, but general trends are summarized by +, +/-, or -.

Bold type alleles indicate I made a Cdc<sup>+</sup> version of the allele (except for mec1-1 for which Cdc<sup>+</sup> versions were already available).
mec1-9

I decided to take one strain from each class where I might find separation of function, and examine them in more detail. I started by examining the mec1-9 strain. Although it is proficient for all functions listed on Table A-2, it was originally identified as being sensitive to cdc13 damage. Therefore, I reexamined the G2/M arrest in the strain after cdc13 damage. mec1-9 cells seem substantially more sensitive (arrest defective) to cdc13 damage than to X-ray damage which may suggest that different types of damage are recognized differently by Mec1p. However, the results from testing mec1-9 cells by themselves may be too weak to support such a claim.

Conclusion

It is still possible that in the original collection of mec1 alleles, there are alleles that demonstrate separation of function. In fact there are still some alleles that may be useful to study, such as mec1-11, which is S/M and G2/M checkpoint deficient, but is not very sensitive to HU or to MMS after short exposures. After having spent much time studying mec1-9 and ultimately learning little, I moved on to other projects and did not resume the testing of the alleles. However, from the experiments I did, it is clear that no alleles in the collection separated MEC1 functions to a large degree. Others in the lab have continued studying this collection of alleles, looking for more subtle separation of function, and also have found nothing of much significance. However, a current project involves new
screen(s) for MEC1 separation of function alleles and the results are promising.
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