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Analyses of the MEC1 DNA Damage Pathway in Saccharomyces cerevisiae

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ANALYSES OF THE \textit{MEC1} DNA DAMAGE PATHWAY IN \textit{SACCHAROMYCES CEREVISIAE}

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Gordon A. and Mary Cain Department of Chemical Engineering

by

William Barrett Ainsworth
B.S., Louisiana State University, 2008
December 2015
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Eukaryotic organisms implement conserved surveillance machinery to sense and respond to DNA damage. Fundamental to the repair process is coordinated regulation of repair genes and initiation of cell cycle arrest protocols. Failure to preserve these checkpoints results in accumulation of mutated DNA and aberrant cell phenotypes that are characteristic of human disease. The yeast, *Saccharomyces cerevisiae*, utilizes the *MEC1* checkpoint pathway to regulate the DNA damage response. This study addressed two overarching themes of transcriptional and post-translational regulation within the *MEC1* pathway. We first applied our understanding of the *MEC1* DNA damage transcriptional response to develop advanced luciferase whole cell biosensors that could detect a broad range of carcinogens using the promoter sequence of the *MEC1* DNA repair gene, *HUG1*. The enhanced whole cell yeast biosensor exhibited improved sensitivity and dynamic range when compared to fluorescent-based biosensors while reducing reporter read-out processing time through a one-step, *in vivo* measurement regime.

Previous global transcription studies performed in our lab identified a dose-dependent biphasic response of *MEC1* repair genes to alkylating agents. The origin of this unique profile, however, remained unknown. Using a GFP promoter-reporter construct placed under *MEC1* pathway genes, we found that the biphasic response persists through the *MEC1* pathway, and that neither reactive oxygen species accumulation nor pro-apoptotic genes contributed to the expression profile. Cell cycle analysis revealed that cells immediately enter a senescent state after experiencing high alkylating concentrations which we proposed was the root cause of the *MEC1* pathway gene repression.
The role of a functionally uncharacterized $MEC1$ DNA repair protein, $HUG1$, in the DNA damage response was also explored. Using overexpression phenotype and subcellular localization assays, we demonstrated that $HUG1$ is a negative regulator of the $MEC1$ pathway and that its co-localization with the positive $MEC1$ effector, Rnr2p, was likely the source of its regulation. Protein affinity assays confirmed the Hug1p-Rnr2p interaction while mutagenesis analysis probed domains within Hug1p to determine regions necessary to its inhibitory action. Finally, we discovered that Hug1p also interacts with human ribonucleotide reductase homologs, p53R2 and hRRM2, demonstrating that Hug1p uses a conserved interaction motif for its inhibition.
CHAPTER 1. INTRODUCTION

Cells in the human body experience tens of thousands of DNA lesions per day, evolving out of both errors in DNA replication mechanisms (i.e., endogenous damage) and through environmental stresses (i.e., exogenous damage) such as UV light or chemical carcinogens. (Ciccia & Elledge, 2010; Harper & Elledge, 2007; Lindahl & Barnes, 2000; Zhou & Elledge, 2000) To counteract this mutagenic damage and preserve genome fidelity, eukaryotic organisms employ sophisticated cellular machinery that regulate and halt cell growth to allow DNA repair to occur. Paramount to this response is the arrest of cells at particular stages in the cell cycle (also known as checkpoints) to prevent mutated DNA from being passed on to daughter generations. Failure to properly activate a checkpoint response results in accumulation of mutagenized DNA which inevitably causes aberrant gene expression and protein formation that leads to human disease. (Ciccia & Elledge, 2010; Di Domenico et al., 2014; Halazonetis et al., 2008; Harper & Elledge, 2007; Lord & Ashworth, 2012; Zhou & Elledge, 2000)

The complexity of human disease progression is a confounding factor in counteracting and treating aberrations in checkpoint pathway genes. Advances in next-generation sequencing (NGS) technologies have revolutionized high-throughput detection of genes involved in disease progression and have even been used to assess treatment efficacy. (Desmedt et al., 2012) However, as NGS is an emerging technology, cost restricts its application. Likewise, a fundamental understanding of underlying mechanisms is required to establish disease onset, prevention, and treatments. (Xuan et al., 2013) A basic science approach that analyzes distinct genetic and proteomic contributions to complex interacting system is thus imperative to elucidating mutation processing, disease initiation, and disease evolution.
One such basic science approach involves analysis of an arm of the checkpoint activation cascade: the DNA damage response (DDR). Insights into DDR gene transcription and enzymatic mechanisms have set the foundation for current human disease therapeutics. (Davar et al., 2012; Woods & Turchi, 2013). However, the mammalian DDR is incredibly intricate, with differential expression of cellular machinery that is cell type-, damage extent-, and cell cycle-dependent. (Jackson & Bartek, 2009) The complexity of this response is a burden on discovery, as conclusions are constrained to the experiment conditions and cellular system utilized. Simple model organisms offer a valuable alternative to the complex mammalian system, as they generally implement simpler (but conserved) pathways while being more cost effective and easier to maintain.

As one of the simplest eukaryotic models, Saccharomyces cerevisiae offer several advantages over other eukaryotic systems in modeling DDR and DDR-related disease. The budding yeast are genetically tractable, have a well-understood and annotated genome, and are inexpensive and easy to grow in rich media. (Botstein et al., 1997; Botstein & Fink, 2011) Most importantly, however, proteins in S. cerevisiae retain homology with other eukaryotic systems as most of the genome has a high degree of evolutionary conservation. In fact, 31% of potential protein-encoding genes in the yeast genome also share a homolog in the mammalian genome, despite yeast having a compact genome size that is roughly 1/250th the size of the human genome. (Botstein et al., 1997) This resounding similarity in functional genes sequence lead to discoveries that not apply to the yeast specific responses, but can also be applied to analogous roles in other eukaryotes, including humans. The DDR response exemplifies this idea of evolutionary conservation, as many DDR proteins serve similar function across the eukaryotic domain (discussed at length in Section 1.1.4 and 1.1.5).
A particular advantage of the model yeast system is its damage checkpoint response has been extensively studied. Similar to the human ATR pathway, the yeast \(MEC1\) pathway mounts a protein response to single and double strand DNA breaks that involves regulates several downstream factors at the transcriptional and post-translational scale to aid in cell cycle arrest and DNA repair (discussed in Section 1.1.5). (Ciccia & Elledge, 2010; Harper & Elledge, 2007; Zhou & Elledge, 2000; Zou & Elledge, 2003) Previous work on \(MEC1\) gene transcription in response to DNA damage has revealed a substantial and dynamic response to various types of DNA damaging agents, making it an ideal candidate for DNA damage based whole cell biosensors (discussed in Sections 0 and 0). (Benton, M. G. \textit{et al.}, 2007) Likewise, \(MEC1\)’s differential response to a broad spectrum of DNA damage has revealed new protein functionality within the \(MEC1\) response that considerably impact the regulation of the DDR. (Benton, M. G. \textit{et al.}, 2006; Gasch \textit{et al.}, 2000; Jelinsky, S. A. \textit{et al.}, 2000)

Despite this, many aspects of the \(MEC1\) checkpoint response still remain uncharacterized at the transcriptional and post-translational level. For instance, transcripts of \(MEC1\) pathway genes undergoes an acute biphasic response to increasing DNA damage by the alkylating agent, MMS; however, the origin of this unique profile is currently undefined. Uncovering the novel response mechanisms within the yeast DNA damage network has implications in analogous eukaryotic pathways through an extensive level of evolutionary conservation in the DNA damage response pathways. Likewise, a thorough understanding of underlying DNA damage mechanisms offers exciting new opportunities to develop improved whole-cell yeast biosensors with advanced sensitivity, dynamic range, and response time. Finally, several functionally uncharacterized \(MEC1\) pathway targets, such as \(HUG1\), appear to exert a control over the \(MEC1\) DNA damage response, but their mechanism and roles within \(MEC1\) remain unclassified. (Basrai
et al., 1999) The absence of a human homolog to the yeast protein, HUG1, suggests it may be a new DDR inhibitor with effects on MEC1 regulatory activity and with applicability to novel drug design.

This chapter will focus on providing background necessary to understand and establish the research objectives addressed in the proceeding chapters of this dissertation. To frame the long-term goal of this project, cancer statistics and state-of-the-art will be briefly reviewed, followed by a brief overview of the applicability of the yeast eukaryotic model in advances to human disease. The yeast DNA damage response will then be dissected at both a transcriptional and post-translational level to provide a basis for the two overarching themes of this project. DNA damage transcriptional activity will be explored to establish both its use in the development of biosensor technologies as well as being applied towards understanding a unique transcriptional response within the yeast DNA damage response. Finally, MEC1 downstream post-translational targets will be discussed with emphasis on protein function unique to Saccharomyces cerevisiae to contextualize significant findings and to introduce foundational research that inspired the current work.

1.1. Literature Review

1.1.1. A primer on DNA

DNA is an essential macromolecule that carries all of the genetic information necessary to develop and sustain life. In most organisms, DNA is composed two anti-parallel helixes; each strand is, in the most basic sense, a simple polymer composed of alternating deoxyribonucleotide monomers. Each deoxyribonucleotide consists of a nucleotide base and 5-carbon deoxyribose sugar with a phosphate group required for covalent linkage. Four different types of nucleotides are found in DNA: adenine (A), thymine (T), cytosine (C), and guanine (G), and are further
subdivided into two groups, purines (A and G) and pyrimidines (C and T), characterized by the rings structure of the corresponding nucleotide. (Alberts et al., 2002)

DNA contains a highly stable backbone that is the result of covalent phosphate linkages between the deoxyribose sugars. The characteristic DNA double helix is the result of hydrogen bonding between complementary nucleotides on an adjacent, anti-parallel DNA strand. These weak hydrogen forces between nucleotides are the foundation of DNA’s utility, as the bonds are easily broken and allow the DNA strands to be pulled apart without disrupting the integrity of the phosphate backbone. This allows for many vital cellular processes (such as DNA replication and transcription) to occur without disturbing the fidelity of the DNA itself and is key to the conserved and stable transfer of the genetic code to subsequent generations. (Alberts et al., 2002)

The DNA sequence encodes the blueprint for all cellular machinery required for life. Through a process known as transcription, specific DNA sequences known as genes can be transcoded into another molecule, known as messenger RNA (mRNA), which serves as a template for the creation of proteins through the process of translation. This sequence forms the central dogma of molecular biology and is the origin of all cellular activities required for developing, maintaining, and sustaining an organism throughout its lifespan. (Alberts et al., 2002)

The DNA sequence itself contributes to the regulation of gene expression. Specific DNA sequences act as sites for protein interaction whereby gene expression can be repressed or activated depending upon the needs of the cells. This transcriptional control allows organisms to maintain strict modular control over the types and quantities of genes being expressed during any given moment and allows for immediate and global adaptations to environmental stimuli. (Alberts et al., 2002)
1.1.2. A briefing on DNA damage and the generalized DNA damage response

Inevitably, DNA will incur damage due to internal errors in replication or external genotoxic factors such as UV-radiation. Unrepaired DNA damage non-specifically alters the genetic sequence encoded by DNA, a change which have drastic effects on vital processes within the cell. Alterations to gene expression, in particular those involved in metabolism and cell cycle, pose significant risk to genome stability and can lead to aberrant cell function and growth.(Ciccia & Elledge, 2010; Harper & Elledge, 2007; Zhou & Elledge, 2000) As such, organisms have developed a sophisticated network of cellular responses to counteract damage and preserve the genome fidelity. These checkpoint responses (discussed in detail in Section 1.1.5) prevent cell growth and repair DNA prior to duplication, however checkpoint disruption by DNA mutagenesis can negatively affect the cells ability to promote arrest and regulate gene expression.(Halazonetis et al., 2008; Harper & Elledge, 2007; Hendry et al., 2015; Jackson & Bartek, 2009; Lindahl & Barnes, 2000; Lord & Ashworth, 2012; Zhou & Elledge, 2000)

Compounding effects of DNA mutagenesis by accumulated DNA damage, including checkpoint response deactivation, tumor-suppressor genes repression, and proto-oncogene activation, inevitably lead to the onset of cancer phenotypes.(Hanahan & Weinberg, 2000; Hanahan, D. & Weinberg, R. A., 2011) As DNA damage response is a vital cellular system that mitigates DNA mutagenesis and prevents aberrant phenotypes from evolving, a thorough appreciation of its intricate role in genome maintenance is paramount to understanding the onset and progression of genetic diseases like cancer.

1.1.3. Cancer and the DNA damage response

Cancer accounts for one in four deaths in the United States and is the second leading cause of death, second only to heart disease. (Elkin & Bach, 2010; Mariotto et al., 2011; Siegel et al., 2013) The domestic cost of care was estimated at $124.5 billion in 2010. Unfortunately a
growing, treatable population and escalating chemotherapeutic costs means the economic burden of cancer care will rise by at least 27% by 2020.(Elkin & Bach, 2010; Mariotto et al., 2011; Siegel et al., 2013) Significant improvements in early detection and treatment quality have reduced cancer-related mortality rates over the past several decades, with a survival rates rising from 49% in 1975-1977 to 68% in 2004-2010.(Society, 2015) However, the development of more effective and inexpensive therapeutics will further improve survivability and help attenuate the financial concerns associated with cancer treatment.

A thorough understanding of cancer initiation and progression is imperative to advancing next-generation therapeutics and reducing overall treatment costs. In healthy cells, the DNA damage response (DDR) is responsible for preserving genomic fidelity and prevention of mutated gene passage to successive generations.(Harper & Elledge, 2007) Consequently, eukaryotic organisms have evolved highly conserved DNA damage checkpoint systems to signal DNA damage and coordinate subcellular mechanisms to ensure cell cycle arrest, DNA repair, and damage recovery. However, mutations to critical DDR genes often lead to increased genomic instability, a major contributor to cancer. To combat DDR dysfunction, diverse classes of cytotoxic and cytostatic therapeutics have been designed to discriminately target and eliminate tumors by initiating cell death via apoptosis, necrosis, mitotic catastrophe, autophagy, and/or senescence.(Hanahan, D. & Weinberg, Robert A., 2011; Ricci & Zong, 2006) Despite the initial success of these treatments, therapy-induced acquired resistance that subverts the intended chemotherapeutic response leads to increased malignancy and reduced drug efficacy. Indeed, both intrinsic and acquired resistances are believed to cause 90% of treatment failure in patients with metastatic cancer.(Hanahan, D. & Weinberg, Robert A., 2011; Ricci & Zong, 2006)

Interestingly, DDR genes responsible for cell cycle arrest, DNA damage repair, and apoptosis are
often mutated in cancers with increased drug resistance. (Hanahan, D. & Weinberg, Robert A., 2011; Hartwell et al., 1997; Lord & Ashworth, 2012; Ricci & Zong, 2006) Thus, mutated DDR genes implicated in genomic instability or drug resistance provide promising targets for future chemotherapeutics.

1.1.4. Yeast as a model for human disease

Due to the high complexity of the human DDR response and interactions, determination of ideal targets in the DDR is not trivial. Fortunately, model eukaryotic organisms, such as the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe, can provide insights into analogous functional roles of DDR proteins in cancer. (Simon et al., 2000) Yeasts in particular offer distinct advantages as model organisms in that they have a small and well-annotated genome, are genetically tractable, have short doubling times with low nutrient requirements, and share homology with several DDR genes implicated in tumorigenesis (Table 1.1). (Foury, 1997;)

Table 1.1.* Human genes altered in tumors and respective genes in the model eukaryote, Saccharomyces cerevisiae. Functionally but not structurally analogous genes are given in brackets.

<table>
<thead>
<tr>
<th>Function</th>
<th>Human genes</th>
<th>System analogs or structural homologs</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA damage checkpoint</td>
<td>p53, ATR, ATM</td>
<td>[RAD9], MEC1, TEL1</td>
</tr>
<tr>
<td>DNA mismatch repair</td>
<td>MSH2, MLH1</td>
<td>MSH2, MLH1</td>
</tr>
<tr>
<td>Nucleotide excision repair</td>
<td>XP-A, XP-B</td>
<td>RAD14, RAD25</td>
</tr>
<tr>
<td>Double-strand break repair</td>
<td>BRCA2, BRCA1</td>
<td>[RAD51], [RAD54]</td>
</tr>
<tr>
<td>Cell cycle control</td>
<td>Cyclin D, Cyclin E</td>
<td>CLN1, CLN2</td>
</tr>
<tr>
<td></td>
<td>p27kip1</td>
<td>[SIC1]</td>
</tr>
</tbody>
</table>

In fact, there have been numerous cancer treatment advances based on yeast studies. For example, by exploiting protein functional homology and the genetic malleability in yeast, researchers have devised high-throughput screening methods to identify anti-cancer treatments with high therapeutic advantage and putative targets for therapeutic treatments.

Likewise, 692 yeast genes were identified which may contribute to mutant chromosome instability (CIN) phenotypes in human disease. (Buschini et al., 2003; Simon et al., 2000) Isogenic yeast strains modeling defects in DNA repair, cell cycle checkpoints, and cell cycle regulation were used as models of cancer cells and screened for drug efficacy. (Buschini et al., 2003; Simon et al., 2000) In our lab, we have sought to further characterize yeast CIN gene targets with high chemotherapeutic potential and further elucidate DNA damage mechanisms and cellular responses induced by the yeast DNA damage stress.

1.1.5. On understanding the DNA damage response

Cells in the human body incur tens of thousands of DNA damaging lesions per a day. (Lindahl & Barnes, 2000) When exposed to exogenous and endogenous DNA damage, eukaryotes employ a sophisticated, evolutionarily-conserved surveillance network to identify DNA lesions and replication stresses. Organisms invoke a canonical protocol upon detection of DNA damage that coordinates cellular activities to ensure aberrant DNA repair prior to the cell dividing (Figure 1.1). (Zhou & Elledge, 2000) Repairing DNA prior to mitosis and meiosis ensures that a stable and high-fidelity genome is replicated to the next cellular generation. Generally speaking, cells detect DNA damage through the use of damage sensing proteins; these “sensors” bind DNA and activate a series of transducers with highly specific activity and function. Transducers are capable of turning on genes or modifying proteins to enable desired effects. The downstream targets activated by transducers are known as “effectors” and act as a
diverse class of proteins that mediate or induce cell protocols, such as initiating cell cycle arrest or increasing availability of DNA monomers to aid in DNA repair. The process is highly coordinated with a series of cellular checkpoints to ensure DNA damage is managed appropriately prior to proceeding through cell.

Mammalian and yeast cells implement a functionally analogous mechanism to respond to DNA damage, however specific proteins utilized in the response vary (Table 1.2). (Hustedt et al., 2013; MacQueen & Hochwagen, 2011) The DNA damage checkpoint is broadly regulated by two response systems: the ATM-mediated double-strand DNA break (DSB) response and the

---

In Saccharomyces cerevisiae, Tel1 (ATM) is largely involved in telomere maintenance and DSB recognition while Mec1 (ATR) has a role in regulating both ssDNA and DSB checkpoint response (Baldo et al., 2008; Di Domenico et al., 2014; Naiki et al., 2004).

**Table 1.2.** Conserved checkpoint proteins and their function. (Hustedt et al., 2013)

<table>
<thead>
<tr>
<th><strong>S. cerevisiae</strong></th>
<th><strong>H. sapiens</strong></th>
<th><strong>Function</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rad24-RFC</td>
<td>RAD17-RFC</td>
<td>RFC-like complex, 9-1-1 clamp loader</td>
</tr>
<tr>
<td>Ddc1-Rad17-Mec3</td>
<td>RAD9-RAD1-HUS1</td>
<td>9-1-1 complex, DNA damage checkpoint clamp, Mec1 activation</td>
</tr>
<tr>
<td>Dpb11</td>
<td>TOPBP1</td>
<td>Mec1 ATR activation</td>
</tr>
<tr>
<td>Dna2</td>
<td>DNA2</td>
<td>Mec1 activation in S phase</td>
</tr>
<tr>
<td>Mre11-Rad50-Xrs2</td>
<td>MRE11-RAD50-NBS1</td>
<td>MRX/MRN complex, DSB resection, Tel1/ATM recruitment</td>
</tr>
<tr>
<td>Mec1-Ddc2</td>
<td>ATR-ATRIP</td>
<td>checkpoint signaling kinase</td>
</tr>
<tr>
<td>Tel1</td>
<td>ATM</td>
<td>checkpoint signaling kinase</td>
</tr>
<tr>
<td>Mrc1</td>
<td>Claspin</td>
<td>fork-associated, checkpoint mediator</td>
</tr>
<tr>
<td>Rad9</td>
<td>53BP1, BRCA1</td>
<td>checkpoint mediator</td>
</tr>
<tr>
<td>Sgs1</td>
<td>BLM, WRN</td>
<td>fork-associated, Rad53 activation</td>
</tr>
<tr>
<td>Rad53</td>
<td>CHK2</td>
<td>effector kinase</td>
</tr>
<tr>
<td>Chk1</td>
<td>CHK1</td>
<td>effector kinase</td>
</tr>
</tbody>
</table>

Gene name abbreviations: Rad24 (radiation sensitive 24), RFC (replication factor c), Ddc1 (DNA damage checkpoint 1), Mec3 (Mitosis entry checkpoint 3), HUS1 (hydroxyurea sensitive 1), Dpb11 (DNA polymerase B11), TOPBP1 (DNA topoisomerase 2 binding protein 1), Dna2 (DNA synthesis defective 2), Mre11 (meiotic recombination 11), Xrs2 (X-ray sensitive 2), Nbs1 (Nijmegen breakage syndrome 1), ATR (ATM and Rad3-related), ATRIP (ATR interacting protein), Tel1 (telomere maintenance 1), ATM (Ataxia telangiectasia mutated), Mrc1 (mediator of the replication checkpoint 1), 53BP1 (tumor suppressor p53 binding protein 1), BRCA1 (breast cancer 1, early-onset), Sgs1 (slow growth suppressor 1), BLM (Bloom syndrome protein), WRN (Werner syndrome ATP-dependent helicase), CHK2 (checkpoint kinase 2), CHK1 (checkpoint kinase 1).

ATR-mediated single-stranded DNA (ssDNA) detection network. (Abraham, 2001) In *Saccharomyces cerevisiae*, Tel1 (ATM) is largely involved in telomere maintenance and DSB recognition while Mec1 (ATR) has a role in regulating both ssDNA and DSB checkpoint response (Baldo et al., 2008; Di Domenico et al., 2014; Naiki et al., 2004).

‡ Yeast proteins are given with their respective human homologs in parentheses.

§ Table 1.2 is adapted from Hustedt, N., S. Gasser, and K. Shimada. *Replication Checkpoint: Tuning and Coordination of Replication Forks in S Phase*. Genes, 2013. 4(3): p. 388. The article and its contents are open access and distributed under the terms and conditions of the Creative Commons Attribution 3.0 license.
Activation of the *MEC1* (ATR) signal transduction cascade induces translational and post-translational activity that initiates cell cycle arrest protocols and DNA repair mechanisms. The DNA damage checkpoint is proposed to be triggered once threshold levels of DNA lesions are detected and replication forks slow or stall. (Minca & Kowalski, 2011) In *Saccharomyces cerevisiae*, the resulting stalled replication forks expose single-stranded DNA (ssDNA) which act to prime DNA damage sensing complexes (Figure 1.2) (Cimprich & Cortez, 2008; Hustedt et al., 2013) Replication protein A (RPA) coat and stabilize bare ssDNA which recruit constitutively associated Ddc2p-Mec1p (ATRIP-ATR) to DNA damage sites (Hustedt et al., 2013; Rouse & Jackson, 2002; Zou & Elledge, 2003).

Mec1p (ATR) is subsequently activated by the heterotrimeric proliferating cell antigen (PCNA)-like 9-1-1 complex, Rad17-Ddc1-Mec3(RAD9-RAD1-HUS1) (Majka, J. & Burgers, 2003; Majka, Jerzy et al., 2006) which coordinates G1 arrest, but is also involved in S- or G2/M arrest mediated by Dna2p (DNA2) (Kumar & Burgers, 2013) or Dpb11 (TOPBP1) (Ogiwara et al., 2006) activity, respectively. (Figure 1.2) (Hustedt et al., 2013). Successful activation of the *MEC1* (ATR) signal transduction pathway initiates phosphorylation events that activate downstream effector kinases, Rad53p (CHK2), Chk1p (CHK1), and Dun1p. These effector kinases trigger further downstream targets that regulate origin firing, gene transcription, and dNTP pools and act together to arrest the cell and repair DNA before cellular reentry into the normal cell cycle programming. (Harper & Elledge, 2007; Hustedt et al., 2013; Zhou & Elledge, 2000; Zou & Elledge, 2003)

1.1.6. *MEC1* pathway transcriptional response

This dissertation explores two overarching themes with respect to the *MEC1* pathway: *MEC1* regulation at the translational level (i.e., *MEC1* gene expression profiling and uses thereof) and at the post-translational level (i.e., effects of *MEC1* target proteins within the DNA
damage response. Chapters 2 and 3 highlight key discoveries in *MEC1* gene expression as it applies towards whole cell biosensor development and in defining the origins of a unique *MEC1* DNA damage gene response at increasing DNA damage dosages. This section serves a primer to these chapters, describing literature necessary in understanding the role of *MEC1* gene expression in biosensor technologies as well as discussing yeast global transcriptional response to DNA damage and how the results contribute to establishing Chapter 3 objectives. Section

**Figure 1.2.** "*MEC1* checkpoint activation by DNA damage and replication stress. (Hustedt et al., 2013)"
1.1.7 discusses post-translational aspects of the *MECl* pathway, including literature related to *MECl* DNA damage targets and their role in regulating the *MECl* response.

The *MECl* checkpoint initiates a vast, differential network of target genes with dependence on cell cycle stage and the quantity and type of DNA damage. Through total RNA extractions and microarray analysis, global transcriptional analysis has been undertaken to define *Saccharomyces cerevisiae* gene expression profiles in response to an assortment of cellular stress agents, genotoxic chemicals, and ionizing and UV radiation. (Benton, M. G. *et al.*, 2006; Fry *et al.*, 2005; Gasch *et al.*, 2000; Jelinsky, S. A. *et al.*, 2000). Most surprisingly, DNA damage induced a differential transcript response in nearly 2500 of the total 6200 open-reading frames in *Saccharomyces cerevisiae*. (Jelinsky, S. A. *et al.*, 2000) A single, low dosage of the DNA alkylating agent, methyl methanesulfonate (MMS), induced differential expression of nearly ~325 gene transcripts (5% of the genome), (Jelinsky, Scott A. & Samson, 1999) and this response was later shown to be specific to the MMS titer. (Jelinsky, S. A. *et al.*, 2000) Likewise, a similar alkylating agent N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) was shown to provoke an entirely different array of genes in response to damage. (Jelinsky, S. A. *et al.*, 2000) A survey of DNA damaging agents including 4-nitroquinoline 1-oxide (4-NQO), γ-irradiation, mitomycin C (MMC), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), tert-butyl hydroperoxide (t-BuOOH), were investigated for their response and shown provoke vast gene transcript effects independent of overall toxicity. (Jelinsky, S. A. *et al.*, 2000)

A similar microarray study performed by our lab has also revealed that *MECl* DNA repair pathway genes exhibit a dose dependent response to MMS response. (Benton, M. G. *et al.*, 2006) Transcripts of *DUN1* (a *MECl* effector kinase), *RNR2* and *RNR4* (*MECl* positive effectors and subunits of the ribonucleotide reductase enzyme which synthesizes DNA
monomers – dNTPs - for DNA repair and synthesis), and HUG1 (MEC1 target protein with unknown function; reviewed in section) were show an increase at low- to mid-dosages of MMS (0.001-0.01% v/v final concentration) and sharply decrease at higher MMS dosages (0.1% v/v final concentration). (Benton, M. G. et al., 2006) Correlatively, genes induced at the 0.1% DNA damage dosage level correspond with genes expressed upon entry in stationary phase. (Jelinsky, S. A. et al., 2000) These intriguing results taken together suggest DNA damage checkpoint pathway genes fail to induce or are repression through a shared general stress response that is experienced during stationary phase or at high threshold levels of MMS, however no data exists that unify these two stages of cellular response. (Jelinsky, S. A. et al., 2000) Chapter 3 of this dissertation explores potential mechanisms that may be influencing this unique biphasic gene response, including DNA damage induced dose-dependent response contributions from DNA damage extent, reactive oxygen species accumulation, pro-apoptotic genes, and cell cycle phase.

The MEC1 DNA damage response is extremely dynamic in sensing DNA damage type and extent. Likewise, downstream MEC1 target genes, such as HUG1, are some of the highest differentially expressed genes in the yeast genome. (Basrai et al., 1999) These characteristics, alongside its genetic tractability and ease in maintenance, make yeast an ideal candidate for use as whole cell biosensors. The term “biosensor” applies to a broad class of analytical devices that use living organisms or biomolecules (such as enzymes or antibodies) to detect environmental perturbations or increased titers of a target analyte. (Turner, 2015) In the case of whole cell biosensors, yeast cultures are utilized as the sensing conduit through which signals activate a detectable reporter. Reporters are placed under a gene’s promoter for the express purpose of activating and reporting gene expression once stimulated by certain environmental conditions. Promoter–reporter systems have been designed to detect a broad array of environmental
perturbations, including the presence of heavy metals (Mascorro-Gallardo et al., 1996; Virta et al., 1995), organic pollutants (Selifonova & Eaton, 1996), sugar sources (Chambers et al., 2004; Marrakchi et al., 2008) and DNA-damaging agents (Benton, Michael G. et al., 2007; Boronat & Pina, 2006).

Reporter choice plays an important role in a biosensing system and typically includes use of one of three types of biological molecules: biological fluorophores, such as green fluorescent protein (GFP) which can be read by a fluorometer, chromophores such as β-galactosidase (which produces a blue color after reacting with the lactose analog, X-gal) and are read by a spectrophotometer, or enzymatic complexes that perform chemical reactions to provide a read-out (such as luciferase’s reaction with D-luciferin to emit a photon of light) and are detected by luminometers or scintillators. (Daunert et al., 2000). Several design considerations must be made when selecting a reporter. Chromophore and enzymatic reporters generally require cell lysates to obtain accurate measurements; the extraction process is typically harsh and results in reporter deactivation. Fluorophore reporters, on the other hand, require expensive equipment (such as an epifluorescent microscope or flow cytometer) for detection and are typically not amenable to field-use. (Garrido et al., 1994; McNabb et al., 2005)

An ideal biosensor maintains quantitative accuracy while achieving high dynamic range when reporting analyte detection. Previous work in our lab have demonstrated the utility of a GFP reporter under promoter control of the MEC1 pathway gene, HUG1 to identify a survey of DNA damaging agents, including alkylating agents MMS, ethyl methanesulfonate (EMS), and 1,2-dimethylhydrazine (SDMH), replication stress agent hydroxyurea (HU), cellular stress agents phleomycin, camptothecin, and 4-NQO, and DNA damaging agent γ-radiation.(Benton, M. G. et al., 2007) Sensitivity of the HUG1 DNA damage promoter-reporter system to the alkylating
agent MMS was also increased after deletion of genes required for repairing alkylated DNA, *MRE11* and *MAG1*. (Benton, Michael G. *et al.*, 2008) These experiments exposed the power of whole cell yeast biosensors and their distinct advantages in genetic tractability. Through rational design of yeast biosensors, highly sensitive and specific reporter systems can be engineered to sense a dynamic range of target analytes, including carcinogenic targets as detected by reporters constructed in DNA damage response promoter elements.

One of the advantages of the luciferase system is its sensitivity in detection, however, as the luciferase enzyme requires extraction prior to taking measurements, its adoption in field-use has been limited. Recent advances in the luciferase system have improved sensitivity and reproducibility of signaling *in vivo*, have eliminated the rate-limiting extraction step, and have allowed for a quick and accurate one-step measurement of bioluminescence. (Leskinen *et al.*, 2003; Tauriainen *et al.*, 1999; Vieites *et al.*, 1994). In our study (Chapter 2), we explored the utility of the advanced luciferase as a reporter for carcinogens by expressing it under promotion of the DNA damage response gene, *HUG1*. We first developed a new plasmid construct that allows for one-step, PCR mediated promoter-reporter engineering with the advanced luciferase reporter. We then characterized detection parameters (such as response time and sensitivity) of the new HUG1 promoter-luciferase reporter system and compared these findings to the established *HUG1* promoter, GFP reporter system.

1.1.7. *MECl* DNA damage response targets

Post-translational modification of proteins act as another mechanism of control over gene expression and protein functionality in the DNA damage response. DNA repair proteins and their regulation are of particular interest to this project, and as such are extensively overviewed to provide context for the project objectives. These sections introduce features of the DNA repair genes in order to establish the motives behind the project objectives. It will also serve to
contextualize experimental approach, design, and interpretations explored in Chapters 4 and 5. These chapters are particularly focused on identifying the functional and mechanistic role of a previously uncharacterized DNA damage response protein, \textit{HUG1}.

Our investigations into the yeast \textit{MEC1} DNA damage response revealed that the \textit{MEC1} downstream effectors, ribonucleotide reductase subunits (\textit{RNR1}, \textit{RNR2}, and \textit{RNR4}) are CIN genes and demonstrate prominent sequence and functional homology to the human RNR complex.\cite{Hendry2015, Stirling2011} Interestingly, dysfunctional levels of a yeast \textit{RNR2} analog, human p53R2, have been observed in several cancer types, including melanoma,\cite{Matsushita2012} esophageal squamous cell carcinoma,\cite{Yokomakura2007} and non-small cell lung cancer.\cite{Uramoto2006} Likewise, mRNA silencing experiments designed to target and inhibit human \textit{RNR2} analogues were successful in reducing tumor progression and decreasing tumor resistance to DNA damage inducing chemotherapeutics.\cite{Halazonetis2008, Kunos2009, Matsushita2012, Shao2006} As such, RNR genes offer specific and viable targets for chemotherapeutic design.

Ribonucleotide reductase (RNR) is an evolutionarily conserved enzyme that is utilized by all domains of life - from bacteria to higher eukaryotes. RNR is responsible for the rate-limiting enzymatic reduction of ribonucleotides to the DNA monomers, deoxyribonucleotides. RNR is typically active only during the DNA synthesis phase (S-phase) as dNTPs are required for DNA replication. However, an increase in RNR expression is generally seen upon DNA damage, as more dNTPs are required for DNA strand repair. As such, RNR is a fundamental component of the DNA damage response.\cite{Elledge1989, Hendry2015, Huang2014, Kolberg2004, Nguyen1999, Torrents2014}
Though RNR function is strictly evolutionarily conserved, three distinct classes of RNRs have been discovered that differ by metal cofactors and thyl radical generation chemistry which, in all three classes, coordinate to produce essential thyl radicals at the active sites of the enzyme. Class I reductases (found in almost all eukaryotes, some prokaryotes, and some viruses) form active complexes containing two distinct multimeric subunits, R1 (also known as α) and R2 (also known as β), which require oxygen to generate stable tyrosyl radicals at the β2 diiron-oxygen cluster (Figure 1.3). (Cotruvo & Stubbe, 2011; Kolberg et al., 2004)

The active form of the *Saccharomyces cerevisiae* RNR utilizes a distinct type of R2 subunit (known *RNR4* or β’) which forms a heterodimer with the β subunit. The β’ subunit lacks iron the capacity to binding and therefore is incapable of forming the tyrosyl radical. (Huang & Elledge, 1997; Wang et al., 1997) *RNR4* deletions result in severe growth inhibition lethality and found to be defective in β subunit iron loading, revealing its vital role in the yeast RNR diiron cluster assembly. (Chabes et al., 2000; Nguyen et al., 1999; Zhang et al., 2011) Yeast also express a DNA damage-inducible form of the large subunit, Rnr3p, which demonstrates strong synergism in catalytic activity when combined with the α large subunit, Rnr1p. (Domkin et al., 2002) Rnr3p functional role in the DNA damage response still remains unclear, however it has been proposed that it may be necessary when dNTP production is low due to lower Rnr1p expression. (Domkin et al., 2002)

Class II reductases, on the other hand, contain a single R1 subunit that form active monomers (α) or dimers (α2). The thyl radical is produced by cleavage of adenosylcobalamin, forming a deoxyadenosyl radical which fulfills an analogous function to R2 of other RNR classes. (Kolberg et al., 2004; Torrents, 2014) Class III reductases are typically found in anaerobic organisms and are thereby repressed in the presence of oxygen. Instead, it uses an iron
sulfur cluster alongside S-adenosylmethionine to generate free radicals required for reduction chemistry. (Nordlund & Reichard, 2006)

Key to maintaining a stable genome is the proper regulation of dNTP pools throughout DNA synthesis and repair. In fact, excessive dNTP production has been linked to increased mutation rates, while low dNTP levels stall DNA synthesis and, as a result, cell division. (Chabes et al., 2003; Chabes & Stillman, 2007; Poli et al., 2012). Due to this, RNR is tightly regulated by a complex allosteric feedback mechanism regulated by substrate availability and product concentration. (Hofer et al., 2012).

Allosteric regulations occur in the R1 subunit at the both the specificity site and the activity site (S- and A-site, respectively; Figure 1.3.A). Allosteric regulation starts in the S-site, which can bind ATP and all dNTPs with the exception of dCTP. When dNTPs bind into the S-

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††Figure 1.3 is adapted from Hofer, A., Crona, M., Logan, D. T., & Sjöberg, B.-M. (2012). DNA building blocks: keeping control of manufacture. Critical Reviews in Biochemistry and Molecular Biology, 47(1), 50-63. The article and its contents are open access and distributed under the terms and conditions of the Creative Commons Attribution 3.0 license.
site, a conformational change induces alterations to the catalytic site that affects substrate reduction. Binding of ATP and dATP to the S-site changes substrate specificity to CDP and UDP, which is subsequently reduced to dCDP and dUDP respectively (.B). Interaction with dTTP at the S-site, on the other hand, stimulates GDP to dGDP reduction, while dGTP stimulates ADP to dADP reduction (Figure 1.3.B). The A-site is an enzyme on/off switch that monitors relative amounts of dATP-to-ATP. At high dATP concentrations, RNR activity is switched off by the A-site dATP feedback mechanism (Figure 1.3.B).

Interestingly, Saccharomyces cerevisiae exhibit a relaxed dATP feedback mechanism that allows for increased survival to DNA damage due to elevated dNTP levels. (Chabes et al., 2003) As this study focuses on the yeast, Saccharomyces cerevisiae, and its RNR complex, the dNTP reduction chemistry will be framed according to its particular enzyme class, Class Ia RNR

The ribonucleotide reduction process involves the conversion of a 2’-hydroxyl group on the ribonucleotide monomer to a hydrogen by radical reduction chemistry (Figure 1.4). First, a thiy radical is generated by long range radical transfer from the tyrosyl radical in the ribonucleotide reductase small subunit, R2. The thiy radical then reacts with ribonucleotides to extract the 3’-hydrogen from the ribose ring, resulting in a free radical intermediate (Figure 1.4.1 to Figure 1.4.2). The reactive intermediate makes the 2’-hydroxyl group more reactive, which becomes protonated and leaves as a water molecule leaving behind a 2’-ketyl radical (Figure 1.4.3).

Two active site cysteines are then oxidized and form a disulfide anion radical after transferring a hydrogen to the radical ribose intermediate (Figure 1.4.4). Radical electron transfer then occurs through a hydrogen bond network whereby the radical relocates to the intermediate ribose 2’-position (Figure 1.4.5). The radical then abstracts a hydrogen from the
nearby cysteine, and the final product – deoxyribonucleotide (dNTP) – leaves the active site (Figure 1.4.6). Long range radical transfer through the electron transport chain in R2 carries the radical back to the R2 subunit to form the stable tyrosyl radical. Regeneration of the reduced active site cysteines evolves from the redox reaction between the cysteine pairs, thioredoxin, and thioredoxin reductase to reduce free NADPH. (Kolberg et al., 2004)

Despite being functionally analogous to the human RNR complex, the yeast RNR complex undergoes considerably different transcriptional and post-translational regulations. However, these protein deregulation mechanisms provide interesting opportunities for

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*Figure 1.4 is adapted from Kolberg, M., K.R. Strand, P. Graff, and K. Kristoffer Andersson, Structure, function, and mechanism of ribonucleotide reductases. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 2004. 1699(1–2): p. 1-34. It is reprinted by permission of Elsevier.*
discovering novel RNR binding and interaction motives that may be exploited in designing future chemotherapeutics.

Transcription of RNR subunits, RNR2, RNR3, and RNR4 is repressed during normal cell growth by the DNA binding protein, Crt1p (Figure 1.5), which recognizes specific X-box sequences in the RNR2, RNR3, and RNR4 gene promoter sequences. Crt1p recruits the Ssn6p-Tup1p which act to repress RNR gene expression. Once the cell experiences DNA damage, Crt1p is hyperphosphorylated and detaches from the RNR promoter region, enabling transcription of RNR genes. Also in response to DNA damage, an HMG-box binding protein, Ixr1p, is recruited to the RNR1 promoter and activates its transcription.

Yeast also utilize a unique post-translational method to sequence and inhibit RNR subunits. Yeast RNR subunit negative regulators, DIF1 and SML1, reduce RNR complex activity during normal cell growth through nuclear redistribution of the Rnr2p-Rnr4p subunit and inhibition of the Rnr1p homodimer, respectively. Upon DNA damage or replication arrest, however, Sml1p and Dif1p are phosphorylated and degraded. This, along with the transcriptional induction of RNR genes and localization of the RNR complex to the cytoplasm, serves to increase dNTP pools.

Post-translational regulation by Dif1p and Sml1p are unique to the yeast response mechanism. As a negative regulator of the yeast DDR, Sml1p inhibition of Rnr1p appeared promising as a chemotherapeutic candidate. Immediately following the discovery of SML1’s functional role, experiments were undertaken to test the capabilities of SML1 inhibiting the
murine \textit{RNR1} homolog, R1. Binding affinity assays demonstrated weak Sml1p association to murine R1 that was greatly affected by the dNTP feedback mechanisms and competition with murine R2 binding. (Andreson \textit{et al.}, 2010; Chabes \textit{et al.}, 1999) Due to these weak interaction characteristics, no further Sml1p studies have examined its potential chemotherapeutic fitness. Despite extensive analysis of \textit{MEC1} targets in DNA repair, several \textit{MEC1} pathway targets remain functionally uncharacterized. Interestingly, though it is the most differentially expressed gene in the \textit{MEC1} DNA damage response to hydroxyurea, UV- and γ-radiation, and

\footnote{Figure 1.5 is adapted from Sanvisens, N., de Llanos, R., & Puig, S. (2013). Function and regulation of yeast ribonucleotide reductase: cell cycle, genotoxic stress, and iron bioavailability. \textit{Biomed J}, 36(2), 51-58.}
MMS, (Basrai et al., 1999) Hug1p still remains functionally and mechanistically undefined. hug1Δ mutants have been shown to recover the synthetic lethality of mec1Δ and rad53Δ deletion strains. (Basrai et al., 1999) This is similar to other negative regulators of the MEC1 pathway, including DIF1 and SML1. (Andreson et al., 2010; Chabes et al., 1999; Lee et al., 2008; Wu & Huang, 2008; Zhao, X. & Rothstein, 2002) Likewise, DIF1 and HUG1 share considerable sequence homology as result of gene duplication. (Lee et al., 2008)

When the homologous “Hug” domain was mutated in DIF1, Dif1p lost the ability to localize the Rnr2p-Rnr4p subunit to the nucleus. (Lee et al., 2008) This strongly suggests a potentially inhibitory interaction between Hug1p and Rnr2p-Rnr4p as Dif1p shows weak inhibition of RNR activity in vitro. (Lee et al., 2008; Wu & Huang, 2008) Elucidating Hug1p’s role in the MEC1 pathway and defining its putative interaction with proteins in the DNA damage response will work towards defining novel MEC1 inhibitory mechanisms with applications in RNR inhibition in higher eukaryotic systems.

1.2. Objective

The eukaryotic DNA damage surveillance machinery is incredibly complex and highly specific towards the extent and type of DNA damage it responds to. The vast protein network initiated upon genotoxic stresses is a hallmark of the cell’s dynamic action against a broad spectrum of environmental perturbations and carcinogens. The material presented in this dissertation follows two overarching themes of the MEC1 DNA damage response in the budding yeast, Saccharomyces cerevisiae. The first involves understanding and utilizing unique features of MEC1 gene transcription. MEC1 transcriptional signatures provide ideal gene candidates that serve to advance sensitivity and broaden sensing capabilities of whole cell yeast biosensors to carcinogens. In conjunction with this theme, this study also examines a unique biphasic transcriptional response of MEC1 DNA repair genes and establishes the genesis of this
characteristic profile. Chapters 2 and 3, respectively, specifically address the theme of transcriptional. Hallmark achievements of the individual chapters include:

- **Chapter 2** - Current fluorescent biosensors reporting DNA damage response genes have been shown to be sensitive to genotoxic agents, but lack efficient and inexpensive methods of detection. By combining yeast *MEC1* pathway genes promoters with a yeast optimized luciferase enzyme reporter system, an enhanced yeast whole cell biosensor was designed that detected carcinogenic compounds with improved sensitivity and dynamic range while shortening detection time when compared to comparable fluorescent-based reporters. A new vector set was also developed to allow facile, PCR mediated promoter-reporter construction with the optimized luciferase enzyme.

- **Chapter 3** - *MEC1* DNA repair genes exhibit a dose-dependent biphasic response to alkylating agents, however the origin of this response is unknown. A GFP promoter-reporter system was used to identify the transcriptional response of major contributors to the *MEC1* response to a survey of 9 cellular stress and DNA damaging agents. External pathways to *MEC1* were also examined for potential epigenetic regulation of *MEC1* gene transcription. Finally, cell cycle analysis was run to identify if cellular stage and arrest contributed to the *MEC1* biphasic response.

The second theme encompasses investigations into post-translational regulation of *MEC1* pathway genes. As the MEC1 pathway is so complex, several target protein functional and mechanism roles have yet to be determined. One such protein, *HUG1*, has characteristics of a *MEC1* negative regulator and may be of clinical applicability, however little remains is known about its role in DNA repair response. This study first establishes *HUG1*’s function in the MEC1 pathway and further identifies the mechanism and protein features utilized by *HUG1* in
its recovery role. Chapters 4 and 5, respectively, specifically address the theme of post translational regulation of the \textit{MEC1} repair process. Hallmark achievements of the individual chapters include:

- \textbf{Chapter 4} - \textit{HUG1} has an uncharacterized role within the MEC1 pathway, despite exhibiting characteristics of \textit{MEC1} negative regulators. Overexpression phenotyping assays were carried out to identify Hug1p effects on cells experiencing DNA damage and replication arrest. Temporal expression and subcellular localization studies were also carried out to identify putative roles for \textit{HUG1} in the \textit{MEC1} response.

- \textbf{Chapter 5} - After characterizing \textit{HUG1} as a negative regulator of the \textit{MEC1} pathway, Hug1’s mechanistic role of inhibition was further examined. Protein interaction assays and dNTP analysis were carried out to identify \textit{HUG1}’s interaction partners and inhibitory mechanism. Mutagenesis was then carried out across HUG1 to isolate regions necessary to Hug1p’s inhibitory activity.

The final chapter summarizes major findings in this work and discusses future work based on these discoveries.

\section*{1.3. References}


2.1. Introduction

When cells are exposed to environmental perturbation, transcription is modulated in a specific manner, ultimately leading to the production of proteins that can increase the likelihood of survival. Assessing the transcriptional level of one or more of these responding proteins provides valuable information regarding the cellular surroundings. In fact, many researchers have exploited this sensitivity to a transient environment to develop a tool to explicitly determine cellular reactions to a wide range of stimuli. These ‘sensors’ typically consist of an easily detectable reporter gene regulated by an environmentally sensitive protein's promoter. Reporters such as β-galactosidase, GFP and luciferase have proved useful in these promoter–reporter systems, due to their considerable dynamic range and ease of use with multiple promoters (Daunert et al., 2000). Previously these promoter–reporter systems have been used to detect the presence of heavy metals (Mascorro-Gallardo et al., 1996; Virta et al., 1995), organic pollutants (Selifonova & Eaton, 1996), sugar sources (Chambers et al., 2004; Marrakchi et al., 2008) and DNA-damaging agents (Benton et al., 2007; Boronat & Pina, 2006).

Despite the ubiquity of use, each sensor suffers from deficits that must be considered prior to implementation. β-Galactosidase- and luciferase-based systems require enzyme extraction for measurements, a process in which enzyme loss/deactivation and experimental time are major concerns (Garrido et al., 1994; McNabb et al., 2005). Fluorophore-derived reporter systems do not require enzyme extraction; however, applications in high-throughput systems are

limited due to the necessity of whole-cell separation and expensive equipment required for measurement (e.g. flow cytometry).

Improvements to each of these systems has increased the viability of these assays in high-throughput systems (Huang & Shusta, 2005; Osipov et al., 2011). Advances to luciferase systems – most notably, a transition from in vitro to in vivo assays (Leskinen et al., 2003) – has been a watershed event in promoter–reporter systems. Detection remains inexpensive (a major advantage of the original detection method), while retaining large, accurate and quantifiable dynamic ranges without the variability reported between in vitro samplings (Tauriainen et al., 1999; Vieites et al., 1994). Most importantly, measurement of in vivo bioluminescence can be taken quickly, accurately and in one-step (Michelini et al., 2005), without the additional lysis step commonly required of other luciferase assays (Liu et al., 2008; McNabb et al., 2005). Additionally, recently discovered secrétable luciferases show promise as next-generation reporters that remain compatible with current detection techniques (Ochi et al., 2011; Tochigi et al., 2010).

Applications of the new luciferase reporters hinge heavily on the method of introduction to host systems. Current luciferase systems utilize one of two common methods of transformation: yeast integrative plasmids (YIps) (Leskinen et al., 2003) and yeast centromere plasmids (YCps) (Ochi et al., 2011; Tochigi et al., 2010), both of which present advantages and disadvantages. YIps directly integrate reporters into the genome at the promoter locus, which preserves transcriptional regulation and accurately reflects native expression levels. Thus, YIps are typically employed when quantitative native promoter expression is desired. YIps, however, are specific to the promoter for which they were created, and are thereby largely inflexible when extending reporter functionality to other promoters. YCps are self-replicating, external plasmids
which express reporters under promoter control within the plasmid. As such, YCps do not knock out functional genes and expression reflects an unaltered phenotype of the host. Thus, YCps are best employed when seeking qualitative promoter response information regarding promoter responses. Despite the replication stability offered by the presence of the CEN/ARS fragments, these plasmids may exist in multiple copies inside the host, thereby exaggerating true promoter expression levels. In the context of biosensors, complete knowledge of active promoter regions must also be ascertained to effectively express a reporter using this system. Finally, plasmid loss remains a concern, as hosts must be maintained under constant selective pressure (Romanos et al., 1992; Rose & Broach, 1991; Stearns et al., 1990).

For promoter–reporter applications requiring stable, accurate reporter quantification with promoter flexibility, a new transformation system is required. PCR-mediated gene deletion/fusion techniques have been developed for fluorophore-based reporter systems and show promise for extension to luciferase reporters (Benton et al., 2007; Longtine et al., 1998; Wach et al., 1997; Wach et al., 1994). Similarly to YIps, this method integrates directly into the genome, but does so via a simple, one-step PCR-mediated gene cassette containing the reporter of interest. In-frame insertions are directed by 5′ and 3′ homologous regions directly up- and downstream of the gene, expressing the reporter under direct promoter control. Primers containing these 5′ and 3′ homologous regions can be tailored according to the promoter of interest, allowing for promoter flexibility without prior knowledge of the promoter's sequence (Longtine et al., 1998).

In this study, a novel plasmid, which introduces a cytosolic expressing firefly luciferase, \(LUC^*\text{(-SKL)}\), capable of one-step, in vivo measurements (Leskinen et al., 2003) into a promoter–reporter system via PCR-based gene deletion (Longtine et al., 1998), was engineered.
The plasmid was utilized to introduce \( \text{LUC}^{*}(-\text{SKL}) \) under \( \text{HUG1} \) promoter control and to confirm cytosolic localization by fluorescence microscopy. The dose–response of this novel construct was compared with that of a similar \( \text{HUG1A}:y\text{EGFP1} \) promoter–reporter system and showed a similar response pattern. To demonstrate the flexibility of the plasmid construct under multiple promoters and as a C-terminal fusion construct, an \( \text{RNR3} \) knockout and a \( \text{TEF1} \) fusion construct were also developed and confirmed to show functional luciferase activity. Thus, the newly constructed plasmid demonstrates utility for simple and accurate one-step reporting under a diverse set of promoters where gene expression and protein data are desired.

2.2. Materials and methods

2.2.1. Plasmid and strain construction

A modified luciferase gene absent restriction enzyme sites (\( \text{LUC}^{*} \)) was amplified from plasmid pBluc* (Bonin et al., 1994) using primers pBluc*–SKL SmaI Cass_F and pBluc*–SKL AscI Cass_R (Table 2.1). The reverse primer contains a C-terminal truncation of the peroxisome-localization tag, SKL, which leads to cytosolic expression of Luc*(−SKL)p (Gould et al., 1988; Leskinen et al., 2003). A region containing the restriction enzyme sites SmaI and PacI in direct tandem and an additional cytosine base to ensure proper reading frame were added directly upstream of the \( \text{LUC}^{*} \) sequence start codon. This region preserves a conservative primer-binding sequence, such that previously described PCR primers (Longtine et al., 1998) can be extended for use in this plasmid. A PacI region was not exclusively used, as \( \text{LUC}^{*} \) contains a PacI restriction site beginning at base 1321 of pBluc*. A restriction site encoding AscI was added to the reverse primer sequence, directly downstream of the stop codon of \( \text{LUC}^{*} \). PCR products were combined, phenol–chloroform extracted once, then digested with SmaI and AscI restriction enzymes for 10 min on a 37°C heat block. Plasmid pFA6-GFP(S65T)-His3MX6 (Longtine et al., 1998) was also digested with SmaI and AscI and the 4.0 kb fragment was excised from a gel,
using a Thermo Scientific Fermentas GeneJET Extraction Kit (cat. no. K0691, Thermo Scientific, Glen Burnie, MD, USA).

The digested PCR product and plasmid fragment were ligated using T4 ligase in a 3:1 molar ratio, respectively, at 4°C overnight. 5 µl ligant were added to DH5α cells (cat. no.18265-017, Invitrogen, Grand Island, NY, USA) and transformed according to the manufacturer's protocol. Transformants were plated on ampicillin (AMP)-selection plates and allowed to grow until colony formation (approximately 16 h). Colonies picked from selection plates were grown in Luria broth (LB)–AMP broth for ~16 h, after which the cells were harvested and plasmid DNA prepared by the alkaline lysis method (Ehrt & Schnappinger, 2003). Successful ligation was confirmed by PCR and Big Terminator Dye sequencing. The resulting plasmid was named pFA6-LUC*(−SKL)-His3MX6 (AddGene, Plasmid ID 40233).

To create a PCR-based gene fusion/deletion-compatible yEGFP1 plasmid, plasmid pKT128 (EUROSCARF) was digested with restriction enzymes PacI and AscI to remove the linker sequence (Sheff & Thorn, 2004). The yEGFP1 fragment was extracted from a gel and subsequently ligated into the 4.0 kb fragment of a PacI–AscI-digested pFA6-GFP(S65T)-His3MX6, as mentioned above, recovering compatibility with the PCR-based gene fusion/deletion primers. Transformation and verification were performed as described above. The new plasmid was named pFA6a-yEGFP1-His3MX6 (AddGene, Plasmid ID 40231). All DNA manipulations were performed using standard techniques unless otherwise stated (Sambrook & Russell, 2001).

To verify Luc*(−SKL)p activity, a promoter–reporter system was developed using PCR-mediated gene deletion (Longtine et al., 1998). Forward primer Hug1 KO Cass _F and reverse primer Hug1 KO Cass_R (Table 2.1) were designed to create a cassette containing the
LUC*(−SKL) and HIS3MX6 fusion flanked by 45 bp of homologous regions up- and downstream of the HUG1 ORF. The resulting PCR product was transformed into the yeast strain BY4743 (MATa/MATa his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/met15Δ0 ura3Δ0/ura3Δ0) (Open Biosystems, Lafayette, CO, USA) using the lithium acetate/single-stranded carrier DNA/polyethylene glycol (PEG) method (Gietz & Schiestl, 2007), resulting in the new strain, ABY700 (MATa/MATa his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/met15Δ0 ura3Δ0/ura3Δ0 hug1Δ::LUC*(−SKL)/HUG1). Transformants were plated on synthetic complete media minus histidine (SC–HIS) selective plates and grown until colony formation. Colonies were picked into liquid SC–HIS media and grown overnight, after which cells were collected and DNA extracted via the bust n’ grab method (Harju et al., 2004). Successful integration into the host genome was confirmed by PCR, using primers Hug1 KO Ver_F and pBluc* Ver_R (Table 2.1).

Strain ABY1101 (MATa/MATa his3Δ1/ his3Δ1 leu2Δ0/ leu2Δ0 met15Δ0/ met15Δ0 ura3Δ0/ ura3Δ0 hug1Δ::yEGFP1/HUG1) was constructed and confirmed identically to ABY700, with the exception that the PCR template was pFA6a-yEGFP1-HIS3MX6, resulting in the knockout of HUG1 with yEGFP1.

In order to demonstrate the flexibility of the luciferase reporter system to other promoters and utility in C-terminal fusion constructs, RNR3 was selected for further examination of gene deletion and TEF1 was chosen for gene fusion. RNR3 gene deletion and TEF1 C-terminal fusion both utilized pFA6a-LUC*(−SKL)-HIS3MX6 as construct templates. Primers used for RNR3 gene deletion were RNR3 KO Cass _F and RNR3 KO Cass_R; primers used for TEF1 C-terminal fusion were TEF1 Fusion Cass_F and TEF1 Fusion Cass_R (Table 2.1). The cassettes were transformed as previously described, resulting in the creation of strains ABY707.
Table 2.1. Sequence of primers used to construct and confirm all plasmids

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide Sequence (5' -- 3’)</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluc*-skl SmaI Cass_F</td>
<td>GCG GCG CCC GGG TTA ATT AAC ATG GAA GAC GCC AAA AAC ATA</td>
<td>PCR-Mediated Gene Deletion/Fusion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plasmid Construction</td>
</tr>
<tr>
<td>pBluc*-skl AscI Cass_R</td>
<td>GTC TGG CGC GCC TTA AAG CTT CTT TCC GCC CTT</td>
<td></td>
</tr>
<tr>
<td>pFA6 MCS US _F</td>
<td>CGC CAG CTT AAG CTT CGT A</td>
<td>GAL1 CEN/ARS Plasmid Construction</td>
</tr>
<tr>
<td>pFA6 MCS DS SalI _R</td>
<td>CGC GTC GAC ATT CGC TTA TTT AGA AGT GG</td>
<td></td>
</tr>
<tr>
<td>Hug1 KO Cass _F</td>
<td>TAT ATA TAA CTA CAA ACC ACA TCA GCA ATA AAA AAA AAC TAT ATG CGG ATC CCC GGG TTA ATT AA</td>
<td></td>
</tr>
<tr>
<td>Hug1 KO Cass_R</td>
<td>GTT CTT TCC TAT CAT TGG CCT ACA AAA AAA AAG AGA AGC ATG CTC GAA TTC GAG CTC GTT TAA AC</td>
<td>PCR-Mediated Gene Deletion</td>
</tr>
<tr>
<td>RNR3 KO Cass _F</td>
<td>AGC AAG AAT AGC AGC AGC AAT AAA TCA AAT ACT CCC ACA CAA ATG CGG ATC CCC GGG TTA ATT AA</td>
<td></td>
</tr>
<tr>
<td>RNR3 KO Cass_R</td>
<td>CGC TCC AAG TTA GAT AAG GAA AGG GAA AAA TGC CAC CAG AAA GAA GAA TTC GAG CTC GTT TAA AC</td>
<td></td>
</tr>
<tr>
<td>TEF1 Fusion Cass_F</td>
<td>C GCT AAG GGT ACC AAG GCT GCT CAA AAG GCT GCT AAG AAA CGG ATC CCC GGG TTA ATT AA</td>
<td>PCR-Mediated Gene Fusion</td>
</tr>
<tr>
<td>TEF1 Fusion Cass_R</td>
<td>A TAT AAA AGA TAT GCA ACT AGA AAA GTC TTA TCA ATC TCC GAA TTC GAG CTC GTT TAA AC</td>
<td></td>
</tr>
<tr>
<td>pBluc* Ver_F</td>
<td>AGA GAT ACG CCC TGG TTC CT</td>
<td>PCR Verification</td>
</tr>
<tr>
<td>pBluc* Ver_R</td>
<td>AAT CTG ACG CAG GCA GTT CT</td>
<td></td>
</tr>
<tr>
<td>Hug1 KO Ver_F</td>
<td>TCG GAC TTA CTC AAA GGG TTG</td>
<td></td>
</tr>
<tr>
<td>Rnr3 KO Ver_F</td>
<td>TTC GTC ACT TGG CTC AAC AG</td>
<td></td>
</tr>
<tr>
<td>TEF1 Fusion Ver_F</td>
<td>CGT CTG TGG TGA CGC TAA GA</td>
<td></td>
</tr>
<tr>
<td>pRS-GAL1 luc* Ver_F</td>
<td>CAT CGA CTG AAA TCC CTG GT</td>
<td></td>
</tr>
<tr>
<td>pRS-GAL1 yEGFP1 Ver_F</td>
<td>CAA GAC TGG ACC ATC ACC AA</td>
<td></td>
</tr>
<tr>
<td>pRS-GAL1 Ver_R</td>
<td>CCC TCC GAA GGA AGA CTC TC</td>
<td></td>
</tr>
</tbody>
</table>

Restriction sites are underlined. Conserved primer binding regions are in italics.
(MATa/MATa his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/met15Δ0 ura3Δ0/ura3Δ0 met15Δ0/met15Δ0 ura3Δ0/ura3Δ0 rnr3Δ::LUC*(−SKL)/HUG1) and MGBY710 (MATa/MATa his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/met15Δ0 ura3Δ0/ura3Δ0 TEF1–LUC*(−SKL)–HIS3/TEF1). Successful integration into the host genome was confirmed by PCR using primer pBluc* Ver_R and either primer Rnr3 KO Ver_F for RNR3 deletion or primer TEF1 Fusion Ver_F for TEF1 C-terminal fusion.

For protein half-life studies, strict regulation of reporter expression was required. pRS413-GAL1 (Mumberg et al., 1995), a YCp containing the GAL1 promoter with a CEN/ARS fragment, allows for induction of the GAL1 promoter in the presence of galactose and a non-inducing, non-repressive carbon source, such as raffinose. However, when switched to glucose, the transcription from the GAL1 promoter is strongly repressed. Utilizing this feature, reporters were placed under GAL1 control and monitored for protein degradation. To create P_GAL1-LUC*(−SKL), the LUC*(−SKL) fragment of pFA6a-LUC*(−SKL)-His3MX6 was copied by PCR using primers pFA6 MCS US _F and pFA6 MCS DS SalI _R (Table 2.1). These primers were designed such that the 5’-end of the fragment contained a portion of the MCS of the pFA6 plasmid, while the 3’ end introduced a SalI restriction enzyme site. The resulting PCR fragment was extracted once with phenol:chloroform and subsequently digested with SmaI and SalI restriction enzymes. The digested product was then ligated into a SmaI–SalI digested pRS413, as outlined above. 5 µl ligant were then transformed into DH5α cells in accordance with the manufacturer's protocol and transformants selected as described above. Successful ligation of the LUC*(−SKL) reporter under the GAL1 promoter was confirmed using primers pRS-GAL1 luc* Ver_F and pRS-GAL1 Ver_R (Table 2.1). The new plasmid was named pRS413-P_GAL1-LUC* (AddGene, Plasmid ID 40234). The P_GAL1-yEGFP1 plasmid was constructed in a similar manner with a few exceptions. Template pFA6a-yEGFP1-HIS3MX6 was used in place of pFA6a-
LUC*(−SKL)-HIS3MX6 to create the yEGFP1 cassette. Successful ligation of yEGFP1 under the GAL1 promoter was confirmed by primers pRS-GAL1 yEGFP1 Ver_F and pRS-GAL1 Ver_R. The new plasmid was named pRS413- P_GAL1-yEGFP1 (AddGene, Plasmid ID 40235).

Both pRS413- P_GAL1-LUC* and pRS413- P_GAL1-yEGFP1 were transformed into the BY4741 parent strain, resulting in the creation of strains MGBY001 and MGBY003, respectively. Transformants were selected as described above, and confirmed using the primers pRS-GAL1 Ver_R and either pRS-GAL1 yEGFP1 Ver_F or pRS-GAL1 luc* Ver_F, respectively.

2.2.2. Cell culture

Methyl methanesulphonate (MMS) (cat. no. 129925, Sigma-Aldrich, St. Louis, MO, USA) and was added to the cultures as stated below. For both LUC*(−SKL) and yEGFP1 expression assays, 10 ml preculture was grown in YPD overnight in a shaking incubator at 30°C and 150 rpm. For RNR3 knockout luciferase assays, log-phase cultures were diluted to OD600 = ~0.1 and subjected to either YPD for controls (no expression) or MMS for promoter induction. For HUG1 knockout luciferase assays, log-phase cultures were diluted in YPD to OD600 = ~0.1. MMS and/or YPD was added to the cultures, such that the final MMS concentrations were 0.00%, 0.005%, 0.01% and 0.05%. The cultures were grown for 2, 4, 6 and 8 h, then prepared for expression analysis. TEF1–LUC* fusion strains were grown overnight in YPD, diluted to OD = ~0.1 and allowed to grow to mid-log-phase (OD = ~0.6). The cultures were then diluted to OD = ~0.2 in YPD prior to taking measurements.

2.2.3. Fluorescence microscopy

To determine the localization of the Luc*(−SKL)p and yEGFP1p, control and induced populations were grown for 8 h as described in Materials and methods. At the indicated time, the entirety of the population was harvested by centrifugation. For Luc*(−SKL)p, an
immunofluorescent assay on fixed cells was then performed as described (Pringle et al., 1991), with no modification. Goat anti-luciferase primary antibody (cat. no. AB3256, Millipore, Temecula, CA, USA) was used at a dilution of 1:2000. Rhodamine-conjugated rabbit anti-goat secondary antibody (cat. no. AP106R, Millipore, Temecula, CA, USA) was reconstituted to 1 mg/ml in double-distilled water (ddH2O) and an equal volume of 100% glycerol and stored in a −20°C freezer until used. A dilution of 1:100 was used for the secondary antibody. Hoechst 33348 (cat. no. I34406, Molecular Probes, Eugene, OR, USA) was added to a final concentration of 1 μm as a nuclear stain. For yEGFP1p localization, intact cells were resuspended in phosphate-buffered saline (PBS). Hoechst 33342 was added to a final concentration of 1 μm as a nuclear stain. The cells were then wet-mounted on a microscope slide and imaged.

Images were acquired using a Zeiss Axio Observer Z1 inverted microscope fitted with an AxioCam MRm camera, and Pln APO 63×/NA 1.4 oil immersion objective with Optiovar 1.6×. Nuclear staining was imaged using Zeiss Filter set 49 (EX365, EM445/50); yEGFP1p was imaged using Zeiss Filter set 38 HE (EX470/40, EM525/50); and antibody-conjugated Luc*(−SKL)p was imaged using Zeiss Filter set 20 (EX546/12, EM575-640). Exposure times were set to 1000, 200 and 200 ms, respectively.

Images were analysed and deconvolved using ImageJ. The point-spread function (PSF) was calculated using the ImageJ plugin, Diffraction PSF 3D. The resulting PSF image was used as the PSF (kernel) image in the ImageJ plug-in, Iterative Deconvolve 3D (Dougherty, 2005), where the default/suggested settings were used.

2.2.4. FACS Analysis of hug1Δ::yEGFP1 expression

For each replicate, approximately 30 000 cells were counted using an Accuri C6 Flow Cytometer (BD Accuri Cytometers, Ann Arbor, MI, USA). GFP induction analysis was performed using Accuri C6 Sampler Analysis software and GraphPad Prism 5 software. Intact
cells were defined as whole cells (measured by forward light scattered) with intact membranes (measured by propidium iodide exclusion). yEGFP1p expression was determined by gating around these parameters.

2.2.5. Luciferase assay

D-Luciferin, sodium salt (cat. no. L37080, RPI Corp., Mount Prospect, IL, USA) was resuspended at a concentration of 20 mM in 50 mM citrate buffer, pH 5.0 (Gomori, 1955) and stored at −20°C until used. For intact cell readings, 100 µl each sample was removed from the growing culture in medium and added to a 12 × 75 mm polystyrene tube, then D-luciferin (100 µl, 2 mM) in 50 mM citrate buffer, pH 5.0, was added to the sample, briefly vortexed and read in an Optocomp I luminometer (MGM Instruments, Hamden, CT, USA). Results from the luminometer's data streaming function indicated maximum luciferase activity 25–35 s after addition of luciferin substrate (Figure 2.3.A). Thus, a 25 s delay and 10 s integration time was used to record the luminometer's arbitrary light unit (RLU) data. Over three independent trials, dosage was read in triplicate and only replicates with a coefficient of variance < 5% were kept.

Previous studies indicate that cell permeabilization may enhance luciferase activity in dual-luciferase assay systems (Liu et al., 2008; McNabb et al., 2005). To test the effect of permeabilization on the luciferase promoter–reporter construct, an assay similar to one previously established (McNabb et al., 2005), utilizing a passive lysis buffer (PLB; cat. no. E1941, Promega, Madison, WI, USA) and the luciferase assay system (cat. no. E1500, Promega) was developed to compare results received from permeabilization to those of intact cells. PLB and luciferase assay buffer + reagent (LABR) from the luciferase assay system were prepared according to manufacturer's instructions. For the assay, 10 µl cells was removed from a growing culture and added directly to 100 µl 1× PLB. After 10 s of lysis, 10 µl this mixture was
transferred to 100 µl LABR, followed by a 10 s delay and a 10 s integration time. These steps were repeated for three independent trials for all integrated promoter–reporter constructs.

2.2.6. Protein half-life assay

D-Galactose (cat. no. G0750, Sigma-Aldrich, St. Louis, MO, USA), d-raffinose (cat. no. R0514, Sigma-Aldrich) and dextrose anhydrous (cat. no. BP350, Fisher BioReagents) were suspended in ddH2O to a final concentration of 20% and filter-sterilized before use. To maintain the plasmid throughout growth, cultures containing pRS413-GAL1 were grown in selective media. Strains were also grown under constant galactose induction to ensure steady-state expression of the reporter, whereupon constant levels of reporter are achieved due to the equilibrium of reporter degradation and production. Thus, when GAL1 promoter transcription is ultimately terminated upon transfer to dextrose, no additional reporter is made and loss of protein signal can be attributed to protein degradation. This method has been successfully utilized in other studies to determine the half-life of both firefly luciferase (McNabb et al., 2005) and cyan-fluorescent proteins (Hackett et al., 2006).

Starter cultures of strains MGBY001 and MGBY003 were seeded into 5 ml SC–HIS plus 2% D-galactose and 1% D-raffinose and grown overnight. The next morning, cultures were diluted to an OD600 = ~0.2 in 5 ml SC–HIS plus 2% D-galactose and 1% D-raffinose and grown for an additional 4 h. At this point, the entirety of the culture was centrifuged, washed twice in equal volumes of PBS, and diluted in SC–HIS plus 2% dextrose to a final OD600 = 0.2. Samples were taken immediately after switching to dextrose. For luminescence assays, triplicate samples were read every 30 min. Prior to reading, the sample volume was adjusted using SC–HIS plus 2% dextrose, such that the final OD600 = ~0.2. Once adjusted, luciferase activity was read as described above. For fluorescence assays, triplicate samples were harvested every hour. On the hour, exactly 1 ml culture was centrifuged, washed twice in PBS, resuspended in 1 ml PBS and
immediately taken to be analysed by flow cytometry. Gating parameters for yEGFP1 were identical to those previously described.

2.2.7. Statistical analysis

For promoter induction assays, yEGFP1p fluorescence as a function of dose and time was determined via flow cytometry, by taking the geometric mean fluorescence intensity in channel FL1 for at least three independent trials. Fold induction was defined as the ratio of the geometric mean in the green channel (FL1) of induced populations over control (no induction) populations for each trial. A Tukey's multiple comparison test with repeated measures was used to determine the p value for GFP fold induction values across all trials and doses for each individual time. The GFP induction was then averaged for all trials with the same dose and time of exposure, and the standard deviation (SD) was calculated. Criteria for positive results (i.e. no auto- or media fluorescence) were established as GFP fold induction > 2 and p ≤ 0.05.

Luciferase activity was calculated using the raw RLU value from the luminometer for at least three independent trials. Fold induction was defined as the ratio of RLU of induced populations with addition of D-luciferin substrate over RLU of control (no induction) populations, with addition of D-luciferin substrate for each trial. Statistical analysis utilized for yEGFP1p data was also performed on the luciferase activity data. Criteria for a positive result were established as luciferase fold induction > 2 and p ≤ 0.05.

For yEGFP1 protein half-life determinations, absolute fluorescence was obtained via flow cytometry and determined as the summation of the FL1 values for all events for at least three independent trials. Since identical cell counts were used in analysis, this value represents the total fluorescence value for a constant population of cells. A decrease in absolute fluorescence intensity can thus be correlated to the loss of protein due to degradation. The percentage of initial fluorescence was then determined to be the ratio of the absolute
fluorescence through time to the absolute fluorescence of the initial time point. Protein degradation was found to be first order by fit to a non-linear, exponential decay equation.

Similar to yEGFP1p, luciferase protein half-life determinations were performed on a culture of a constant cell count by adjusting the sample to OD = 0.2 prior to reading. Luciferase activity was calculated using the raw RLU value from the luminometer for at least three independent trials. The percentage of initial luminescence was calculated as the ratio of the raw RLU value through time vs the initial raw RLU value. Luc* (−SKL) protein degradation was also found to be first order by fit to a non-linear, exponential decay equation.

2.3. Results and discussion

2.3.1. Successful integration and functional expression of cytosolic-expressing luciferase into the PCR-mediated gene deletion/fusion plasmid

Previous works have suggested that deletion of the C-terminal peroxisome localization signal, SKL, leads to cytosolic localization of luciferase (Gould et al., 1988; Leskinen et al., 2003). In this study, this cytosolic-expressing luciferase was introduced into a PCR-mediated gene deletion/fusion plasmid construct (Longtine et al., 1998). The one-step PCR gene deletion/fusion process used to construct knock-out or fusion strains provides a simple method of integrating single copies of reporters into the genome under promoter control, without prior knowledge of the promoter sequence. This method also allows direct comparisons of sensitivity and expression levels to fluorophore-based sensors created in the same manner. Sequencing data (Figure 2.1) demonstrates successful integration of LUC* (−SKL) into the gene deletion plasmid. Biosensor reporter systems are typically designed to respond to environmental stresses, such as heavy metals (Corbisier et al., 1999) and DNA damage (Afanassiev et al., 2000; Benton et al., 2007). Characterizations of temporal and dosage responses of Hug1-based GFP reporters have been established in great detail for multiple DNA damaging agents (Benton et al., 2007). Of the
DNA-damaging agents studied, methyl methanesulphonate (MMS) induces a highly characteristic dose–response from $P_{HUG1}$-GFP reporter constructs, where expression levels show a biphasic response centred around the 0.01% MMS dosage (Benton et al., 2007).

Due to this distinctive response, the $HUG1$ promoter was chosen for the $LUC^*(−SKL)$ reporter construct. Utilizing the engineered plasmid as a template, further confirmation of compatibility with PCR-mediated gene deletion was undertaken by knocking out $HUG1$ and replacing it with the $LUC^*(−SKL)$ reporter, thereby placing the reporter under genomic control of the $HUG1$ promoter. To demonstrate promoter flexibility of the plasmid constructs, the $LUC^*(−SKL)$ reporter was also placed under RNR3 promoter control by gene deletion. Also, yEGFP1 was placed under $HUG1$ promoter control as a positive control for strain-specific sensitivity and expression levels.

Similar to $HUG1$, RNR3 shows a peak of expression when exposed to 0.01% MMS for ≥ 6 h (Ochi et al., 2011). These conditions were utilized to demonstrate functional expression of Luc*(−SKL)p from the RNR3 promoter. Finally, $LUC^*(−SKL)$ was integrated at the C-terminal of $TEF1$ to validate reporter utility in fusion constructs. Due to the constitutive expression of the $TEF1$ promoter, fusions to the C-terminal of $TEF1$ express high levels of reporter in functional constructs. Functional luciferase activity was reported in all three constructs (Table 2.2). Briefly, $hug1Δ::LUC^*$ and $rnr3Δ::LUC^*$ gene deletions show a 38-fold and 29-fold increase over the control samples, respectively. $TEF1–LUC^*$ also shows clear expression of functional luciferase, with RLU values nearly twice as large as induced $hug1Δ::LUC^*$ deletion strains. Other studies have indicated that permeabilization of samples prior to reading increases the sensitivity of the luciferase assay (Liu et al., 2008; McNabb et al., 2005; Vieites et al., 1994). Permeabilization of yeast cultures using proprietary detergents has
been extensively investigated and found to have an immediate improvement on luciferase activity, but a long-term detrimental effect if permeabilization extends outside of the ideal read-interval (McNabb et al., 2005).

This observation was consistent with experiments performed on the construct developed (data not shown). To further investigate permeabilization effects on luciferase activity, experiments were performed within the ideal read-time indicated by McNabb et al. (McNabb et al., 2005). Results given in Table 2.2 show a large increase (9–40-fold) in the RLU values of permeabilized cells when normalized to method-related dilutions. Thus, the sensitivity of permeabilized cultures is significantly higher than intact cell readings when viewing raw RLU data. However, when RLU values were normalized to control samples for fold induction analysis, the difference between the two methods was less pronounced. As such, permeabilization appears to increase the lower detection limit and may prove more useful than

![Sequencing data and general characteristics of plasmid pFA6a-LUC*(−SKL)-His3MX6: CFBS, conserved forward binding site; CRBS, conserved reverse binding site.](image)

Figure 2.1. Sequencing data and general characteristics of plasmid pFA6a-LUC*(−SKL)-His3MX6: CFBS, conserved forward binding site; CRBS, conserved reverse binding site.
the intact cell method for detecting weak promoters. However, if relative data of moderately-expressing promoters is required, the intact cell method provides accurate data in an equally efficient and inexpensive manner. Further, trends obtained through the use of one-step, intact cell readings coincide well with data previously reported for other genotoxicity assays using the dual luciferase system (Liu et al., 2008). Most importantly, previous implementation of the permeabilization technique was performed in the context of the dual-luciferase assay, where access to both the firefly luciferase inhibitor and Renilla luciferase add additional concerns. Clearly, in these types of measurement, permeabilization is required for accurate readings. However, our study focuses on the creation of constructs capable of integrating a single

Table 2.2. Relative light units (RLU), fold induction (FI), and sensitivity data of Luc*(−SKL)p expressed as a promoter-reporter and C-terminal fusion

<table>
<thead>
<tr>
<th>Strain</th>
<th>Promoter Control</th>
<th>Permeabilization (RLU/1e4)a</th>
<th>Sensitivityb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em><em>hug1Δ::LUC</em>(−SKL)</em>*</td>
<td>No induction</td>
<td>2.2 ± 0.2</td>
<td>52.9 ± 7.2</td>
</tr>
<tr>
<td></td>
<td>MMS-induced FIc</td>
<td>88.1 ± 5.6</td>
<td>802 ± 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>38 ± 3</td>
<td>15 ± 3</td>
</tr>
<tr>
<td><em><em>rnr3Δ::LUC</em>(−SKL)</em>*</td>
<td>No induction</td>
<td>0.9 ± 0.1</td>
<td>11.3 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>MMS-induced FIc</td>
<td>25.6 ± 1.5</td>
<td>402 ± 50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29 ± 5</td>
<td>38 ± 10</td>
</tr>
<tr>
<td><em><em>TEF1-LUC</em>(−SKL)</em>*</td>
<td>Constitutive</td>
<td>90.6 ± 3.3</td>
<td>3620 ± 230</td>
</tr>
</tbody>
</table>

a RLU values are expressed as the raw RLU value from the luminometer multiplied by the method-related dilution factor.
b Sensitivity is calculated as the ratio of RLU of permeabilized samples versus RLU of intact sample.
c Fold induction is calculated as the ratio of MMS-induced samples vs samples with no induction.
luciferase gene into the genome and therefore does not have the additional restrictions of the dual-luciferase assay.

2.3.2. Confirmation and localization of Luc*(−SKL)p and yEGFP1 by fluorescence microscopy

Cytosolic localization increases the luciferase activity in intact yeast cells, presumably due to increased availability of luciferase to its substrate (Leskinen et al., 2003). Thus, any modified luciferase must be evaluated for proper localization to ensure that optimal activity will be obtained. To confirm localization of Luc*(−SKL)p to the cytoplasm, an immunofluorescent assay was performed on control populations and populations induced with 0.01% MMS for 8 h. Antibody-conjugated Luc*(−SKL)p appears to be evenly distributed in the cell cytoplasm, with little organelle-specific localization (Figure 2.2), consistent with previous reports (Leskinen et al., 2003). Some nuclear localization was noted; however, this appears to be a bleed-through artifact from out-of-focus light (Figure 2.2, arrow). Expression levels were also notably higher than control populations (Figure 2.2). Similarly, confirmation and localization of a successful hug1Δ::yEGFP1 construct was determined in MMS-induced and uninduced strains under the same conditions. yEGFP1p is evenly distributed throughout the cell; unlike Luc*(−SKL)p, yEGFP1p demonstrates clear nuclear localization (Figure 2.2, arrow). This distribution is consistent with GFP expression patterns seen in other eukaryotes (Ogawa et al., 1995).

2.3.3. Dose–response of hug1Δ::LUC*(−SKL) and hug1Δ::yEGFP1 to MMS

To determine the effectiveness of the construct as a DNA-damage reporter, luciferase expression in response to MMS was monitored using the hug1Δ::LUC*(−SKL) promoter–reporter system and compared to HUG1::yEGFP1. As previously reported, HUG1 promoter–reporter systems demonstrate a biphasic response to MMS exposure, with a maximum centred at 0.01% MMS (Benton et al., 2007). Dosage levels of 0.005%, 0.01% and 0.05% MMS were chosen, as these levels are sufficient in resolving the biphasic response. A biphasic response of
the hug1Δ::LUC*(−SKL) construct can be seen within 2 h of induction, and continues through the 8 h time points (Figure 2.3.B). Similarly, a biphasic response of the hug1Δ::yEGFP1 construct can be resolved within 2 h of induction, continuing for the duration of the 8 h experiment. Both constructs displayed maximum induction at 0.01% MMS. Clearly, the LUC*(−SKL) reporter accurately depicts qualitative information comparable to the yEGFP1 positive control constructs. More importantly, the luciferase assay is significantly quicker to perform, requiring no processing prior to data obtainment. Real-time acquisition of luciferase activity took in the order of minutes per sample, while yEGFP1 constructs required hours for processing and flow-cytometry data acquisition. The simplicity and ease of the luciferase assay is therefore more accommodating for high-throughput assays. Significant differences of temporal response were noted between the hug1Δ::yEGFP1 positive control and the hug1Δ::LUC*(−SKL) strain. A two-way ANOVA with Bonferroni multiple comparisons was performed to analyse any
significant changes across time. If no significant differences were noted, the population was considered to have reached an expression level steady state.

After the 2 h time point, the $hug1\Delta::LUC^*(-SKL)$ construct reveals no statistical difference for $p < 0.05$ of the 0.005% dose fold induction. The $hug1\Delta::yEGFP1$ construct, on the other hand, shows no statistical difference for $p < 0.05$ after the 4 h time point. Therefore, for the 0.005% dosage, Luc*($-SKL$)p reaches a steady-state much earlier than yEGFP1p. For the 0.01% dose, $hug1\Delta::LUC^*(-SKL)$ shows significance to $p < 0.001$ throughout the 8 h experiment. $hug1\Delta::yEGFP1$, on the other hand, shows no significant difference for $p < 0.05$ after the 6 h time point. Unlike at the lower dosage, yEGFP1p appears to reach a steady-state expression before luc*–SKL. For the 0.05% dose, $hug1\Delta::LUC^*(-SKL)$ also shows no significant
difference for \( p < 0.05 \) of fold inductions after the 6 h time point. \( hug1A:yEGFP1 \) demonstrates significance for \( p < 0.0001 \) throughout the experiment. The highest dosage, therefore, mirrors the lowest dosage with respect to steady-state expression levels, with \( \text{Luc}^*(-SKL)p \) reaching steady state before \( yEGFP1p \).

Differences between the reporters may be attributed to the half-life of the respective reporter. Firefly luciferase has been shown to have a half-life of approximate 96 min in vivo (McNabb et al., 2005), while GFP variants have a half-life which lasts anywhere from 3.3 h (Chambers et al., 2004) to 7 h in vivo (Mateus & Avery, 2000; Natarajan et al., 1998). For half-life determinations of \( \text{Luc}^*(-SKL)p \) and \( yEGFP1p \) in the strains utilized in this study, an assay similar to one used to determine the half-life of cyan fluorescent proteins (CFP) (Hackett et al., 2006) was implemented. In short, \( LUC^*(-SKL) \) and \( yEGFP1 \) were placed under \( GAL1 \) promoter control and assayed using galactose–glucose promoter regulation. Prior to \( GAL1 \) half-life experiments, the minimal induction time for \( LUC^*(-SKL) \) after induction with galactose was determined to be approximately 40 min (Figure 2.4.A). However, after 120 min, luminescence continued to increase without reaching a stability limit. Thus, in order to achieve steady-state expression, cultures were grown overnight in galactose induction and diluted into galactose-containing medium the next morning. Time points immediately following the carbon shift were taken and used to calculate the half-lives of the reporters.

A lag of approximately 1 h was seen in both luciferase and fluorescence assays performed (data not shown). Data received from both assays were plotted (Figure 2.4.B, C) and fitted to first-order exponential decay kinetics. Results from these analyses indicate a half-life for \( \text{Luc}^*(-SKL)p \) of 98 min, which correlates well with the 96 min previously reported (McNabb et al., 2005). \( yEGFP1p \) has a half-life of 184 min, which correlates closely with other reports of
3.3h using GFP and alternative carbon source induction (Chambers et al., 2004), but is significantly less than the 7h half-life reported using yEGFP3 and cycloheximide (Mateus & Avery, 2000). Despite this, luciferase appears to degrade nearly twice as quickly as yEGFP1p in vivo, according to these data. yEGFP variants, therefore, may show a high level of accumulation prior to reaching an expression steady state. As luciferase has a much shorter half-life, it is conceivable that these reporter constructs would reach the expression steady state more quickly than the yEGFP1 counterpart. This trend can be seen in a comparison of the significance values of 0.005% and 0.05% dose in the two reporters. Luc*(−SKL)p appears to reach an expression steady-state at the 2h time point for 0.005% dosage and the 6h time point for 0.05% dosage.

yEGFP1p, on the other hand, reaches an expression steady state at the 4h time point for the 0.005% dosage and does not reach a steady state for the 0.05% dosage. yEGFP1p's high stability in vivo leads to a pronounced accumulation effect under constant induction. Luc*(−SKL)p, on the other hand, readily degrades between assay time points. Due to this, each reporter assumes a specific application in the context of biosensors. If real-time data of a promoter system are required, Luc*(−SKL)p would be ideal, due to its relatively short half-life in vivo. Alternatively, yEGFP1 constructs amplify promoter signals through the accumulation
effect. This could be useful if the promoter itself is weak or the promoter experiences weak induction.

We have successfully constructed a plasmid that allows for both simple, one-step PCR-mediated gene deletion and fusion and luciferase expression under a genomic promoter of choice, and a one-step measurement of luciferase from intact yeast cells. Results are shown to be highly reproducible and correlate well with comparable reporter constructs. We have applied this construct directly to DNA damage biosensing; however, its use extends to any application where promoter response levels are desired.

2.4. References


CHAPTER 3. CYTOPLASMIC LOCALIZATION OF HUG1P, A NEGATIVE REGULATOR OF THE MEC1 PATHWAY, COINCIDES WITH THE COMPARTMENTALIZATION OF RNR2P-RNR4P

3.1. Introduction

Cellular survival in response to DNA lesions and replication arrest requires the coordination of checkpoint-mediated mechanisms to ensure DNA damage repair, cell cycle arrest, and recovery for genome stability. Checkpoint pathways regulate the expression of protein kinases, which mediate a transcriptional response and cell cycle arrest through downstream effectors. In *Saccharomyces cerevisiae*, the evolutionarily conserved *MEC1* (ortholog to the human ataxia telangiectasia mutated- and Rad3-related – ATR – protein) checkpoint pathway regulates origin firing, fork progression, and DNA repair and recovery (reviewed in (Nyberg et al., 2002)).

Mec1p and its effector kinases, Rad53p and Dun1p, activate both positive and negative effectors that regulate deoxyribonucleotide (dNTP) pools, cell cycle arrest, and recovery (Nyberg et al., 2002). The activity of the positive effector RNRs (Ribonucleotide reductases), which are responsible for the rate-limiting conversion step of ribonucleotides (rNDPs) to dNTPs, is tightly regulated. The homodimer Rnr1p and the heterodimers Rnr2p and Rnr4p, which compose the Rnr complex, are transcriptionally repressed by Crt1p (Basrai et al., 1999; Huang et al., 1998) while Rnr1p contains binding sites for dATP allosteric inhibition (Nguyen et al., 1999). In the absence of DNA damage, negative regulators such as Sml1p and Dif1p regulate Rnr complex activity through inhibition of the Rnr subunit, Rnr1p, and by subcellular compartmentalization of the Rnr2p–Rnr4p subunits to the nucleus (Lee et al., 2008; Wu & Huang, 2008). However, in

response to DNA damage or replication arrest, Sml1p and Dif1p are phosphorylated and degraded (Andreson et al., 2010; Lee et al., 2008). This, along with the transcriptional induction of RNRs and localization of the Rnr complex to the cytoplasm, serves to increase dNTP pools (Andreson et al., 2010; Lee et al., 2008; Zhao, X. & Rothstein, 2002). Checkpoint mediated response to DNA damage and replication arrest has been studied extensively, however we do not fully understand how cells recover from checkpoint arrest and downregulate Rnr activity to maintain genome stability.

In this paper, we examined the role of Hug1p (Hydroxyurea, Ultraviolet, Gamma) as a negative regulator of the MECl pathway. HUG1 is one of the most differentially expressed genes identified in a screen for gene expression in response to HU treatment (Velculescu et al., 1997). Unlike SML1 and DIF1, the transcription of HUG1 is induced in cells treated with HU or exposed to ultraviolet or gamma radiation in a MECl-dependent manner (Basrai et al., 1999). A deletion of HUG1 has been shown to rescue lethality due to a MECl null allele and suppress the HU sensitivity of dun1Δ mutants (Basrai et al., 1999). Studies with HUG1 have primarily focused on its transcriptional response to replication arrest and DNA damage (Ainsworth et al., 2012; Basrai et al., 1999; Benton et al., 2007; Sharma et al., 2007; Wei et al., 2013). Using a polyclonal serum to Hug1p, we have shown that HUG1 encodes for a protein. Our results for HUG1 overexpression phenotypes, a delayed induction pattern of Hug1p in response to HU treatment, and the MECl-dependent compartmentalization of Hug1p in response to replication arrest define a novel role for Hug1p as a negative regulator of the MECl-checkpoint response through its compartmentalization with Rnr2p–Rnr4p (Yao et al., 2003).
3.2. Materials and methods

3.2.1. Strains, plasmids, cell cycle arrest with HU and growth sensitivity to HU, MMS and BLM

Strains and plasmids are described in Table 3.1. Transformations, cloning, and cell culture were performed using standard methods as previously described (Adams et al., 1997; Basrai et al., 1999; Mumberg et al., 1994; Zhao, Xiaolan et al., 1998). Additional strain and expression vector construction procedures are outlined in the Supplementary materials and methods. Primer sequences are available upon request. Cell cycle arrest with 0.1 M HU (Fluka Chemika) was as described (Basrai et al., 1999). Cells (>90%) exhibited a large budded phenotype with S-phase DNA content, as determined by flow cytometry using an Accuri C6 flow cytometer (BD Accuri Cytometers) (Carter et al., 2005). Serial dilutions of cells grown in medium selective for the plasmid were assayed for growth with dextrose (2%) or raffinose (2%) plus galactose (2%) with 0.1 M HU, 0.01% MMS (Sigma–Aldrich), or 5 mU/mL BLM (Bristol-Myers Squibb) as described (Carter et al., 2005).

3.2.2. Western blots and subcellular fractionation

Western blots for Hug1p, Hug1p-GFP, Rnr3p-HA, Sir2p, Pgk1p, Tub2p and subcellular fractionation were performed as described (Carter et al., 2005; Liang & Stillman, 1997) using anti-HA (12CA5 Roche), -GFP (A11122 Invitrogen), -Sir2p (yN-19) (sc-6666 Santa Cruz), -Pgk1p (459250 Invitrogen), -Hug1p and -Tub2p (antisera generated in Basrai Laboratory).

3.2.3. Localization of Hug1p-GFP

Hug1p-GFP expressing strains were grown to exponential phase in YPD and treated with 0.1 M HU for 3.5 h. For localization of GAL1-HUG1-GFP, cells were grown to exponential phase in synthetic medium with raffinose (2%) followed by growth in galactose (2%) medium for 2 h, shifted to dextrose (2%) medium with or without 0.1 M HU for 3.5 h. Harvested cells were prepared for microscopy as described (Pringle et al., 1991) except that paraformaldehyde
was the only fixative and Hoechst 33342 (Thermo Scientific) was used for nuclear staining.

Images were acquired using a Zeiss Axio Observer Z1 microscope.

Image deconvolution and analysis were performed in ImageJ using plug-ins Diffraction PSF 3D to calculate the point-spread-function and Iterative Deconvolve 3D (Dougherty, 2005) for deconvolution. Localization analysis was performed as described (Malinovska et al., 2012). Cytoplasmic localization was determined empirically to be a nuclear-to-cytoplasmic

Table 3.1. Genotypes of strains and plasmids.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>U952-3C</td>
<td>W1588-4A * sml1Δ::HIS3</td>
<td>(Zhao, Xiaolan et al., 1998)</td>
</tr>
<tr>
<td>U953-61D</td>
<td>W1588-4A mec1Δ::TRP1 sml1Δ::HIS3</td>
<td>(Zhao, Xiaolan et al., 1998)</td>
</tr>
<tr>
<td>U960-5C</td>
<td>W1588-4A rad53Δ::HIS3 sml1-1</td>
<td>(Zhao, Xiaolan et al., 1998)</td>
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<tr>
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<td>(Zhao, Xiaolan et al., 1998)</td>
</tr>
<tr>
<td>W1588-4A</td>
<td>MATα ade2-1 can1-100 leu2,3-112 his3-11,15 trp1 ura3-1 RAD5</td>
<td>(Zhao, Xiaolan et al., 1998)</td>
</tr>
<tr>
<td>Y217</td>
<td>MATα ade2-1 his3 leu2-3,112 lys2 trp1 ura3-A100 rrn3::RNR3-URA3-TRP1 crt4-2/tup1</td>
<td>(Huang et al., 1998)</td>
</tr>
<tr>
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<td>(Huang et al., 1998)</td>
</tr>
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<td>YPH499 huglΔ2::HIS3</td>
<td>This study</td>
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<td>YPH499 * HUG1-GFP-HIS3</td>
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<tr>
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<tr>
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<td>MATα ade2-101 leu2-Δ1 lys2-801 his3-Δ200 trp1-Δ63 ura3-52</td>
<td>(Sikorski &amp; Hieter, 1989)</td>
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<tr>
<th>Plasmids</th>
<th>Genotype</th>
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<td>pMB368</td>
<td>pRS426-P<em>GAL1-HUG1</em> (URA3, 2μ)</td>
<td>This study</td>
</tr>
<tr>
<td>pMB379</td>
<td>pRS426-P*GAL1-HUG1 (URA3, 2μ)</td>
<td>This study</td>
</tr>
<tr>
<td>pMB394</td>
<td>pRS424-P*GAL1-HUG1 (TRP1, 2μ)</td>
<td>This study</td>
</tr>
<tr>
<td>pMB444</td>
<td>pRS316-P*HUG1-HUG1 (URA3, CEN)</td>
<td>This study</td>
</tr>
<tr>
<td>pMB588</td>
<td>pRS415-HUG1-GFP (LEU2, CEN)</td>
<td>This study</td>
</tr>
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<td>pMB734</td>
<td>pRS414-P*GAL1-HUG1-GFP (TRP1, CEN)</td>
<td>This study</td>
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<tr>
<td>pMB830</td>
<td>pRS415-P*GAL1-HUG1-GFP (LEU2, CEN)</td>
<td>This study</td>
</tr>
</tbody>
</table>

was the only fixative and Hoechst 33342 (Thermo Scientific) was used for nuclear staining.

Images were acquired using a Zeiss Axio Observer Z1 microscope.

Image deconvolution and analysis were performed in ImageJ using plug-ins Diffraction PSF 3D to calculate the point-spread-function and Iterative Deconvolve 3D (Dougherty, 2005) for deconvolution. Localization analysis was performed as described (Malinovska et al., 2012). Cytoplasmic localization was determined empirically to be a nuclear-to-cytoplasmic

††‡ Table 3.1 appears in the original publication as Supplementary Table 1.
intensiometric ratio below 0.9, even distribution between 0.9 and 1.1, and nuclear localization above 1.1. For each strain, at least 100 large budding cells with a nucleus at the bud neck were counted. Statistical analysis on the subcellular localization data was performed in SAS 9.3 using three-way factorial analysis of variance (ANOVA) with Tukey’s multiple comparison range test. Additional statistical analysis is provided in the supplementary materials and methods.

3.3. Results

3.3.1. Overexpression of HUG1 increases the sensitivity of wild-type strains to HU, MMS and BLM

We have previously shown that a deletion of HUG1 suppresses the viability of mec1Δ strains and HU sensitivity of dun1Δ strains (Basrai et al., 1999). Similar results have been reported in the HUG1 paralogs, DIF1 and SML1, both of which are negative regulators of the Rnr complex and the checkpoint response (Andreson et al., 2010; Lee et al., 2008; Zhao, Xiaolan et al., 1998). To determine if Hug1p acts as a negative regulator of the MEC1 pathway, wild-type strains overexpressing HUG1 were assayed for growth on media containing HU and DNA damaging agents. GAL1-HUG1 was found to increase the sensitivity of wild-type strains to HU on medium containing galactose (GAL) and 0.15 M HU (Figure 3.1 A, Row 2). GAL1-HUG1 or vector (Figure 3.1 A, Rows 2 and 1, respectively) did not show growth defects on dextrose (DEX) plates with and without HU and GAL plates without HU. The phenotype was specifically due to expression of Hug1p, as a frame-shift mutation in the HUG1 open reading frame (GAL1-HUG1*) abolished the dosage lethality phenotype (data not shown).

Since the viability of mec1Δ strains is suppressed by sml1-1 or sml1Δ (Andreson et al., 2010; Lee et al., 2008; Zhao, Xiaolan et al., 1998), the dosage lethality of GAL1-HUG1 in wild-type strains was examined for dependence on SML1. Similar to the wild-type strain, the sml1Δ strain with GAL1-HUG1 exhibited growth inhibition on HU containing medium (Figure 3.1 A,
Row 6). Results verifying that SML1 is not required for the dosage lethality of strains containing GAL1-HUG1 are supported by recent work describing the ubiquitylation and subsequent degradation of Sm1p in response to DNA damage (Andreson et al., 2010).

As expected, the mec1Δsml1Δ strain was sensitive to growth on HU containing plates with or without GAL1-HUG1 (Figure 3.1.A, Rows 3 and 4). mec1Δsml1Δ strains expressing GAL1-HUG1 also showed a slow growth phenotype even in the absence of HU (Figure 3.1.A, Row 4, center panel). These results are similar to the negative regulator, DIF1, which displays dosage lethality in mec1Δsml1Δ strains (Lee et al., 2008).

In addition to HU sensitivity, GAL1-HUG1 strains exhibited significant growth inhibition on MMS and BLM containing media (Figure 3.1.B, Row 3) when compared with empty-vector strains (Figure 3.1.B, Row 2). As expected, the mec1Δsml1Δ strain displayed growth inhibition.

Figure 3.1. Overexpression of HUG1 sensitizes growth to HU, BLM and MMS. (A) Serial dilutions of wild-type strain (WT, W1588-4A), mec1Δsml1Δ (U953-61A) and sml1Δ (U952-3B) with vector (pRS316) or GAL1-HUG1 (pMB379) were grown on plates with dextrose (DEX), galactose (GAL) or galactose with 0.15 M HU (GAL + HU) and incubated at 30° for 2–3 days. (B) Serial dilutions of mec1Δsml1Δ (U953-61A) or hug1Δ (YMB847) with vector (pRS414-GAL1) or GAL1-HUG1 (pMB394) were grown on plates with galactose (GAL), galactose with 5 mU/mL BLM (GAL + BLM), or 0.01% MMS (GAL + MMS) and incubated at 30° for 2–3 days.
on plates containing MMS and BLM (Figure 3.1.B, Row 1). Taken together, the synthetic dosage lethality of GAL1-HUG1 strains along with previous data support a role for Hug1p as a negative regulator of the MEC1-mediated checkpoint response to DNA damage and replication arrest.

3.3.2. Expression of Hug1p shows delayed induction to 0.1 M HU compared to Rnr3p, a positive effector of the MEC1 pathway

Using transcriptome profiling, we previously reported that HUG1 represents one of the most highly differentially expressed genes in the yeast genome (Basrai et al., 1999; Velculescu et al., 1997). Initial genome sequencing efforts annotated all ORFs of at least 100 contiguous codons, hence HUG1 was not annotated as it encodes for a protein of 68 amino acids. To validate that HUG1 encodes for a protein, a rabbit polyclonal serum specific to Hug1p was generated. Results from Western blot analysis corroborated results of Northern blot analysis (Basrai et al., 1999), as Hug1p expression was observed in a wild-type strain treated with HU (Figure 3.2.A, Lane 2). The control includes a hug1Δ strain that shows Hug1p expression when transformed with a plasmid expressing HUG1 from its own promoter (pHUG1; Figure 3.2.A, Lane 6). In agreement with previous results, tup1 and crt1Δ strains constitutively expressed Hug1p (Figure 3.2.A, Lanes 7–10). Crt1p, Tup1p and Ssn6p are transcriptional repressors that bind to X-box sequences in the promoter of HUG1 and RNRs in the absence of DNA damage and replication arrest (Basrai et al., 1999). The polyclonal serum also showed that, in agreement with previous Northern blot analysis, no HU induced expression of Hug1p was detected in mec1Δsml1Δ strains (Figure 3.2.B, Lane 6) and SML1 was not required for the expression of Hug1p (Figure 3.2.B, Lane 4).

To gain further insight into the role of Hug1p, HU induced expression of Hug1p was compared with Rnr3p, a positive regulator of the MEC1 pathway. Hug1p expression was detected 1.5 h post-HU addition and increased until approximately 3.5 h post-HU addition after
Figure 3.2. Genes in the *MEC1* pathway are required for HU induced expression of Hug1p and delayed induction of Hug1p compared to Rnr3p. (A) Western blot analysis of wild-type (WT, YPH499), *hug1A* (YMB847), *hug1A* (YMB847) with pHUG1 (pMB444), *tup1A* (Y217), *crt1A* (Y577) grown with or without 0.1 M HU for 3.5 h and probed with anti-Hug1p or -Pgk1p (loading control). (B) Western blot analysis of wild-type (WT, W1588-4A), *sml1A* (U952-3C), *mec1A sml1A* (U953-61D) grown with or without 0.1 M HU for 3.5 h (C) Western blot analysis of *RNR3-HA* strains (YMB1657) after treatment with 0.1 M HU for various times and probed with anti-Hug1p, -HA (Rnr3p-HA) and -Tub2p (loading control).
which no further induction was apparent (Figure 3.2.C). Consistent with previous reports (Huang et al., 1998), Rnr3p-HA was detected 30 min post-HU addition, increased until 90–120 min post-HU addition, and subsequently declined (Figure 3.2.C). The delayed induction of Hug1p with high levels present at 3.5–5 h post-HU addition resembles the profile of Crt1p (Huang et al., 1998), a negative regulator of RNRs and HUG1 gene expression.

3.3.3. Hug1p-GFP localizes to the cytoplasm in HU treated cells

The subcellular localization of Hug1p-GFP was analyzed by fusing GFP to the C-terminus of Hug1p expressed from its native promoter at the chromosomal locus in the genome. Western blot analysis showed expression of Hug1p-GFP in cells treated with HU (Figure 3.3.A). Fluorescence microscopy of Hug1p-GFP cells without HU treatment showed only background fluorescence (Figure 3.3.B, left column).

However, upon treatment with HU, Hug1p-GFP was enriched in the cytoplasm and was notably excluded from the nucleus in 96.3 ± 3.1% of the cells (Figure 3.3.B, right column). DNA content measurement by FACS and nuclear morphology of the cells confirmed S-phase arrest of the HU treated cells (data not shown). To rule out artifacts in localization due to GFP tagging of Hug1p, the data were corroborated by subcellular fractionation of cells expressing non-epitope tagged Hug1p expressed from its native promoter.

Total, nuclear (Nuc) and cytoplasmic (Cyto) fractions of cells with or without HU were analyzed by Western blot using anti-Sir2p (nuclear marker), -Pgk1p (cytoplasmic marker) or -Hug1p. Sir2p was enriched in the nuclear fraction (Figure 3.3.C, Lane 5) and Pgk1p in the cytoplasmic fraction (Figure 3.3.C, Lane 6) in HU treated cells. Hug1p was only observed in the cytoplasmic fraction of the HU treated cells and was excluded from the nucleus (Figure 3.3.C, Lane 6). The enrichment of Hug1p in the cytoplasmic fraction supports the data showing Hug1p localization to the cytoplasm in HU treated cells.
3.3.4. Cytoplasmic localization of Hug1p in response to HU treatment is not merely due to overexpression of the protein

Since HUG1 expression is induced in response to DNA damage and replication arrest in a checkpoint dependent manner (Basrai et al., 1999), we examined if the cytoplasmic localization of Hug1p under these conditions may reflect its high level of expression using cells expressing GAL1-HUG1-GFP. Western blot analysis showed that GAL1-HUG1-GFP was expressed in
wild-type, meclΔsml1Δ, and sml1Δ strains grown in galactose medium (Figure 3.5.A). Cells grown in the presence of galactose for 2 h, followed by growth in glucose medium with or without HU were examined for nuclear morphology and localization of GAL1-HUG1-GFP. Nuclear-to-cytoplasmic intensiometric ratios were quantified as described (Malinovska et al., 2012) to determine Hug1p-GFP subcellular compartmentalization for all strains (Figure 3.4). In the absence of HU, Hug1p-GFP was primarily localized to the nucleus (88.2 ± 2.0%) whereas a majority of the HU treated cells (86.6 ± 3.3%) exhibited cytoplasmic localization (Figure 3.5.B, WT). The similar localization pattern of GAL1-HUG1-GFP to that of HUG1-GFP expressed under the native HUG1 promoter revealed that the cytoplasmic localization of Hug1p-GFP is independent of protein expression levels.

3.3.5. Cytoplasmic localization of Hug1p-GFP is MEC1-dependent and coincides with the compartmentalization of Rnr2p–Rnr4p to the cytoplasm

Since genes in the MEC1 pathway are required for the DNA damage and replication arrest induced expression of Hug1p (Basrai et al., 1999), we examined whether the cytoplasmic localization of Hug1p is dependent on the MEC1 effector kinases, MEC1, RAD53, and DUN1. In the absence of HU, Hug1p-GFP mainly localized to the nucleus in meclΔ sml1Δ strains (92.1 ± 8.5%), similar to that observed in wild-type strain (88.2 ± 2.0%).

However, contrary to the cytoplasmic localization of Hug1-GFP in wild-type cells (86.6 ± 3.3%), very few of the meclΔsml1Δ cells showed localization to the cytoplasm (8.5 ± 1.1%) in response to HU treatment. The majority of Hug1p-GFP in the meclΔsml1Δ cells was nuclear (64.0 ± 7.3%) or was evenly distributed throughout the cell (27.5 ± 7.0%). The localization pattern observed in meclΔsml1Δ strains was independent of SML1 as sml1Δ strains exhibited a localization pattern more closely resembling wild-type strains. In the sml1Δ strain, Hug1p-GFP

§§§ Figure 3.3 appears in the original publication as Supplementary Figure 1.
localized to the nucleus \((97.1 \pm 1.0\%\) in the absence of HU and to the cytoplasm \((85.5 \pm 2.3\%\) in the presence HU (Figure 3.5.B).

We next examined the localization of \(GAL1\)-\(HUG1\)-\(GFP\) in \(rad53\Delta\) and \(dun1\Delta\) strains. In the absence of HU, both \(rad53\Delta\) \((74.8 \pm 11.2\%)\) and \(dun1\Delta\) \((83.9 \pm 0.5\%)\) strains showed nuclear localization of Hug1p-GFP similar to that observed in the wild-type strain (Figure 3.5.B).

However, unlike the wild-type cells, in the presence of HU, only a small fraction of \(rad53\Delta\) cells localized to the cytoplasm \((15.7 \pm 4.9\%)\) with a majority of the cells exhibiting an even distribution \((46.3 \pm 4.5\%)\) or nuclear localization \((37.9 \pm 9.3\%)\) of Hug1p-GFP signal.

In the presence of HU, the \(dun1\Delta\) strains exhibited a cytoplasmic localization profile of Hug1p-GFP that was intermediate to the pattern in \(mec1\Delta sml1\Delta\) and wild-type strains.

Cytoplasmic localization was observed in approximately half the population \((52.5 \pm 5.0\%)\) of \(dun1\Delta\) cells whereas the remaining cells had either an even distribution \((45.3 \pm 4.1\%)\) or nuclear localization \((1.9 \pm 0.9\%)\) of Hug1p-GFP. Taken together, these data indicate that Hug1p-GFP
locally to the cytoplasm in response to HU treatment and this localization is dependent on

**MEC1, RAD53, and DUN1** and is independent of **SML1**.

### 3.4. Discussion

Checkpoint mediated recovery from DNA damage and replication arrest is in part mediated by stringent regulation of Rnr activity. Negative effectors of the **MEC1** pathway, namely **SML1** and **DIF1**, interact with Rnr complex subunits and regulate its activity and
subcellular compartmentalization. The downregulation of Dif1p and Sml1p in response to DNA damage or replication arrest increases dNTP pools (Andreson et al., 2010; Lee et al., 2008; Wu & Huang, 2008). However, after recovery from checkpoint arrest, Rnr activity must be attenuated by negative regulators for normal cell cycle progression (Andreson et al., 2010; Chabes & Stillman, 2007; Kim & Siede, 2011; Lee et al., 2008; Poli et al., 2012; Wu & Huang, 2008; Zhao, Xiaolan et al., 1998; Zhao, X. & Rothstein, 2002). We propose that Hug1p is a negative regulator of the MEC1 pathway, which unlike DIF1 and SML1, is induced in response to DNA damage and replication arrest. This is based on our results which show that: (a) strains expressing GAL1-HUG1 are sensitized to growth in the presence of HU and DNA damaging agents, (b) the temporal pattern of Hug1p expression in the presence of HU exhibits a lag when compared with Rnr3p, a positive regulator of the MEC1 pathway, and resembles that of Crt1p, a negative regulator of the MEC1 pathway, and (c) suppression of lethality of mec1Δ and HU sensitivity of dun1Δ strains by deletion of HUG1.

We propose that Hug1p may serve to negatively regulate the MEC1 pathway by co-compartmentalization with Rnr2p–Rnr4p to the cytoplasm in response to HU treatment. The cytosolic localization is not simply due to overexpression of Hug1p as corroborated by localization analysis of GAL1-HUG1-GFP. Consistent with a requirement of MEC1 pathway genes for the induction of HUG1, cytoplasmic localization of Hug1p was dependent on MEC1, RAD53, and DUN1. Interestingly, Hug1p and Rnr2p–Rnr4p subcellular compartmentalization data share similar dependencies on the MEC1 pathway genes (Yao et al., 2003). The localization to the same cellular compartment may allow Hug1p to interact with Rnr2p–Rnr4p through an undetermined, potentially inhibitory mechanism (Figure 3.5.C) and downregulate Rnr activity.
As seen in the model, in cycling cells, Dif1p mediates the localization of Rnr2p–Rnr4p to the nucleus where Wtm1p anchors it, while Sml1p inhibits the activity of Rnr1p in the cytoplasm. After 1.5–2 h of HU induction, Dif1p and Sml1p are phosphorylated and degraded; Rnr2p–Rnr4p is exported from the nucleus to the cytoplasm where it forms the active Rnr complex with the Rnr1p homodimer. After 3.5 h of HU treatment, the high level of Hug1p expression and its localization to the cytoplasm and co-compartmentalization with the Rnr complex serve to downregulate Rnr activity and, potentially, dNTP pools. The delayed expression of Hug1p to replication arrest and co-compartmentalization with Rnr2p–Rnr4p may act to negatively regulate Rnr activity in the absence of negative MEC1 effectors, DIF1 and SML1, and permit cellular recovery in post-stress conditions. Taken together, our data define a novel role for HUG1 in the DNA damage and replication arrest pathway.

3.5. Supplementary materials and methods

3.5.1. Construction of expression vectors and strains

Plasmid pMB368 constructed from pMB379 contains a frame-shift mutation in the HUG1 ORF at codon 16 (aspartic acid to valine) with a premature stop at position 26. pMB588 contains the HUG1 open reading frame with a C-terminal GFP fusion in pRS415 (Mumberg et al., 1994; Niedenthal et al., 1996). Plasmid pMB734 contains a SnaBI fragment of HUG1-GFP from pMB588 cloned into pRS414. The GAL-HUG1-GFP fragment from pMB734 was cloned into pRS415 (Mumberg et al., 1994) to create pMB830. YMB1657 and YMB1618 were constructed using a PCR-mediated gene fusion method (Longtine et al., 1998).

3.5.2. Statistical Analysis

Statistical analysis of the subcellular localization profile data was performed using three-way factorial analysis of variance (ANOVA) with Tukey’s multiple comparison range test in SAS 9.3. A three-way factorial ANOVA yielded a main effect for localization, F (2,90)=417.72,
p<0.0001, however strain and HU treatment effects were non-significant. Interaction effects between strain and localization and HU treatment and localization were also significant, $F(8,90)=87.99$, $p<0.0001$ and $F(2,90)=80.17$, $p<0.0001$, respectively. Interaction effect between all main effects was also significant, $F(8,90)=80.17$, $p<0.0001$.

### 3.6. References


CHAPTER 4. ALKYLATING AGENTS INDUCE BIPHASIC TRANSCRIPTION OF MECl PATHWAY GENES WITH CELL CYCLE DEPENDENCE

4.1. Preface

This chapter is currently under peer review for publication. In response to critical levels of DNA damage and cellular stress, the MECl DNA damage checkpoint pathway coordinates cell cycle arrest and induces effector targets which work to preserve genomic stability. Microarray and promoter-reporter analysis of MECl effector transcription profiles has revealed a sophisticated and differential expression network response to increasing DNA damaging agents, however the actors and mechanism of this response remain unknown. In this study, we investigated the transcriptional response of five MECl pathway genes (MECl, RAD53, DUN1, RNR3, and HUG1) to a panel of DNA damaging and cellular stress agents using a GFP promoter-reporter system. We discovered a unique biphasic response of MECl and its effectors to alkylating agents that persisted through the MECl cascade. We determined that the biphasic signal was not the result of reactive oxygen species accumulation. Interestingly the early phase alkylating agent response shows dependence on pro-apoptotic yeast proteins, AIF1 and MCA1, however the late phase did not show similar dependencies. Finally, cell cycle analysis reveals a differential phase arrest that was dependent on alkylating agent titers and correlates with the biphasic response. These results demonstrate new insights regarding cellular fate and the MECl response when differentially exposed to alkylating DNA damaging agents.

4.2. Introduction

Faithful duplication of genomic information is of paramount importance to cellular survival and stable cell growth, however exogenous and endogenous DNA damage constantly challenge genome stability. To counteract these threats and preserve genomic integrity,
eukaryotic organisms have evolved a conserved and sophisticated surveillance system (known as checkpoints) to identify DNA lesions and mount a response. (Ciccia & Elledge, 2010; Harper & Elledge, 2007) Detection of DNA damage by sensor proteins leads to the stimulation of a conserved cascade of adaptors, transducers and effectors that, upon activation, trigger an array of cellular activities which act to preserve genome fidelity through replication fork stabilization, cell cycle arrest, DNA repair, and (depending on the severity of damage) apoptosis. (Branzei & Foiani, 2006; Ciccia & Elledge, 2010; Harper & Elledge, 2007; Zhou & Elledge, 2000)

The DNA damage checkpoint in eukaryotes is broadly regulated by two response systems: the ATM-mediated double-strand DNA break (DSB) repair cascade and the ATR-mediated single-stranded DNA (ssDNA) repair network. (Abraham, 2001) In *Saccharomyces cerevisiae*, Tel1p (ATM) is largely involved in telomere maintenance and DSB recognition while Mec1p (ATR) has a role in regulating both ssDNA and DSB checkpoint response (Baldo *et al*., 2008; Di Domenico *et al*., 2014; Naiki *et al*., 2004). Activation of the *MECI* signal transduction cascade induces translational and post-translational activity that initiates cell cycle arrest protocols and DNA repair mechanisms. (Huang *et al*., 1998; Lee *et al*., 2008; Sanchez *et al*., 1996; Zhao & Rothstein, 2002) The DNA damage checkpoint is proposed to be triggered once threshold levels of DNA lesions and stalled replication forks are detected. (Minca & Kowalski, 2011; Shimada *et al*., 2002) The resulting stalled replication forks expose single-stranded DNA (ssDNA) which act to prime DNA damage sensing complexes. (Cimprich & Cortez, 2008) For *S. cerevisiae*, replication protein A (RPA) coats and stabilizes bare ssDNA which recruits the heterotrimeric proliferating cell antigen (PCNA)-like 9-1-1 complex (Rad17p, Ddc1p, and Mec3p) and the constitutively associated Ddc2p- Mec1p to DNA damage sites (Rouse & Jackson, 2002; Zou & Elledge, 2003). Mec1p is subsequently activated by the Rad9p
mediator and the 9-1-1 complex (Rad17p, Ddc1p, and Mec3p) (Majka, J. & Burgers, P. M., 2003; Majka et al., 2006) that coordinates checkpoint activation through Dna2p- (Kumar & Burgers, 2013) or Dpb11p-associated (Ogiwara et al., 2006) activity.

Stimulation of Mec1p via the DNA damage sensing mechanism initiates the MEC1 checkpoint pathway, which exerts control over the DNA damage response through transcriptional and post-translational modifications to gene and protein targets. (Huang et al., 1998; Lee et al., 2008; Sanchez et al., 1996; Zhao & Rothstein, 2002) A systematic study of protein abundance and localization in response to DNA damaging agents methyl methanesulfonate (MMS) and replication stress agent hydroxyurea (HU) has uncovered the significance of protein subcellular compartmentalization with respect to DNA damage and replication stress type. (Tkach et al., 2012) Similarly, global transcription studies on the Saccharomyces cerevisiae response to alkylating, ionizing, and other physical and chemical cellular stresses have revealed a vast network of genetic responses exhibiting dose- and agent dependencies. (Benton et al., 2007; Benton et al., 2006; Fry et al., 2005; Gasch, A. P. et al., 2000; Jelinsky & Samson, 1999) In fact, over 2500 of the approximately 6200 Saccharomyces cerevisiae genes showed significant changes in transcript levels in response to carcinogen-induced cell damage. (Jelinsky et al., 2000) Gene clustering information from the differential transcriptomic expression results has led to discoveries of previously uncharacterized pathways and proteins with imperative function in the DNA damage response. (Fry et al., 2005; Gasch, A. P. et al., 2000; Jelinsky et al., 2000; Jelinsky & Samson, 1999)

Recent studies utilizing mRNA transcript profiling and a GFP promoter-reporter assay have identified a unique biphasic expression profile of MEC1 pathway genes to increasing alkylating agent concentrations (Benton et al., 2007; Benton et al., 2006). One study suggests
this profile may arise from differential cellular responses to changing MMS dosages, methylation and degradation of proteins required for \textit{MEC1} gene expression, or methylation and inactivation of the ribonucleotide reductase enzyme leading to deoxyribonucleotide reduction and \textit{MEC1} pathway exit, however little evidence yet exists to confirm these theories. (Benton et al., 2006)

Thus, the origin of the biphasic \textit{MEC1} gene response remains unknown. In this study, we sought to uncover the elements responsible for the biphasic \textit{MEC1} pathway gene expression. Using several GFP promoter-reporter constructs of \textit{MEC1} pathway genes, we examined the differential and temporal expression of \textit{HUG1}, \textit{RNR3}, \textit{DUN1}, \textit{RAD53}, and \textit{MEC1} to various DNA damage and cellular stress agents to determine if the origin of the biphasic response arose from genes within the \textit{MEC1} pathway. Pro-apoptotic genes and reactive oxygen species accumulation were also examined as potential external contributors to the unique response. Finally, cell cycle arrest assays were performed to assess variability in cell phase arrest under increasing MMS concentrations.

\textbf{4.3. Materials and methods}

\subsection*{4.3.1. Plasmid and strain construction}

The strains used in this study are outlined in Supplementary Table 4.2. Promoter-reporter constructs for \textit{MEC1}, \textit{RAD53}, \textit{DUN1}, \textit{HUG1}, and \textit{RNR3} were created using PCR-mediated gene deletion (Longtine \textit{et al.}, 1998) and the diploid parent strain BY4743 (GE Dhharmacon; YSC1050) to prevent the synthetic lethality of \textit{MEC1} or \textit{RAD53} gene deletion (Desany \textit{et al.}, 1998). Plasmid pFA6a-GFP(S65T)-HIS3MX6 (a kind gift of John Pringle) was used as template for construction of all promoter-reporter constructs using primers listed in Supplementary Table 4.3. Primers with flanking 45bp of homology immediately up- and downstream of the respective gene ORF were used to amplify a PCR cassette that includes a fusion of GFP(S65T) and HIS3MX6. PCR products were purified and directly transformed into BY4743 using a lithium-
acetate transformation method (Gietz & Schiestl, 2007a, 2007b). Transformants were selected on synthetic defined media lacking histidine and verified by colony PCR using primers binding into the promoter region of the indicated gene and into the GFP(S65T) ORF (Supplementary Table 4.3).

4.3.2. DNA damage exposure and cell stress experiments

DNA damage exposure and cell stress experiments were performed as previously described (Benton et al., 2007). Agent concentrations were limited to exposures that maintained a minimum 90% cell viability after eight hours as determined by propidium iodide (PI) live/dead cell staining. All DNA damaging experiments for each promoter-reporter system consisted of at least three independent trials. Strains were treated with each DNA damaging agent at the indicated concentrations and sampled in two hour intervals for a total exposure time of eight hours. At the each time point, samples were washed in phosphate buffered saline (PBS) and resuspended at final cell densities empirically determined to reduce flow cytometry read errors resulting from detector occlusion. Samples were stored in the dark no more than one day at 4°C until analyzed by flow cytometry.

4.3.3. Free radical detection and N-acetyl-L-cysteine (NAC) experiments

Dihydrorhodamine 123 (DHR123) was used to detect free radical formation in accordance with previous studies (Madeo et al., 1999; Wysocki & Kron, 2004) with some modifications. Prior to DHR123 addition, the DNA damage induction was performed as described. DHR123 at a final concentration of 5µg/mL was then added to aliquots of the induced strains two hours before harvesting. The strains were sampled every two hours for a total induction time of 8 hours. Samples were washed and resuspended in PBS and stored at 4°C prior to flow cytometric analysis.
N-acetyl-L-cysteine (NAC) pretreatment of cultures was performed as previously described (Kim et al., 2013) with some modifications. Log phase cultures were diluted to OD600=0.05 in pre-warmed YPD supplemented with 50mM NAC and grown with shaking at 30°C for two hours. DNA damage exposure and flow cytometry was then carried out as described in the previous section.

4.3.4. DNA analysis and cell cycle arrest

To analyze cell cycle progression in response to MMS, log-phase wild type (BY4741) cultures were exposed to the MMS dosages described in the previous section. Samples were harvested at the indicated times, washed once in distilled water, and then fixed with ethanol. DNA staining with SYBR Green I (Invitrogen) was then carried out in accordance with previously described methods without modification (Fortuna et al., 2001).

4.3.5. Flow cytometry analysis

Flow cytometry data for the promoter-reporter system were collected by Accuri C6 Flow Cytometer using the C6 Sampler software whereby at least 10,000 gated events per sample were counted. To prevent inclusion of undesirable small particle contaminants, samples were gated in the forward scatter channel (FSC) to remove small event sizes less than 50,000 relative units. Cell viability was determined using propidium iodide (PI) live/dead cell staining. Viable cells were defined as whole cells with PI impermeable, intact membranes. Inviable cells exhibiting PI permeable membranes were differentiated by large red fluorescence shifts in the FL3 channel (488nm laser with 670nm longpass filter) and were gate excluded from the total cell count. GFP signal of the promoter-reporter response was measured from channel FL1 (488nm laser with 530/30nm filter) with all prior gating settings applied. Data was analyzed using the Accuri C6 Sampler and FlowJo software.
4.3.6. Data analysis and statistics

The mean GFP fluorescent intensity was used to assess promoter-reporter responses to drug induction. The fold induction (FI) for the promoter-GFP reporters was calculated as the mean fluorescent intensity of drug-induced strains vs the mean fluorescent intensity of an uninduced control. Fold inductions were averaged across independent trials with matching DNA induction agent, time, and dosage. A Student t-test was performed on all induced strains relative to the untreated control strains to calculate a p-value for GFP fold induction. To eliminate false positives due to auto-fluorescence, positive GFP signal was established to be fold inductions greater than 2 and a p-value less than or equal to 0.05 when compared to controls. One-way analysis of variance (ANOVA) was used to determine the significant differences of temporal deviations of corresponding drug-induced strains. Dunnett’s test was used as the post-hoc test to identify strains with significant differences from the control.

4.4. Results

4.4.1. Differential dosage response of MEC1 pathway genes to DNA damage and cell stress agents using GFP promoter-reporter construct

Previous studies have demonstrated a global, differential transcriptional response that is highly specific to the genotoxic or cellular stress agent. (Benton et al., 2006; Fry et al., 2005; Gasch, A. P. et al., 2000) A unique biphasic response of the MEC1 repair pathway genes, DUN1, RNR2, RNR4, and HUG1, was identified in a study surveying the transcription outputs after exposing cultures to MMS dosages spanning three-orders of magnitude. (Benton et al., 2006) However, it remains unknown whether the source of this response is due to regulation within the MEC1 pathway or is the result of external repression controls.

To investigate MEC1 internal transcription signaling, we implemented a GFP(S65T) promoter-reporter gene deletion construct (see Materials and Methods) to fuse GFP under the
Table 4.1. DNA damage, replication arrest, and cellular stress agents implemented in MEC1 GFP promoter-reporter assays.

<table>
<thead>
<tr>
<th>AGENT</th>
<th>MECHANISM</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl methanesulfonate (MMS)</td>
<td>S&lt;sub&gt;N&lt;/sub&gt;2-type methylation of 7-deoxyguanosine and 3-deoxyadenosine blocks DNA synthesis</td>
<td>(Tracey, 2013; Xiao &amp; Chow, 1998)</td>
</tr>
<tr>
<td>Ethyl methanesulfonate (EMS)</td>
<td>Mixed S&lt;sub&gt;N&lt;/sub&gt;1/S&lt;sub&gt;N&lt;/sub&gt;2 ethylation of nucleophilic base; ethylation of guanine in DNA to form O6-ethylguanine leads to thymine mispairing by DNA polymerase</td>
<td>(Sega, 1984; Tracey, 2013)</td>
</tr>
<tr>
<td>Symmetrical dimethylhydrazine (SDMH)</td>
<td>Carbocation mediated methylation of purine bases; increases mismatched base pairing during replication</td>
<td>(Likhachev et al., 1978)</td>
</tr>
<tr>
<td>Hydroxyurea (HU)</td>
<td>Inhibitor of the ribonucleotide reductase by tyrosyl free radical scavenging; reduces deoxyribonucleotide availability to DNA polymerase</td>
<td>(Koc et al., 2004; Yarburo, 1992)</td>
</tr>
<tr>
<td>Phleomycin</td>
<td>Inhibitor of DNA synthesis through DNA binding double strand break formation in the presence of metal ions and oxygen</td>
<td>(Earhart, 1979; Suzuki et al., 1970)</td>
</tr>
<tr>
<td>4-nitroquinoline 1-oxide (4-NQO)</td>
<td>4-NQO metabolite reacts with DNA to form adducts to N6- on deoxyadenine and C8- or N2- on deoxyguanine, which induces single stranded DNA breaks</td>
<td>(Bailleul et al., 1989)</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>Binds and inhibits topoisomerase I cleavage and re-ligation activity; leads to replication fork breakage and arrest</td>
<td>(Liu et al., 2000)</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>Forms single strand breaks and DNA/protein crosslinks</td>
<td>(Magaña-Schwenccke &amp; Moustacchi, 1980)</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>Reactive oxygen species that induces DNA single- and double-strand breaks and apurinic/apyrimidinic lesions</td>
<td>(COOKE et al., 2003; Salmon et al., 2004)</td>
</tr>
<tr>
<td>Hydrochloric acid (HCl)</td>
<td>General cellular stress agent that increases environment acidity</td>
<td>(Carmelo et al., 1998)</td>
</tr>
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</table>

promoter control of MEC1, DUN1, RAD53, RNR3, and HUG1 in the BY4743 diploid parent strain. These genes were chosen to span the MEC1 cascade in order to identify the source of the distinctive biphasic transcriptional signal should it occur within the MEC1 pathway. GFP
promoter-reporter strains were then exposed to an array of DNA damage and stress agents (outlined in Table 4.1) and harvested every 2hrs for a total exposure time of 8hrs. The mean GFP intensity of at least 30,000 cells at each time point was then measured by flow cytometry.

GFP response curves for the five _MEC1_ pathway genes are given in Figure 4.1 and Supplementary Figure 4.5, Figure 4.6, Figure 4.7, Figure 4.8, and Figure 4.9. MMS provoked the highest reporter induction in all knockout strains with maximal peak intensities occurring after 8 hour exposure for all strains at the 0.05% v/v concentration (Figure 4.1.A). Expression patterns resulting from MMS exposure are consistent with previously reported global transcriptional profiles (Benton _et al._, 2006; Fry _et al._, 2005; Gasch, A. P. _et al._, 2000; Jelinsky & Samson, 1999) and are reflective of signal amplification through the _MEC1_ phosphorylation cascade, as effectors _HUG1_ and _RNR3_ demonstrated the highest signaling across doses and time, while transducers _DUN1, RAD53_ and _MEC1_ had decreasing response intensities, respectively, when compared to comparable dose and times.

Interestingly, all genes exhibited the distinct biphasic response originally observed in the mRNA transcript profiling of _HUG1, RNR3_, and _DUN1_ at differential doses of MMS (Benton _et al._, 2007; Benton _et al._, 2006), indicating that the drastic reduction in expression across all _MEC1_ genes at the highest two MMS dosages is likely the result of a repression event occurring upstream or outside of the _MEC1_ pathway.

The _hug1Δ::GFP_ fold induction for all MMS dosages was notably attenuated relative to previous reports; likewise, the peak intensity of the MMS dosage curve shifted from 0.01% to 0.05%. (Benton _et al._, 2007) This discrepancy can be attributed to construction of the promoter-reporter system in diploid instead of haploid strains, as similar GFP reporters created in the isogenic haploid strain yield anticipated reporter intensity and peak dosage (see Figure 4.3.A).
Similar profiles were obtained when strains were exposed to the alkylating agent, EMS (Figure 4.1.B), whereby the biphasic profile persisted in all genes tested. *hug1Δ::GFP* signals were again attenuated relative to comparable EMS induction in haploid strains strain (Benton et al., 2007), however the peak dose intensity occurred at the same time point and dosage (8hrs and
Expression levels for the 0.1% v/v EMS dosage were considerably higher for the *hug1Δ::GFP* than in the haploid strain (Benton *et al.*, 2007), which again can be attributed to the diploid strain constructs utilized in this study (data not shown).

SDMH, hydroxyurea, phleomycin, and 4-NQO (Figure 4.1.C-F) all exhibited monotonically increasing dose response to the respective agents. Notably, the alkylating agent SDMH did not show the biphasic induction patterns of MMS and EMS despite previous studies reporting otherwise (Figure 4.1.C) (Benton *et al.*, 2007).

Small increases in SDMH dosages above 1% w/v resulted in substantial (>85%) cellular death as indicated propidium iodide staining (data not shown), despite comparable dosages eliciting a non-lethal (>90% cell survival by propidium iodide staining) response in similar GFP reporter strains (Benton *et al.*, 2007). For SDMH, a significant fold induction in reporter signal was only identified at the highest dosage (1%; Figure 4.1.C), however the fold induction (1.7±0.4) was below our established positive GFP expression threshold.

SDMH is also the only agent that did not follow the predicted phosphorylation cascade expression levels, whereby only *dun1Δ::GFP* met the criteria for significant fold induction (2.1±0.2). At the 1% dosage and 6hr exposure time, there was a significant effect due to gene promoter on the fold induction of the GFP reporter as determined by a one-way ANOVA [F(4,10)=5.20, p<0.02], however a Tukey HSD post-test (α=0.05) revealed a significant difference only between the *dun1Δ::GFP* and *mec1Δ::GFP* reporter, but no statistical difference between other gene reporters. Thus, with the exception of the *dun1Δ::GFP* and *mec1Δ::GFP* comparison, genes exposed to SDMH have indistinguishable reporter expression at the 1% w/v dosage level at 6hr exposure.
Hydroxyurea, phleomycin, and 4-NQO (Figure 4.1.D-F) induced gene expression were consistent with phosphorylation cascade signal amplification, as *HUG1* and *RNR3* showing highest fold induction to increasing dosages, followed respectively by *DUN1*, *RAD53*, and *MEC1*. Despite a monotonic increase in GFP signal across the strains, peak and significant GFP detection thresholds differed between various strains and dosages. Significant fold increases were noted in response to hydroxyurea dosages at 10mM and above for *hug1Δ::GFP* and *rnr3::GFP*, 50mM and above for *dun1Δ::GFP* and *rad53Δ::GFP*, and 100mM for *mec1Δ::GFP* from the 4hr exposure onward (Figure 4.1.D). For phleomycin, dosages below 0.05µg/mL did not show significant fold increase in GFP signal. Only *RNR3* and *HUG1* exhibited significant GFP expression at the 0.05µg/mL phleomycin dosage.

However, significant signal was noted for all strains at the 5µg/mL concentration (Figure 4.1.E). 4-NQO did not have significant increases in GFP signaling for dosages at or below 0.1µg/mL; however a significant monotonic increase in all strains was seen in higher dosages (Figure 4.1.F). Camptothecin, hydrogen peroxide, and formaldehyde (Figure 4.1.G-I) did not elicit a significant fluorescent response in any strain with the thresholding values described in the Materials and methods.

4.4.2. Reactive oxygen species (ROS) accumulation in wild-type cells exposed to differential MMS

The GFP promoter-reporter results for *mec1Δ::GFP* show a measurable reduction in *MEC1* transcription at the highest dosages of MMS, indicating that the biphasic signal induced by alkylating agents MMS and EMS does not originate within the *MEC1* repair pathway. To determine other putative contributing factors, we investigated cellular pathways shown to be capable of epigenetic regulation in response to catastrophic levels of DNA damage. In *Saccharomyces cerevisiae*, increases in DNA damage by MMS and UV-C have been shown to
increase accumulation of reactive oxygen species (ROS) (Rowe et al., 2008); ROS can also act as signaling molecules that trigger a variety of cellular stress responses, including apoptosis and necrosis. (Carmona-Gutierrez et al., 2010; D’Autreaux & Toledano, 2007; Eisenberg et al., 2010; Fleury et al., 2002) To investigate the ROS accumulation at the higher MMS concentrations, we exposed wild-type (BY4741) strains to varying dosages of MMS for 2, 4, 6, and 8 hours and monitored ROS accumulation using dihydorhodamine 123, a general reactive oxygen species stain (Figure 4.2).

For every exposure time (2, 4, 6, and 8 hours), all but the highest MMS dosage tested (0.2% v/v) had a slight but distinguishable increase in DHR123 staining when compared to control strains (Figure 4.2.A, D). However, the highest MMS dosage tested (0.2% v/v) demonstrated considerable ROS accumulation beginning at 4hrs exposure and increased until the final 8hr exposure time (Figure 4.2.A-C). Interestingly, the highest MMS dosage at the 6hr exposure had bimodal staining characteristics whereby approximately 50% of the measure events expressed little ROS accumulation.

Despite these intriguing results, the deficiency in ROS accumulation at lower dosages of MMS indicate that ROS accumulation levels are not the driving factor behind the MECl pathway gene reduction. Likewise, pre-incubation of hug1Δ::GFP cultures with the free radical scavenger, N-acetylcysteine (NAC), prior to MMS induction did not result in a significant change in peak intensity or maximum induction dosage (Supplementary Figure 3), thereby suggesting ROS signaling had little effect on the overall MECl repair pathway response.

4.4.3. Differential MMS dosage response of the hug1Δ::GFP promoter-reporter in AIF1 and MCA1 deletion strains

The ROS increase at the highest MMS concentration warranted further investigation due in large part to the starkness of ROS response. ROS has been shown to be a key regulator of
Figure 4.2. Reactive oxygen species (ROS) accumulation in wild-type (BY4741) strains at varying MMS concentrations. (A) ROS accumulation histograms as measured by flow cytometry using dihydrorhodamine 123 staining of MMS dosages ranging from 0 (control) to 0.2% v/v at 2 hr (top left panel), 4 hr (top right panel), 6 hr (bottom left panel), and 8 hr (bottom right panel) exposure times. (B) ROS accumulation histograms demonstrating fluorescent peak shift of the highest (0.2% v/v) MMS dosage for exposure times 2, 4, 6 and 8 hrs. (C) Average fold induction of ROS accumulation in wild-type strains continuously exposed to 0.005 to 0.2% v/v MMS at 2, 4, 6, and 8hrs. Results are the average of at least three independent trials with error bars representing the standard deviation.
yeast apoptosis signaling (Madeo et al., 1999). Oxygen radical formation induced by low levels of hydrogen peroxide and cellular stress caused by acetic acid are known to initiate apoptosis in yeast.(Ludovico et al., 2001; Madeo et al., 1999)

Strains containing deletions of the mitochondrial cell death effector, AIF1 (apoptosis inducing factor-1) and the yeast metacaspase, MCA1 (formally YCA1), were more resistant to hydrogen peroxide, indicating a regulatory role in the free-radical, apoptotic response.(Wissing et al., 2004) Likewise, activation of MCA1 has also been show to coincide with the MEC1-RAD53 DNA damage response. (Weinberger et al., 2005)

Due to the increased ROS accumulation measured at high MMS dosages, we investigated the role of the apoptotic proteins, MCA1 and AIF1, in the MMS DNA damage response. The hug1Δ::GFP reporter construct was introduced into aif1Δ and mca1Δ haploid deletion strains and expression induced at varying MMS dosages. The GFP intensity was then measured via flow cytometry.

Unexpectedly, the aif1Δ and mca1Δ both affected GFP reporter peak dosage and intensity relative to the hug1Δ::GFP strain. Peak dosage for hug1Δ::GFP appears at 0.01% MMS while the peaks in aif1Δ hug1Δ::GFP and mca1Δ hug1Δ::GFP both shift to a higher 0.05% MMS concentration. Likewise, GFP signaling is considerably attenuated at all exposure times in the aif1Δ hug1Δ::GFP and mca1Δ hug1Δ::GFP strains relative to hug1Δ::GFP alone. Despite the peak dosage shift, all strains exhibit reporter repression at all time points and the two highest MMS doses (0.1 and 0.2% MMS), and respective fold inductions were statistically equivalent. These results are the first to implicate yeast apoptotic markers AIF1 and MCA1 in MEC1 signal amplification, however it is clear that they have no contribution towards initiating MEC1 gene repression at higher MMS doses.
4.4.4. Cell cycle arrest profiles in response to increasing MMS dosages

Previous reports have shown that transcripts of yeast cultures exposed to 0.1% MMS mirrored transcripts of cultures arrested in stationary phase, (Jelinsky et al., 2000) while 0.015% MMS has been shown to slow cell progression and arrest cells in the S-phase.(Gasch, Audrey P. et al., 2001; Paulovich & Hartwell, 1995) Taken together, these data suggest a cell-phase dependency for the DNA damage response and the respective gene expression. We sought to characterize the cell cycle transitions of wild-type (BY4741) strains exposed to MMS dosage ranges utilized in the reporter assays in order to identify potential overlap between cell cycle progression and MEC1 reporter expression. Wild-type strains were induced with MMS for short and long time intervals, whereupon they were fixed in ethanol and the DNA stained with SYBR Green I. DNA content was then measured by flow cytometry and cell cycle phase determined using the FlowJo cell cycle application and gating software.

Cultures induced with no to little (0-0.005% v/v) MMS proceeded normally through the cell cycle with clear phase transitions and no delay or S-phase arrest noted (Figure 4.4.A-D).
Figure 4.4. Cell cycle analysis of wild-type (BY4741) strains treated with differential MMS concentrations through a range of exposure time points. (A-H) Wild-type strains were exposed to MMS concentrations 0, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, and 0.5% v/v, respectively, for 25, 50, 60, 80, 100, 120, 240, 360, and 480 minutes to monitor cell cycle phase by SYBR Green I staining and flow cytometry. (I) A representative DNA content histogram is given with indicative gating for cell cycle phase determinations.
Mid-range MMS dosages (0.01% and 0.05% v/v) showed S-phase arrest as early as 100 minutes exposure time and persisted throughout the remaining 8hr exposure.

Of the total cells counted for the 0.01% and 0.05% dosages, 34% and 84% were arrested at the end of the experiment, respectively, which is consistent with previously reported data (Paulovich & Hartwell, 1995). Most interestingly, the two highest dosages of MMS (0.1 and 0.2% v/v) resulted in an immediate freeze in cellular phase (Figure 4.4.G, H). Notably, cultures experiencing this high dose did not undergo a measurable cell cycle phase transition, starting from the earliest time point (20 minutes). This immediate cell cycle arrest stage persisted for the remaining run time of the experiment.

4.5. Discussion

The eukaryotic response to exogenous and endogenous DNA damage requires sophisticated machinery that ensures genomic stability through coordinated cell cycle arrest and DNA repair. Understanding the intricacies of this machinery is paramount to assessing the cell’s molecular response to environmental changes. Global transcription analysis of the yeast response to alkylating agents, ionizing radiation, and other cellular stresses have revealed a complex and differential network of gene expression.(Fry et al., 2005; Gasch, A. P. et al., 2000; Jelinsky et al., 2000; Jelinsky & Samson, 1999) These studies have also uncovered novel transcriptional profiles within the DNA damage response - including the unique biphasic response of MECl repair pathway genes DUN1, HUG1, RNR2 and RNR4 to alkylating agents (Benton et al., 2007; Benton et al., 2006) – whose regulatory origins remain unknown. Characterizing the source of this unusual cellular response to alkylating agents was the fundamental purpose of this study.

We first set out to identify if the MECl biphasic response originated within the MECl phosphorylation cascade. In the Saccharomyces cerevisiae DNA damage response, DNA
damage sensors detect DNA lesions and activate Mec1p kinase. (Abraham, 2001; Cimprich & Cortez, 2008; Craven et al., 2002; Di Domenico et al., 2014; Majka, J. & Burgers, P. M., 2003; Majka et al., 2006; Minca & Kowalski, 2011; Ogiwara et al., 2006; Rouse & Jackson, 2002) Mec1p-mediated hyperphosphorylation of effector kinase, Rad53p, then provokes a phosphorylation cascade involving transducer Dun1p activation that relieves DNA repair protein transcriptional repression and post-translational inhibition. (Basrai et al., 1999; Chen et al., 2007; Lee et al., 2008; Zhao & Rothstein, 2002) As previous reports have demonstrated a dose dependent transcriptional repression of DUN1, HUG1, RNR2, and RNR4 when challenged with extreme doses of MMS, we extended the genes studied to those further upstream in the MEC1 phosphorylation cascade, MEC1 and RAD53. Using a GFP promoter-reporter detection system, we assayed the transcript specific response to a survey of DNA damaging agents (Table 4.1) at varying dosages and times (Figure 4.1 and Supplementary Figure 3). Our results confirmed that the alkylating agent-mediated biphasic expression profile persisted through effectors HUG1 and RNR3 to the upstream transducers DUN1, RAD53, and MEC1, and that this signaling was specific to the alkylating agents MMS and EMS (Figure 4.1). Therefore, we have concluded that MEC1 gene repression at extreme alkylating agent dosages must occur outside of the MEC1 phosphorylation cascade. Coinciding with this finding, MMS-induced global protein degradation was shown to be independent of the MEC1 DNA repair pathway and intermediate repair pathways. (Burgis & Samson, 2007) However, deletions of RPN4, a MMS-responsive transcriptional activator (Jelinsky et al., 2000), did not show significant effects on total protein degradation. (Burgis & Samson, 2007) We also created GFP promoter-reporter constructs to monitor the transcriptional profiles of genes involved in double stranded DNA break repair (MRE11 and RIF1) (Chamankhah & Xiao, 1999; Martina et al., 2014), PCNA-like DNA clamp
loading (DDC1) (Bonilla et al., 2008; Majka, J. & Burgers, P. M. J., 2003), and Mec1p activation and DNA damage site localization (DDC2) (Bandhu et al., 2014; Rouse & Jackson, 2000), however resulting expression levels were below the detection thresholds for the GFP reporter assay (data not shown).

We then sought to identify extrinsic pathways with putative involvement in MEC1 repair pathway repression. At high doses of the cellular stress agents, hydrogen peroxide and acetic acid, reactive oxygen species (ROS) signal an apoptotic response that triggers a global response that modulates response protein expression levels and abundance (Carmona-Gutierrez et al., 2010). We theorized that a similar apoptotic-dependent pathway may execute in response to high titers of alkylating agents; therefore, ROS accumulation and pro-apoptotic proteins offer prospective factors involved in the suppression of the MEC1 repair response. Interestingly though, ROS accumulation (as measured by dihydrorhodamine 123 staining and flow cytometry) showed nominal increases at all except the highest MMS dosage, despite MEC1 pathway gene expression reduction at the lower levels of MMS (Figure 4.2). Knockouts of two apoptotic genes, AIF1 and MCA1, also did not affect MEC1 gene repression at high dosages, though these knockouts did show a considerable shift in GFP peak intensity and attenuation of signal intensity (Figure 4.3). Previous reports have demonstrated reduced sensitivity of mca1 deletions to hydrogen peroxide; therefore, it is likely that the mca1 and aif1 deletions exert reduced sensitivity effects for other DNA damaging through a similar mechanism. Moreover, apoptotic signals in yeast have been shown to be MEC1-dependent (Qi et al., 2003). Our findings further extend this observation, as the attenuated GFP signaling in the aif1 and mca1 deletion mutants implicate AIF1 and MCA1 in the feedback of MEC1-mediated signal propagation.
Intra-S phase arrest is a hallmark of the MMS-induced DNA damage response, as cellular machinery restrict cell cycle progression to prevent duplication of damaged DNA. The concerted, adaptor-mediated *MEC1* activation of Rad53p ensures late origin firing and slowed S-phase progression. (Ciccia & Elledge, 2010; Harper & Elledge, 2007; Huang *et al.*, 1998; Zhou & Elledge, 2000) Checkpoint activation has been shown to require threshold levels of damage to adequately phosphorylate Rad53p to initiate the DNA damage response. (Shimada *et al.*, 2002) Likewise, global transcriptional analysis of *S. cerevisiae* exposed to high concentrations (0.1%) of MMS show reduced signatures of genes in the DNA damage response and an increase in genes characteristically expressed during stationary phase. (Benton *et al.*, 2007; Benton *et al.*, 2006; Jelinsky *et al.*, 2000) which further emphasizes the importance of cell phase on gene expression. Our cell cycle analysis corroborates previous data showing slowed and arrested S-phase arrest (Figure 4.4.E, F). High alkylation damage, however, did not show characteristic intra-S-phase arrest. Instead, cultures showed no indications of cell cycle progression and remained in the quiescent state for the entirety of the 8h experiment. This result was unexpected, but aligns with previous reports expressing similarities between stationary phase and 0.1% MMS-treated log-phase cells gene expression. (Jelinsky *et al.*, 2000) Likewise, unusual cell phase arrest occurs under the exact conditions as the right-handed portion of the *MEC1* reporter biphasic peak, suggesting a link between the extreme alkylation, immediate arrest, and repression of *MEC1* repair pathway genes. Our ROS and apoptotic gene response data indicate the mechanisms responsible for these profiles are independent of apoptosis commitment subroutines. Interestingly, quiescent state commitment is immediate and acute in the highest two alkylation dosages, suggesting the DNA damage response and, by extension, the *MEC1* checkpoint response fails to activate. As MMS alkylates proteins as well as DNA and RNA
(Boffa & Bolognesi, 1985), it is possible that critical MMS levels alkylate proteins upstream of the *MEC1* response and targets them to the proteasome. Previous studies have shown that genes required for protein degradation are upregulated while protein synthesis are reduced in response to MMS (Jelinsky *et al.*, 2000; Jelinsky & Samson, 1999); likewise, a significant increase in global protein degradation was reported in response to high MMS dosages (0.3%).(Burgis & Samson, 2007) However, the reason behind the increase in stationary gene transcript expression and our corroborating evidence showing immediate quiescent cell arrest at critical MMS dosages remains uncharacterized and warrants further investigation.

### 4.6. Conclusion

In this study, we have characterized the differential and temporal response of five yeast checkpoint pathway genes (*MEC1*, *RAD53*, *DUN1*, *HUG1*, and *RNR3*) to a survey of DNA damaging, physical and replicative stress agents. The biphasic response of *MEC1* and genes in its phosphorylation cascade was shown to be specific to the alkylating agents, MMS and EMS. *MEC1* pathway gene repression at high alkylating dosages was shown to occur outside of the *MEC1-RAD53-DUN1* pathway and is independent of reactive oxygen species accumulation and apoptotic genes, *AIF1* and *MCA1*, though the knockouts of the latter genes did show effects on *MEC1* signal amplification. Cell cycle analysis on wild-type strains exposed to mildly toxic MMS dosages corroborates previous reports of S-phase arrest, however, at high MMS dosages, cells experienced an immediate and enduring arrest in a quiescent state. This suggests high levels of alkylating damage provoke an alternate quiescent arrest pathway marked by stationary phase gene expression.
4.7. Supplementary tables and figures

4.7.1. List of strains used in the study

Table 4.2. List of strains used in the study

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### 4.7.2. List of primers used in this study

Table 4.3. List of primers used for PCR-mediated gene deletion and verification

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<td>RNR3-KO_R</td>
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**Colony PCR Verification Primers**

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<td>DUN1-Ver_F</td>
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Underline represents primer binding site on PCR mediate gene deletion vector for GFP(S65T) promoter-reporter construction
4.7.3. *mec1Δ::GFP* promoter-reporter system data

Figure 4.5. *mec1Δ::GFP* promoter-reporter temporal and dose dependent response to (A) MMS, (B) EMS, (C) SDMH, (D) hydroxyurea, (E) phleomycin, (F) 4-NQO, (G) camptothecin, (H) hydrogen peroxide (**H**2**O**2), and (I) formaldehyde.
4.7.4. *rad53Δ::GFP* promoter-reporter system data

Figure 4.6. *rad53Δ::GFP* promoter-reporter temporal and dose dependent response to (A) MMS, (B) EMS, (C) SDMH, (D) hydroxyurea, (E) phleomycin, (F) 4-NQO, (G) camptothecin, (H) hydrogen peroxide (H$_2$O$_2$), and (I) formaldehyde. Exposure times are denoted as follows: 2 hr (■), 4hr (▲), 6hr (△), and 8hr (○).
4.7.5. *dun1Δ:*GFP promoter-reporter system data

![Graphs showing temporal and dose dependent response to various agents.](image)

Figure 4.7. *dun1Δ:*GFP promoter-reporter temporal and dose dependent response to (A) MMS, (B) EMS, (C) SDMH, (D) hydroxyurea, (E) phleomycin, (F) 4-NQO, (G) camptothecin, (H) hydrogen peroxide (H₂O₂), and (I) formaldehyde. Exposure times are denoted as follows: 2 hr (■), 4hr (△), 6hr (□), and 8hr (○).
4.7.6. *hug1Δ::GFP* promoter-reporter system data

Figure 4.8. *hug1Δ::GFP* promoter-reporter temporal and dose dependent response to (A) MMS, (B) EMS, (C) SDMH, (D) hydroxyurea, (E) phleomycin, (F) 4-NQO, (G) camptothecin, (H) hydrogen peroxide (H₂O₂), and (I) formaldehyde. Exposure times are denoted as follows: 2 hr ( ), 4hr ( ), 6hr ( ), and 8hr ( ).
4.7.7. rnr3Δ::GFP promoter-reporter system data

Figure 4.9. rnr3Δ::GFP promoter-reporter temporal and dose dependent response to (A) MMS, (B) EMS, (C) SDMH, (D) hydroxyurea, (E) phleomycin, (F) 4-NQO, (G) camptothecin, (H) hydrogen peroxide (H₂O₂), and (I) formaldehyde. Exposure times are denoted as follows: 2 hr (■), 4hr (■■), 6hr (□□□□), and 8hr (△△△△)
4.8. References


5.1. Preface

This chapter is a continuation of major discoveries made Chapter 4, which established \textit{HUG1} as a negative regulator of the \textit{MEC1} checkpoint pathway. In Chapter 4, we proposed \textit{HUG1}’s inhibitory activity was the result of its co-localization with the small subunit of ribonucleotide reductase, \textit{RNR2}. In this chapter, we will further examine the putative interaction with between \textit{HUG1} and \textit{RNR2} using an array of yeast-specific protein interaction techniques, including co-immunoprecipitation assays and traditional and fluorescent yeast two-hybrid systems. Further, we will explore the effects of \textit{HUG1} mutations on the ability of \textit{HUG1} to perform its inhibitory function. Finally, we will discuss the discovery of new \textit{HUG1} interaction partners, the human \textit{RNR2} homologues p53R2 and hRRM2.

5.2. Introduction

Eukaryotic organisms employ sophisticated surveillance machinery to identify and respond to endogenous and exogenous sources of DNA damage.(Ciccia & Elledge, 2010) To preserve DNA fidelity, cells coordinate a complex network of signal sensing and transduction mechanisms to mend DNA lesions.(Harper & Elledge, 2007) Prevention of unstable genome inheritance is paramount to the DNA damage response, and, as such, effective repair requires the initiation of arrest stages (termed “checkpoints”) to halt cell cycle progression. (Zhou, B. B. & Elledge, 2000) Alongside initiating cell cycle arrest, checkpoint pathways induce a host of cellular activities, including transcriptional activation of genes required for repair (Huang \textit{et al.}, 1998), regulation of replication origin firing (reviewed in (Yekezare \textit{et al.}, 2013)), and, in the

In the budding yeast, *Saccharomyces cerevisiae*, the DNA damage response is mainly controlled by a highly conserved transduction pathway, known as the *MEC1* checkpoint response. Upon its activation, *MEC1* (homolog to the human Ataxia telangiectasia and Rad3 related – ATR) phosphorylates a cascade of effector kinases, such as Rad53p and Dun1p, which are required for transcript induction and repair target activation. (Ainsworth *et al.*, 2013; Chabes *et al.*, 1999; Chabes *et al.*, 2003; Chen *et al.*, 2007; Ciccia & Elledge, 2010; Harper & Elledge, 2007; Huang *et al.*, 1998; Lee *et al.*, 2008; Zhou, B. B. & Elledge, 2000) An essential element of the DNA repair response is the ability to supply adequate levels of DNA monomers, dNTPs, for DNA lesion repair. Ribonucleotide reductase (RNR) genes, the enzyme that performs the rate-limiting dNTP synthesis step, is a downstream target of the *MEC1* pathway and is fundamental to maintaining cellular dNTP pools during normal and genotoxic stress conditions. (Chabes *et al.*, 2000; Sanvisens *et al.*, 2013; Sommerhalter *et al.*, 2004) The active RNR complex is composed of a homodimer large subunit (Rnr1p), which houses the catalytic and two allosteric sites, (Xu, Faber, Uchiki, Fairman, *et al.*, 2006) and a heterodimer small subunit (Rnr2p-Rnr4p), which contains the diferric-tyrosyl radical cofactor necessary for NTP reduction chemistry. (Chabes *et al.*, 2000; Sommerhalter *et al.*, 2004) However, tight regulation of RNR activity is required as suboptimal or excessive dNTP concentrations have been linked to increased mutagenesis rates. (Chabes & Stillman, 2007; Kumar, D. *et al.*, 2010)

*S. cerevisiae* implement a unique, *MEC1*-mediated regulation regime to control RNR activity at both the transcriptional and post-translational levels. During the normal cell cycle, RNR subunit genes, *RNR2, RNR3*, and *RNR4*, are repressed by the DNA binding protein, Ctr1p,
which recruits the Ssn6p and Tup1p repressor complex to RNR gene promoter sites. (Huang et al., 1998). After DNA damage is sensed, however, MEC1-RAD53 mediated hyper phosphorylation of Crt1p detaches the repression complex from the RNR promoters and allows transcription to be initiated. (Huang et al., 1998) RNRI transcription is also regulated by a DNA binding protein, Ixr1p, which has been shown to be required for normal RNRI expression. (Tsaponina et al., 2011)

The RNR complex is also repressed during normal cell growth by two MEC1 negative regulator proteins unique to S. cerevisiae. The large RNR homodimer subunit (Rnr1p) is inhibited by the protein, Sml1p, which interacts through a conserved Rnr1 interaction domain domain. (Chabes et al., 1999; Zhao et al., 2000) The small RNR heterodimer subunit, Rnr2p-Rnr4p, is inhibited through Dif1p-mediated nuclear localization whereby it is anchored by Wtm1p. This effectively sequesters the small subunit and prevents formation of the active RNR complex in the cytoplasm. (Lee et al., 2008; Wu & Huang, 2008) In response to DNA damage, Sml1p and Dif1p are phosphorylated at a conserved phosphodegron site and degraded. Active RNR complex then readily assembles in the cytoplasm as Sml1p no longer inhibits the Rnr1p homodimer and the Rnr2p-Rnr4p heterodimer is relieved from Dif1p-Wtm1p mediated nuclear sequestration. (Chabes et al., 1999; Lee et al., 2008; Wu & Huang, 2008; Zhao et al., 2000)

Our lab has recently determined that the MEC1 target, HUG1, also negatively regulates the MEC1 response. (Ainsworth et al., 2013) Sequence alignments between the Saccharomyces cerevisiae negative regulators HUG1, DIF1 and SML1 and the functionally analogous Schizosaccharomyces pombe SPD1 and Ashbya gossypii Aer122c genes revealed a series of conserved domains that is likely the result of ancestral gene duplication of the Aer122c gene. DIF1 and SML1 shared the phosphodegron site required for protein degradation upon genotoxic
stress induction. SPD1, which serves dual function to inhibit large RNR subunit and sequester the small RNR subunit, had a conserved “Sml” domain also present in SML1 which is required for large subunit inhibition. Most interestingly, DIF1, HUG1, and SPD1 all contained homology in the “Hug” domain, a region originally identified in DIF1 as the region necessary for small subunit nuclear localization. (Lee et al., 2008)

Corroborating this data, our lab examined the subcellular compartmentalization of Hug1p in response to replication stress agent, hydroxyurea, and found that its localization profile correlated to that the small subunit heterodimer, Rnr2p-Rnr4p. (Ainsworth et al., 2013) Taken together with data demonstrating Hug1p’s delayed response to hydroxyurea relative to the positive MEC1 effector, Rnr3p, we proposed Hug1p to be a negative regulator of the MEC1 pathway that functions in cellular recovery through its co-localization with Rnr2p-Rnr4p. (Ainsworth et al., 2013)

Despite this, the mechanism by which Hug1p regulates the MEC1 pathway remains undetermined.**** Utilizing a fluorescent yeast-two hybrid system and co-immunoprecipitation assays, we determined that Hug1p indeed interacts with the RNR small subunit, Rnr2p-Rnr4p. dNTP pools were also measured with and without Hug1p overexpression to assess the Hug1p’s putative RNR inhibitory role. Several biochemical techniques were then utilized to determine the amino acid regions required for this interaction, including error-prone PCR and EMS mutagenesis and site-directed scanning alanine mutagenesis of the HUG1 ORF. Overexpression plating and liquid growth analysis were performed on the resulting mutants to determine the

**** Hug1’s interaction with Rnr2p-Rnr4p has since been confirmed and interaction domains identified in the publication, Meurisse, J., Bacquin, A., Richet, N., Charbonnier, J.-B., Ochsenbein, F., & Peyroche, A. (2014). Hug1 is an intrinsically disordered protein that inhibits ribonucleotide reductase activity by directly binding Rnr2 subunit. Nucleic Acids Res. (Meurisse et al., 2014). These findings and their relation to this study will be discussed in the Conclusions section.
overall effects of the mutations on Hug1p’s inhibitory activity. Western blot analysis and co-immunoprecipitation assays were also carried out to assess mutant expression levels and identify mutation-related loss-of-interaction. Finally, the positive interaction between intact Hug1p and two human RNR2 homologs, p53R2 and hRRM2, was identified using the fluorescent yeast-two hybrid system. These discoveries further clarified Hug1p’s role in the MEC1 recovery process and identified new domains required for its interaction with the RNR small heterodimeric subunit, Rnr2p-Rnr4p.

5.3. Materials and methods

5.3.1. Strains and plasmids construction

Plasmid and primer sequences used throughout this study are available upon request. pMB379 was created as described in previous studies.(Basrai et al., 1999) Plasmids pGAD-C1 and pGBD-C1 were used to create the traditional yeast two hybrid expression vectors to test HUG1 and RNR2 interaction.(James et al., 1996). HUG1 was inserted by restriction enzyme digestion and ligation into the pGAD-C1 vector by amplifying its ORF from the BY4741 genomic DNA extractions using high-fidelity PCR ad mutagenic primers containing a BamHI and SalI sites on the forward and reverse primers respectively. RNR2 was inserted into the pGBD-C1 vector using a similar method, with the exception PstI restriction enzyme site was used in the reverse primer.

The fluorescent yeast two-hybrid system was designed in accordance with previous split GFP constructs.(Barnard et al., 2008) yEGFP1 (pKT218; EUROSCRAF) was used as the template for split GFP fragment construction. Overlap extension PCR was used to construct a poly-GGGS linker sequence at the N- and C-terminus, depending on split GFP fragment fusion location desired. Primers were designed to truncate yEGFP1 from amino acids 1-157 (N-terminal yEGFP1 fragment, or NyEGFP1) and amino acids 158-238 (C-terminal yEGFP1
fragment, or CyEGFP1). Two vector sets were developed to allow PCR mediated gene deletion and fusion with the NyEGFP1 and CyEGFP1 fragments (pFA6a-NyEGFP1-KANMX6 and pFA6a-CyEGFP1-HIS)(Longtine et al., 1998) The pRS-plasmid vector set was used to fuse CyEGFP1 and NyEGFP1 fragments N- and C-terminally to HUG1, RNR2, p53R2 (synthesized using idtDNA’s gBlock technology from GenBank accession number AB036063), hRRM2 (amplified from pET-hRRM2.T2; a kind gift of Dr. Andres Chabes)(Guittet et al., 2001), and DNA2 (amplified from the yeast BY4741 genome) and placed under GAL, HUG1, or RNR2 promoter control where indicated.(Mumberg et al., 1995)

Strains utilized in HUG1 overexpression phenotyping analysis and dNTP determinations were constructed in accordance with previous investigations (Ainsworth et al., 2013; Basrai et al., 1999). PJ69-4A and PJ69-4a (kind gifts of Dr. Munira Basrai and Dr. James P. Halladay) were used for the traditional yeast two-hybrid assays as described previously (James et al., 1996). Fluorescent yeast two-hybrid strains were constructed from BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0). hug1Δ knockouts and C-terminal fusions of the split GFP fragments and HA-epitope tag (from plasmid pFA6a-3xHA-KANMX6) were performed using PCR mediated gene deletion and gene fusion protocols as described.(Longtine et al., 1998) Strains Y300 (MATa can1-100 ade2-1 his3Δ11,15 leu2-3,112 trp1-1 ura3-1) and MHY343 (Y300 rnr2::PRNR2-FLAG-RNR2-kan) were provide by Dr. Mingxia Huang and constructed as previously described.(An et al., 2006) All yeast transformations were performed using the high efficiency lithium acetate / single-stranded carrier DNA / PEG transformation method. (Gietz & Schiestl, 2007a, 2007b)
5.3.2. Quantitation of dNTP pools††††

dNTP extraction, HPLC measurements, and calculation of dNTP pools were performed as described previously.(Chabes et al., 2003; Chabes & Stillman, 2007)

5.3.3. Western blot and co-immunoprecipitation assays‡‡‡‡

Protein extraction, western blotting, and co-immunoprecipitation assays were carried out as described with slight modifications.(Basrai et al., 1999; Mishra et al., 2015; Ohkuni et al., 2015) Briefly, strains expressing the HUG1-HA construct under native promoter control were induced with hydroxyurea for 1.5 and 3.5 hours and whole cell extracts (WCE) collected. WCE were then added to anti-HA beads to pulldown the HUG1-HA. Western blots were then run using both WCE and anti-HA pulldown elutions. Anti-HA antibodies were used to identify Hug1p and anti-RNR2 (a kind gift of Dr. Joanne Stubbe) were used to stain Rnr2p pulled down by the anti-HA beads.

5.3.4. Overexpression phenotype plating and liquid growth assays

Overexpression phenotype plating assays were performed according to previous methods (Ainsworth et al., 2013; Basrai et al., 1999). Serial dilutions of log-phase cultures were spotted at a dilution factor of five and allowed to grow for three to seven days, as indicated.

Overexpression liquid growth assays were carried out as described (Toussaint & Conconi, 2006) with slight modifications. Triplicate cultures were grown overnight in selective media containing 2% raffinose and reseeded the following day in fresh media to an absorbance of OD600 of 0.1. Cultures were then incubated additional 5 hours to ensure log-phase growth,

†††† dNTP experiments were performed in collaboration with Dr. Sushma Sharma and Dr. Andrei Chabes at Umeå University in Umeå, Sweden.

‡‡‡‡ Whole cell protein extractions, Western blots, and co-immunoprecipitation assays were performed in collaboration with Dr. Munira Basrai and Dr. Wei Au at the Center for Cancer Research, National Cancer Institute in Bethesda, MD.
whereupon 95µL of each sample was transferred to a low-evaporation 96-well plate. 5µL of galactose media with or without hydroxyurea was added to each well to give a final galactose concentration of 2% and a final hydroxyurea concentration of 150mM. The plate was wrapped in parafilm and immediately placed in the SpectraMax M5 microplate reader. The plate reader was set to take kinetic measurements of the absorbance at 660nm every 10 minutes for 48 hours with shaking occurring 2 minutes immediately before reading. Data was exported to and analyzed in Microsoft Excel. For data analysis, background was subtracted from all sample measurements using blank wells containing selective media only. Growth rates were determined as the slope of five consecutive OD points in time. Maximal growth rates were averaged from at least three replicates and used to determine the effect of intact and mutant HUG1 overexpression and hydroxyurea induction.

5.3.5. Random and site-directed mutagenesis

Error-prone PCR was performed on the HUG1 ORF in accordance with previous protocols (McCullum et al., 2010) whereby the mutated HUG1 was placed under HUG1 and GPD promoter controls in the respective pRS-vector sets by co-transformation and plasmid reconstruction by homologous recombination. (Ma et al., 1987; Mumberg et al., 1995) Intact HUG1 PCR fragments were also mutagenized by EMS mutagenesis to reduce base mutation bias.(Winston, 2008) All mutants were transformed into yPP8 with a hug1 deletion (MATα ade2 ade3 leu2 ura3 trp1 mec1A::TRP1 his3 hug1A::HIS3 [pEF208=URA3 ADE3 MEC1 CEN].(Basrai et al., 1999) Transformants were plated on selective media with low adenine concentrations to allow for red-white colony selection, in accordance with previous protocols.(Ma et al., 1987; Zhao et al., 2000) All white and red-white segregated colonies were replica plated onto selective media with low adenine prior to plasmid extraction and sequencing.
Site-directed mutagenesis was performed using the Phusion site-directed mutagenesis kit (Thermo Scientific) in accordance with manufacturer’s instructions. 22 primer sets were designed to mutate three to four amino acids to alanines and to add a PstI digestion site for preliminary selection. The pUC18-HUG1 used as the mutagenesis template contained homology to the pRS- GAL promoter and CYC1 terminator up-and downstream of the HUG1 ORF. After verification of the mutants by sequencing, MHY343 hug1Δ2::HIS3 knockouts were transformed with the HUG1 mutants and linearized vector set, whereby the HUG1* mutants were inserted under GAL promoter control by plasmid reconstruction by homologous recombination (Ma et al., 1987). Overexpression assays were then carried out as described.

5.3.6. Fluorescent yeast two hybrid (split GFP) assay

Strains expressing fusions of the NyEGFP1 and CyEGFP1 fragments were grown overnight in selective media containing 2% raffinose. The following day, strains were reseeded to an OD_{600} of 0.1 and allowed to grow for an additional 3.5 hours to ensure log-phase growth. At that time, 2% galactose was added to induce expression from the galactose promoter and incubated at 30°C for 3.5 hours. For strains under HUG1 promoter control, hydroxyurea was then added to a final concentration of 150mM and allowed to incubate for 3.5 hours. Samples were washed in PBS and prepared for microscopy as previously described (Ainsworth et al., 2013).

5.4. Results and discussion

5.4.1. Effects of HUG1 overexpression on cellular dNTP levels

We have previously shown that Hug1p is a negative regulator of the MEC1 DNA damage checkpoint pathway and proposed that the interaction serves as a mediator in cellular recovery after DNA damage (Ainsworth et al., 2013; Basrai et al., 1999). We also observed similar subcellular co-compartamentalization of Hug1p and Rnr2p when exposed to the replication stress agent,
hydroxyurea, which suggests Hug1p’s activity putatively arises from its interaction with Rnr2p (Ainsworth et al., 2013). As Rnr2p contains a diferric tyrosyl radical cofactor required for de novo radical reduction of ribonucleotides (NTPs) to deoxyribonucleotides (dNTPs) (Chabes et al., 2000; Sommerhalter et al., 2004), we proposed that the Hug1p’s negative regulation activity may arise from its inhibition of dNTP production by RNR.

To examine the effects of Hug1p expression on dNTP levels, we overexpressed Hug1p in wild-type (BY4741) strains transformed with an episomal plasmid containing intact HUG1 under a galactose-inducible promoter (P_GAL1), as described in previous overexpression phenotype plating assays (Ainsworth et al., 2013; Basrai et al., 1999). Galactose-induced strains were also simultaneously exposed to hydroxyurea to produce conditions of growth inhibition synergism between Hug1p overexpression and dNTP depletion as seen in our previous overexpression plating analysis (Ainsworth et al., 2013). After 3.5 hours under these conditions, dNTPs were purified and analyzed as previously described (Chabes et al., 2003). Normalized dNTP levels determined with or without galactose overexpression of HUG1 at various induction times and with or without the replication stress agent, hydroxyurea, are given in Figure 5.1. Temporal overexpression of HUG1 with galactose shows a qualitative drop in dNTPs pools (Figure 5.1.A), however a one-way ANOVA reveals that there is no significant difference between dNTPs (dCTP, dTTP, dATP, and dGTP) of the wild-type and that of the 1, 2, and 3hr galactose-induced HUG1 (p=0.552, 0.437, 0.470, and 0.213 for dCTP, dTTP, dATP, and dGTP, respectively).

dNTP measurements were also collected for strains overexpressing HUG1 in the absence or presence of hydroxyurea (Figure 5.1.B). One-way ANOVA revealed significant differences between wild-type and HUG1 overexpression strains with and without hydroxyurea for all
dTNPs (F(3,4)=2.360; p=0.231). A significant reduction was seen in all dNTPs when HU was included in the media for both no (Figure 5.1.B; WT+HU) and overexpression of HUG1 strains (Figure 5.1.B; GAL1-HUG1+HU) as determined by a one-way ANOVA with Bonferroni post-hoc analysis (p≤0.005 for all dNTPs). Bonferroni post-hoc was used as a small number of mean comparisons was performed (Field, 2005). No significant different was seen between wild-type strains and strains overexpressing HUG1 for dCTP and dTTP (p>0.160); however, significant differences were noted for dATP and dGTP (p<0.005). Despite a qualitative reduction in dCTP values between wild-type and HUG1 overexpressed strains induced with hydroxyurea (Figure 5.1.B; WT+HU and GAL1-HUG1+HU, respectively), the Bonferroni post-hoc test revealed no significant differences between the two (p=0.567).

Figure 5.1.†††† Effects of HUG1 overexpression on wild-type (WT; BY4741) stain dNTP levels with and without hydroxyurea for 1, 2, and 3hrs. (A) WT strains with and without galactose-inducible HUG1 (WT HUG1 and GAL1-HUG1, respectively) were grown in galactose for 1, 2, and 3hrs and dNTP levels (dCTP, dTTP, dATP, and dGTP) measured by HPLC. (B) WT strains with and without the galactose-inducible HUG1 (WT HUG1 and GAL1-HUG1, respectively) were grown in the presence of galactose with and without 200mM hydroxyurea (HU) for 3.5 hours whereby the dNTP levels (dCTP, dTTP, dATP, and dGTP) were analyzed by HPLC. Duplicate biological measurements were run for each sample. Bars represent the mean of the dNTP measurements and error bars indicate the standard deviation of the mean.
Similarly, dTTP, dATP, and dGTP values also showed no significant differences between wild-type and HUG1 overexpressed strains induced with hydroxyurea (Figure 5.1.B; p>0.168). Due to the rigor of performing the experiment, the sample size (n=2) is very small, and thus these statistical results may not be reflective of HUG1’s true effect on dNTP concentrations. Likewise, hydroxyurea reduces dNTP levels to concentrations that approach the dNTP extraction’s lower detection threshold (Andrei Chabes, personal correspondence). A new DNA damaging agent that does not dramatically and directly affect cellular dNTP levels should be used for future experiments.

5.4.2. Traditional yeast two-hybrid fails to verify the Hug1p-Rnr2p interaction

One of the ways to verify the Hug1p-Rnr2p interaction is to use the traditional yeast two-hybrid assay (Y2H). The traditional Y2H system requires the fusion of two putatively interacting proteins to the GAL4 activating domain (GAL4-AD) and the GAL4 binding domain (GAL4-BD). The GAL4-BD binds to the upstream activating sequence of GAL1 (UAS-GAL1) and, upon activation by the activating domain, begins transcribing genes under GAL1 promoter control. Protein fusions to the GAL4 binding and activating domains can determine protein interaction propensity, as the proximity of interacting proteins allows for the GAL4 activator domain to trigger binding domain mediated GAL1 promoter gene transcription. Designer stains, such as PJ69-4a, have been engineered to include HIS3 selection under the GAL1 promoter to enable facile selection of strains with interacting proteins; however, the leakiness of the HIS3 selection marker requires media to contain a HIS3 competitive inhibitor, 3-amino-1,2,4-triazole to reduce false positives or an additional, stringent selection marker (for instance, PJ69-4a/α uses ADE2 as the extra selection marker under the GAL2 promoter, which activates downstream of GAL1 and requires GAL1 promoter activation for its expression). (James et al., 1996)
Thus, to test the Hug1p-Rnr2p interaction using the traditional Y2H, we fused the GAL4 activation domain to the N-terminus of Rnr2p (GAL4-AD-RNR2) and the GAL4 binding domain to the Hug1p N-terminus (GAL4-BD-HUG1). BigDye® Terminator sequencing was used to verify correct reading frame of all plasmid constructs. We then co-transformed GAL4-AD-RNR2 and GAL4-BD-HUG1 into the traditional Y2H-compatible designer strain, PJ69-4a (kind gifts of Dr. Munira Basrai and Dr. James P. Halladay), whereby positive uptake of the plasmids was verified by colony PCR. Strains were then grown to log phase and plated in serial dilutions on selective media lacking either histidine (SD-HIS) or histidine and adenine (SD-HIS-ADE). Likewise, strains were also plated on selective media lacking histidine and containing titrations 5, 10, 25, and 100mM 3-AT (SD-HIS+3AT). Strains were allowed to grow for several days to allow colony growth on the selective media. However, after seven days of growth, the plates demonstrated no strain new growth on even on the lowest selection stringency, SD-HIS, despite normal growth on media selective for the GAL4-AD and BD plasmids alone (SD-URA-LEU). Attempts at switching the fusions (AD-HUG1 and BD-RNR2) also proved unsuccessful as similar results were obtained for both constructs.

There are many potential reasons as to why this assay did not initially function. First, in order to express appropriate levels of HUG1 in the pRS-vector sets, at least 35 bases of the upstream HUG1 promoter sequence and 66 bases of the HUG1 terminator sequence are required. (Basrai et al., 1999) Likewise, an even higher induction can be achieved in GAL-HUG1 vectors if replication stress agent hydroxyurea or DNA damaging agent, MMS, are included with the galactose induction. Interestingly, full derepression of the HUG1 and other DNA damage response genes requires the transcriptional derepressor, Asf1p. (Minard et al., 2011) Thus, when placing the HUG1 under different promoter controls, the Asf1p depression sequence is lost.
HUG1 expression is therefore severely reduced as it no longer contains the depressor binding site to relieve transcriptional repression machinery.

5.4.3. Fluorescent yeast two-hybrid reveals the Hug1p-Rnr2p in vivo

Failure of the traditional Y2H system in the above section may be the result of poor expression of the HUG1 and RNR2 constructs from their respective plasmids. In fact, proper expression of HUG1 in plasmid constructs requires the inclusion of at least 35 bases of the HUG1 upstream promoter region for proper expression (Dr. Munira Basrai; personal communication). To mitigate these expression effects, we required a system that allowed use of intact fusion proteins under native promotion.

The fluorescent yeast two hybrid system (also known as the split GFP system) was chosen as the ideal candidate for protein interaction as it allowed both genomic fusion construct creation and it offered redundant verification of our previous Hug1p-GFP subcellular localization results. Likewise, signal-to-noise ratios for the split GFP assay are high which, as a result, reduces the likely of false positive identification.

The split GFP system works similarly to the traditional Y2H system described in the previous section and is diagrammed in Figure 5.2.A. Briefly, the yeast enhanced green fluorescent protein (yEGFP1) is split into two fragments: the N-terminal fragment, NyEGFP1, contains the M1 through Q157 amino acids while the C-terminal fragment, CyEGFP1, contains amino acids K158 to K238.

These fragments are then fused to the respective potential proteins of interest and expression induced in wild-type strains. If the proteins interact, the yEGFP1 fragments will come in close proximity of each other and refold into the fluorescent yEGFP1. Otherwise, no fluorescence will be seen.(Barnard et al., 2008; Park et al., 2007) Due to steric hindrances introduced by the fragment fusion and/or proximity requirements of the fragments, yEGFP1
fragment fusions to the N- and C-terminus of both putative interacting proteins must be designed and empirically confirmed verify a non-functioning split GFP assay (Figure 5.2.B).

We first constructed a galactose-inducible, C-terminal fusion of the yEGFP1 fragments, NyEGFP1 and CyEGFP1, to Rnr2p and Dif1p respectively to verify correct construction and function of the split GFP assay. However, upon inducing with galactose, the resulting PCR verified strains did not exhibit fluorescence when examined by epifluorescent microscopy (data not shown). Since Rnr2p’s C-terminus contains a binding region required for its proper interaction and folding with the Rnr1 homodimer (Chabes et al., 1999; Xu, Faber, Uchiki, Racca, 

![Diagram of the split GFP system and conformation variants using yeast enhanced green fluorescent protein (yEGFP1).](image_url)

**Figure 5.2.** Diagram of the split GFP system and conformation variants using yeast enhanced green fluorescent protein (yEGFP1). (A) yEGFP1 is split at amino acids 1-157 (NyEGFP1) and 158-238 (CyEGFP1) and fused to potential interacting partners. If proteins interact, the split yEGFP1 fragments refold to form active yEGFP1. If they do not interact, no fluorescence is seen. (B) Due to steric hindrances and the required proximity of the yEGFP1 fragments, various positional fusion constructs are necessary to confirm interaction via yEGFP1 refolding. These positions include: C-terminal fusion of both yEGFP1 fragments (C-C fusion), N-terminal fusion of NyEGFP1 and C-terminal fusion of CyEGFP1 (N-C fusion), C-terminal fusion of NyEGFP1 and N-terminal fusion of CyEGFP1 (C-N fusion), and N-terminal fusion of both yEGFP1 fragments to putative interacting proteins.
et al., 2006), we theorized that the Rnr2p-NyEGFP1 construct was likely nonfunctional and therefore unlikely to correctly fold and bind to Dif1p-CyEGFP1. Further, when we genomically integrated NyEGFP1 at the C-terminus of RNR2 in haploid strains, resulting PCR verified constructs grew significantly slower than wild type strains (3 hours vs 1.5 hours, respectively), indicating a partial disruption in the Rnr2p activity.

We thus reconstructed the Rnr2p construct with an N-terminal fusion of NyEGFP1 and placed this under galactose promoter control. The resulting plasmid was co-transformed with the galactose inducible Dif1p-CyEGFP1 and positive constructs containing both plasmids were verified by colony PCR. A negative construct was also designed to include only the two split GFP fragments under galactose promoter control. Both strains were then induced with galactose for 6 hours, after which strains were washed, resuspended in PBS, and prepared for microscopy as described previously (Ainsworth et al., 2013). Epifluorescent microscopy was used to verify the expression levels of the split GFP system; representative images of the resulting GFP fluorescence for NyEGFP1/CyEGFP1 only and Dif1p-CyEGFP1/NyEGFP1-Rnr2p are given in Figure 5.3. The negative control (Figure 5.3, top row) shows little to no fluorescence in the GFP channel. Similarly, all strains grown in glucose for 6 hours also had little to no fluorescence in the GFP, indicating successful repression of the galactose promoter in these constructs (data not shown). Meanwhile, clear fluorescence is seen in the GFP channel for the Dif1p-CyEGFP1/NyEGFP1-Rnr2p constructs, indicating that the split GFP system is functional. Interestingly, the Dif1p/Rnr2p GFP signal is localized to the nucleus, which corroborates previous results demonstrating Dif1p’s role in sequestering Rnr2p-Rnr4p to the nucleus (Lee et al., 2008).
As the split GFP was proven to successfully capture the Dif1p/Rnr2p interaction, we then created a galactose inducible Hug1p-CyEGFP1 plasmid for use in testing the Hug1p-Rnr2p interaction. This construct was then co-transformed with galactose inducible NyEGFP1-Rnr2p and colony PCR was used to verify the uptake of both constructs. The new Hug1p-CyEGFP1/NyEGFP1-Rnr2p strain was then induced with galactose for 6 hours and prepared and analyzed by microscopy as described above. A noticeable, but slight increase in GFP signal was noted in GFP channel for the galactose induced Hug1p-CyEGFP1/NyEGFP1-Rnr2p strains when

![GFP, DAPI, MERGE images](image)

**Figure 5.3.** Representative images of split GFP assay measured by fluorescent microscopy showing resulting fluorescence of various bait and prey constructs under galactose inducible promoter control. Strains were induced with galactose for 6hr and imaged using a Zeiss AxioCam MRm microscope using GFP and DAPI filter sets (left and center column, respectively). (Top row) Negative control strain containing NyEGFP1 and CyEGFP1 fragments only under galactose promotion. (Middle row) Positive control containing bait Dif1p-CyEGFP1 and prey NyEGFP1-Rnr2p under galactose promotion. (Bottom row) Galactose-induced co-expression of bait Hug1p-CyEGFP1 and prey NyEGFP1-Rnr2p induces GFP fluorescence. Scale bars represent 20µm.
compared with negative controls (Figure 5.3, bottom row), demonstrating that Hug1p does in fact interact with Rnr2p.

As the GFP signal was low in the Hug1p-CyEGFP1/NyEGFP1-Rnr2p strains described above, we attempted to improve the signal quality by fusing the CyEGFP1 fragment at the C-terminus of the \( HUG1 \) in the genome (i.e., under native \( HUG1 \) promoter control). This was performed using the PCR gene fusion method as described with the CyEGFP1 fragment created in the pFA6a series plasmids (Longtine et al., 1998). The genomic Hug1p-CyEGFP1 construct was co-transformed with \( P_{GAL1} \)-NyEGFP1-RNR2 used in the previous experiments to allow galactose induction of the NyEGFP1-Rnr2p construct. Successful transformations were confirmed by colony PCR.

Strains containing the genomic \( HUG1 \)-CyEGFP1 and \( P_{GAL1} \)-NyEGFP1-RNR2 constructs were first grown in galactose for 6 hours to induce NyEGFP1-Rnr2p expression, followed by transfer to glucose media with or without 150mM hydroxyurea for 3.5 hours to induce Hug1p-CyEGFP1 expression. Samples were then washed and prepared for microscopy as described above. In strains not induced with hydroxyurea, no fluorescence is noted in the GFP channel (Figure 5.4, top row), consistent with the lack of Hug1p-CyEGFP1 expression. However, upon induction with hydroxyurea, bright fluorescence is noted in the GFP channel in several cells in the representative and total image (Figure 5.4, bottom row). This further serves as confirmation of the Hug1p-Rnr2p interaction, with physiological conditions inducing enough Hug1p expression to be detected by the split GFP assay.

For each construct described above, at least 300 cells were counted to determine the efficiency of the split GFP construct in for the combinations of bait/prey and expression systems. Cells containing appreciable fluorescent signal (2-fold or greater relative to control) as
determined by GFP signal densiometric analysis were deemed “GFP positive”. Any cells failing to meet this criteria were considered to be GFP negative. The galactose inducible constructs containing NyEGFP1 and CyEGFP1 only had the lowest percent positive cells at 1%. Similarly, constructs with genomic HUG1-CyEGFP1 and P\textsubscript{GAL}-NyEGFP1-RNR2 induced with only galactose also exhibited 1% GFP positive cells. The positive split GFP control strain containing P\textsubscript{GAL}-DIF1-CyEGFP1 and P\textsubscript{GAL}-NyEGFP1-RNR2 plasmid constructs had 25% GFP positive, while the unknown test strain containing P\textsubscript{GAL}-HUG1-CyEGFP1 and P\textsubscript{GAL}-NyEGFP1-RNR2 plasmid constructs exhibited only 17% GFP positive. A two-fold increase in percent positive (34%) was seen when the genomic HUG1-CyEGFP1 and P\textsubscript{GAL}-NyEGFP1-RNR2 were induced with both galactose and hydroxyurea when compared to the same split GFP constructs placed under galactose promoter control.

Figure 5.4. Representative images of split GFP assay measured by fluorescent microscopy showing resulting fluorescence from galactose and hydroxyurea (HU) induction. Fluorescence in the GFP channel indicates successful detection of GFP refolding using the split GFP bait Hug1p-CyEGFP1 under native promoter control and prey NyEGFP1-Rnr2p under galactose promoter control without (top row) and with (bottom row) 150mM HU induction. Strains were induced with galactose for 6hr, transferred to glucose media with or without 150mM HU, and incubated for an additional 3.5 hours. Images were taken using a Zeiss AxioCam MRm microscope with GFP and DAPI filter sets (left and center column, respectively). Scale bars represent 20µm.
Co-immunoprecipitation of HUG1-HA and Rnr2p corroborates fluorescent Y2H results

Though the split GFP system successfully verified the Hug1p-Rnr2p interaction, further proof is required to corroborate the fluorescence results. As such, a co-immunoprecipitation assay was run on strains containing a C-terminal fusion of the epitope tag, hemagglutinin A (HA), to genomic HUG1 and intact (unmodified) RNR2. The procedure was run as described in (Mishra et al., 2015; Ohkuni et al., 2015) WCE probed with anti-RNR2 and anti-HA antibodies demonstrate significant staining of the Rnr2p and Hug1p-HA proteins after 1.5 hours and continuing to 3.5 hours post-HU induction, respectively, and show successful extraction of the desired proteins. WCE do not contain Hug1p-HA at the initial 0 hour induction time point, which is consistent with DNA damage or replication arrest induction of Hug1p (Basrai et al., 1999).

anti-HA pulldown elutions also showed enrichment of Hug1p-HA as given by positive anti-HA staining at 1.5 and 3.5 hours post-HU (Figure 5.5, right column). Again, no Hug1p-HA was noted at the 0 hour induction point as Hug1p is only expressed in response to replication arrest or DNA damage (Basrai et al., 1999). Finally, positive enrichment of Rnr2p was seen when probed with anti-RNR2. This evidence corroborates the split GFP results which originally identified the Hug1p-Rnr2p interaction and indicates a successful pulldown of Rnr2p via its interaction with Hug1p-HA.

<table>
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<tr>
<th>BAIT</th>
<th>PREY</th>
<th>PROMOTER (BAIT/PREY)</th>
<th>INDUCTION TYPE / TIME</th>
<th>CELLS COUNTED</th>
<th>CELLS WITH POSITIVE GFP</th>
<th>PERCENT POSITIVE</th>
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<td>GAL / HUG1</td>
<td>GAL / 3.5hr</td>
<td>374</td>
<td>4</td>
<td>1%</td>
</tr>
<tr>
<td>RNR2</td>
<td>HUG1</td>
<td>GAL / HUG1</td>
<td>GAL+HU / 6hr+3.5hr</td>
<td>212</td>
<td>72</td>
<td>34%</td>
</tr>
</tbody>
</table>

Table 5.1. Results of split GFP experiment for various bait, prey, and expression systems
5.4.5. Determination and effects of large-domain HUG1 knockout targets on HUG1 inhibitory activity

Confirmation of the Hug1p-Rnr2p interaction is a major initial finding that elucidates Hug1p’s inhibitory mechanism; however, this discovery does not provide insight into whether the Hug1p-Rnr2p binding event alone is required for its inhibition or if Hug1p has distinct domains separately involved in Rnr2p binding and inhibition. Indeed, our previous studies have shown that galactose-induced expression of Hug1p-GFP retains the propensity to co-localize with Rnr2p, despite overexpression plating analysis demonstrating a loss of Hug1p inhibitory activity (Ainsworth et al., 2013). These results support the model whereby Hug1p utilizes independent domains to bind and inhibit, however more work to identify these regions is required.

A typical method to classify necessary domains is to perform scanning alanine mutagenesis, whereby amino acid regions believed to be involved in protein activity are mutated to alanine and protein function tested by enzyme specific assays (Lefèvre et al., 1997).
However, determining these regions for HUG1 is not trivial, as global protein alignment software (e.g., Protein BLAST) and conserved domain databases (e.g., NCBI Conserved Domain Search) fail to find sequences with adequate homology to the HUG1 primary sequence.

Interestingly, a recent study has found that the S. cerevisiae protein, Dif1p, contains a domain that is required for Rnr2p-Rnr4p nuclear localization; this domain has been termed the “Hug domain” due to its local sequence similarity to HUG1. Likewise, it was found that HUG1 arose from DIF1 gene duplication and, thus, likely retains characteristic features of DIF1, including the ability to bind (and inhibit) Rnr2p (Lee et al., 2008). The comparable Dif1-hug1 mutant region in Hug1p offers an ideal candidate as a necessary HUG1 domain (Figure 5.6.B) and was thus selected for scanning alanine mutagenesis (Figure 5.6.G).

Despite the absence of Hug1p structural data and the failure of local conserved domain databases to identify domain similarity within Hug1p primary sequence, secondary sequence protein prediction software offers a valuable alternative to finding unverified but probable secondary structures within proteins lacking requisite homology and/or structural information.

The primary sequence of HUG1 (Figure 5.6.A) was used to poll a panel of secondary structure prediction software to identify potential secondary structures within HUG1. Slight differences between the individual software’s prediction capabilities results in variable secondary structure outputs that, when aligned with other prediction software outputs, results in a consensus of probable secondary structures. Our survey of prediction software included: I-TASSER (Iterative Threading ASSEmbly Refinement) (Roy et al., 2010; Yang et al., 2015; Zhang, Y., 2008) (Figure 5.6.C), RaptorX (Peng & Xu, 2011) (Figure 5.6.D), Phyre2 (Figure 5.6.E) (Kelley et al., 2015), and PSIPRED (Buchan et al., 2013) (Figure 5.6.F). An α-helix spanning Hug1.Pro9 to Hug1.Lys35 was identified by all secondary structure prediction programs (Figure
5.6.C-F). Using overlapping regions from all α-helix predictions, the optimal region within 

*HUG1* chosen for alanine mutagenesis was the region spanning Hug1.Val17 to Hug1.Cys24

(Figure 5.6.G; HUG1.D1). A β-sheet was also predicted by RaptorX (Figure 5.6.D) at 

Hug1.Tyr38 to Hug1.Asp43; therefore, comparable alanine mutagenesis was performed at 

Hug1.Lys35 to Hug1.Phe40 (Figure 5.6.I; HUG1.D3) to probe potential β-sheet involvement in 

*HUG1* activity. The β-sheet predicted by both RaptorX (Figure 5.6.D) and PSIPRED (Figure 

5.6.F) between Hug1.Ile50 and Hug1.Gly57 (Figure 5.6.J; HUG1.D4) provided the last domain 

to be targeted for scanning alanine.

Scanning alanine mutagenesis was performed on each domain as described in (Lefèvre et 

al., 1997; Zhao et al., 2000) and resulting galactose-inducible plasmids expressing HUG1.D1- 

HUG1.D4 were transformed into *hug1A::KANMX6* deletion strains. Overexpression plating 

analysis was then carried out as previously described (Ainsworth et al., 2013). All strains 

demonstrated constant and unimpeded growth on dextrose-containing media (Figure 5.7; left 

panel) and galactose media (Figure 5.7; center panel), which is consistent with glucose 

repression of the GAL promoter (and, by extension, repression of *HUG1* expression) and
previous results showing galactose-induced *HUG1* overexpression is not sufficient in causing growth inhibition phenotypes, respectively (Ainsworth *et al.*, 2013; Basrai *et al.*, 1999).

Whenever hydroxyurea is included in the media (Figure 5.7; right panel), significant growth differences are noted between *GAL1-HUG1* and the mutant variants.

![Figure 5.7](image.png)

**Figure 5.7.** Overexpression phenotype plating assay of galactose-inducible *HUG1* (*GAL1-HUG1*) and four mutant *HUG1* variants (*GAL1-HUG1.D1-D4*) in *hug1A* deletion strains. Log phase cultures were serially diluted on plates supplemented with dextrose (DEX; left panel), galactose (GAL; center panel), and galactose with 150mM hydroxyurea (GAL+150mM HU; right panel). Plates were incubated at 30°C for 4 days and imaged.

Consistent with previous reports, *hug1A* strains with GAL vector only (Figure 5.7.A) shows the least growth inhibition while *hug1A* strains expressing intact *HUG1* under galactose promotion (Figure 5.7.B) shows the strongest inhibition (Ainsworth *et al.*, 2013; Basrai *et al.*, 1999). All four HUG1 mutants, HUG1.D1-D4 (Figure 5.7.C-F, respectively), demonstrate no growth effects and grow at similar rates to the GAL vector only control (Figure 5.7.A). These results clearly indicate that all scanning alanine mutations in *HUG1* had significant effects on Hug1p functionality. However, since these mutations altered at least 10% of the total amino acids within *HUG1*, true domain features and distinct amino acids functionality in Hug1p activity cannot be resolved. Thus, a more focused approach is required to identify key functional elements within Hug1p.
Error-prone PCR and EMS-induced random mutagenesis of HUG1 fails to identify required HUG1 interaction regions

As the previous scanning alanine mutagenesis process lacked robustness in identifying independent interaction or binding domains, we explored alternative options to introduce and screen for mutations affecting HUG1 activity. To verify regions required for Sml1p’s inhibition of its binding partner, Rnr1p, Zhao et al utilized error-prone PCR mutagenesis and plasmid reconstruction by homologous recombination to uncover amino acids required for Sml1p’s inhibitory action (Zhao et al., 2000).

Transformation and plasmid recombination into strains carrying mec1Δ sml1Δ deletions and complementation plasmid ADE3-P_{MEC1}-MEC1 allowed for colorimetric selection of colonies bearing the mutagenized Sml1p, as dysfunctional Sml1p mutants exhibit a mec1Δ sml1Δ phenotype and the complementary ADE3-P_{MEC1}-MEC1 plasmid is lost in division as it is no longer required for cell progression. The colorimetric assay leverages ade3Δ ade2Δ designer strains and the ADE3 gene (which is epistatic to ADE2 in the metabolic pathway) whose expression in ade3Δ ade2Δ strains results in red colonies and absence results in the formation of white colonies (Forsburg, 2001). Thus, when the complementary ADE3-P_{MEC1}-MEC1 plasmid is lost in division, white-red segmented colonies form that can easily be identified by inspection (Forsburg, 2001; Zhao et al., 2000). Segmented colonies can then be picked, plasmid extracted, and sequence verified to determine the mutated amino acids that affected expression (Forsburg, 2001). This exact approach was applied towards determining important regions in the HUG1.

The specifics of strain creation is outlined in detail in the Materials and methods.

A diagram of the mutagenesis in given in Figure 5.8 and discussed briefly here. Using a parent plasmid containing intact HUG1, primers binding up- and down-stream of HUG1 are designed with at least 45bp of homology to the target vector (Figure 5.8.A; up- and downstream
Figure 5.8. Diagram of HUG1 mutagenesis by error-prone PCR or EMS mutagenesis with outcomes for HUG1 mutations with and without effect on inhibitory activity.
homologous regions given as orange and blue rectangles, respectively). Error-prone PCR is then run to mutagenize the HUG1 insert (McCullum et al., 2010). Alternatively, EMS was added after low-fidelity PCR amplification to induce mutagenesis in the fragment and provide alternate base conversions for broader potential base exchanges (Lai et al., 2004). Mutagenized PCR fragments (HUG1*) were then purified and co-transformed into mec1Δ hug1Δ strains bearing an ADE3-PMEC1-MEC1 plasmid using a linearized vector to place the HUG1* mutants under HUG1 promoter control via the 45bp of homology flanking the HUG1* fragments.

Previous reports have indicated that hug1Δ deletions recover the synthetic lethality of mec1Δ (Basrai et al., 1999). Using similar logic applied to Sml1p functional inhibition determinations, we reasoned that HUG1* mutations which affect HUG1 functionality will present with the mec1Δ hug1Δ phenotype, and thereby discard the ADE3-PMEC1-MEC1 plasmid, while functional HUG1* will require the ADE3-PMEC1-MEC1 plasmid to avoid synthetic lethality of the mec1Δ. We thus anticipated white colonies to contain mutagenized HUG1* with significant effects on HUG1’s inhibitory function, while red colonies retained functional HUG1. After plating on selective media with low adenine concentrations, an assortment of red and white colonies were seen after 5 days of growth at 30°C. White and white-red segmented colonies were replica plated onto selective media with low adenine to ensure white colony formation (Figure 5.9).

After growing for an additional 3 days, white colonies were reseeded into selective media, grown overnight, and plasmids extracted. Extracted plasmids were then transformed into E. coli competent cells whereby plasmid was re-extracted for DNA sequencing. Of the eight strains verified by BigDye® terminator sequencing, one strain was positively mutated within the HUG1* open-reading frame. The remaining seven strains all contained intact HUG1. Thus the
assay as designed was considered to be insufficient at finding ineffective HUG1* mutants. We also attempted to exchange the HUG1 promoter with that of the high expression and constitutive GPD/TDH3 promoter to improve the expression of HUG1* mutants and increase the likelihood of intact HUG1-mediated synthetic lethality, however all five new white colonies tested by sequencing containing intact HUG1. The assay was thereby abandoned as it yielded HUG1* mutants only 8% of the time and was not stringent enough to detect mutational effects on HUG1* inhibitory activity.

5.4.7. Site-directed mutagenesis of HUG1

Failure of the error-prone PCR mutagenesis prompted a new approach to solving HUG1’s domain dependencies. Similar to error-prone PCR, site-directed mutagenesis (SDM) utilizes PCR to create desired mutational constructs in gene ORFs. The major difference, however, is
that SDM allows for selective, rationally-designed (i.e., non-random) mutations in gene products. Likewise, mutations can be engineered to incorporate desired amino acid modifications to change global protein features such as solubility as well as local effects to amino acid such as size, charge, and polarity.

In section 5.4.5, scanning alanine mutagenesis (a type of SDM) was employed to mutate large domains within HUG1 to identify regions of secondary structure with functional importance. However, mutations were too large to discriminately identify specific effects on HUG1’s inhibitory activity. To mitigate these shortfalls, we redesigned the scanning alanine mutagenesis protocol to only modify three to four amino acids to alanine, and iterated this process across the entire HUG1 protein to create a total of 22 HUG1* mutants (HUG1 contains a total of 67 amino acids excluding start and stop codons).

Figure 5.10 outlines the design of the 22 HUG1* mutant plasmids using plasmid reconstruction by homologous recombination. Briefly, intact HUG1 (flanked by 35bp of the HUG1 promoter on the 5’ end and 66bp of the HUG1 terminator on the 3’ end) was inserted into a small shuttle plasmid to serve as template for the scanning alanine mutagenesis. Mutagenic primers were designed to flank the site of the desired mutation and contain sequences coding for three alanines at the 5’ ends.

Primers were then phosphorylated and high-fidelity PCR used to amplify the entire plasmid, thereby creating a large, linear strand of DNA with phosphorylated ends (Figure 5.10.A). T4 ligase was then used to re-circularize the linear strand into a functional plasmid which was subsequently transformed into E. coli competent cells (Figure 5.10.B). This effectively forms a mutated HUG1* where the mutation is directed by the binding site of the original mutagenic primers.
To create a galactose inducible form of the HUG1*, HUG1* was amplified from the newly-created plasmid by high-fidelity PCR and co-transformed into hug1Δ P_RNR2∷FLAG∷RNR2 strains with a linearized target vector containing the GAL promoter and homologous ends used for plasmid reconstruction by homologous recombination (Figure 5.10.C; orange and green rectangles). Strains containing an N-terminally FLAG epitope tagged RNR2 (P_RNR2∷FLAG∷RNR2) and the hug1Δ deletion were chosen to facilitate downstream expression and co-immunoprecipitation assays (sections 5.4.9 and 5.4.10, respectively). Strains positive for
$P_{\text{GAL-}HUG1^*}$ uptake were verified by selection and colony PCR. Notably, mutagenesis to three alanines also results in the introduction of a $PstI$ restriction enzyme site which was used for more extensive verification prior to plasmid sequencing. To sequence the new constructs, plasmids were extracted from yeast, transformed into E. coli competent cells, purified, then sequenced by BigDye® terminator sequencing. Successful transformants were used in overexpression plating analysis, western blot expression experiments, and co-immunoprecipitation assays described in Sections 5.4.8, 5.4.9, and 5.4.10.

5.4.8. Overexpression phenotyping assay reveal region-specific growth variability across $HUG1^*$ mutations

$HUG1$ overexpression growth phenotyping assays have previously been used to demonstrate $HUG1$’s negative role in the $MEC1$ pathway (Ainsworth et al., 2013; Basrai et al., 1999) as well as determine the effects of large-domain $HUG1$ knockouts on growth inhibition (Section 5.4.5). The new $HUG1^*$ SDM mutants were placed under identical promoter control and are compatible with the overexpression plating assays described previously. As such, strains containing all 22 $HUG1^*$ SDM mutants were grown, treated, and serial dilutions plated as described previously (Ainsworth et al., 2013; Basrai et al., 1999). After seven days of growth, a large variation in growth effects were observed across the mutants (Figure 5.11.A). Strains expressing $HUG1^*$.1-7 exhibited similar growth patterns to the intact $HUG1$ control ($GAL-HUG1$) whereby density of cells at higher dilutions was considerably lower than the vector control. Interestingly, a clear loss of growth inhibition was seen in $HUG1^*$ mutants, $HUG1^*$.6, 8, 10, 12-17, and 19. This region is clustered with the Dif1-hug mutation and exhibited a high

§§§§ Note that $HUG1^*$ mutants are annotated with the mutant notation followed by the respective mutant number (Hug1*.1p). The mutant number is indicative of the amino acids altered in sets of three starting with Hug1p’s second amino acid, Thr2. For example, Hug1*.14p refers to the amino acid change from P41K42D43 to A41A42A43. The only exception is Hug1*.22p, which mutates the final four amino acids in the Hug1p to alanines.
density of hydrophobic amino acids (common to protein interaction motifs). More importantly, suggests an independent and identifiable domain that is vital to \textit{HUG1} functionality. The remaining mutants showed little growth variation with respect to the intact \textit{HUG1} control.

To corroborate these results, we also performed a complementary overexpression growth assay in liquid media as described previously (Toussaint & Conconi, 2006) with some modifications. Briefly, log-phase culture dilutions of the same strains used in the overexpression
plating phenotype assay were aliquoted into 96 wells in triplicate and HUG1* mutant expression induced with galactose and 150mM hydroxyurea. A spectrophotometric plate reader was used to monitor the 660nm absorbance wavelength (OD\textsubscript{660}; giving an approximation of cell concentration) for all cultures every ten minutes for 48 hours. The growth rate of each sample was determined by measuring the differential OD\textsubscript{660} versus time at five consecutive points. The maximal growth rates (μ\textsubscript{max}) for each strain replicate was found and averaged. Maximal growth rate means clearly vary according to strain type (Figure 5.11.B), however considerable variability exists for the maximal growth rate between replicates. A one-way ANOVA comparing the all means of strain maximal growth rates shows no significant difference (F(23,48)=0.624; p=0.889). Thus, the overexpression liquid phenotype assay was not sensitive enough to capture the growth related variations of overexpressed HUG1* mutant strains when exposed to hydroxyurea.

5.4.9. Western blotting assays uncovers expression problems in the HUG1 mutant constructs

Verification of the unique HUG1* growth inhibition profile identified in Figure 5.11.A required corroboration with proteomic data to rule out false reads due to variable protein expression levels. As such, strains with and without HUG1* mutations were run via Western blot to provide protein quantities upon induction of galactose and hydroxyurea. Log-phase strains containing GAL1 only, GAL1-HUG1 and GAL1-HUG1* mutants 1-4, 7, were transferred to selective media supplemented with galactose and 100mM hydroxyurea and expression induced for 3 hours prior to collecting whole cell extracts. Whole cell extracts were then loaded on an SDS-PAGE gel and run. Western blot analysis was then run on the resulting gel using antibodies to Hug1p (anti-HUG1). Clear deviations in Hug1p and Hug1*p cellular expression levels was seen across the mutants tested. While the wild-type Hug1p exhibited normal levels of expression (Figure 5.12.A.1), the Hug1p mutants, Hug1*.1p and Hug1*.3p, both showed no
measurable expression under similar conditions (Figure 5.12.A.3 and Figure 5.12.A.5).

Hug1*.2p and Hug1*.4p, on the other hand, both exhibited appreciable levels of expression (Figure 5.12.A.4 and Figure 5.12.A.6), with Hug1*.4p demonstrating a higher level of expression than the intact Hug1p control.

![Western blot expression analysis of GAL1-HUG1 and the GAL1-HUG1* mutant strains. (A) Whole cell extracts were collected from strains expressing intact HUG1 (GAL1-HUG1), HUG1* mutants (GAL1-HUG1*,1-4), or a vector control (GAL only) after induction with galactose and 100mM hydroxyurea for 3 hours and a Western blot performed with antibodies binding to Hug1p (anti-HUG1) and Tub1p (anti-TUB1; loading control). (B) Whole cell extracts were collected from strains expressing intact HUG1 (GAL1-HUG1), HUG1* mutants (GAL1-HUG1*,7, 10, 12, and 21), or a vector control (GAL only) after induction with galactose and 100mM hydroxyurea for 3 hours and a Western blot performed with antibodies binding to Hug1p (anti-HUG1) and the FLAG epitope (anti-FLAG) to analyze FLAG-Rnr2p expression.](image1)

Similar expression variability was seen in other Hug1p mutant strains, including low expression in the Hug1*.7p and Hug1*.12p (Figure 5.12.B.3 and Figure 5.12.B.5, respectively) relative to their respective control (Figure 5.12.B.1) and normal expression in the Hug1*.10p and Hug1*.21p strains (Figure 5.12.B.4 and Figure 5.12.B.6). Clearly, these expression differences can cause significant problems in interpreting the overexpression plating analysis results, whereby low mutant expression prevents inhibition effects regardless of truly modified inhibition activity of the Hug1p mutants.

Interestingly, low expression was seen in the Hug1*.7p mutant strain, but the comparable strain in the overexpression plating analysis demonstrates significant reduction in growth and appears to be acting as wild-type Hug1p. This conflicting result suggests a more nuanced and
complex relationship between Hug1p expression levels and its inhibition activity, though it also indicates that protein expression levels may not be the major contributing factor to Hug1p-mediated growth inhibition seen in the plating assays.

5.4.10. Co-immunoprecipitation assays demonstrate weak interaction between select Hug1p mutants and Rnr2p

Despite the poor expression seen in the Western blotting assays from the previous section, co-immunoprecipitation assays were carried out on the galactose and hydroxyurea-induced HUG1 mutant strains to examine the effects of mutations on Hug1p’s interaction with Rnr2p. Unlike the previous co-immunoprecipitation assay (Section 5.4.4), these assays were designed to pulldown Rnr2p via the FLAG epitope tag fused to its N-terminus. Intact HUG1 and HUG1* mutant strains were prepared as described in the previous section and whole cell extracts collected. Whole cell extracts were then run through an anti-FLAG column to bind FLAG-RNR2. Elutions were subjected to Western blotting analysis whereby anti-FLAG and anti-HUG1 antibodies were used for staining.

To account for variable Hug1p concentrations, Western blots were loaded with respective to total protein concentrations as well as Hug1p and Hug1*p normalized

![Figure 5.13](image-url) Co-immunoprecipitation analysis of FLAG-Rnr2p and intact Hug1p or select Hug1p mutants. Western blots of FLAG pulldown assay elutions were stained with antibodies to Hug1p (anti-HUG1) and to the FLAG epitope (anti-FLAG) with protein loading normalized to (A) total protein concentration and (B) relative Hug1p and Hug1*p mutant protein concentrations.
concentrations. Westerns run with total protein concentration loaded (Figure 5.13.A) stained with large amounts of FLAG-Rnr2p (Figure 5.13.A; bottom row), however no staining was evident for the wild-type Hug1p control (GAL1-HUG1; Figure 5.13.A.1). No Hug1p was pulled down for mutant strains Hug1*.10p and Hug1*.12p (Figure 5.13.A.4 and Figure 5.13.A.6, respectively) while only very faint staining of Hug1*.7p and Hug1*.21p (Figure 5.13.A.3 and Figure 5.13.A.5, respectively) were noted. When protein loading was normalized to the relative Hug1p or mutant concentrations, slightly different results were obtained. All strains containing a form of Hug1p (intact or mutant) stained positive for Hug1p (Figure 5.13.B), but were all very faint with the exception of Hug1*.7p Figure 5.13.B.3.

These results further confound our understanding of the Hug1p-Rnr2p interaction mechanism. As Rnr2p is known to interact with other proteins, it is likely that pulling down Rnr2p instead of Hug1p results in significantly lower instances of Rnr2p-Hug1p complexes. Indeed, when Hug1p was used as the pulldown target, considerably higher concentrations of both Hug1p and Rnr2p were observed. Taken together, these results suggest improvements to the co-elution can be achieved by co-expressing FLAG-Rnr2p and Hug1p-HA and running co-immunoprecipitations using anti-HA beads to pulldown Hug1p-HA.

5.4.11. Fluorescent yeast two-hybrid assay uncovers interactions between HUG1 and two human RNR2 homologues

Eukaryotes utilize an evolutionarily conserved mechanism to respond to DNA damage. Proteins expressed in the ATR damage pathway share both functional and sequence similarity to those in the yeast MEC1 pathway, including effector kinases ATR (Mec1p in S. cerevisiae), ATRIP (Ddc2p in yeast) and the ribonucleotide reductase small subunits, R1 (Rnr1p in yeast) and R2/hRRM2 (Rnr2p in yeast). (Ciccia & Elledge, 2010; Harper & Elledge, 2007; Zhou, B. B. & Elledge, 2000) Humans express two isoforms of the ribonucleotide reductase small subunit:
hRRM2, whose expression is restricted to S-phase where it produces dNTPs for DNA synthesis, and p53R2, whose expression can be found in G0/G1 phase and early S-phase, but is more importantly significantly induced in response to DNA damage by a p53 (a human tumor suppressor gene). (Engstrom et al., 1985; Nakano et al., 2000; Tanaka et al., 2000; Xue et al., 2003; Yamaguchi et al., 2001) Overexpression of hRRM2 has been correlated to increased pancreatic adenocarcinoma invasiveness (Duxbury & Whang, 2007) and implicated in tumor angiogenesis (Zhang, K. et al., 2009). Both p53R2 and hRRM2 serve as prognostic biomarkers for colon cancer metastasis (Liu et al., 2007) and Stage I and II Non-small cell lung cancers (Hsu et al., 2011)

Interestingly, sequence alignment of hRRM2 and p53R2 to the yeast RNR2 using EMBOSS Needle global alignment (Supplementary Figure 5.15 and Figure 5.17, respectively) showed the yeast RNR2 shared approximately 70% sequence similarity between the human homologs. This is especially remarkable considering p53R2 and hRRM2 share 76% sequence similarity between each other using the same metric (Supplementary Figure 5.16). As Hug1p has been discovered to interact and inhibit the yeast Rnr2p, we reasoned that it may also serve similar function when expressed alongside the human Rnr2p homologs since the sequence homology is similar.

Thus, the p53R2 and hRRM2 cDNA sequences were both fused to the NyEGFP1 split GFP fragment and transformed into strains containing HUG1-CyEGFP1 under its native promotion. Cultures were then grown and induced with galactose and hydroxyurea as described for the original Hug1p-Rnr2p split GFP experiment and imaged by fluorescent microscopy. In both p53R2 and hRRM2, faint but detectable signal was observed (Figure 5.14). Interestingly, p53R2 (Figure 5.14, bottom row) demonstrated roughly 2 fold brighter fluorescent intensity
when compared to hRRM2 (Figure 5.14, top row) split GFP constructs as determined by densiometric analysis. This is likely due to slight sequence variations between p53R2 and hRRM2 that results in more favorable binding conditions for Hug1p into p53R2. A recent structural analysis of the two proteins has shown that a key tyrosine (Tyr221) in hRRM2 involved in coordinating the hydrogen bond network for the dityrosyl radical transfer is replaced by phenylalanine (Phe183) in p53R2 which results in significant effects to the respective RNR’s activity. (Zhou, B. et al., 2010)

Sequence alignments of the yeast RNR2 to p53R22 reveals that the yeast also express a phenylalanine at the comparable site (Phe228; Figure 5.17, yellow highlight). This similarity provides a tenuous link between the brighter fluorescence seen in p53R2 and the yeast RNR2 split GFP assays. Interestingly, when we mutated Rnr2p.Phe228 to Tyr228, we saw no growth effects in the mutant strains. However, overexpression phenotype assays revealed no significant disruption in Hug1’s ability to inhibit cell growth in the mutant Rnr2p.Tyr228 stain (data not

Figure 5.14. Fluorescent yeast two hybrid analysis of Hug1-CyEGFP1 and human ribonucleotide reductase small subunit (hRRM2; top row) and human p53-inducible ribonucleotide reductase small subunit (p53R2; bottom row) with and without 2% galactose and/or 150mM hydroxyurea. Arrows indicate positive fluorescence as detected by densiometric analysis.
shown). As such, further studies are required to identify the Hug1 interaction regions within yeast Rnr2p and the human RNR homologs.

5.5. Conclusions

In our previous work, we established HUG1 as a negative regulator of the MEC1 DNA damage response pathway. We proposed that Hug1p’s inhibitory action was the result of its co-localization with the RNR small subunit heterodimer, Rnr2p-Rnr4p based on sequence homology and fluorescent microscopy data. (Ainsworth et al., 2013) In this study, we extended the findings of our previous work to examine if Hug1p interacts with Rnr2p and, if so, which domains within Hug1p are important to the interaction.

Overexpression of Hug1p did appear to significantly affect dNTP levels in the presence of hydroxyurea. However, as hydroxyurea already drastically reduces dNTP concentrations, it is likely that, though Hug1p may be affecting dNTP pools, dNTP levels were at the lower limit of HPLC detection thereby making it difficult to quantitate actual Hug1p effects.

The traditional yeast two-hybrid system - commonly used to quickly detect protein-protein interactions - failed to detect an interaction between HUG1 and RNR2. However, these results may be explained by the disruption of the ASF1 depressor sequence when the HUG1 ORF is transferred into the new vector constructs, resulting in significantly reduced HUG1 expression.

An alternate method of protein interaction was therefore pursued. Using the fluorescent yeast two-hybrid system (split GFP), C- and N-terminal yEGFP1 fragments were fused to HUG1 and RNR2 and placed under native and GAL promoter control, respectively. Hydroxyurea and galactose induced expression of the constructs revealed bright fluorescence indicative of a positive Hug1p-Rnr2p interaction. These results were further verified by a co-immunoprecipitation assay demonstrating that Rnr2p co-elutes with Hug1p.
We next sought to identify the regions in the Hug1p sequence required for its interaction with Rnr2p. First, error-prone PCR and EMS mutagenesis was first attempted to randomly mutate *HUG1*, however the selection system for *HUG1* mutants failed to identify true positives as determined by sequencing. Instead, 22 *HUG1* mutants spanning the *HUG1* gene were generated by site-directed mutagenesis and growth inhibition effects confirmed by overexpression plating analysis. A novel region spanning amino acids Hug1p.Lys23 to Hug1p.Gly58 was found to affect Hug1p’s inhibitory, however overexpression liquid growth analysis cold not corroborate the profiles due to large variances in the maximal growth rate means. Likewise, western blot analysis revealed variable protein expression levels that may be influencing the overexpression results. Co-immunoprecipitation of FLAG-Rnr2p also demonstrated inconsistent co-elution of Hug1p, Hug1*p, and Rnr2p. Currently, the *HUG1* mutants are being redesigned to incorporate a C-terminal fusion of the HA-epitope tag to allow for direct pulldown of Hug1*p mutants and counterstaining to the FLAG-Rnr2p. We anticipate these new constructs will more accurately detect the Hug1p-Rnr2p as Hug1p likely binds Rnr2p stoichiometrically (resulting in equal protein staining when Hug1p-HA is pulled down) while Rnr2p interacts with other partners (resulting in fewer bound Hug1p and, by extension, less detected in the FLAG-Rnr2p pulldown assay).

Finally, the *HUG1* split GFP was used to test the interaction between two human RNR2 homologs, p53R2 and hRRM2. Surprisingly, fluorescence was observed for both human homologs, indicating a positive interaction between Hug1p and p53R2, and Hug1p and hRRM2. The interaction signal, however, was weaker than that of the Rnr2p-Hug1p split GFP experiment which suggests that though may be a common interaction motif in between the RNR homologs, slight sequence variations and amino acid composition result in weaker interactions.
A recently released study has confirmed the Hug1p-Rnr2p interaction through co-elution assays similar to the co-immunoprecipitation assays performed in this study. Also, circular dichroism, size exclusion chromatography, and NMR structural analysis revealed that Hug1p is an intrinsically disordered protein that is natively “pre-molten globule like” with weak helical propensity between amino acids Hug1p.V17-S27. NMR was also used to determine regions within Hug1p that were perturbed by titrating Rnr2p-Rnr4p with $^{15}$N labeled Hug1p and monitoring intensity shifts. They found that the region spanning amino acids Hug1p.Asn28 to Hug1p.Gly57 were slightly-to-highly perturbed by the titrations, and proposed that this domain was likely involved in Rnr2p binding. Excitingly, our overexpression phenotype analysis corroborates these results; we show that the amino acids covered by Hug1p*.10-16 are indeed necessary for Hug1p’s inhibitory activity. Not perturbed during the NMR titration experiments was the weak helical structural region. Meurisse et al did not perform mutations within the helical structure to verify the NMR perturbation results. Interestingly, our overexpression analysis detected a small region in the helical structure that is important in Hug1p inhibition (Figure 5.7; Hug1p*.8). Meurisse et al further performed mutagenesis on HUG1 and defined four distinct mutations that affected Hug1p’s ability to bind Rnr2p: Hug1p.R29A, Hug1p.G37AY38A, Hug1p.Y38L39E, and I50E/I51E. Again, our overexpression plating assay correctly identified these mutations as important to Hug1p’s inhibitory activity (Hug1p*.10, Hug1p*.10, Hug1p*.12 and 13, and Hug1p*.17). Finally, Meurisse et al utilized a traditional yeast two-hybrid system to identify a separate Hug1p binding partner, Dna2. Though no further analysis was performed with respect to HUG1 mutational analysis, the result is intriguing in that Dna2p has been shown to activate the MEC1 response in S-phase.(Kumar, S. & Burgers, 2013) This finding may offer new insights into an independent Hug1p function whereby inhibition of
Dna2p works to deactivate the MECl response after DNA damage has been repaired or upon completion of S-phase.

5.6. Supplementary material

5.6.1. EMBOSS Needle global protein alignment between yeast RNR2 and the human homolog, hRRM2

```
# 1: RNR2
# 2: hRRM2 (human RNR2)
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
# Length: 408
# Identity: 236/408 (57.8%)
# Similarity: 285/408 (69.9%)
# Gaps: 27/408 (6.6%)
# Score: 1187.5

#=======================================
RNR2  1   --------MPKETPSKAAADLSDEIJKDSKSLNKELETLREENRVKSD  42
     .|...|......|....| |....| ...||.:. hRRM2  1  MLSLRVPLAPITDPQQQLSPLKGLSLVD-KENTPPAL----SGTRVLS  45
RNR2  43  MLKEKLSKDAENHKAYLKHQVHRHKLKEMKEEPEPLNEDKERTVLFPIK  92
5.6.2. EMBOSS Needle global protein alignment between human homologs p53R2 and hRRM2

# 1: BAA92434.1
# 2: hRRM2
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
# Identity: 273/406 (67.2%)
# Similarity: 310/406 (76.4%)
# Gaps: 72/406 (17.7%)
# Score: 1450.5
# ========================================

p53R2 1 1MLSLRVPLAPITDPQQQLQLSPLKGLVLDKENTFPAISGTRVLASKTARR 0
hRRM2 1 MLSTLRVPLAPITDPQQQLQLSPLKGLVLDKENTFPAISGTRVLASKTARR 50
p53R2 1 -MGDPERFE----AAGLDDQDERSSDDTNEIEIKSN73EPLRKR3RRFVIF 45
hRRM2 51 IFQEFTEPKTAAAPGVE----------DEPLLENPRRFVIF 83
p53R2 46 PIQYPDWKMKYQAQQASFWTAEVE DLSKDLFHWNLKDAEK YFISHLAF 95
hRRM2 84 PIYHDIWQYKKAESASFWTAEVE DLSKDIQH WESLK PEE YFISHLAF 133
p53R2 96 FAASDGIVNENLVERFSQEVQ PEARCFYGFQIL ENVHSEMYSLIDTY 145
hRRM2 134 FAASDGIVNENLVERFSQEVQITEARCFYGFQIAMENIHEMLDITYLI 183
p53R2 146 IRDPKKREFLFNAIETMPYKKKAD沃尔FLASKTIFGERVVF AAAVEG 195
hRRM2 184 IKDPKEREFLFNAIETMPVKKKAD沃尔FLASKTIFGERVVF AAAVEG 233
p53R2 196 VFFSGSAFAIFWLKKRGLMPGLTFSNELISRDEGLHCDFACL MFQYLVNVK 245
hRRM2 234 IFFGSFSASFLWKKKRGLMPG LFSNELISRDEGLHCDFACL MFKHLVHK 283
p53R2 246 PSEEVEVREIIVD AVKEQEFLE TPVGLG NCMILKQ YEFVADRLLV 295
hRRM2 284 PSEEVEVREIINAVR IQEFLTE PVLG NCMILKQ YEFVADRLML 333
p53R2 296 ELGFSKVFQAENPFD MENISLEGK TNNFKEKR VSEYQ FRVAEMTTDNVF 345
hRRM2 334 ELGFSKVF VRVENPFD MENISLEGK TNNFKEKR VGEYQR MGVMSSPTENS F 383
p53R2 346 TLDADF 351
hRRM2 384 TLDADF 389

Figure 5.16. EMBOSS Needle global protein alignment between p53R2 and hRRM2.
5.6.3. EMBOSS Needle global protein alignment between yeast RNR2 and the human homolog, p53R2

Figure 5.17. EMBOSS Needle global protein alignment between yeast RNR2 and the human homolog, p53R2.
5.7. References


CHAPTER 6. CONCLUSION AND FUTURE WORK

6.1. Closing Remarks

Themes of transcriptional and post-translational control of the *MEC1* DNA damage response were explored throughout this study. Utilizing our understanding of the *MEC1* DNA damage transcriptional response, a survey of *MEC1* checkpoint pathway gene candidates were examined for their potential as transcript reporters in a carcinogen-sensing yeast whole cell biosensor. The *MEC1* DNA damage response, *HUG1*, as the most differentially expressed gene in response to DNA damage and replication stress (Basrai *et al.*, 1999), prevailed as the ideal candidate for use in biosensor development. We thus inserted a yeast optimized luciferase reporter under *HUG1* promoter control and analyzed its response to the DNA alkylating agent, methyl methanesulfonate (MMS). Compared to established fluorescent biosensors, the new luciferase whole cell biosensor exhibited improved sensitivity and dynamic range in detecting low titers of carcinogen. Likewise, we designed the system to reduce sample preparation time by establishing a simple one-step, *in vivo* protocol. Finally, a new vector system was generated to allow simple transfer of the improved luciferase reporter gene to new promoters systems.

Global transcription studies had previously revealed a unique transcriptional profile of *MEC1* DNA repair genes to increasing concentrations of alkylating agents. We first sought to further characterize this response using a GFP promoter-reporter system coincidentally with *MEC1* pathway genes. We then exposed the *MEC1/GFP* promoter reporters to an assortment of alkylating agents, and cellular and replicative stress inducers and monitored the activity of the GFP response by flow cytometry. We found that the biphasic gene response was reproduced only upon exposure to alkylating agents. All other DNA damaging and replicative stress agents either did not induce a response or demonstrated a monotonically increasing GFP reporter response without a characteristic decline in gene expression. Furthermore, the biphasic response
was found to persist through the MEC1 cascade, indicating that external epigenetic regulation was the cause of the unique profile.

To identify the origin of this response, we then examined accumulation of reactive oxygen species (ROS) as a potential cause as ROS accumulation has been linked to inhibition of stress response genes. Dihydrorhodamine 123 staining of ROS revealed that insignificant accumulation of ROS at low to mid-high ranges of MMS, and a large accumulation of ROS at the highest dosages. As MEC1 gene loss-of-induction occurs prior severe ROS accumulation, we determined that ROS were not the causative factor behind MEC1 gene repression. Apoptotic gene knockdowns were also performed to examine the role of apoptosis in MEC1 gene regulation. Despite a significant attenuation in MEC1 gene signaling, repression of MEC1 genes still occurred at the highest alkylating dosages, and we therefore concluded that apoptotic genes did not contribute to the MEC1 biphasic response.

Cell cycle analysis revealed that cells experiencing low to low-mid range alkylating agents proceed normally through the cell cycle, while those experiencing mid-high levels of alkylating agent arrest in the characteristic intra-S phase. At the highest MMS dosage, cells did not exit the cellular stage upon alkylating induction and instead entered a senescent state. This finding corroborates previous results which show that high MMS dosages induce gene transcription seen in the yeast stationary phase. We thus suggested that the MEC1 biphasic response was the result of an uncharacterized repression mechanism induced upon cellular entry into senescence.

Though demonstrating characteristics of a MEC1 negative regulator, the role of Hug1p in the MEC1 DNA damage response remained unclear. Using overexpression plating analysis, we discovered that HUG1 overexpression sensitized yeast strains to replication arrest by
hydroxyurea and DNA damage by MMS and bleomycin. Comparison of time-dependence of the positive *MEC1* regulator, Rnr3p, to Hug1p revealed that Hug1p shows a delayed response to replication stress and its temporal expression profile was reflective of *MEC1* negative regulators. Subcellular localization studies using GFP fused to the *HUG1* C-terminus demonstrated that Hug1p-GFP localizes to the nucleus in the absence of DNA damage, but shuttles to the cytoplasm in the presence of replication stress. This unique subcellular redistribution was found to be dependent on genes in the *MEC1* pathway and correlated with the localization of the *MEC1* positive effector, Rnr2p. These results suggested that Hug1p’s negative regulation was likely the result of its co-localization with Rnr2p.

As Hug1p shares sequence homology with Dif1p, a known Rnr2p inhibitor, we further investigated the putative interaction between Hug1p and Rnr2p. Traditional yeast two-hybrid assays were found to be ineffective at detecting the interaction, likely due to the disruption of the *HUG1* depression mechanism when engineering the plasmid constructs. An alternative yeast two-hybrid system - the split GFP assay – was developed to allow for the fusion of the split GFP fragment to *HUG1* while retaining *HUG1*’s promoter sequence. Using the fluorescent yeast two-hybrid system, we discovered that Hug1p does interact with Rnr2p as corroborated by Hug1p pulldown assays. We next set out to define regions within Hug1p that were necessary for its inhibition activity. Random mutagenesis of the *HUG1* ORF by error-prone PCR and EMS mutagenesis failed to identify new regimes within *HUG1*. Site-directed mutagenesis was then utilized to generate a total of 22 mutants which spanned the *HUG1* ORF. Overexpression plating analysis of strains expressing the *HUG1* mutants demonstrated uncovered a series of amino acids required for Hug1p inhibitory activity. However, attempts to corroborate these findings through co-immunoprecipitation assays have been unsuccessful, as Hug1p expression is too variable to
reliably assess Hug1p-Rnr2p co-elution efficiency. Finally, the split GFP system was used to test
Hug1p’s interaction capacity with human RNR2 homologs, p53R2 and hRRM2, and found to
successfully (but weakly) interact with both homologs. These results indicate that Hug1p
inhibits the ribonucleotide small subunit through a conserved interaction mechanism.

6.2. Future Work

Discoveries made throughout this dissertation brought about new and exciting questions
to be addressed. Below is a brief outline of potential extensions to this project.

6.2.1. Next-generation biosensors based on the secretable Gaussia luciferase

Our study utilized a single firefly luciferase in the DNA damage experiments whereby
signal was normalized to cell concentration. Though effective, a dual luciferase provides a
considerably better alternative to signal normalization. In a dual luciferase system, a Renilla
luciferase (Rluc) is typically fused under a constitutive promoter, such as PGK1 or ADH1, which
stably expresses Rluc while firefly luciferase (Fluc) is fused to the promoter of the gene-of-
interest. As the Rluc and Fluc utilize different substrates (coelenterazine and D-luciferin,
respectively), luminescence from either enzyme can be easily differentiated with little cross-
signal contamination.(McNabb et al., 2005)

A new luciferase has recently been discovered that has several exciting new properties.
The Gaussia luciferase (Gluc) is naturally secreted, relatively small (19kDa), incredibly stable,
utilizes the staple coelenterazine substrate, and is tremendously bright (nearly 1000-times
brighter than Rluc and Fluc).(Wu et al., 2015) All of these properties make it an ideal reporter
for use in yeast whole cell carcinogen biosensing. The next stage of the enhanced luciferase
detection system lies in the combination of Gluc with the Fluc system described in this
dissertation. Instead of using the Fluc as a reporter, however, Fluc can be incorporated under a
constitutive promoter and serve a normalization control while Gluc is placed under the desired promoter and acts as the reporter.

6.2.2. Connecting MMS-induced senescence and stationary phase genes

We have found that the MECl biphasic induction in response to alkylating agents is the result of cells entering a senescent state, however the genes required for that repression and the mechanisms by which the cells shut down the DNA damage response are still unknown. However, potential genes involved in that process may be able to be determined by a thorough revisiting of the global transcription analysis performed under high alkylating agent conditions. (Benton et al., 2006; Jelinsky et al., 2000; Jelinsky & Samson, 1999) Candidate genes can then be tested for effects on the MECl biphasic response by using the hug1Δ::GFP reporter system we implemented in this work.

6.2.3. Extending the split GFP system to the yeast HUG1* mutants and human RNR2 homologs

We have demonstrated the utility of the split GFP system in determining Hug1p interactions with Rnr2p, p53R2, and hRRM2. The next logical step for this project is to utilize the split GFP system in conjunction with the HUG1* mutant constructs to determine whether the Hug1p inhibition activity is lost due to disruption in its Rnr2p binding ability or disruption of an as-yet characterized inhibition role. These data can be further corroborated with co-immunoprecipitation assays that pulldown the Hug1*p mutants and assess Rnr2p co-elution. Likewise, the HUG1* mutant split GFP system can also be used to probe mutant interactions with p53R2 and hRRM2 through a similar experiment. The results from these studies can be used to clarify whether a conserved region within Hug1p is being used to bind across the RNR2 homologs. It is important to note that the split GFP system is amenable to high-throughput systems, such as flow cytometry, which can be used to quickly and quantitatively assess the strength of the split GFP interactions.
6.2.4. The novel interaction between \textit{HUG1} and \textit{DNA2}

A recent study has shown that Hug1p also binds to Dna2p, a helicase/nuclease involved in S-phase activation of \textit{MEC1}, and that the binding region is independent of the one used in the Rnr2p interaction. (Meurisse \textit{et al.}, 2014) However, little remains known about the mechanism or function of the Dna2p-Hug1p interaction. Utilizing the split-GFP system, we can confirm that Hug1p indeed interacts with Dna2p. Likewise, we can use the mutant \textit{HUG1}* in combination with the split GFP system to define the specific regions within Hug1p necessary for its interaction. More importantly, Hug1p-mediated inhibition needs to be determined. This is considerably more complex than determining the Hug1p’s inhibition of Rnr2p (i.e., dNTP measurements and overexpression plating analysis). However, an assay can be designed that leverages Dna2p stimulation of Mec1p activity \textit{in vitro} to assess Hug1p’s potential inhibition of this mechanism. (Kumar & Burgers, 2013) Briefly, the Mec1p-Dna2p phosphorylation assay is performed as previously described, with the exception that increasing titers of purified Hug1p are added to the assay. Hug1p’s inhibitory action against Dna2p can then be determined by the relative reduction in Mec1p stimulation. (Kumar & Burgers, 2013) Hug1p inhibition of Dna2p has potential to be a significant discovery, as it would further explain Hug1p’s recovery role in the DNA damage response and would provide new evidence for Hug1p’s involvement in the downregulation of the \textit{MEC1} response.

6.3. References


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William Barrett Ainsworth was born in Lafayette, Louisiana in 1985. He attended high school at Fontainebleau High School in Mandeville, Louisiana and graduated in 2004. He then matriculated at Louisiana State University where he completed his Bachelor of Science degree in Biochemistry with a minor in Chemistry in 2008. After transitioning into the Department of Chemical Engineering undergraduate program in August 2008 to obtain his second B.S. degree, Barrett was exposed to the department’s diversity in research, including the yeast DNA damage response work of his future mentor, Dr. Michael Benton. Upon enrolling into graduate school at Louisiana State University in January 2010, he focused his doctoral studies on uncovering novel features of the yeast MEC1 checkpoint pathway. This work resulted in the development of improved MEC1-specific whole-cell biosensors, revealed new cell cycle regulatory mechanisms of MEC1 pathway genes, and uncovered the function and mechanism of a previously uncharacterized protein in the MEC1 pathway. He will fulfill the requirements for the Doctor of Philosophy degree in Chemical Engineering in December 2015. He hopes to secure a research scientist position within the pharmaceutical industry where he can apply his knowledge of the DNA damage response towards the advancement of next generation cancer treatments.