# Dia2 mediates Mrc1 degradation to promote checkpoint recovery from DNA damage in *Saccharomyces cerevisiae*

A DISSERTATION SUBMITTED
TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA BY

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IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Adviser

August, 2012

# Acknowledgement

I would like to thank the following people from the science community for all the help during the course of my graduate career:

Deanna - for all the guidance on my graduate career and thesis project; for all the patience she had with me even when I was difficult to deal with at times and her trust in me in taking control over my projects (and for not giving me too much of a hard time when I decide to not pursue a career in research anymore!)

Undergraduates - for helping me with my projects; not only did they contribute to this thesis, but also that they taught me a lot about mentoring and team work. Their experimental contributions are as (roughly) indicated below:

Alisha B. - setup and optimization of the suppressor screen

Allison B. and Matthew S. - complementation of the suppressors

Owen S. - assistance with the Dia2/Mrc1 project, especially the suppressor *mrc1* allele

Ashwini A. - assistance with the Dia2/Mrc1 project, especially crucial results and figures for my manuscript

Past and current members of Koepp Lab - for all the help over the years with my experiments

Department of GCD and thesis committee members - for all the help with course works, seminars, and advice

Stephen J. Elledge (Harvard Medical School) and Masato Kanemaki (Osaka University) groups - for providing reagents used in this thesis

I also would like to thank family and friends who help me get through difficult times during my graduate career:

My parents and sister: for all the support and understanding throughout the years. I am sorry that I did not spend more time with you because of my hectic schedule. But, thank you for always caring for me and supporting me no matter what (for the most part). Special thanks to them for understanding that I want a major change for my career. Now if my parents will refrain from spoiling me too much it would even be better!

My friends: for all the help both inside and outside of the lab. Thank you for joining me to movies and fine-dining experiences (yum croissant), that is to help me relax, while I was constantly under stress from work. I would like to especially thank Deanna and Jay, Allison and Chris, Euhan and John, as well as Nicole and Haink for taking care of me during the time I was recovering (spoiler: you will see the word recover/recovery a lot in this thesis) from my injuries and surgeries (yes plural; I happened to fracture the fibula of each ankle in an eight-month span and I had never injured myself before until then).

My coaches and friends (Page, Karen, Ari, Amber, Viktors, Raleigh, and many others) from Parade Ice Garden: for helping me maintain ~~my sanity~~ a healthy work/life balance throughout my graduate career. While they do not take part in helping me with my projects, they have taught me valuable life lessons that were especially useful to me during my last few years of graduate school. Special thanks to Page for all your guidance and patience with me.

This thesis is dedicated to my late grandma Lee, Suk Jan and my late friend Kevin John Stoner (and I am very sorry that I did not visit you one last time before you left).

# Abstract

Cell cycle progression is monitored by checkpoint pathways that pause the cell cycle when stress arises to threaten the integrity of the genome. Although activation of checkpoint pathways has been extensively studied, our understanding of how cells resume the cell cycle when the stress is resolved is relatively limited. In this thesis, I identify the F-box protein Dia2 as a novel player in the S-phase checkpoint recovery pathway. Dia2 is required for robust deactivation of the Rad53 checkpoint kinase and timely completion of DNA replication during recovery from DNA damage induced by methyl-methanesulfonate (MMS). Aiming to identify the substrate of $SCF^{Dia2}$ (Skp1/Cul1/F-box Dia2) in checkpoint recovery, I performed a genetic screen to identify suppressors of *dia2*Δ cells. The screen identified a new checkpoint-defective allele of *MRC1* truncated at the C-terminus. I found that checkpoint-defective *mrc1* alleles suppress the MMS sensitivity and the checkpoint recovery defect of *dia2*Δ cells. In addition, Dia2 is required for Mrc1 degradation during S-phase checkpoint recovery. Furthermore, induced degradation of checkpoint-functional Mrc1 is critical to checkpoint recovery of *dia2*Δ cells. These data support a model in which Dia2 mediates Mrc1 degradation for cells to resume the cell cycle during recovery from MMS-induced DNA damage in S-phase.

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# List of Abbreviations

αF = alpha factor
ARS = autonomously replicating sequence
ATP = adenosine triphosphate
ATR = ataxia telangiectasia, mutated and Rad3-related
Bp = base pairs
Cdc = cell division cycle
CDK = cyclin-dependent kinase
CHX = cycloheximide
Ctf = chromosome transmission fidelity
Cul = Cullin
DIA = digs into agar
DNA = deoxyribonucleic acid
Dpb = DNA polymerase B (II)
G1 = Gap phase 1
G2 = Gap phase 2
GINS = go, ichi, ni, san
HU = hydroxyurea
Ino = inositol requiring
LRR = leucine-rich repeat
M = mitosis
Mcm = minichromosome maintenance
Mec = mitosis entry checkpoint
MMS = methyl-methanesulfonate
MRC = mediator of replication checkpoint
Noc = Nocodazole
ORC = origin of replication complex
PCNA = proliferating cell nuclear antigen
PCR = polymerase chain reactions
Pol = polymerase
Pre-RC = pre-replication complex
Ptc = phosphatase two C
Psy = platinum sensitivity
RFC = replication factor C
RPA = replication protein A
Rad = radiation sensitive
Rbx = RING box
RING = really interesting new gene
Pph = protein phosphatase
Rtt = regulator of Ty1 transposition
S = synthesis
SCF = Skp1/Cdc53/(Rbx1)/F-box
skp = suppressor of kinetochore protein mutant
sld = synthetically lethal with Dpb11

slx = synthetic lethal of unknown (X) function
ssDNA = single stranded DNA
SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA = trichloroacetic acid
TPR = tetratricopeptide repeat
WT = wildtype

# Experimental Contributions

**Chapter II:** Identification of putative Dia2 substrate(s) by a genetic suppressor screen

D.M. Koepp designed the genetic suppressor screen. C.M. Fong optimized and performed the screen. A. Bailey, A. Bock, O. Smith, A. Arumugam, and M. Schwartz assisted C.M. Fong in performing the screen.

**Chapter III:** Characterization of the suppressor *mrc1* allele

C. M. Fong designed and performed all of the experiments with inputs from D.M. Koepp. O.K. Smith and A. Arumugam assisted C.M. Fong in performing the experiments.

**Chapter IV**: Dia2 is a mediator of S-phase checkpoint recovery

C. M. Fong designed and performed most of the experiments with inputs from D.M. Koepp. A. Arumugam and O.K. Smith assisted C.M. Fong in performing the experiments. A. Arumugam performed experiments in Fig. 2D, 3B, and 6B. O.K. Smith performed experiments in Fig. 2A and 2B.

# Chapter I

# Introduction to cell cycle, ubiquitin-proteasome system, genome duplication and maintenance

## Cell Division Cycle

The cell is the structural and functional building block of all organisms. In order for the cell to survive, it must grow and replicate its contents so that it can divide into two daughter cells with equally distributed genome content. This is essential for the propagation of organisms no matter whether they are unicellular or multi-cellular organisms. In eukaroytes, each cell division cycle consists of four major stages: G1, S (Synthesis), G2, and M (Mitosis). The cell cycle is regulated so that each stage only occurs once per cycle in an irreversible order of direction. G1 is the first phase of the cell cycle where the cell grows, makes new proteins and organelles, as well as readies itself to enter the Synthesis phase of the cell cycle when conditions allow. During the Synthesis phase, the cell replicates its genome to allow propagation of genetic information to subsequent generations of cells. Next comes the G2 phase where the final preparation of cell division takes place before the cell enters Mitosis. In Mitosis, the duplicated chromosomes are condensed, aligned, and separated before cytokinesis takes place to divide the cell into two daughter cells each harboring one copy of the genetic content. This thesis is focused on the (Synthesis) S-phase of the cell division cycle, particularly on the regulation of DNA replication to maintain overall genomic integrity of the cell, using baking yeast *Saccharomyces cerevisiae* as the model organism. Baking yeast is an

excellent model for cell cycle study because 1) genetic, cellular, and molecular biology tools are well developed for cell cycle study and 2) the regulatory mechanisms are often conserved from yeast to humans. One major mechanism of cell cycle control is the ubiquitin-proteasome pathway, which will be discussed in the following section.

**Ubiquitin-proteasome pathway**

The cell cycle is constantly in a dynamic state of protein synthesis and degradation. Proteins are synthesized to perform their specific roles in a particular stage of the cell cycle. In many cases some of these proteins are to be degraded in order for cells to proceed to the next stage of the cell cycle. The ubiquitin-proteasome pathway provides the cell specificity in protein degradation for carrying out distinct roles in the cell cycle (Fig. 1).

The ubiquitin-proteasome pathway consists of two major steps: 1) the conjugation of multiple ubiquitin molecules to the protein to be degraded [1-3], and 2) the recognition and degradation of the substrate protein by the 26S proteasome [4] (Fig. 1). In the first step, ubiquitin-activating enzyme (E1) activates and binds to an ubiquitin in an ATP-dependent reaction [1]. The ubiquitin is then transferred to ubiquitin-conjugating enzyme (E2) [2]. With the help of ubiquitin ligase (E3), the ubiquitin is then conjugated to the substrate protein [3]. If the E3 is of the RING (Really Interesting New Gene) finger domain family, the ubiquitin is transferred directly from the E2 to lysine residue(s) of the E3-bound substrate [5-7]. If the E3 is of the HECT (Homologous to the E6-AP COOH Terminal) domain family, the ubiquitin is first transferred to a cysteine residue of the E3 before conjugating to the substrate [7, 8]. Multiple rounds of these reactions result in a

polyubiquitin chain conjugated to the substrate protein. In the next step of the ubiquitin-proteasome pathway, the polyubiquitinated substrate is recognized and degraded by the 26S proteasome complex [4].

*S. cerevisiae* is reported to have at least one E1, 16 E2, and 42 E3 enzymes [9, 10]. Different combinations of these enzymes provide a hierarchy structure to the ubiquitin-proteasome pathway. At the top of the hierarchy, the E1 activates ubiquitin for all the ubiquitin conjugation reactions as it can interact with all E2 enzymes in the cell. Each E2 has a set of E3 enzymes that it can interact with and conjugate ubiquitin to the E3-bound substrates. Because the interaction of an E3 and its substrate protein is specific, E3 enzymes dictate which proteins are targeted for ubiquitin-mediated degradation by the proteasome.

One of the prominent examples of E3 in cell cycle control is the SCF family of ubiquitin ligases. An SCF ubiquitin ligase complex consists of Skp1, Cul1/Cdc53, Rbx1, and an F-box protein [11-15]. The F-box protein provides specificity to the complex by interacting with the substrate protein, thereby dictating which protein is to be targeted for degradation by the 26S proteasome [13]. The F-box protein possess an F-box domain that is required for association with Skp1 and therefore the rest of the complex [11]. Domain structure characterization revealed that protein-protein interaction domains are often found in many F-box proteins which include leucine-rich repeats (LRR), tetratricopeptide repeats (TPR), and WD40 repeats (WD stands for tryptophan-aspartic acid) [16, 17]. These repeats potentially serve as docking sites for either regulators of the F-box protein or the substrate protein to be targeted for degradation.

## Genome maintenance

Genome maintenance is critical to the survival and well-being of the cell and subsequent generations of cells. In the case of higher eukaroytes, genome instability could lead to pathological disorder or cancer development [18]. Genome instability exists in the form of aneuploidy, chromosome mutation, rearrangement, deletion, or breakage, to name a few [19]. In addition to being an essential process to duplicate and propagate genetic information for all organisms, DNA replication is also considered a delicate cellular event because of the inherent risks of alteration to the genetic information during the process even in the absence of environmental stress. DNA replication must therefore be tightly regulated to prevent genome instability from threatening faithful duplication and subsequent segregation of the genome.

## DNA replication

### *Initiation*

DNA replication is initiated at specific locations of the chromosomes in *S. cerevisiae* during S-phase of the cell cycle. These replication origins are termed ARS (autonomously replicating sequences) and contain conserved sequences that are critical to the initiation event [20, 21]. Yeast has over 300 replication origins throughout the genome and they differ from each other in the time of initiation during S-phase as well as the efficiency of utilization [21]. Before DNA replication is initiated at any given replication origin, the origin must first be licensed by a series of protein complexes (Fig. 2).

The first step of origin licensing is accomplished by the binding of the Origin Replication Complex (ORC) to a replication origin. The ORC complex consists of six associated proteins (Orc1-6) and binds to conserved sequences within an ARS in an ATP-dependent manner [22-24]. With ORC at the origin, two addition key proteins, Cdc6 and Cdt1, are both recruited to the chromatin through association with ORC [25-27]. Next, Mcm2-7 (minichromosome maintenance complex components 2-7) complex is recruited to the origin and both Cdc6 and Cdt1 are independently required for the recruitment [25, 28]. The collective complex consists of ORC, Cdc6, Cdt1, and Mcm2-7 is termed the pre-Replication Complex (pre-RC). The formation of pre-RC at an origin indicates that the origin is licensed and is ready for the initiation of DNA replication. Intriguingly, some of the pre-RC components are regulated by the ubiquitin-proteasome pathway. For example, the Cdc6 protein is targeted for degradation to ensure that replication only occurs once in S-phase [29, 30], whereas the Cdt1 protein is targeted for degradation in response to DNA damage induced by Ultraviolet (UV) irradiation [31].

Unwinding of DNA at origins, stabilization of single-stranded DNA, and loading of DNA polymerases are essential to the initiation of DNA replication. Additional components are recruited to the pre-RC at the origin to facilitate these events before DNA initiation takes place. The replication factors required for the initiation event include Mcm10, Sld2, Sld3, Ctf4, GINS, Cdc45, RPA, and replication polymerases. The kinase activities of CDKs (cyclin dependent kinases) and the DDK (Dbf4 dependent kinase) mark the start of the initiation event [21, 32-36]. Among all of the factors required for initiation, the assembly and function of each identified replication factor will be discussed in this section.

The Mcm10 protein is one of the earliest factors recruited to the pre-RC. The recruitment of the replication factor Cdc45 to the pre-RC is dependent on Mcm10 [37-39]. Mcm10 interacts with Mcm2-7 and the initiation of DNA replication is dependent on this interaction [40-43]. Interestingly, Mcm10 also promotes phosphorylation of Mcm2-7 components by DDK [44]. In addition to its role in recruiting factors for initiation, Mcm10 was recently reported to have a role in unwinding DNA at replication origins [45].

The GINS (Go, Gchi, Ni, San) complex consists of Sld5, Psf1, Psf2, and Psf3 in yeast [46]. Activation of CDKs and DDK recruits the GINS complex to the pre-RC during initiation [47]. The association of Cdc45 with the pre-RC is also regulated by CDKs and DDK activities. Specifically, CDK phosphorylates Sld2 and Sld3 to promote interaction with replication factors Dpb11 and Cdc45, respectively. The Sld2-Dpb11 and Sld3-Cdc45 complex are then recruited to the complex in order to initiate DNA replication [48-52]. In the case of DDK, the recruitment of replication factor Cdc45 to the pre-RC may be linked to the DDK-driven phosphorylation of Mcm2-7 at the pre-RC [53, 54]. Cdc45 forms a complex with Mcm2-7 and GINS (termed CMG complex). In humans, Mcm10 and Ctf4 are required for the assembly of the CMG complex [55]. The CMG complex is proposed to be the DNA helicase required for chromosome unwinding [56, 57].

Upon DNA unwinding at origins by the Cdc45-MCM-GINS complex, RPA (Replication Protein A) binds and stabilizes the resulting single-stranded DNA [58]. The origin unwinding also allows for the recruitment of DNA replication polymerases [58], which indicates the final step of the initiation event. First recruited to origins is DNA

polymerase α, which is responsible for making short RNA primers for leading and lagging strand DNA synthesis [59]. Intriguingly, the recruitment of pol α is dependent on Cdc45, RPA, Ctf4, and Mcm10 proteins [60-63]. Additional replication factors including Mrc1 and Tof1 that have critical roles in regulating DNA replication are also recruited to the initiation complex [64, 65], and their roles will be discussed in the next section.

*Elongation*

The three major polymerases that have been identified for DNA replication are pol α, pol δ, and pol ε. Their roles in DNA synthesis was first elucidated using an *in vitro* SV40 virus system [66]. DNA pol α synthesizes short RNA primers so that pol δ and pol ε can perform lagging and leading strand DNA synthesis, respectively [59]. The loading of DNA pol α is followed by the recruitment of the clamp loader Replication Factor C (RFC), which is required for the loading of sliding clamp PCNA to the DNA [59, 67]. According to the current model, DNA pol α is replaced by pol δ and pol ε upon the loading of PCNA to the DNA and PCNA acts as a processivity factor for pol δ and pol ε in DNA synthesis [59].

Besides helicase and polymerases that drive DNA synthesis, additional players are required to regulate the interactions between factors within the replication machinery and fork progression in order to prevent mishaps in DNA replication and to maintain genome integrity. Replication factors Ctf4 and Mrc1 both travel with the replication fork and have key roles in DNA replication. Yeast cells that are deleted of both *CTF4* and *MRC1* genes are inviable, suggesting that they provide redundant but crucial roles in DNA replication. Ctf4 is essential in coupling Mcm2-7 and GINS to DNA pol α to coordinate

DNA replication [63, 68]. Cells that lack both the Mrc1 protein and Ctf4 association with GINS and pol α are also inviable, suggesting that the coupling of Mcm2-7 and GINS to pol α is key to Ctf4 function in coordinated DNA replication [63].

The Mrc1 protein was first identified as a mediator of the replication checkpoint [69]. It was later determined that Mrc1 also has a functional role in DNA replication, which is separable from its role in replication checkpoint [64]. The recruitment of Mrc1 to the replication machinery is dependent on Tof1, and the two proteins also form a complex together with Csm3 [65, 68]. Tof1 is not required for fork progression in an unperturbed S-phase. Rather, Tof1 but not Mrc1, plays a role in properly pausing the replication machinery when the complex encounters a replication barrier, as defined by a hard-to-replicate DNA region bound heavily by non-nucleosomal proteins [70]. However, replication forks progress slowly in S-phase in cells that lack Mrc1 [71], suggesting that the replication machinery may be compromised in cells lacking Mrc1. Interestingly, Mrc1 interacts with Cdc45, Mcm2-7, and DNA pol ε [72-74]. In addition, pol ε association with replicating DNA is reduced in cells deleted of *MRC1* at least under conditions of depleted nucleotides in S-phase [73]. Whether the Mrc1 role in DNA replication is dependent on the coupling of Cdc45-MCM-GINS helicase complex to pol ε similar to the role of Ctf4 in lagging strand synthesis remains to be determined.

**S-phase checkpoint**

At each stage of the cell division cycle, cells actively monitor progression and pauses the cell cycle should problems arise. For example, cells stall at G2 phase of the cycle when damage to the duplicated DNA is detected [75]. Cells also actively monitor

DNA replication in S-phase and slow down DNA replication should stress occur to threaten genome integrity [76, 77]. This stalling mechanism of the cell cycle is termed the checkpoint system [78].

DNA replication is delicate in the sense that inherent risks of mutations during the replication process in S-phase could threaten genome integrity. The association of factors within the replication machinery must be firmly sustained in the face of road blocks during the replication process. Environmental stress including agents that induce damage to the DNA structure puts extra pressure on the cell to stabilize DNA replication and prevent disassembly of the replication machinery. The S-phase checkpoint is in place to help cells maintain the integrity of the replication machinery in the face of exogenous or endogenous stress. To simulate the stress events, researchers utilize agents such as hydroxyurea (HU) and methyl-methanesulfonate (MMS) to investigate how cells handle DNA replication in the face of replication stress. HU is a replication stress agent that depletes nucleotides available for DNA synthesis, in turn arresting cells in S-phase. MMS is a DNA-damaging agent that alkylates the DNA. Identified pathways that deal with these replication stress events will be discussed in this section.

*Checkpoint activation*

During DNA replication, cells monitor the accumulation of single-stranded DNA as a result of replication stress or DNA damage to activate the S-phase checkpoint [77, 79, 80] (Fig. 3). Specifically, the coating of ssDNA by RPA initiates the S-phase checkpoint [80]. In S. *cerevisiae*, the initial signaling of the S-phase checkpoint leads to activation and recruitment of the 9-1-1 clamp complex (Rad17-Mec3-Ddc1 in yeast) and

Mec1/ATR kinase to the region of stress or damage [64, 81-83]. From there, Mec1 relays the checkpoint signal to downstream effectors through mediators that include Mrc1, Rad9, Tof1, and Csm3 [69, 84-87]. In the case of Mrc1 and Rad9, these mediators are subjected to phosphorylation at Mec1 consensus S/TQ sites, which in turn facilitates the recruitment of a key downstream effector, the Rad53 kinase [64, 69, 85, 88-90]. Once recruited, Rad53 is activated by Mec1 phosphorylation and autophosphorylation in *trans* [85, 91-93]. In the case of Mrc1, in addition to these S/TQ sites, other residues are also required to efficiently mediate checkpoint activation [94]. It was proposed that the S/TQ phosphorylation events are important for the initial enrichment of Mec1 at the site of stress, whereas additional residues are required to relay the signaling to Rad53 [94]. With the activation of Rad53 by the S-phase checkpoint, cells stabilize the replication fork and prevent origins from firing inappropriately [95-99].

*Checkpoint recovery*

As important as it is for cells to activate the S-phase checkpoint in the face of DNA damage, cells must deactivate the checkpoint to resume the cell cycle after exposure to the DNA damage in a process termed checkpoint recovery [100, 101]. Previous studies have identified players that modulate the S-phase checkpoint signaling pathway during recovery from DNA damage. DNA repair scaffold proteins Slx4 and Rtt107 were reported to be important for checkpoint recovery from MMS-induced DNA damage [102, 103]. In the presence of MMS, Mec1 phosphorylates the Slx4-Rtt107 scaffold to allow association of the scaffold with replication factor Dpb11 and replication fork restart during checkpoint recovery from DNA damage [104, 105].

In addition to recruiting the DNA repair scaffold for recovery, cells also act on the S-phase checkpoint effector Rad53 to inactivate checkpoint signaling during recovery. Specifically, phosphatases Pph3 and Ptc2 remove checkpoint phosphates from Rad53 when cells are recovering from MMS-induced DNA damage [106, 107]. Indeed, Rad53 dephosphorylation is sufficient for fork restart during checkpoint recovery [106]. Interestingly, fork recovery from the replication stress agent HU is not dependent on the Rad53 phosphatases [108]. Rather, fork recovery from HU is dependent on the chromatin remodeling complex Ino80 [109], suggesting that cells have distinct pathways to manage recovery from different sources of replication stress in S-phase.

**Linkage between the ubiquitin-proteasome pathway and the S-phase checkpoint**

Our group has identified a previously-uncharacterized linkage between the replication stress response and the SCF ubiquitin-proteasome pathway [110]. We recently found that the proteolysis of the *S. cerevisiae* F-box protein Dia2 is regulated by the S-phase checkpoint [110], which suggests a possible role of Dia2 in the checkpoint. However, what role does Dia2 play in the S-phase checkpoint remains an open question.

Dia2 contains two protein-protein interaction domains on each side of the F-box domain: TPR at the N-terminus and LRR at the C-terminus [111]. Dia2 is a chromatin-bound protein that binds replication origins [112]. Dia2 was recently reported to bind the replication machinery through interaction with replication factors Mrc1 and Ctf4, and the N-terminal TPR repeats of Dia2 are important for this association [113, 114].

Dia2 is required for genome maintenance in *S. cerevisiae*. *DIA2* genetically interacts with many genes that are involved in the S-phase checkpoint [115]. Cells deleted of the

*DIA2* gene exhibit genome instability including chromosome loss, rearrangement, and breakage [115, 116]. Furthermore, *dia2* null (*dia2*Δ) cells are sensitive to replication stress and DNA damaging agents HU and MMS [112, 115]. Interestingly, Dia2 is required for normal DNA replication progression in the presence of MMS [115]. Dia2 is an unstable protein, but the protein is stabilized by the activation of the MMS-induced S-phase checkpoint [110]. These results suggest that Dia2 may play a role in the S-phase checkpoint. Because Rad53 is constitutively phosphorylated in the absence of Dia2 [116], it seems unlikely that Dia2 is required for the initial signaling of checkpoint activation. Rather, we speculated that Dia2 may be involved in checkpoint deactivation for cells to resume the cell cycle during recovery.

The Mrc1 protein has recently been identified as a ubiquitin-mediated degradation substrate of $SCF^{Dia2}$ [114]. As mentioned, Mrc1 has roles in both DNA replication and the S-phase checkpoint activation [64, 71]. The degradation of Mrc1 is most prominent in S-phase cells arrested in HU [114]. However, it remains an open question what the biological relevance of Mrc1 degradation is and whether Mrc1 is degraded for a role in an unperturbed S-phase or in response to the S-phase checkpoint activation. Intriguingly, the human homolog of Mrc1, Claspin, is targeted for proteasome degradation by the $SCF^{\beta\text{-}TrCP}$ complex during recovery from replication stress or DNA damage before mitotic entry [117, 118]. This degradation is regulated by Polo-like kinase-1 (Plk1) phosphorylation which precedes the interaction between $SCF^{\beta\text{-}TrCP}$ and Claspin [117, 119].

To better understand the function of Dia2 in the S-phase checkpoint, this thesis study aimed to identify substrate(s) of Dia2 and investigate a possible role for Dia2 in checkpoint recovery from MMS-induced DNA damage. A better understanding of the

regulation of DNA replication in the face of DNA damage will likely be beneficial to the development of treatment to pathological disorders that are stemmed from genome instability. For example, the S-phase checkpoint is a target of interest for antitumor therapies because chemotherapy and radiotherapy induce genotoxic stress to trigger cell death in cancer cells. Inhibitors of checkpoint activators are currently being explored as treatments to overload cancer cells with genotoxic stress [120]. Furthermore, checkpoint recovery components may serve as alternative targets of treatment to sensitize cancer cells to antitumor therapies.

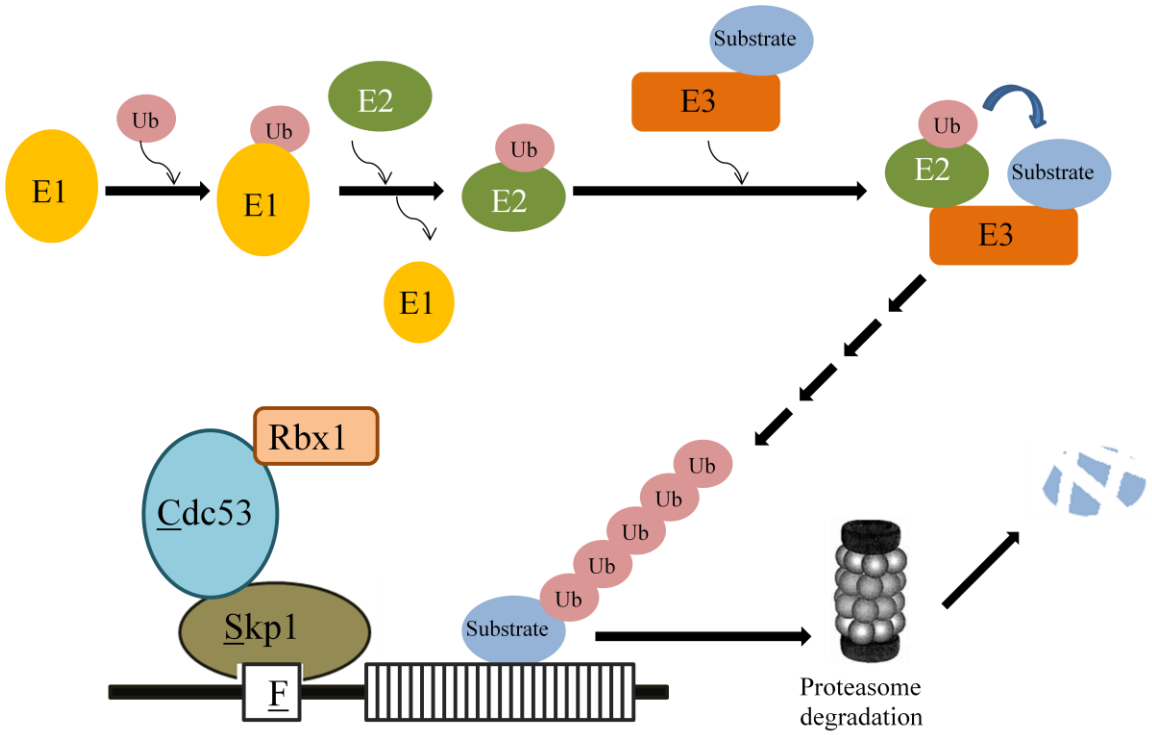


**Fig. 1.** The ubiquitin-proteasome pathway. A series of enzymatic reactions carried out by an E1 (ubiquitin-activating enzyme), an E2 (ubiquitin-conjugating enzyme), and an E3 (ubiquitin ligase) lead to the transfer of a ubiquitin to the substrate protein. Multiple rounds of these reactions conjugate a polyubiquitin chain to the substrate, resulting in substrate degradation by the 26S proteasome. The example of E3 shown here is an SCF (Skp1/Cdc53/Rbx1/F-box) complex.

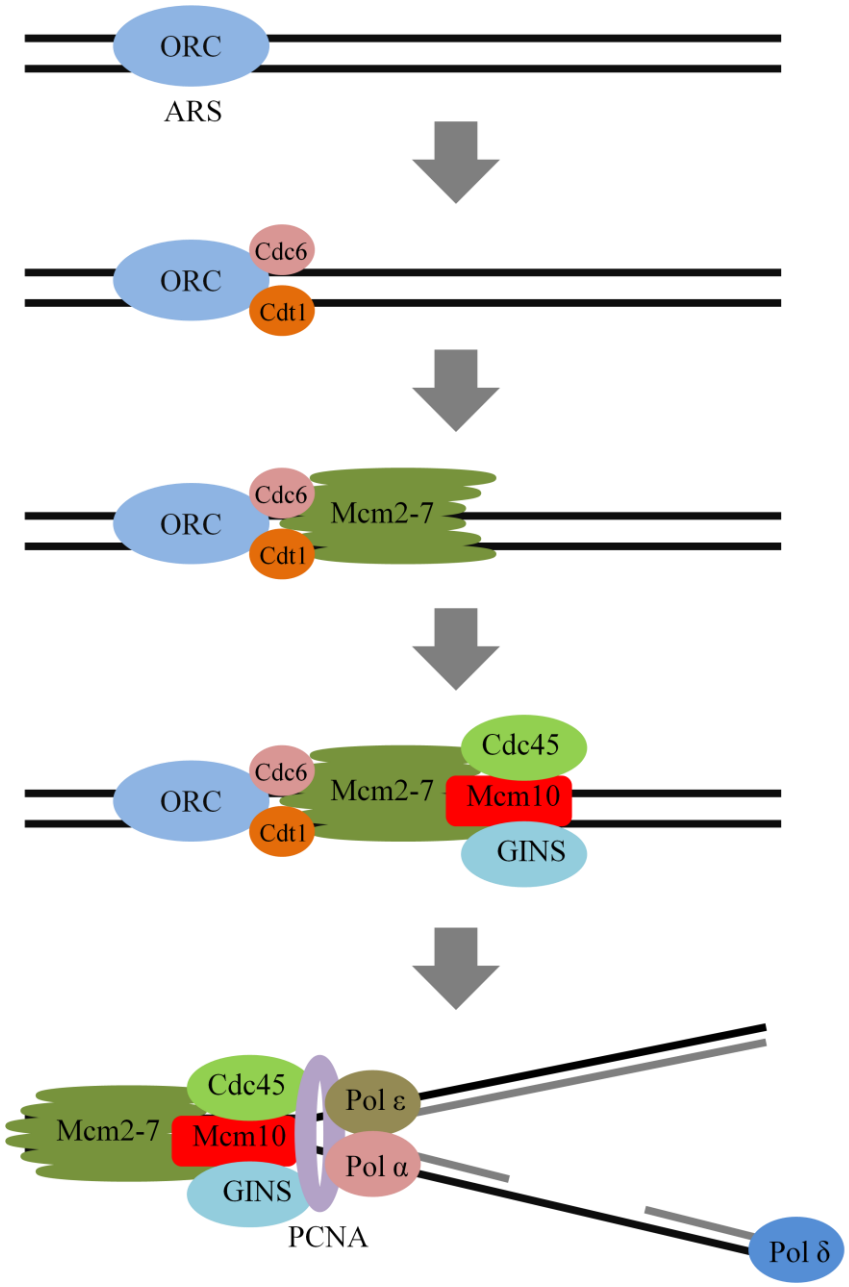


**Fig. 2.** Initiation of DNA replication. A replication origin (ARS in yeast) is licensed by the sequential assembly of the pre-RC, which includes ORC, Cdc6, Cdt1, and Mcm2-7. Upon formation of the pre-RC, additional replication factors including Mcm10, Cdc45, and GINS are recruited to form the initiation complex. DNA polymerases are then recruited to the initiation complex to initiate DNA replication. This is a simplified

schematic of the initiation of DNA replication that does not include all the proteins important for the initiation event.

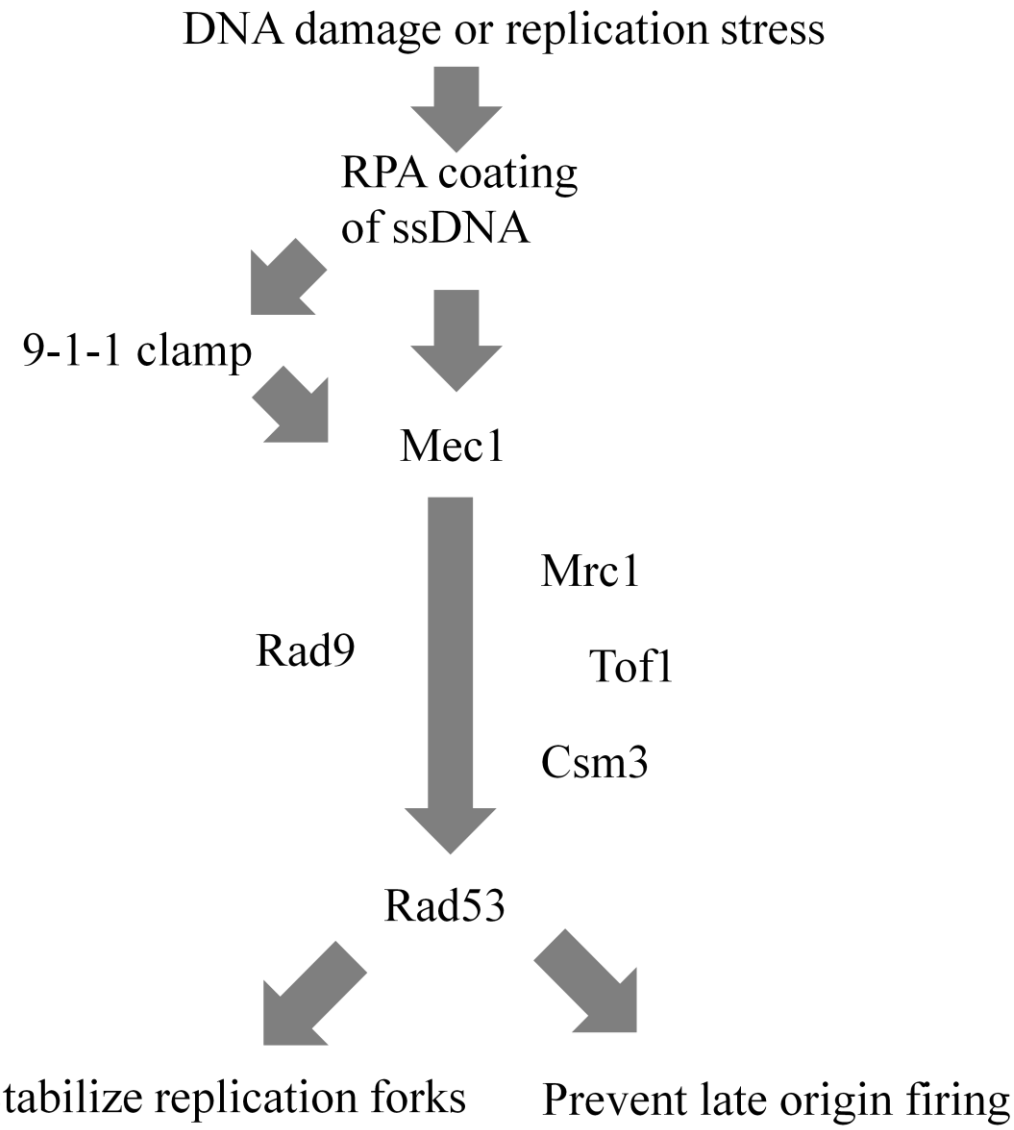


**Fig. 3.** The S-phase checkpoint pathway. The coating of ssDNA by RPA (Replication Protein A) leads to activation of the 9-1-1 clamp and Mec1 kinase. Mec1 kinase in turns activates and phosphorylates Rad53 kinase with the help of mediators that include Rad9, Mrc1, Tof1, and Csm3. The activation of Rad53 results in events that include stabilization of replication forks, as well as prevention of late origin firing.

# Chapter II

# Identification of putative Dia2 substrate(s) by a genetic suppressor screen

**Summary**

Dia2 is an F-box protein of an SCF (Skp1/Cdc53/Rbx1/F-box) ubiquitin ligase complex. Dia2 was proposed to have a role in the S-phase checkpoint. To elucidate the role of Dia2, I performed a genetic suppressor screen to identify genes that share the same biological pathway as *DIA2*. Specifically, mutants that suppressed *dia2*Δ sensitivity to MMS were isolated. The screen isolated six suppressor candidates. Three of the candidates were tested further and determined to possess a single-gene suppressor mutation. Complementary tests confirmed that one candidate was *mrc1* (mediator of replication checkpoint), consistent with an earlier study reporting that Mrc1 is an ubiquitin-mediated substrate of Dia2. However, the biological role of Dia2-mediated Mrc1 degradation remains an open question. This suppressor allele of *mrc1* could be key to elucidate the role of Dia2 in the S-phase checkpoint.

**Introduction**

Dia2 is an F-box protein of an SCF (Skp1/Cdc53/Rbx1/F-box) ubiquitin ligase complex [111]. The F-box protein is required for genome maintenance. Cells that lack *DIA2* exhibited chromosome breakage, loss, and rearrangement [115]. *dia2*Δ cells are also hyper-sensitive to DNA damaging agent MMS. Furthermore, *DIA2* genetically interacts with genes involved in the MMS-induced S-phase checkpoint [115]. Our group has recently shown that Dia2 is an unstable protein. However, the F-box protein is stabilized by the activation of the S-phase checkpoint in the presence of MMS [110]. These results suggest that Dia2 plays a role in the S-phase checkpoint. Because Dia2 is an F-box protein of an ubiquitin ligase, I hypothesized that Dia2 mediates ubiquitination of substrate protein(s) which is key to Dia2 role in the S-phase checkpoint.

As an unbiased approach to determine the function of Dia2 in the S-phase checkpoint, I performed a genetic suppressor screen in the presence of MMS to identify genes that are in the same pathway as Dia2. With this approach, it is possible that putative substrate(s) of Dia2 will be identified. I predicted that the substrate protein(s) accumulate in *dia2*Δ cells, resulting in the MMS sensitivity phenotype. The rationale is that mutations to the substrate protein(s) could alleviate the requirement of Dia2 in the S-phase checkpoint.

## Methods and Materials

*Yeast cultures*

Yeast cultures were grown according to standard protocols [121]. Rich media used in this study is YPD (Yeast Peptone Dextrose).

*Genetic suppressor screen*

*dia2*Δ suppressors were selected on media containing 0.007% MMS. MMS resistant candidates were backcrossed to the *dia2*Δ strain to identify recessive and single-gene mutants. Mutants that exhibited higher MMS resistance than wildtype in a *DIA2* background were eliminated. Candidates were crossed to the *mrc1*Δ and *ctf4*Δ strains for verification purposes.

*Strain construction*

Tables 1, 2, and 3 contain a list of strains, plasmids, and oligonucleotides used in this study. Deletion strains were generated by standard PCR replacement approaches [121]. To construct *mrc1*Δ*::HIS3*, the *HIS3* gene flanked by 5' and 3' UTRs of *MRC1* was amplified using pRS403 as the DNA template and primers CMF024 and CMF025. The PCR product was purified and transformed into the Y80 strain (wildtype). Transformants were selected on media lacking histidine. Candidates were then tested on media containing hydroxyurea (HU) because *mrc1*Δ is very sensitive to HU. Because of size difference in locus between *MRC1* and *HIS3*, the *mrc1*Δ strain was confirmed by genomic DNA PCR using primers CMF024 and CMF025. Other deletion strains used in this thesis work were generated in a similar manner.

*Growth assays*

10-fold serial dilutions of 2 x $10^7$ to 2 x $10^3$ cells were spotted onto media using a replica plater. Plates were incubated at 30ºC for two to three days.

Table 1. A list of strains used in this study.

| Strain | Genotype | Reference |
|---|---|---|
| Y80 | *can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 MAT**a*** | [112] |
| DKY194 | As Y80 but *dia2*Δ*::KanMX* | [112] |
| AKY159 | As Y80 but *dia2*Δ*::KanMX ctf4Δ*::*KanMX* | Koepp, unpublished |
| DKY645 | As Y80 but *dia2*Δ*::KanMX mrc1*Δ*::HIS3* | This study |

Table 2. A list of plasmids used in this study.

| Plasmid | Relevant Features | Reference |
|---|---|---|
| pRS403 | *HIS3* $Amp^r$ | [122] |
| pMRC1 | *CEN URA3 MRC1* $Amp^r$ | [69] |
| pDMK465 | *CEN URA3 DIA2* $Amp^r$ | [112] |

Table 3. A list of oligonucleotides used in this study.

| Oligo-nucleotide | Sequence (5' to 3') |
|---|---|
| CMF024 | GAAGTTCGTTATTCGCTTTTGAACTTATCACCAAATATTT TAGTGGGCCTCCTCTAGTACACTC |
| CMF025 | AGCTTCTGGAGTTCAATCAACTTCTTCGGAAAAGATAAA AAACCAGCGCGCCTCGTTCAGAATG |

## Results

*Suppressor screen conditions*

A genetic suppressor screen was performed to identify mutations that would suppress the MMS sensitivity phenotype of *dia2*Δ cells. I first optimized the percentage of MMS used in the screen so that wildtype cells would grow well whereas *dia2*Δ cells would not. Wildtype and *dia2*Δ cells were cultured and plated on rich media containing 0.005%, 0.007%, and 0.01% MMS. As shown in Fig. 1A, growth of wildtype and *dia2*Δ cells was indistinguishable on 0.005% MMS, whereas 0.01% MMS was too harsh even for wildtype cells. However, wildtype cells grew well on 0.007% MMS, whereas *dia2*Δ cells did not. Colony forming units (CFU) of *dia2*Δ mutants that were similar in size compared to wildtype were picked for further testing. To confirm the MMS resistance of these CFU, 10-fold serial dilutions of each candidate were spotted onto rich media containing 0.007% MMS. For example, all of the suppressor candidates shown in Fig. 1B were confirmed to be MMS resistance. A total of 105 MMS resistance candidates were confirmed.

*Dominant versus recessive mutation*

The 105 MMS resistance candidates were tested to determine whether they harbor dominant or recessive suppressor mutations. Each candidate was backcrossed to the *dia2*Δ strain, which has a wildtype copy of the candidate suppressor gene. The MMS sensitivity of the resulting candidate diploids was compared to the *DIA2/DIA2*, *DIA2/dia2*Δ, and *dia2*Δ*/dia2*Δ controls. If a suppressor mutation is recessive, the candidate diploid would "revert" back to MMS sensitive. For example, suppressor

mutations in candidates 1 and 4 are recessive, whereas the other candidates tested have dominant mutations (Fig. 2). Nine candidates were determined to be recessive and selected for further testing. I picked recessive candidates because 1) substrate mutation is likely to be recessive, and 2) suppressor genes are to be identified using complementation.

*dia2*Δ *suppressor or MMS hyper-resistant mutant*

Next, I tested whether the nine recessive candidates are *dia2*Δ suppressors or they simply have mutations that confer hyper-resistant to MMS. Each of the candidate was transformed with a plasmid that contains a wildtype copy of the *DIA2* gene. If any candidate with the *DIA2* plasmid grows better than wildtype on high concentration of MMS, it would suggest that the candidate has an MMS hyper-resistant mutation, rather than a mutation that specifically suppresses the *dia2*Δ strain. I found that three of the nine candidates grew better than wildtype at 0.02% and 0.025% MMS, whereas the rest did not (Fig. 3). I proceeded to the next step of the screen with the six *dia2*Δ suppressors - they are candidate 11, 20, 29, 36, 38, and 102.

*Suppressor: single- or multi-hit mutation*

Before I could identify the suppressor gene of each candidate through complementation, I determined whether the mutation is a single-hit. Each of the six *dia2*Δ suppressors was backcrossed to the *dia2*Δ strain. The diploid heterozygous of the suppressor mutation(s) was induced to undergo meiosis to form four haploid cells. The haploid cells were then assayed for growth on rich media containing MMS. If the suppressor mutation is a single-hit, two of the haploid cells would be MMS sensitive

*(dia2*Δ *SUP*$^{+}$) and the other two would be MMS resistant (*dia2*Δ *sup*$^{-}$) (Fig. 4). I tested candidate 29, 36, and 38 and all of them are single-hit suppressors.

*Complementation: a candidate approach*

I then determined the complementation group of each of the three candidates (29, 36, and 38) by crossing them with each other. For example, if candidate 36 and 38 possess different suppressor gene mutations, the diploid (36 x 38) would have a wildtype copy of each suppressor mutation and result in complementation of the suppression phenotype. In that case, the diploid would behave as *dia2*Δ and be MMS sensitive (Fig. 5). I found that all of the three suppressor candidates belong to different complementation groups.

Because Mrc1 and Ctf4 were reported to be substrates of Dia2 [114], I asked whether any of the three suppressors are alleles of *mrc1* or *ctf4*. Candidate 29, 36, and 38 were crossed to the *dia2*Δ *mrc1*Δ and *dia2*Δ *ctf4*Δ strains and the diploids were assayed for growth on rich media containing MMS. As shown in Fig. 6A, suppressor 38 is neither an allele of *mrc1* or *ctf4* due to complementation by a wildtype copy of the suppressor mutation from the parental *dia2*Δ *mrc1*Δ and *dia2*Δ *ctf4*Δ strains. However, suppressor 36 is an allele of *mrc1* because the suppressor remained MMS resistant after crossing to the *dia2*Δ *mrc1*Δ strain (Fig. 6A). Suppressor 29 was also tested but it is neither an allele of *mrc1* or *ctf4*. I also transformed a *CEN* plasmid containing a wildtype copy of *MRC1* into suppressor 36 to confirm that it is indeed an allele of *mrc1*. As shown in Fig. 6B, the *MRC1* plasmid complemented the suppressor phenotype and the strain was rendered MMS sensitive. I concluded that candidate 36 has a suppressor

mutation in the *MRC1* locus. The progress of the entire suppressor screen is summarized in Fig. 7.

**Discussion**

The genetic suppressor screen isolated three putative *dia2*Δ suppressors. One of them was identified as *mrc1* based on complementation tests. I look forward to future studies investigating on the remaining suppressors.

I found that most of the initial candidates have dominant mutations. I speculated that most of those are unspecific mutations that confer hyper-resistance to MMS, which are likely to be dominant mutations and easier to obtain compared to specific *dia2*Δ suppressors. In contrast, many of the recessive mutants are indeed *dia2*Δ suppressors (six out of nine) as opposed to non-specific MMS resistant mutants.

I attempted to identify some of the suppressors with a genomic DNA library. Briefly, a suppressor candidate in question was transformed with a genomic library. Countable number of cells were spotted onto media without MMS. The CFU were then replica plated to media with MMS. A CFU that picked up a plasmid containing the wildtype copy of the suppressor gene would result in complementation and MMS sensitivity. However, it is extremely difficult to determine MMS sensitivity based on colony size, and spotting assay would not be a viable option considering that hundreds and thousands of CFUs are to be screened. In addition, MMS could induce additional mutations that may interfere with the interpretation of complementation. Furthermore, it is possible that additional copy of gene(s) from the genomic library would affect growth of the transformants. An alternative approach is whole genome sequencing to identify the suppressor mutations. In that case, the suppressor mutants would first be backcrossed several times to segregate out any MMS-induced mutation in the genome that does not play a role in suppressing *dia2*Δ.

I instead used a candidate approach to determine that one suppressor is *mrc1*. Because *DIA2* genetically interacts with genes involved in the DNA replication and S-phase checkpoint, Mrc1 is a mediator of replication checkpoint, and both Mrc1 and Ctf4 have crucial roles in DNA replication, some of these suppressors may be genes in the DNA replication or the S-phase checkpoint pathways. Crossing *dia2*Δ suppressors to mutants in these pathways provides another viable option to identify the remaining suppressors. However, this approach is biased towards the DNA replication and S-phase checkpoint pathways and may be laborious considering that many genes have been implicated in those pathways.

Mrc1 was recently reported to be a substrate of Dia2 [114]. Isolation of a suppressor *mrc1* allele indicates that the suppressor screen is a viable approach to identify putative Dia2 substrate(s). The role of Mrc1 degradation remains an open question. In the next chapter, I characterized this suppressor allele in order to understand the role of Dia2-mediated Mrc1 degradation.

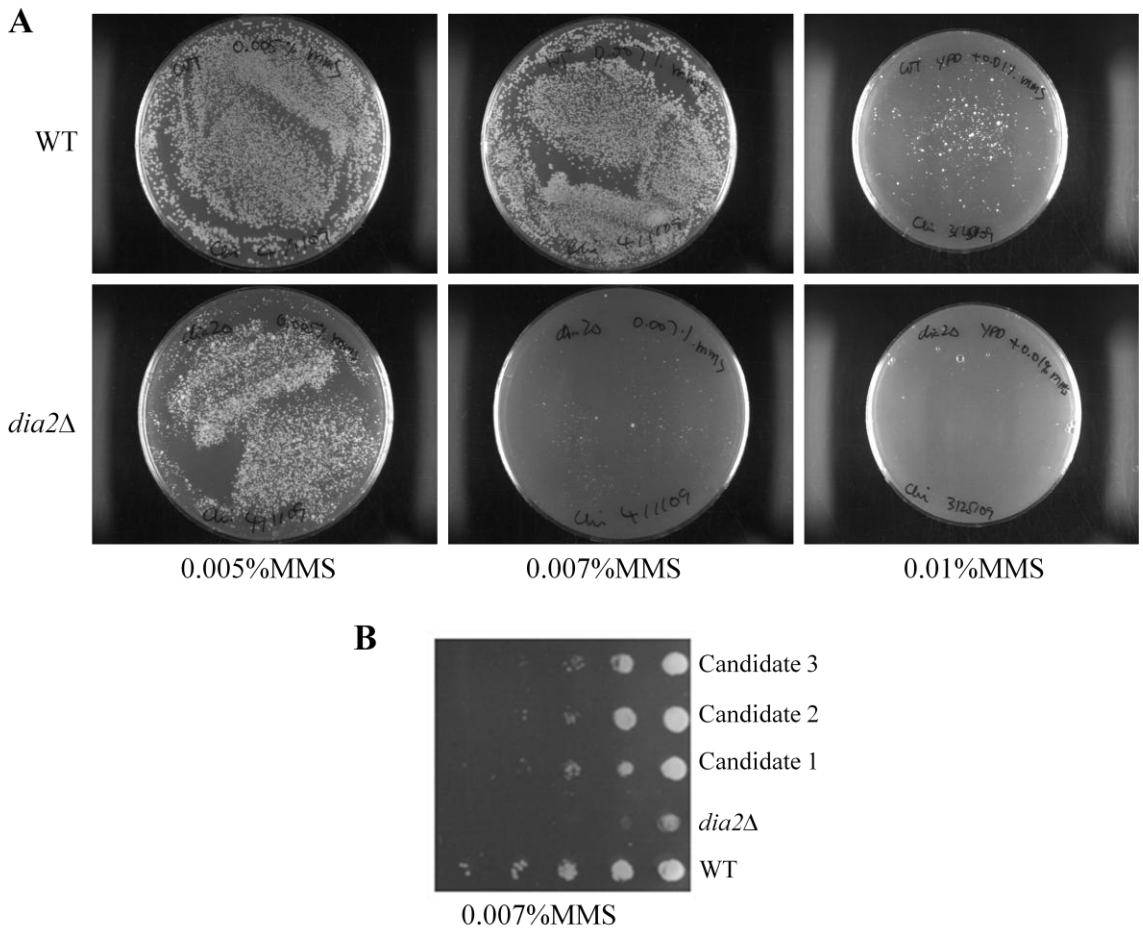


**Fig. 1.** Isolation of MMS resistance *dia2*Δ colonies. (A) Optimization of MMS condition for a genetic suppressor screen. Wildtype (WT) and *dia2*Δ cells were cultured and plated on rich media containing 0.005%, 0.007%, and 0.01% MMS (2 x $10^4$ cells per plate). (B) 10-fold serial dilutions of indicated strains and suppressor candidates were spotted on rich media containing 0.007% MMS.

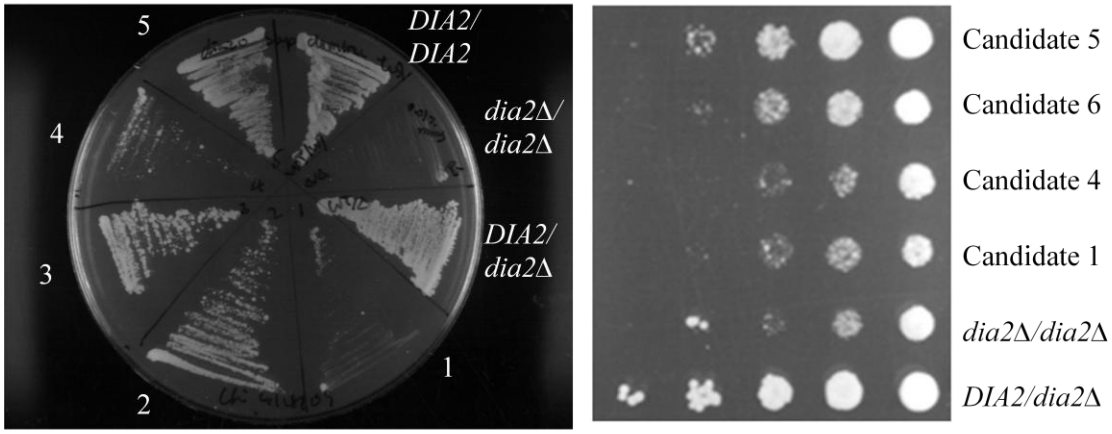


**Fig. 2.** Suppressor mutation: dominant or recessive. Each candidate was backcrossed to the *dia2*Δ strain. Left: The resulting diploids were streaked onto rich media containing 0.01% MMS to screen for recessive mutants. Right: 10-fold serial dilutions of recessive candidates were spotted onto MMS media to confirm MMS sensitivity of recessive diploid mutants.

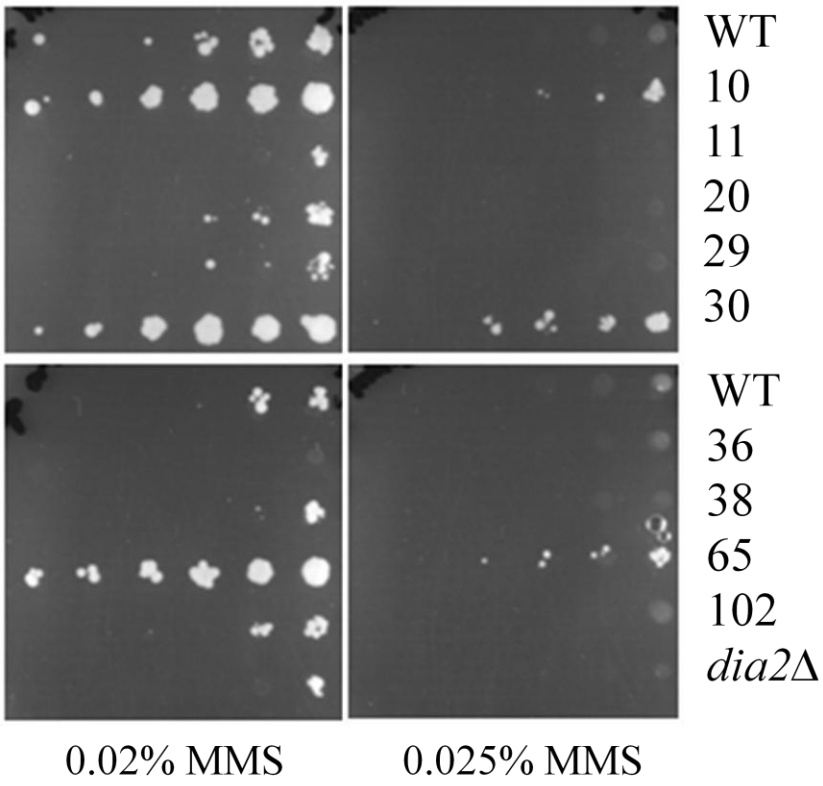


**Fig. 3.** *dia2*Δ suppressor versus MMS hyper-resistant mutant. 10-fold serial dilution of indicated strains and suppressor candidates (as indicated by numbers) were spotted onto rich media containing 0.02% and 0.025% MMS. The suppressor candidates were transformed with a *CEN* plasmid containing a wildtype copy of the *DIA2* gene before they were assayed for growth on media with MMS.

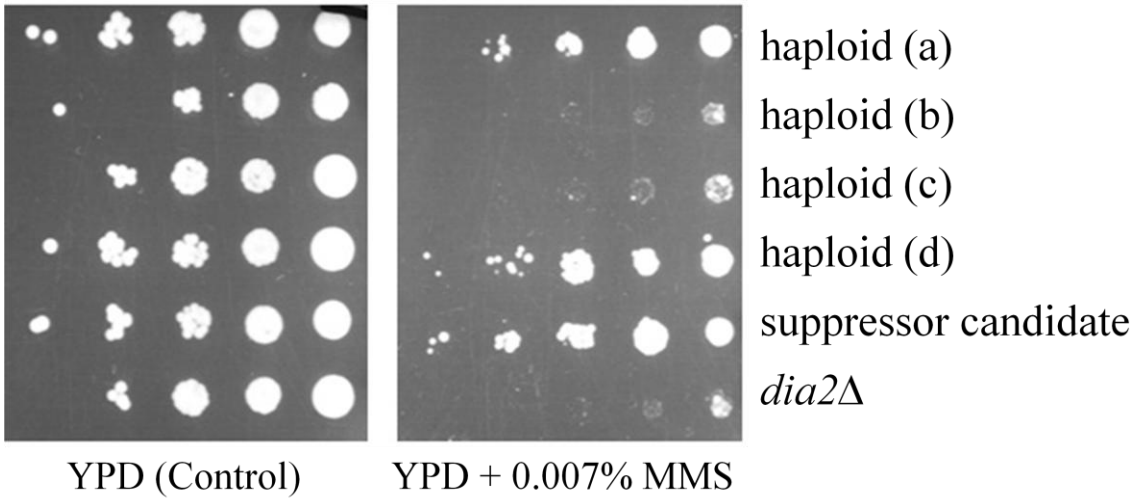


**Fig. 4.** Tetrad dissection of a *dia2*Δ suppressor candidate. Each of the six *dia2*Δ suppressors was backcrossed to the *dia2*Δ strain. The diploid heterozygous of the suppressor mutation(s) was induced to undergo meiosis to form four haploid cells. 10-fold serial dilutions of the four haploid strains were spotted onto rich media (YPD) with and without 0.007% MMS. Shown here is an example of a 2:2: segregation of the single-hit suppressor mutation.

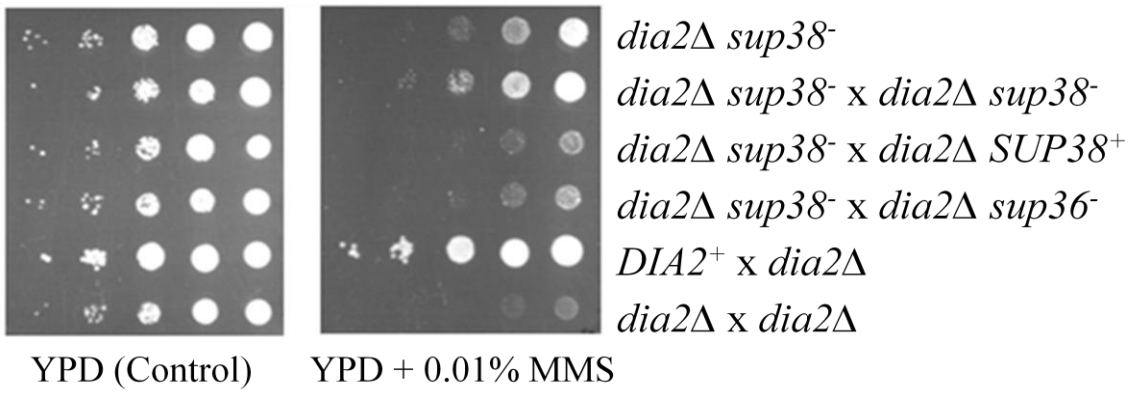


**Fig. 5.** Complementation group of suppressor candidates. Each of the three single-hit suppressors was crossed with each other. The diploids are assayed for growth by spotting 10-fold serial dilutions of indicated strains on rich media (YPD) with and without 0.01% MMS. Shown here is an example of testing whether suppressor 36 and 38 belong to different complementation groups. *sup36*$^{-}$ and *sup38*$^{-}$ indicate the suppressor mutations from candidate 36 and 38, respectively.

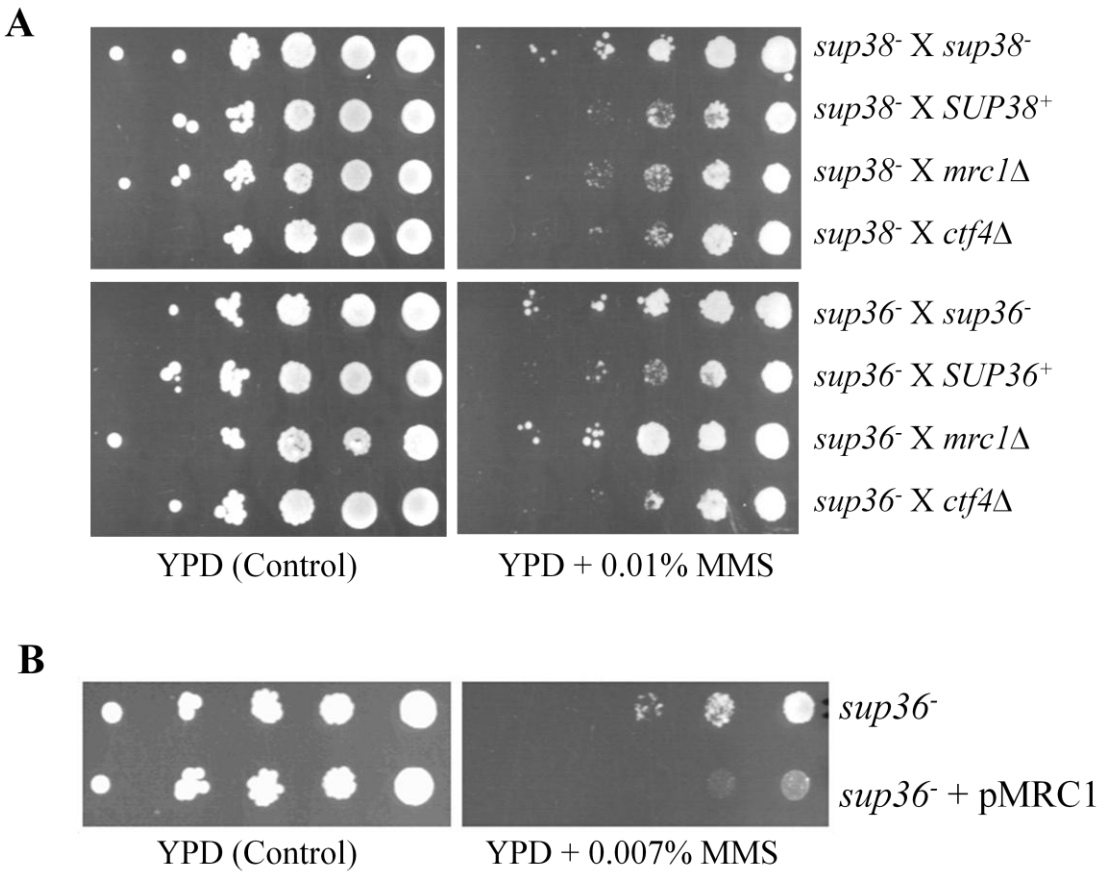


**Fig. 6.** Complementation of *dia2*Δ suppressors with *mrc1*Δ and *ctf4*Δ. All strains shown in this figure are in a *dia2*Δ background. (A) *dia2*Δ suppressors were crossed to the *dia2*Δ *mrc1*Δ and *dia2*Δ *ctf4*Δ strains and the diploids were assayed for growth by spotting 10-fold serial dilutions of the diploids to rich media (YPD) with or without 0.01% MMS. (B) *dia2*Δ suppressor 36 was transformed with a *CEN* plasmid containing wildtype *MRC1*. 10-fold serial dilutions of indicated strains were spotted onto media with or without 0.007% MMS.

**Scheme of *dia2*Δ suppressor screen**

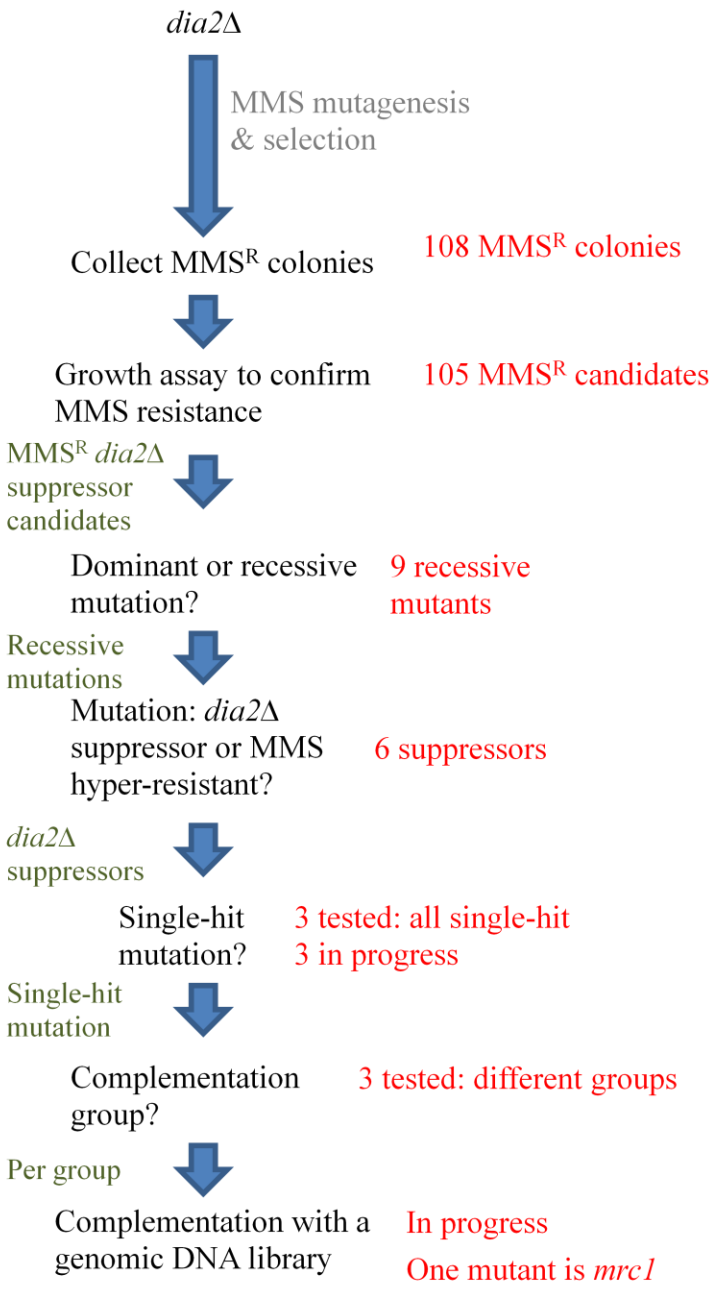


**Fig. 7.** Progress summary of the genetic suppressor screen.

# Chapter III

# Characterization of the suppressor *mrc1* allele

**Summary**

I identified an allele of *mrc1* that suppresses the MMS sensitivity of the *dia2*Δ strain, as described in chapter II. Sequencing results revealed two mutations within the allele: a point mutation of proline (P) to alanine (A) at amino acid residue 263, and an early stop codon glutamine (Q) 966, which is also at the last checkpoint phosphorylation site of Mrc1. The two mutations were separated and I found that the early stop codon is responsible for the suppression phenotype. Further dissection of the early stop mutation indicated that it is the C-terminal truncation, rather than the last checkpoint phosphorylation site, that suppresses *dia2*Δ MMS sensitivity. I generated a variation of the suppressor allele that contains residues 1 to 971 (*mrc1*$_{1\text{-}971}$). This allele was tested to determine whether it is functional in both DNA replication and checkpoint activation. I found that the *mrc1*$_{1\text{-}971}$ allele progresses through an unperturbed S-phase at the same rate as wildtype. However, the *mrc1*$_{1\text{-}971}$ allele genetically interacts with other checkpoint mediator mutants, is defective in mediating the checkpoint phosphorylation of Rad53 kinase, and fails to properly slow down DNA replication in the face of DNA damage. Thus, this allele is functional in DNA replication but defective in checkpoint activation. These results suggest a linkage between the checkpoint function of Mrc1 and the role of Dia2 in the checkpoint.

## Introduction

DNA replication is a delicate cellular event because of the inherent risks of alteration to the genetic information during the process. Environmental stress puts extra pressure on cells to stabilize the replication machinery in order to maintain overall genomic integrity. Mrc1 is a major player in genome maintenance because it has a dual role in DNA replication in an unperturbed S-phase and in mediating the S-phase checkpoint in the face of replication stress or DNA damage [64, 71].

In *S. cerevisiae*, one of the earliest steps in S-phase checkpoint signaling is the activation and the recruitment of Mec1/ATR kinase and co-factor Ddc2/ATRIP to the region of stress or damage [64, 81-83]. From there, the Mec1-Ddc2 complex relays the checkpoint signal through multiple mediators to downstream effector Rad53. The mediators of this pathway includes Rad9, Mrc1, Tof1, and Csm3 [69, 84-87]. In the case of Mrc1, Mec1 kinase phosphorylates Mrc1 at the S/TQ sites. The S/TQ phosphorylation events are important for the initial enrichment of Mec1 at the site of stress and the recruitment of a key downstream effector, the Rad53 kinase [64, 69, 85, 88-90]. Once recruited, Rad53 is activated by Mec1 phosphorylation and autophosphorylation in *trans* [85, 91-93]. Checkpoint phosphorylation of Rad53 leads to stabilization the replication fork and a stall in origin firing, which in turns slow down the overall progression of the fork in the face of DNA damage [95-99]. In addition to checkpoint activation, Mrc1 is required for proper replication fork progression in S-phase [71], and this role is separable from Mrc1 function in the S-phase checkpoint [64]. Previous work suggested that the S/TQ phosphosites play a role in checkpoint activation, whereas the C-terminus of Mrc1 is important for its role of DNA replication [64, 71].

As mentioned in chapter II, I identified a suppresser *mrc1* allele that suppresses the MMS sensitivity of the *dia2*Δ strain. To better understand the suppression mechanism, I sequenced the suppressor *mrc1* allele and determined functionality of the allele in DNA replication and checkpoint activation.

## Methods and Materials

*Yeast cultures and cell cycle*

Yeast cultures were grown according to standard protocols [121]. Rich media used in this study is YPD (Yeast Peptone Dextrose). For checkpoint activation experiments, cells were arrested in late G1 using α–factor (GenScript) and then transferred into YPD containing 0.033% MMS.

*Plasmid and strain construction*

Tables 1, 2 and 3 contain a list of strains, plasmids, and oligonucleotides, respectively, used in this chapter of the thesis. Deletion strains were generated by standard PCR replacement approaches [121]. Using *csm3*Δ*::KanMX* as an example, the *KanMX* gene flanked by 5' and 3' UTRs of *CSM3* was amplified using primers CMF084 and CMF085. The PCR product was purified and transformed into the Y80 strain (wildtype). Transformants were selected on media containing antibiotic G418. The following primer pairs were used to make the other deletion strains: CMF024-CMF025 (*mrc1*Δ*::HIS3*), CMF086-CMF087 (*rad9*Δ*::KanMX*) and CMF103-CMF104 (*tof1*Δ*::URA3*).

To construct pCMF001, the *MRC1* locus was amplified from the Y80 strain using primers CMF013 and CMF014 and ligated to pRS415 using XhoI. To generate pCMF002 ($mrc1_{P263A}$) and pCMF003 ($mrc1_{Q966stop}$), the suppressor *mrc1* allele was amplified from the genomic DNA of the *mrc1* suppressor strain. NdeI-NheI and NheI-PacI regions of pCMF001 were replaced with those of the suppressor allele to generate pCMF002 and pCMF003, respectively. pCMF011 ($mrc1_{S965A}$) was constructed using primer sets CMF014-CMF018 (5’ fragment) and CMF017-CMF013 (3' fragment). The

fragments were combined by PCR before ligating to pRS415 using XhoI. pCMF013 (*mrc1*$_{1\text{-}971}$) was made in a similar fashion using primer sets CMF014-CMF019 and CMF021-CMF013, generating a stop codon mutation at residue 972.

Alleles were integrated using standard homologous recombination approaches [121]. To generate the *mrc1*$_{1\text{-}971}$ strain, the entire XhoI fragment of *mrc1*$_{1\text{-}971}$ was moved from pCMF013 to pRS405 to generate pCMF021. The plasmid was linearized with NdeI and transformed into the *mrc1*Δ strain, and the same is true for the integration of all other *mrc1* alleles used in this study. For N-terminal tagged *MRC1* strains, fragments of *MRC1* 5' UTR, the 3xHA epitope, and the *MRC1* N-terminus ORF (open reading frame) were amplified with primer sets CMF010-CMF044, CMF045-CMF046, and CMF047-CMF043, respectively. The products are combined by PCR and subcloned to pCMF001 and pCMF013 to generate *3HA-MRC1* and *3HA-mrc1*$_{1\text{-}971}$. The XhoI fragments of *3HA-MRC1* and *3HA-mrc1*$_{1\text{-}971}$ were subcloned to pRS405 to generate pCMF022 and pCMF023.

Alleles were integrated using standard homologous recombination approaches [121]. To generate the *mrc1*$_{1\text{-}971}$ strain, the entire XhoI fragment of *mrc1*$_{1\text{-}971}$ was moved from pCMF013 to pRS405 to generate pCMF021. The plasmid was linearized with NdeI and transformed into the *mrc1*Δ strain. To construct *RAD53-3FLAG*, a C-terminal *RAD53* fragment and 3xFLAG were amplified with primer sets CMF077-CMF078 and CMF079-CMF080. The fragments were combined by PCR and ligated to pRS404 using XhoI to generate pCMF028. The plasmid was linearized with SwaI and transformed into the Y80 strain.

*Growth assays*

10-fold serial dilutions of 2 x $10^7$ to 2 x $10^3$ cells were spotted onto media using a replica plater. Plates were incubated at 30ºC for two to three days.

*Flow cytometry*

Harvested cells were fixed with 70% ethanol and resuspended in 1x PBS (Phosphate Buffered Saline). Cells were sonicated to break open clumps and subjected to RNase treatment (100μg/ml) in Tris-EDTA overnight. Samples were stained with propidium iodide (Calbiochem) at a final concentration of 50μg/ml in 1x PBS for one hour and analyzed by flow cytometry using FACSCalibur (BD Biosciences) and FlowJo software (Tree Star). The cell cycle distribution graphs were generated with Deltagraph (Red Rock).

*Protein gel electrophoresis and Western blots*

Protein extracts were prepared using 20% trichloroacetic acid (TCA) precipitation as previously described [110]. Protein samples were resolved in 3-8% Tris-Acetate gels (Invitrogen). 3HA-Mrc1, Rad53-3FLAG and Pgk1 were detected by anti-HA (Covance), anti-FLAG (Sigma), and anti-Pgk1 (Molecular Probes) antibodies, respectively. Secondary antibody incubation, blot development, protein quantification and loading normalization were performed as previously described [110].

Table 1. Strains used in this study.

| Strain | Genotype | Reference |
|---|---|---|
| Y80 | *can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 MAT**a*** | [112] |
| DKY194 | As Y80 but *dia2*Δ*::KanMX* | [112] |
| DKY669 | As Y80 but *mrc1*Δ*::HIS3::mrc1*$_{1\text{-}971}$ *LEU2* | This study |
| DKY643 | As Y80 but *mrc1*Δ*::HIS3* | This study |
| DKY645 | As Y80 but *dia2*Δ*::KanMX mrc1*Δ*::HIS3* | This study |
| DKY781 | As Y80 but *rad9*Δ*::KanMX* | This study |
| DKY820 | As Y80 but *tof1*Δ*::URA3* | This study |
| DKY786 | As Y80 but *csm3*Δ*::KanMX* | This study |
| DKY782 | As Y80 but *rad9*Δ*::KanMX mrc1*Δ*::HIS3::mrc1*$_{1\text{-}971}$ *LEU2* | This study |
| DKY852 | As Y80 but *tof1*Δ*::KanMX mrc1*Δ*::HIS3::mrc1*$_{1\text{-}971}$ *LEU2* | This study |
| DKY800 | As Y80 but *csm3*Δ*::KanMX mrc1*Δ*::HIS3::mrc1*$_{1\text{-}971}$ *LEU2* | This study |
| DKY728 | As Y80 but *mrc1*Δ*::HIS3::3HA-MRC1 LEU2 RAD53::RAD53-3FLAG TRP1* | This study |
| DKY729 | As Y80 but *mrc1*Δ*::HIS3::3HA-mrc1*$_{1\text{-}971}$ *LEU2 RAD53::RAD53-3FLAG TRP1* | This study |
| DKY783 | As Y80 but *rad9*Δ*::KanMX mrc1*Δ*::HIS3::3HA-MRC1 LEU2 RAD53::RAD53-3FLAG TRP1* | This study |
| DKY784 | As Y80 but *rad9*Δ*::KanMX mrc1*Δ*::HIS3::3HA-mrc1*$_{1\text{-}971}$ *LEU2 RAD53::RAD53-3FLAG TRP1* | This study |

Table 2. Plasmids used in this study.

| Plasmid | Relevant Features | Reference |
|---|---|---|
| pRS415 | *CEN LEU2* $Amp^r$ | [122] |
| pCMF001 | *MRC1* genomic locus (22591 to 17555 on chromosome III) in pRS415 | This study |
| pCMF002 | $mrc1_{P263A}$ in pRS415 | This study |
| pCMF003 | $mrc1_{Q966stop}$ in pRS415 | This study |
| pCMF011 | $mrc1_{S965A}$ in pRS415 | This study |
| pCMF013 | $mrc1_{1-971}$ in pRS415 | This study |
| pCMF021 | $mrc1_{1-971}$ in pRS405 | This study |
| pCMF022 | *3HA-MRC1* in pRS405 | This study |
| pCMF023 | *3HA-*$mrc1_{1-971}$ in pRS405 | This study |
| pCMF028 | *RAD53-3FLAG* C-terminal fragment in pRS404 | This study |

Table 3. Oligonucleotides used in this study.

| Oligo-nucleotide | Sequence (5' to 3') |
|---|---|
| CMF010 | GGACACCAACTCTACTGGCTC |
| CMF013 | CCCCTCGAGAACGCATAGAAGACTTGGTTCG |
| CMF014 | CCCCTCGAGAATTGAAAGTGGTGAGTATTTC |
| CMF017 | GTCATTCACAAATGCTCAAACTGATTC |
| CMF018 | GAATCAGTTTGAGCATTTGTGAATGAC |
| CMF019 | GATAAAAAACCAGTTTACTGTTTTTCAAGTGGTCGAATC |
| CMF021 | GAAAAACAGTAAACTGGTTTTTTATCTTTTCCGAAG |
| CMF024 | GAAGTTCGTTATTCGCTTTTGAACTTATCACCAAATATTTTAGT GGGCCTCCTCTAGTACACTC |
| CMF025 | AGCTTCTGGAGTTCAATCAACTTCTTCGGAAAAGATAAAAAAC CAGCGCGCCTCGTTCAGAATG |

| | |
|---|---|
| CMF043 | GAATTTGTTTCCTGCTAGCTTTC |
| CMF044 | GTCGTATGGGTAACCTGCCATCACTAAAATATTTGGT |
| CMF045 | ACCAAATATTTTAGTGATGGCAGGTTACCCATACGAC |
| CMF046 | CAAAGCATGCAAGGCATCATCGGAACGTAGAGAAGCGTAATC |
| CMF047 | GATTACGCTTCTCTACGTTCCGATGATGCCTTGCATGCTTTG |
| CMF077 | AGGTTGTCTCGAGTTCATTGCTTC |
| CMF078 | TTTATAATCGAGCTCCAGCTTCGAAAATTGCAAATTCTCGGGGCC |
| CMF079 | GGCCCCGAGAATTTGCAATTTTCGAAGCTGGAGCTCGATTATAAA |
| CMF080 | CCCCTCGAGGTCCATAAATTCCTGCAGTTACTT |
| CMF084 | GATTAAAATGCCATGAAAACGTGAACAGAAACTTTTATTGAGGTCGTTTAGCTTGCCTCGTCCCCG |
| CMF085 | TAGATGCCCACACGCACGTTTGGATTATTACCTTCAATGACATTGTTAAGGGTTCTCGAGAGCTCG |
| CMF086 | CGCCATAGAAAAGAGCATAGTGAGAAAATCTTCAACATCAGGGCTGTTTAGCTTGCCTCGTCCCCG |
| CMF087 | AATCGTCCCTTTCTATCAATTATGAGTTTATATATTTTTATAATTTTAAGGGTTCTCGAGAGCTCG |
| CMF103 | AGCTTGTGGGGTTTAGTGTATCTTTAATATAGGAGGGCGCACACTAGCTTTTCAATTCAATTCATC |
| CMF104 | CTAAAATTACACGTATTAAAGGGATTAATTACTACATATTCATTCCACACCGCATAGGGTAATAAC |

**Results**

I identified an *mrc1* allele that suppresses *dia2*Δ MMS sensitivity. Sequencing results revealed two mutations within the allele: a point mutation P263A and an early stop codon at Q966 (Fig. 1A). Separate mutants were generated to determine which mutation was responsible for the suppression. Each mutation was subcloned into a *CEN* plasmid containing the *MRC1* gene. The plasmids were transformed into the *dia2*Δ *mrc1*Δ strain and the transformants were tested for growth on rich media containing MMS. As shown in Fig. 1B, both the *dia2*Δ and *dia2*Δ $mrc1_{P263A}$ strains are equally sensitive to MMS, whereas *dia2*Δ $mrc1_{Q966Stop}$ grew better on media with MMS. Thus, the early stop mutation at Q966 suppresses *dia2*Δ MMS sensitivity, whereas the P263A point mutation does not. Because the early stop codon is at the last SQ phosphosite of Mrc1, I investigated whether the last SQ site (S965A) or the C-terminal truncation (1-971) would suppress *dia2*Δ cells. Separate *mrc1* mutants were generated on a *CEN* plasmid and transformed into the *dia2*Δ *mrc1*Δ strain for growth analysis on media with MMS. I found that the $mrc1_{1-971}$ allele suppresses the *dia2*Δ strain's MMS-sensitivity phenotype, whereas the $mrc1_{S965A}$ allele does not (Fig. 1B). I conclude that the C-terminus of Mrc1 is responsible for *dia2*Δ suppression.

As Mrc1 has roles in both DNA replication and checkpoint activation [64, 69, 71], I tested which function was required for suppression. A previous study has shown that deletion of residues beyond 988 do not compromise Mrc1 replication function [94], however the $mrc1_{1-971}$ suppressor allele had a slightly larger deletion. To confirm functionality of DNA replication, wildtype and $mrc1_{1-971}$ cells were synchronized in G1 using α-factor and released into S-phase and DNA replication was monitored by flow

cytometry. I found that the *mrc1*$_{1\text{-}971}$ strain progressed at the same rate as wildtype through DNA replication, with the majority of cells at 2C DNA content by 40 minutes (Fig. 1C). I conclude that *mrc1*$_{1\text{-}971}$ is functional in DNA replication.

To examine the checkpoint function of the *mrc1*$_{1\text{-}971}$ strain, I tested 1) whether other checkpoint mediators are required for *mrc1*$_{1\text{-}971}$ to survive replication stress and DNA damage, 2) Rad53 activation in response to MMS, and 3) if the checkpoint is active in *mrc1*$_{1\text{-}971}$ cells to slow down DNA replication in the presence of MMS. First, the *mrc1*$_{1\text{-}971}$ mutant was crossed to checkpoint mediator mutants *rad9*Δ, *tof1*Δ, and *csm3*Δ, and single and double mutants were examined for sensitivity to HU and MMS. While the *mrc1*$_{1\text{-}971}$ allele alone did not exhibit any noticeable sensitivity to HU and MMS, the *mrc1*$_{1\text{-}971}$ *rad9*Δ double mutant exhibited weaker growth than the *mrc1*$_{1\text{-}971}$ or the *rad9*Δ single mutant on media containing HU or MMS, and the same negative genetic interaction was observed between *mrc1*$_{1\text{-}971}$ and *tof1*Δ, as well as between *mrc1*$_{1\text{-}971}$ and *csm3*Δ (Fig. 1D). Thus, other mediators are required for the *mrc1*$_{1\text{-}971}$ strain to survive replication stress and DNA damage. Second, I compared Rad53 activation in wildtype and *mrc1*$_{1\text{-}971}$ cells in response to MMS. Cells were arrested in late G1 using α-factor and released into media with MMS so that the S-phase checkpoint was activated soon after the cells entered S-phase. Because Rad53 becomes phosphorylated during checkpoint activation [91], the shift from non-phosphorylated to phosphorylated Rad53 was monitored over time in wildtype and *mrc1*$_{1\text{-}971}$ cells. Since Mrc1 and Rad9 both activate Rad53 in response to MMS [64, 69, 85, 89], I also tested Rad53 activation in the *rad9*Δ background. While the difference in Rad53 activation was subtle between wildtype and *mrc1*$_{1\text{-}971}$ cells, the Rad53 phosphorylation shift was mostly abolished in *rad9*Δ *mrc1*$_{1\text{-}971}$

cells (Fig. 1E). This result indicates that $Mrc1_{1\text{-}971}$ is defective in activating Rad53 in response to MMS. Interestingly, the phosphorylation shift of $Mrc1_{1\text{-}971}$ is reduced compared to full length Mrc1 (Fig. 1E), even though the deletion mutant retains all of the S/TQ checkpoint phosphosites (Fig. 1A). Last, I determined if the S-phase checkpoint is intact in *mrc1*$_{1\text{-}971}$ cells. If *mrc1*$_{1\text{-}971}$ is checkpoint defective, I expected that DNA replication in MMS would be faster in the mutant than wildtype. Cells were treated as described for Fig. 1E and DNA replication was monitored by flow cytometry. DNA replication within the first two hours was indistinguishable between wildtype and *mrc1*$_{1\text{-}971}$ cells. However, I noticed a difference in the DNA replication profile between the two strains by the three-hour time point. By four hours, the *mrc1*$_{1\text{-}971}$ strain completed DNA replication, whereas the majority of wildtype cells did not complete DNA replication (Fig. 1F). These data indicate that this novel allele *mrc1*$_{1\text{-}971}$ is functional in DNA replication but partially defective in activating the S-phase checkpoint. These results suggest that the checkpoint function of Mrc1 is linked to the role of Dia2 in the S-phase checkpoint.

**Discussion**

It has been proposed that S/TQ phosphosites of Mrc1 are critical for checkpoint activation, whereas the C-terminus is important for replication fork progression [64, 71]. The suppressor *mrc1*$_{1\text{-}971}$ allele is truncated at the C-terminus but retains all of the S/TQ phosphosites. Surprisingly, this allele is functional in DNA replication but defective in checkpoint activation. I speculate the contradiction is perhaps due to a difference in the methods used to assay for checkpoint activation. An earlier publication constructed a series of C-terminal deletion of *MRC1* to dissect its role in replication versus checkpoint activation [94]. The report found that deletion of residues beyond 988 do not compromise Mrc1 replication function. I confirmed that the same is true for residues beyond 971. To assay for checkpoint function, the same report tested the C-terminal deletion mutants for HU sensitivity and synthetic lethality with *rad9*Δ. Consistent with the report, I did not observe in the *mrc1*$_{1\text{-}971}$ mutant HU sensitivity or a synthetic growth defect with *rad9*Δ. Indeed, I did not find this mutant partially defective in checkpoint activation until I performed more stringent tests such as genetic interactions with *rad9*Δ, *tof1*Δ, and *csm3*Δ on media containing HU or MMS and Rad53 activation in the *rad9*Δ background. These findings suggest that the structure and function relationship of Mrc1 is more complex than originally thought.

One possibility to explain why the *mrc1*$_{1\text{-}971}$ allele is checkpoint defective is that the missing residues may mediate protein-protein interaction(s) important for checkpoint activation. Proteins that interact with Mrc1 that have been implicated in checkpoint function include Mcm6, Pol ε, Tof1, Csm3, Mec1, and Rad53. Mrc1 binding to Mcm6 is important for checkpoint activation in the face of DNA damage [74], and Mrc1

association with Pol ε is changed in the presence of replication stress [73]. However, the missing residues of the *mrc1*$_{1\text{-}971}$ allele are not required for Mrc1 interaction with either Mcm6 or Pol ε [73, 74]. Thus, it is unlikely that either Mcm6 or Pol ε is responsible for the compromised checkpoint function of the *mrc1*$_{1\text{-}971}$ allele. Mrc1 forms a complex with Tof1 and Csm3 and Mrc1-Tof1 is thought to be important for stabilization of replication fork [65, 68]. The recruitment of Mrc1 to chromatin is dependent on Tof1 and Csm3 [65]. While it is not clear which domains of Mrc1 that are required for association with Tof1 and Csm3, it is unlikely that the C-terminal missing residues are critical for the association because the *mrc1*$_{1\text{-}971}$ strain is functional in replication fork progression. If Mrc1$_{1\text{-}971}$ cannot interact with Tof1 and Csm3, it would not be recruited to the chromatin and replication fork progression would suffer as a result. Considering that Mec1 kinase phosphorylates Mrc1 at the S/TQ phosphosites [64] and the phosphorylation of Mrc1$_{1\text{-}971}$ is incomplete, it is possible that the interaction between the Mec1-Ddc2 complex and the Mrc1$_{1\text{-}971}$ protein is compromised. Alternatively, the truncated protein may fold differently than the wildtype counterpart and some of the phosphosites are hindered from Mec1-driven phosphorylation. Because phosphorylation of Mrc1 is key to recruit Rad53 to the site of stress [64], Rad53 is probably not recruited properly in the *mrc1*$_{1\text{-}971}$ strain, resulting in defective activation of Rad53 and the S-phase checkpoint.

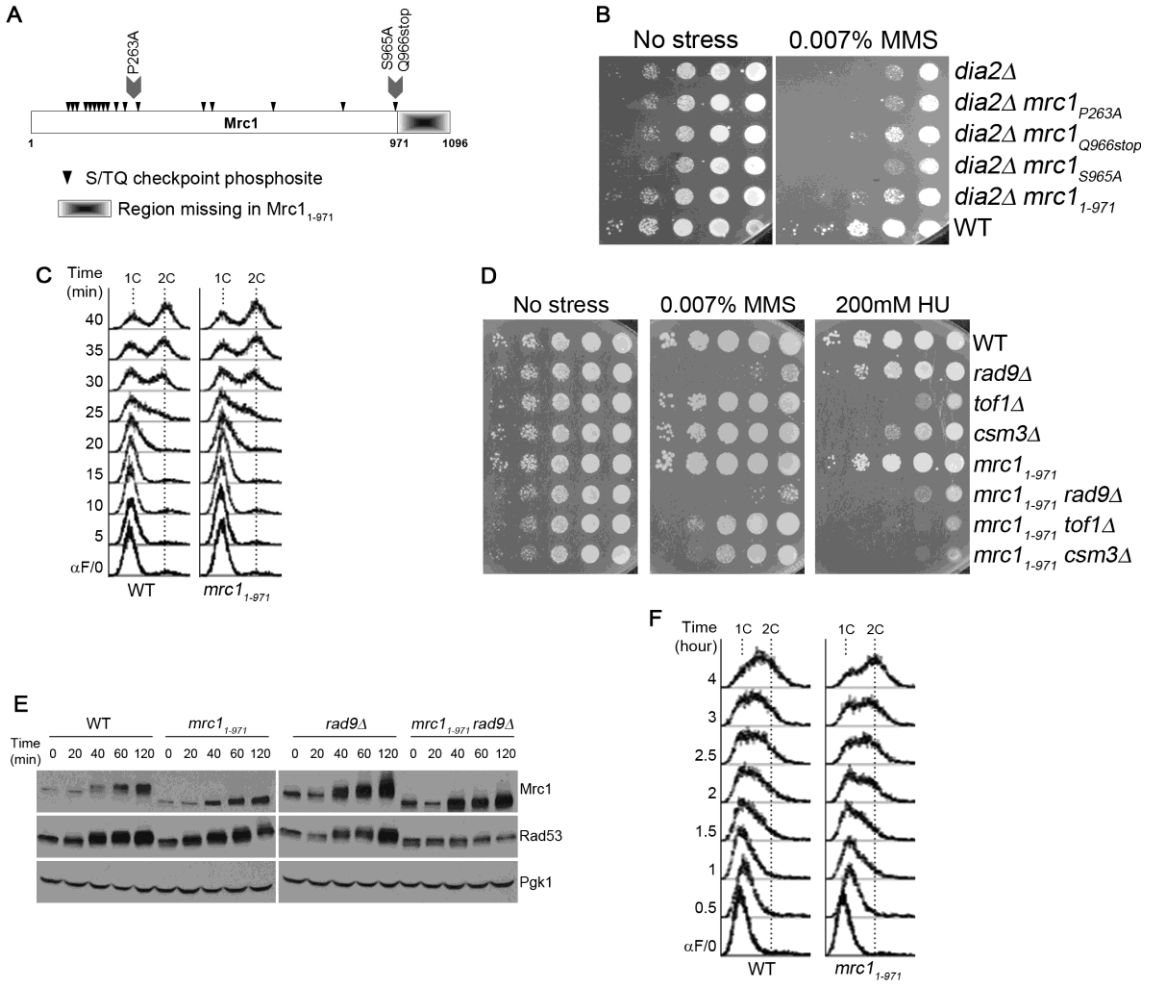


**Fig. 1.** A genetic screen identified a checkpoint-defective allele of *mrc1* that suppresses the MMS sensitivity of *dia2*Δ. (A-B) A C-terminal truncation of *mrc1* allele was identified to suppress *dia2*Δ. (A) Structural schematic of the Mrc1 protein. Arrow heads indicate S/TQ Mec1-directed phosphosites. Mutations used in these studies are marked. (B) $mrc1_{1\text{-}971}$ suppresses the MMS sensitivity of *dia2*Δ. The *dia2*Δ *mrc1*Δ strain carrying the indicated *mrc1* alleles were spotted using 10-fold serial dilutions on selective media with or without 0.007% MMS and incubated at 30ºC. (C-F) $mrc1_{1\text{-}971}$ is a checkpoint-defective allele. (C) $mrc1_{1\text{-}971}$ is functional in DNA replication. Cells were arrested in G1 by α-factor, and released into YPD for cells to enter S-phase at 30ºC. The indicated

time points were analyzed by flow cytometry. 1C and 2C indicate DNA content. (D) The *mrc1*$_{1\text{-}971}$ allele exhibits negative genetic interactions with other S-phase checkpoint mediator mutants. 10-fold serial dilutions of the indicated strains were spotted on YPD, YPD + 0.007% MMS or YPD + 200mM HU, and incubated at 30ºC. (E) Checkpoint activation of Rad53 is compromised in *mrc1*$_{1\text{-}971}$. Cells were arrested in G1 by α-factor, and released into YPD + 0.033% MMS to activate the S-phase checkpoint at 30ºC. Protein samples were taken at the indicated time points. Pgk1 was used as a loading control. The checkpoint activation of Rad53 was measured using the intensity of Rad53 phosphorylation shift. (F) the *mrc1*$_{1\text{-}971}$ allele bypasses checkpoint-activated slowing of DNA replication. Samples were prepared as described in (E) and analyzed at the indicated time points by flow cytometry.

# Chapter IV

# Dia2 is a mediator of S-phase checkpoint recovery

**Summary**

Dia2 is proposed to have a role in the S-phase checkpoint [110, 112, 115]. I found that Dia2 mediates S-phase checkpoint recovery from MMS-induced DNA damage, and it is dependent on the ability of Dia2 to form an SCF ubiquitin ligase complex. Dia2 functions in a parallel pathway to Rad53 phosphatase Pph3 in mediating checkpoint recovery. Rad53 deactivation is slow in the *dia2*Δ strain during checkpoint recovery, which is consistent with the defect of *dia2*Δ in the resumption of DNA replication post-MMS exposure. Intriguingly, checkpoint defective *mrc1* alleles suppress the Rad53 deactivation and checkpoint recovery defects of the *dia2*Δ strain. Consistent with this observation, I found that Mrc1, among Rad53 mediators, is degraded during checkpoint recovery in a Dia2-dependent manner. The degradation of Mrc1 during recovery is partially dependent on S/TQ phosphosites. Induced degradation of Mrc1 rescues the checkpoint recovery defect of the *dia2*Δ strain to almost wildtype kinetics, indicating that the degradation of Mrc1 is key to Dia2-mediated checkpoint recovery from MMS-induced DNA damage.

## Introduction

The cell division cycle is tightly regulated to preserve genomic integrity and the viability of cells. Cells constantly monitor cell cycle progression and employ checkpoints to pause the cell cycle when genome maintenance is threatened by genotoxins [75, 78, 123]. In the face of DNA damage, cells initiates the S-phase checkpoint by first activating the Mec1 kinase, from which the checkpoint signal is relayed to downstream effect Rad53 kinase through mediators that include Rad9, Mrc1, Tof1 and Csm3 [64, 69, 77, 80, 82, 85, 86]. In the case of Mrc1, it is phosphorylated at S/TQ sites by Mec1 to allow recruitment of Rad53 [64]. Additional residues of Mrc1 that are independent of the S/TQ sites are required for Mec1 to activate Rad53 by phosphorylation [94]. These signaling events result in a slowdown of DNA replication while repair pathways are activated to resolve damage [76]. Any unrepaired damage in the newly synthesized DNA will trigger the G2/M DNA damage checkpoint to prevent cells from segregating the genetic material before the DNA damage is resolved [76, 78, 124].

As important as it is for cells to activate the S-phase checkpoint in the face of DNA damage, cells must deactivate the checkpoint to resume the cell cycle after exposure to the DNA damage in a process termed checkpoint recovery [100, 101]. Previous studies have identified DNA repair scaffold proteins Slx4 and Rtt107, as well as Rad53 phosphatases Pph3 (complex with cofactor Psy2) and Ptc2 to be important for checkpoint recovery from [102, 103, 106, 107]. In the case of Rad53 phosphatases, Rad53 dephosphorylation is sufficient for fork restart during checkpoint recovery [107]. Interestingly, fork recovery from replication stress agent hydroxyurea (HU) is not

dependent on the Rad53 phosphatases [108]. Rather, fork recovery from HU is dependent on the chromatin remodeling complex Ino80 [109].

Our group recently identified a previously-uncharacterized linkage between the replication stress response and the SCF ubiquitin-proteasome pathway [110], a system that is better known for its role in protein turnover during cell cycle progression [92]. An SCF ubiquitin ligase complex consists of Skp1, Cul1, Rbx1, and an F-box protein which provides specificity of the complex [12-15]. Interestingly, we found that the proteolysis of the *S. cerevisiae* F-box protein Dia2 is regulated by the S-phase checkpoint. Indeed, Dia2 is highly stabilized when the checkpoint is activated in the presence of MMS [110]. Furthermore, *dia2* null (*dia2*Δ) cells are sensitive to MMS-induced DNA damage [112, 115]. These findings suggest that Dia2 plays a role in the S-phase checkpoint. Because Rad53 is constitutively phosphorylated in the absence of Dia2 [116], it seems unlikely that Dia2 is required for checkpoint activation. Consistent with the data showing hyperactivation of Rad53 in *dia2*Δ cells, DNA replication is slow in *dia2*Δ cells in the presence of MMS [115].

The checkpoint mediator Mrc1 has recently been identified as an ubiquitin-mediated degradation substrate of $SCF^{Dia2}$ [114]. In addition to its role in checkpoint activation, Mrc1 also travels with the replication fork and is required for efficient DNA replication in an unperturbed S-phase [64, 71]. The degradation of Mrc1 is most prominent in S-phase cells arrested in HU [114]. However, it remains an open question what the biological relevance of Mrc1 degradation is and whether Mrc1 is degraded for a role in an unperturbed S-phase or in response to the S-phase checkpoint activation. Intriguingly, the human homolog of Mrc1, Claspin, is targeted for proteasome degradation by the

$SCF^{\beta\text{-}TrCP}$ complex during recovery from replication stress or DNA damage before mitotic entry [117, 118]. This degradation is regulated by Polo-like kinase-1 (Plk1) phosphorylation which precedes the interaction between $SCF^{\beta\text{-}TrCP}$ and Claspin [117, 119].

To better understand the function of Dia2 in the S-phase checkpoint, I investigated a possible role for Dia2 in checkpoint recovery from MMS-induced DNA damage and examined the suppression mechanism behind the checkpoint defect *mrc1*$_{1\text{-}971}$ allele.

## Methods and Materials

*Yeast cultures and cell cycle*

Yeast cultures were grown according to standard protocols [121]. For checkpoint recovery experiments, cells were synchronized in late G1 by α–factor (GenScript) and then transferred into YPD (Yeast Peptone Dextrose) containing 0.033% MMS for 40 minutes in order for cells to enter S-phase and activate the S-phase checkpoint, before transferring into YPD without MMS for checkpoint recovery while cells were still in S-phase. Nocodazole (Sigma) was added to a final concentration of 15μg/ml.

*Plasmid and strain construction*

Tables 1, 2 and 3 contain a list of strains, plasmids, and oligonucleotides, respectively, used in this study. Deletion strains were generated by standard PCR replacement approaches [121], as described in previous chapters of this thesis. The following oligonucleotide pairs were used to make the deletion strains used in this study: CMF024-CMF025 (*mrc1*Δ*::HIS3*), CMF091-CMF092 (*pph3*Δ*::KanMX*), CMF093-CMF094 (*psy2*Δ*::KanMX*), CMF105-CMF106 (*ptc2*Δ*::URA3*), CMF146-CMF147 (*slx4*Δ*::HIS3*) and CMF148-CMF149 (*rtt107*Δ*::HIS3*). pCMF013 ($mrc1_{1\text{-}971}$) was constructed using primer sets CMF014-CMF019 (5’ fragment) and CMF021-CMF013 (3' fragment). The fragments were combined by PCR before ligating to pRS415 using XhoI.

Alleles were integrated using standard homologous recombination approaches [121]. To generate the $mrc1_{1\text{-}971}$ strain, the entire XhoI fragment of $mrc1_{1\text{-}971}$ was moved from pCMF013 to pRS405 to generate pCMF021. The plasmid was linearized with NdeI and transformed into the *mrc1*Δ strain, and the same is true for the integration of all other *mrc1* alleles used in this thesis study. For N-terminal tagged *MRC1* strains, fragments of

*MRC1* 5' UTR, the 3xHA epitope, and the *MRC1* N-terminus ORF (open reading frame) were amplified with primer sets CMF010-CMF044, CMF045-CMF046, and CMF047-CMF043, respectively. The products are combined by PCR and subcloned to pCMF001 and pCMF013 to generate *3HA-MRC1* and *3HA-mrc1*$_{1-971}$. The XhoI fragments of *3HA-MRC1* and *3HA-mrc1*$_{1-971}$ were subcloned to pRS405 to generate pCMF022 and pCMF023. *3HA-mrc1*$_{3SA}$ was generated by first amplifying the NheI-PacI region of *MRC1* with primer sets CMF135-CMF137 and CMF136-CMF138. The fragments were combined by PCR and subcloned into pCMF022 to generate pCMF041. Generation of the *mrc1* mutants deleted of the putative Dia2-binding regions were performed as follows: 5' and 3' fragments of *MRC1* were amplified using two pairs of primers before the products were combined by PCR. Primer sets CMF010-CMF158 and CMF159-CMF160 were used to generate a *mrc1*$_{\Delta 380-430}$ fragment, CMF140-CMF141 and CMF142-CMF143 were used to generate a *mrc1*$_{\Delta 461-557}$ fragment, and CMF140-CMF144 and CMF145-CMF143 were used to generate a *mrc1*$_{\Delta 701-800}$ fragment. The *mrc1*$_{\Delta 701-800}$ fragment was first subcloned into pCMF022 using XbaI to generate *3HA-mrc1*$_{\Delta 701-800}$ in pRS405. The *mrc1*$_{\Delta 380-430}$ fragment was then subcloned into *3HA-mrc1*$_{\Delta 701-800}$ using NdeI and NheI to generate pCMF050, whereas the *mrc1*$_{\Delta 461-557}$ fragment was subcloned into *3HA-mrc1*$_{\Delta 701-800}$ using NdeI and XbaI to generate pCMF045. *3HA-mrc1*$_{AQ}$ was constructed using an untagged *mrc1*$_{AQ}$ plasmid (pAO138 from [64] ). ApaI-PacI fragment of *mrc1*$_{AQ}$ was subcloned into pCMF022 to generate pCMF055.

*MRC1* was conjugated to an auxin-inducible degron (AID) peptide containing amino acid residues 3 - 111 [125, 126]. To generate the *3HA-AID*$_{3-111}$*-MRC* construct, 5' UTR (containing 3xHA) and 5' ORF of *MRC1* were amplified using primer sets CMF10-

CMF129 and CMF166-CMF167. The AID peptide was also amplified using primers CMF130 and CMF165. The three fragments were combined by PCR, and the product was digested with NdeI and NheI enzymes before subcloning into pCMF022 and pCMF023 to generate pCMF053 (*3HA-AID*$_{3\text{-}111}$*-MRC*) and pCMF054 (*3HA-AID*$_{3\text{-}111}$*-mrc1*$_{1\text{-}971}$), respectively.

Epitope-tagged *TOF1* was generated by combining several fragments. The C-terminal *TOF1* ORF, 3xFLAG, and 3' UTR were amplified using primer sets CMF063-CMF072, CMF073-CMF074, and CMF075-CMF061. Those fragments were combined by PCR and ligated to pRS404 using XhoI to generate pCMF026. The plasmid was linearized with NsiI and transformed into the Y80 strain. A similar approach was used to generate *CSM3-3MYC*, using primer sets CMF064-CMF068 (ORF), CMF069-CMF070 (3xMYC), and CMF071-CMF065 (3' UTR). Those fragments were combined by PCR and ligated to pRS404 using XhoI to generate pCMF027. The plasmid was linearized with NsiI and transformed into the Y80 strain. To construct *RAD53-3FLAG*, a C-terminal *RAD53* fragment and 3xFLAG were amplified with primer sets CMF077-CMF078 and CMF079-CMF080. The fragments were combined by PCR and ligated to pRS404 using XhoI to generate pCMF028. The plasmid was linearized with SwaI and transformed into the Y80 strain. *RAD9-3FLAG* was generated in a similar fashion using primer sets CMF081-CMF082 (ORF) and CMF083-CMF080 (3xFLAG). The fragments were combined by PCR and ligated into pRS405 using XhoI to generate pCMF029. The plasmid was linearized with NsiI and transformed into a *bar1*Δ strain in Y80 background.

*Growth and viability assays*

10-fold serial dilutions of 2 x $10^7$ to 2 x $10^3$ cells were spotted onto media using a replica plater. Plates were incubated at 30ºC for two to three days. For viability assays, cells were first grown in YPD to log phase. Equal numbers of cells were plated on YPD containing MMS. Plates were incubated at 30ºC for four days and CFU were counted. The percentage viability of cells was determined by dividing CFU on YPD with MMS by CFU on YPD without MMS.

*Flow cytometry*

Harvested cells were fixed with 70% ethanol and resuspended in 1x PBS (Phosphate Buffered Saline). Cells were sonicated to break open clumps and subjected to RNase treatment (100μg/ml) in Tris-EDTA overnight. Samples were stained with propidium iodide (Calbiochem) at a final concentration of 50μg/ml in 1x PBS for one hour and analyzed by flow cytometry using FACSCalibur (BD Biosciences) and FlowJo software (Tree Star). The cell cycle distribution graphs were generated with Deltagraph (Red Rock).

*Protein gel electrophoresis and Western blots*

Protein extracts were prepared using 20% trichloroacetic acid (TCA) precipitation as previously described [110]. For Rad53 deactivation experiments, protein samples were resolved in 6% Tris-Glycine denaturing protein gels (SDS-PAGE). The proteins were then transferred to a nitrocellulose blot for Western blotting. Proteins of interested were then detected using an ECL (enhanced chemiluminescence) kit. The very top modified band of Rad53 was quantified using ImageJ as a measure of Rad53 deactivation. For all other experiments, protein samples were resolved in 3-8% Tris-Acetate gels (Invitrogen). 3HA-Mrc1, Csm3-3MYC and Pgk1 were detected by anti-HA (Covance), anti-MYC

9E10 (Covance), and anti-Pgk1 (Molecular Probes) antibodies, respectively. Tof1-3FLAG, Rad9-3FLAG, and Rad53-3FLAG were detected by anti-FLAG (Sigma) antibody. Secondary antibody incubation, blot development, protein quantification and loading normalization were performed as previously described [110].

*Stability Assays*

Cells were grown in YPD to log phase before performing arrest and release checkpoint recovery experiments. Cycloheximide (CHX) (Sigma) was added to cell cultures to a final concentration of 200μg/ml. For proteasome inhibitor experiments, the *3HA-MRC1 rpn4*Δ *pdr5*Δ strain was used and MG-132 (American Peptide) was added to the media at a final concentration of 50μM.

*Auxin-induced degradation of Mrc1*

This system was adapted from [125, 126], provided by the Yeast Genetic Resource Center, Osaka University. During checkpoint recovery experiments, either vehicle (100% Ethanol) or 1.5mM indole-3-acetic acid (IAA) (Alfa Aesar) was added to cell cultures in YPD at 30ºC.

Table 1. A list of strains used in this study.

| Strain | Genotype | Reference |
|---|---|---|
| Y80 | *can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 MAT**a*** | [112] |
| DKY194 | As Y80 but *dia2*Δ*::KanMX* | [112] |
| AKY188 | As Y80 but *dia2Δ*::*KanMX*::*9MYC-DIA2-ΔF* (bp Δ670-792) *URA3* | [110] |
| DMK922 | As Y80 but *slx4*Δ*::HIS3* | This study |
| DMK940 | As Y80 but *dia2*Δ*::KanMX slx4*Δ*::HIS3* | This study |
| DMK923 | As Y80 but *rtt107*Δ*::HIS3* | This study |
| DMK941 | As Y80 but *dia2*Δ*::KanMX rtt107*Δ*::HIS3* | This study |
| DKY812 | As Y80 but *pph3*Δ*::KanMX* | This study |
| DKY814 | As Y80 but *psy2*Δ*::KanMX* | This study |
| DKY822 | As Y80 but *ptc2*Δ*::URA3* | This study |
| DKY826 | As Y80 but *dia2*Δ*::KanMX pph3*Δ*::KanMX* | This study |
| DKY832 | As Y80 but *dia2*Δ*::KanMX psy2*Δ*::KanMX* | This study |
| DKY848 | As Y80 but *dia2*Δ*::KanMX ptc2*Δ*::URA3* | This study |
| DKY643 | As Y80 but *mrc1*Δ*::HIS3* | This study |
| DKY645 | As Y80 but *dia2*Δ*::KanMX mrc1*Δ*::HIS3* | This study |
| DKY669 | As Y80 but *mrc1*Δ*::HIS3::mrc1*$_{1\text{-}971}$ *LEU2* | This study |
| Y2298 | *can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 HIS::mrcl*$_{AQ}$*-MYC13 MAT**a*** | [64] |
| DKY769 | As Y80 but *dia2*Δ*::KanMX HIS::mrcl*$_{AQ}$*-MYC13* | This study |
| DKY672 | As Y80 but *dia2*Δ*::KanMX mrc1*Δ*::HIS3::mrc1*$_{1\text{-}971}$ *LEU2* | This study |
| DKY824 | As Y80 but *pph3::KanMX mrc1*Δ*::HIS3::mrc1*$_{1\text{-}971}$ *LEU2* | This study |
| DKY728 | As Y80 but *mrc1*Δ*::HIS3::3HA-MRC1 LEU2 RAD53::RAD53-3FLAG TRP1* | This study |
| DKY729 | As Y80 but *mrc1*Δ*::HIS3::3HA-mrc1*$_{1\text{-}971}$ *LEU2 RAD53::RAD53-3FLAG TRP1* | This study |
| DKY765 | As Y80 but *HIS::mrcl*$_{AQ}$*-MYC13 RAD53::RAD53-3FLAG TRP1* | This study |

| | | |
|---|---|---|
| DKY730 | As Y80 but *dia2*Δ*::KanMX mrc1*Δ*::HIS3::3HA-MRC1 LEU2 RAD53::RAD53-3FLAG TRP1* | This study |
| DKY731 | As Y80 but *dia2*Δ*::KanMX mrc1*Δ*::HIS3::3HA-mrc1*$_{1\text{-}971}$ *LEU2 RAD53::RAD53-3FLAG TRP1* | This study |
| DKY802 | As Y80 but *dia2*Δ*::KanMX HIS::mrcl*$_{AQ}$*-MYC13 RAD53::RAD53-3FLAG TRP1* | This study |
| DKY818 | As Y80 but *dia2*Δ*::KanMX rad9::KanMX* | This study |
| DKY854 | As Y80 but *dia2*Δ*::KanMX tof1::URA3* | This study |
| DKY816 | As Y80 but *dia2*Δ*::KanMX csm3*Δ*::KanMX* | This study |
| DKY688 | As Y80 but *mrc1*Δ*::HIS3::3HA-MRC1 LEU2* | This study |
| DKY698 | As Y80 but *dia2*Δ*::KanMX mrc1*Δ*::HIS3::3HA-MRC1 LEU2* | This study |
| DKY919 | As Y80 but *RAD9::RAD9-3FLAG LEU2 bar1*Δ*::URA3* | This study |
| DKY920 | As Y80 but *dia2*Δ*::KanMX RAD9::RAD9-3FLAG LEU2 bar1*Δ*::URA3* | This study |
| DKY710 | As Y80 but *TOF1::TOF1-3FLAG TRP1 CSM3::CSM3-3MYC TRP1* | This study |
| DKY756 | As Y80 but *dia2*Δ*::KanMX TOF1::TOF1-3FLAG TRP1 CSM3::CSM3-3MYC TRP1* | This study |
| DKY972 | As Y80 but *mrc1*Δ*::HIS3::3HA-mrc1*$_{AQ}$ *LEU2* | This study |
| DKY973 | As Y80 but *dia2*Δ*::KanMX mrc1*Δ*::HIS3::3HA-mrc1*$_{AQ}$ *LEU2* | This study |
| DKY862 | As Y80 but *rpn4*Δ*::HIS3 pdr5*Δ*::LEU2 mrc1::HIS3::3HA-MRC1 LEU2* | This study |
| DKY946 | As Y80 but *mrc1*Δ*::HIS3::3HA-mrc1*$_{\Delta 380\text{-}430,701\text{-}800}$ *LEU2* | This study |
| DKY928 | As Y80 but *mrc1*Δ*::HIS3::3HA-mrc1*$_{\Delta 461\text{-}557,701\text{-}800}$ *LEU2* | This study |
| DKY949 | As Y80 but *dia2*Δ*::KanMX mrc1*Δ*::HIS3::3HA-mrc1*$_{\Delta 380\text{-}430,701\text{-}800}$ *LEU2* | This study |
| DKY931 | As Y80 but *dia2*Δ*::KanMX mrc1*Δ*::HIS3::3HA-mrc1*$_{\Delta 461\text{-}557,701\text{-}800}$ *LEU2* | This study |
| DKY914 | As Y80 but *mrc1*Δ*::HIS3::3HA-mrc1*$_{3SA}$ *LEU2* | This study |
| DKY970 | As Y80 but *mrc1*Δ*::HIS3::3HA-AID*$_{3\text{-}111}$*-MRC1 LEU2 ura3-* | This study |

| | | |
|---|---|---|
| | *1::ADH1-OsTIR1-9MYC URA3* | |
| DKY967 | As Y80 but *dia2*Δ*::KanMX mrc1*Δ*::HIS3::3HA-AID$_{3-111}$-MRC1 LEU2 ura3-1::ADH1-OsTIR1-9MYC URA3* | This study |
| DKY974 | As Y80 but *dia2*Δ*::KanMX mrc1*Δ*::HIS3::3HA-AID$_{3-111}$-mrc1$_{1-971}$ LEU2 ura3-1::ADH1-OsTIR1-9MYC URA3* | This study |

Table 2. A list of plasmids used in this study.

| Plasmid | Relevant Features | Reference |
|---|---|---|
| pRS415 | *CEN LEU2* Amp$^r$ | [122] |
| pCMF013 | *mrc1$_{1-971}$* in pRS415 | This study |
| pCMF021 | *mrc1$_{1-971}$* in pRS405 | This study |
| pCMF022 | *3HA-MRC1* in pRS405 | This study |
| pCMF023 | *3HA-mrc1$_{1-971}$* in pRS405 | This study |
| pCMF026 | *TOF1-3FLAG* C-terminal fragment in pRS404 | This study |
| pCMF027 | *CSM3-3MYC* C-terminal fragment in pRS404 | This study |
| pCMF028 | *RAD53-3FLAG* C-terminal fragment in pRS404 | This study |
| pCMF029 | *RAD9-3FLAG* C-terminal fragment in pRS405 | This study |
| pCMF041 | *3HA-mrc1$_{3SA}$* in pRS405 | This study |
| pCMF045 | *3HA-mrc1$_{\Delta 461-557,701-800}$* in pRS405 | This study |
| pCMF050 | *3HA-mrc1$_{\Delta 380-430,701-800}$* in pRS405 | This study |
| pCMF053 | *3HA-AID$_{3-111}$-MRC1* in pRS405 | This study; Derived from pMK38 [126] |
| pCMF054 | *3HA-AID$_{3-111}$-mrc1$_{1-971}$* in pRS405 | This study |
| pCMF055 | *3HA-mrc1$_{AQ}$* in pRS405 | This study; Derived from pAO138 [64] |

Table 3. A list of oligonucleotides used in this study.

| Oligo-nucleotide | Sequence (5' to 3') |
|---|---|
| CMF010 | GGACACCAACTCTACTGGCTC |
| CMF013 | CCCCTCGAGAACGCATAGAAGACTTGGTTCG |
| CMF014 | CCCCTCGAGAATTGAAAGTGGTGAGTATTTC |
| CMF019 | GATAAAAAACCAGTTTACTGTTTTTCAAGTGGTCGAATC |
| CMF021 | GAAAAACAGTAAACTGGTTTTTTATCTTTTCCGAAG |
| CMF024 | GAAGTTCGTTATTCGCTTTTGAACTTATCACCAAATATTTTAGTG GGCCTCCTCTAGTACACTC |
| CMF025 | AGCTTCTGGAGTTCAATCAACTTCTTCGGAAAAGATAAAAAACC AGCGCGCCTCGTTCAGAATG |
| CMF043 | GAATTTGTTTCCTGCTAGCTTTC |
| CMF044 | GTCGTATGGGTAACCTGCCATCACTAAAATATTTGGT |
| CMF045 | ACCAAATATTTTAGTGATGGCAGGTTACCCATACGAC |
| CMF046 | CAAAGCATGCAAGGCATCATCGGAACGTAGAGAAGCGTAATC |
| CMF047 | GATTACGCTTCTCTACGTTCCGATGATGCCTTGCATGCTTTG |
| CMF061 | CCCCTCGAGGAAGAAGTTACTCCAAGATTTG |
| CMF063 | GGGCTCGAGGGGAAGGAGACGATGATTATG |
| CMF064 | TTTCTCGAGCGAGTTTTGGACGAACGTGGG |
| CMF065 | CCCCTCGAGCCCGTTGGTTATCGAAAATCG |
| CMF068 | GCCGCATAGCTCGAATCCCATAAAGCCCATTTCCTTCATAGC |
| CMF069 | GCTATGAAGGAAATGGGCTTTATGGGATTCGAGCTATGCGGC |
| CMF070 | TTATTACCTTCAATGACATTGCTAGCTACTATTAAGATCCTCCTC |
| CMF071 | GAGGAGGATCTTAATAGTAGCTAGCAATGTCATTGAAGGTAATA A |
| CMF072 | ATCTTTATAATCGAGCTCCAGATCATCACTATCACCTTGGCT |
| CMF073 | AGCCAAGGTGATAGTGATGATCTGGAGCTCGATTATAAAGAT |
| CMF074 | GGATTAATTACTACATATTCATTCCAGTTACTTGTCATCGTCATC |
| CMF075 | GATGACGATGACAAGTAACTGGAATGAATATGTAGTAATTAATC |

C

CMF077 AGGTTGTCTCGAGTTCATTGCTTC

CMF078 TTTATAATCGAGCTCCAGCTTCGAAAATTGCAAATTCTCGGGGC
C

CMF079 GGCCCCGAGAATTTGCAATTTTCGAAGCTGGAGCTCGATTATAA
A

CMF080 CCCCTCGAGGTCCATAAATTCCTGCAGTTACTT

CMF081 CCCCTCGAGCATCCGCTAGCTAAATCTTTAG

CMF082 ATCTTTATAATCGAGCTCCAGTCTAACCTCAGAAATAGTGTTG

CMF083 CAACACTATTTCTGAGGTTAGACTGGAGCTCGATTATAAAGAT

CMF091 AAGTAAAACAGCACGAAAAAAGTGATTACAAATTTCAAGGGAG
ATGTTTAGCTTGCCTCGTCCCCG

CMF092 AAAAAAAGAAAAATGCACTTGACAATTAGAGTGCCTGTTAAAA
ATTTAAGGGTTCTCGAGAGCTCG

CMF093 AGAAATTCTAACTGAAAAGTTTAGGATTTACGTATAGTAAGAGT
AGTTTAGCTTGCCTCGTCCCCG

CMF094 GACCGTTGTGCTAGCTTTTTATTCTTCTTTCCAACAAGGAAAAAC
TTAAGGGTTCTCGAGAGCTCG

CMF105 ACTATTCCATTGTTGTATAAAATATAGAGAACCAGAAAAAGAA
AAAGCTTTTCAATTCAATTCATC

CMF106 GGTTCGTATATAGGTATGTATATATAATGAAGGATGGAAGATCC
TCACACCGCATAGGGTAATAAC

CMF129 CAGATTCAGCTCGACACTGCCGGAACGTAGAGAAGCGTAATC

CMF130 GATTACGCTTCTCTACGTTCCGGCAGTGTCGAGCTGAATCTG

CMF135 GGCGACTATATTAAACCTGAAGGCAAG

CMF136 CGCCCATGATGCCGGTTCTGACGCAGGGTCAGAGGCTTCTGG

CMF137 CCAGAAGCCTCTGACCCTGCGTCAGAACCGGCATCATGGGCG

CMF138 GGAGAAAGAATAAGGGCATGAATGAAGAAC

CMF140 GAGACAAGAATAAATGAGAAAAGGGTTCCAC

CMF141 TTCATTACCACTCAAATTCGGCCTTTGAGACAACTTTTG

| | |
|---|---|
| CMF142 | CAAAGGCCGAATTTGAGTGGTAATGAAATTGCCGATTATG |
| CMF143 | GCAAGATGCTTTGAATACAGAACTGCTG |
| CMF144 | GCTCTAAGCTTTCTTTCTGTTTTAGTTGCAATTTCTC |
| CMF145 | CTAAAACAGAAAGAAAGCTTAGAGCTAGAACTAAGTGATG |
| CMF146 | GTTCCATAATAATAACCAGTAGTTCAGTTGGGGAACTTAATAGG CCTCCTCTAGTACACT |
| CMF147 | TTTGTTTTGTTTTTGTTTTGTCAAATTCTCAATCATTCGGCAGCGC GCCTCGTTCAGAAT |
| CMF148 | GCAAGATTTCAAGGAGGTAGCATACTGGTAAATATACGATCCG GCCTCCTCTAGTACACT |
| CMF149 | TCAGTGTCGTATGTCAGATGACGTAATATATAGTGTCCATTTGC GCGCCTCGTTCAGAAT |
| CMF158 | CTGAGAGATTGGCAAATTTATTCTTGTCTCATCAGTTAG |
| CMF159 | GAGACAAGAATAAATTTGCCAATCTCTCAGTTATCAAAG |
| CMF160 | CACTACCAGATGATTCATAATCGGC |
| CMF165 | CAAAGCATGCAAGGCATCATCCGCCGCCGCCTCCGGGCCACC |
| CMF166 | GGTGGCCCGGAGGCGGCGGCGGATGATGCCTTGCATGCTTTGTC C |
| CMF167 | CTTTCTGATGATCCAGAATTTGTTTCCTGC |

## Results

*Dia2 is required for effective checkpoint recovery from MMS-induced DNA damage*

The mechanistic role of Dia2 in the S-phase checkpoint remains largely unknown. *dia2*Δ cells are sensitive to MMS and it is dependent on Dia2 association with the rest of the SCF complex (Fig. 1A) [112]. Because the Dia2 protein is stabilized when the S-phase checkpoint is activated in the presence of MMS [110] and the checkpoint remains active in *dia2*Δ cells [116], I hypothesized that the role of Dia2 lies downstream of checkpoint activation rather than at the initiation of signal. Furthermore, DNA replication in the presence of MMS was reported to be defective in *dia2*Δ cells [115]. One possibility is that Dia2 is required for DNA replication to resume during checkpoint recovery. Thus, I asked if Dia2 plays a role in checkpoint recovery from MMS-induced DNA damage. Cells were synchronized in late G1 by α-factor, released into media containing MMS to activate the S-phase checkpoint, and then released into media without MMS to observe DNA replication during checkpoint recovery. After cells had been released from media containing MMS, almost 70% of wildtype cells completed DNA replication in 60 minutes, whereas only 58% of *dia2*Δ cells completed DNA replication at the same time point (Fig. 1B). Indeed, only at the 100-minute time point did 70% of *dia2*Δ cells complete DNA replication. I did not observe any difference between wildtype and *dia2*Δ cells completing S-phase in the absence of MMS (Fig. 1C), indicating that DNA replication is impeded during recovery from MMS-induced DNA damage but not in an unperturbed S-phase in *dia2*Δ cells. These results suggest that Dia2 has a role in checkpoint recovery.

I asked if Dia2 is required to form an SCF complex to regulate checkpoint recovery. I used the *dia2*Δ*F-box* mutant as this strain lacks only the F-box domain of Dia2, which is required for binding to Skp1 and therefore the rest of the SCF complex [11]. As shown in Fig. 1A, *dia2*Δ*F-box* cells completed DNA replication at a later time point than wildtype. Indeed, the checkpoint recovery rate was similar between *dia2*Δ and *dia2*Δ*F*-box strains, with about 70% of cells completing DNA replication by 100 minutes. These data suggest that the ubiquitination function of Dia2 may be important for checkpoint recovery from MMS-induced DNA damage.

I asked where Dia2 fits in the checkpoint recovery pathway among players identified to have a role in the pathway. DNA repair scaffold proteins Slx4 and Rtt107 were reported to be important for replication fork recovery from MMS-induced DNA damage [102, 103, 105]. To test whether Dia2 functions in a parallel pathway to Slx4-Rtt107, I constructed *dia2*Δ *slx4*Δ and *dia2*Δ *rtt107*Δ double mutants and examined their growth on MMS compared to *dia2*Δ, *slx4*Δ, and *rtt107*Δ single mutants. Serially diluted cells were spotted onto media with or without MMS to compare growth between wildtype, *slx4*Δ, *dia2*Δ, and *dia2*Δ *slx4*Δ strains (Fig. 2A). The *dia2*Δ *slx4*Δ double mutant is more sensitive than either the *dia2*Δ or the *slx4*Δ strain on media with MMS (Fig. 2A), and the same is true for the *dia2*Δ *rtt107*Δ double mutant compared to the single mutants (Fig. 2B). These results indicate that Dia2 functions in a parallel pathway to the Slx4-Rtt107 complex.

Previous studies showed that Rad53 is deactivated by phosphatases Pph3-Psy2 and Ptc2 during S-phase checkpoint recovery [106, 107]. I generated *dia2*Δ *pph3*Δ, *dia2*Δ *psy2*Δ, and *dia2*Δ *ptc2*Δ double mutant strains and examined them for growth on MMS-

containing media. All of the double mutants tested showed significant weaker growth on media with MMS compared to single mutants (Fig. 2C). These results suggest that Dia2 functions in a parallel pathway to Rad53 phosphatases. I chose to focus on Pph3 to investigate further on the role of Dia2 and Pph3 in response to MMS. First, I tested viability of wildtype, *pph3*Δ, *dia2*Δ, and *dia2*Δ *pph3* strains on MMS-containing media. To examine viability, equal numbers of cells were plated onto media containing various concentrations of MMS. The *dia2*Δ *pph3*Δ mutant exhibited weaker viability than the *dia2*Δ or the *pph3*Δ mutant on media containing MMS (Fig. 2D), consistent with the results observed from growth assays (Fig. 2C). Next, I examined checkpoint recovery with these strains as described in Fig. 1B. As expected, *pph3*Δ [106] and *dia2*Δ cells completed DNA replication at later time points than wildtype cells during checkpoint recovery (Fig. 2E & 2F). Furthermore, the *dia2*Δ *pph3*Δ strain exhibited even slower checkpoint recovery compared to the single mutants. Indeed, the double mutant did not complete DNA replication by the last time point tested in this assay (Fig. 2E & 2F). These data indicate that Dia2 plays an important role in S-phase checkpoint recovery and acts in parallel to the Rad53 phosphatase Pph3.

*Checkpoint-defective mrc1 alleles suppress dia2*Δ *sensitivity to MMS and defects in checkpoint recovery*

Given that Mrc1 was reported to be an ubiquitin-mediated degradation substrate of Dia2 [114], results from this thesis study so far raised the possibility that Dia2 mediates Mrc1 degradation for checkpoint recovery. In this case, I would predict that the absence of the substrate would suppress the MMS sensitivity of *dia2*Δ cells. To test this, I

generated a *dia2*Δ *mrc1*Δ double mutant and examined it for growth on media with or without MMS. As shown in Fig. 3A, the *dia2*Δ *mrc1*Δ mutant exhibited modestly stronger growth than the *dia2*Δ strain on media containing MMS.

Since the suppressor *mrc1* allele was checkpoint defective, I asked if the reduction of Mrc1-mediated checkpoint function was important for the suppression of *dia2*Δ cells. I tested whether $mrc1_{AQ}$, a previously described checkpoint-defective allele in which all S/TQ phosphosites were mutated to AQ [64], would also rescue the MMS sensitivity of the *dia2*Δ strain. Growth and viability of *dia2*Δ, *dia2*Δ $mrc1_{1\text{-}971}$, and *dia2*Δ $mrc1_{AQ}$ cells on MMS-containing media were assayed as previously described. I found that checkpoint-defective $mrc1_{1\text{-}971}$ and $mrc1_{AQ}$ mutants at least modestly enhanced growth and viability of *dia2*Δ cells in the presence of MMS (Fig. 3A and B). The suppression by $mrc1_{1\text{-}971}$ is specific to *dia2*Δ cells as I did not observe $mrc1_{1\text{-}971}$ suppressing the MMS sensitivity of *pph3*Δ (Fig. 3C). I then investigated whether the $mrc1_{1\text{-}971}$ and the $mrc1_{AQ}$ mutants would suppress the checkpoint recovery defect of *dia2*Δ cells. Cells were arrested in G1, released into MMS-containing media to activate the S-phase checkpoint, and then released into media without MMS to observe DNA replication recovery when the checkpoint was deactivated (Fig. 3D). Cells were also released from G1 into S-phase in media without MMS as a control (Fig. 3E). As shown in Fig. 3D, wildtype cells completed DNA replication by 60 minutes, whereas the majority of *dia2*Δ cells did not finish DNA replication until 180 minutes post-MMS exposure. Similar to wildtype cells, the majority of *dia2*Δ $mrc1_{1\text{-}971}$ and *dia2*Δ $mrc1_{AQ}$ cells completed DNA replication by 60 minutes. Thus, the $mrc1_{1\text{-}971}$ and $mrc1_{AQ}$ mutants suppress the checkpoint recovery defect of the *dia2*Δ strain.

Previous work has shown that Rad53 is constitutively hyperphosphorylated in *dia2*Δ cells [116], but it has not been established whether this is a result of failure to deactivate the checkpoint or constant re-initiation of checkpoint signaling. If Dia2 is critical for checkpoint recovery, I would expect to see a defect in Rad53 deactivation in *dia2*Δ cells. To test this, cells were arrested in G1, released into MMS-containing media to activate Rad53, and then released into media containing nocodazole. Nocodazole was used to block the cell cycle at early G2/M to separate Rad53 deactivation during S-phase checkpoint recovery from G2/M checkpoint recovery. As shown in Fig. 3F, the top phosphorylated Rad53 band was more intense in the *dia2*Δ strain compared to wildtype at the 1.5-hour time point. Thus, Rad53 deactivation was slower in *dia2*Δ cells than in wildtype.

Since the $mrc1_{1\text{-}971}$ and the $mrc1_{AQ}$ mutants suppress the checkpoint recovery defect of *dia2*Δ cells, I would expect Rad53 deactivation to be more robust in *dia2*Δ $mrc1_{1\text{-}971}$ and *dia2*Δ $mrc1_{AQ}$ cells because the checkpoint would not be fully activated in the first place, making recovery faster despite the lack of Dia2. Consistent with the *mrc1* alleles being checkpoint-defective, I found fewer phosphorylated Rad53 bands in the $mrc1_{1\text{-}971}$ and $mrc1_{AQ}$ strains compared to wildtype at time zero of checkpoint recovery, and the same was true in the *dia2*Δ $mrc1_{1\text{-}971}$ and the *dia2*Δ $mrc1_{AQ}$ mutants compared to the *dia2*Δ strain. Not surprisingly, the top phosphorylated Rad53 band in *dia2*Δ $mrc1_{1\text{-}971}$ and *dia2*Δ $mrc1_{AQ}$ cells was less intense than that in *dia2*Δ cells at the 1.5-hour time point (Fig. 3F). Indeed, the intensity of the top bands was similar between wildtype, *dia2*Δ $mrc1_{1\text{-}971}$, *dia2*Δ $mrc1_{AQ}$ cells. These data suggest that checkpoint recovery is faster in the

*dia2*Δ *mrc1* double mutants because the initial activation of the S-phase checkpoint is not as robust.

Since Rad9, Tof1, and Csm3 are mediators of Rad53 checkpoint phosphorylation in addition to Mrc1, I would predict that *rad9*, *tof1*, and *csm3* mutants would also suppress *dia2*Δ due to a lower level of Rad53 checkpoint activation. To test this, *dia2*Δ, *dia2*Δ *rad9*Δ, *dia2*Δ *tof1*Δ, and *dia2*Δ *csm3*Δ cells were analyzed for growth on MMS-containing media (Fig. 4A) and checkpoint recovery by flow cytometry (Fig. 4B). As expected, *rad9*Δ, *tof1*Δ, and *csm3*Δ mutants all, at least partially, suppress *dia2*Δ MMS sensitivity and checkpoint recovery defects (Fig. 4A & B). These data are consistent with the earlier observation that less robust checkpoint activation of Rad53 suppresses *dia2*Δ cells.

*Dia2 targets Mrc1 for degradation during checkpoint recovery*

Results from this thesis study so far raised the possibility that Dia2 mediates Mrc1 degradation for checkpoint recovery. If this is the case, I would predict that Dia2 targets Mrc1 for degradation to facilitate inactivation of checkpoint signaling and return to the cell cycle. To test this hypothesis, I monitored the stability of Mrc1 in wildtype and *dia2*Δ cells during S-phase checkpoint recovery. Cells were arrested in G1, released into MMS-containing media for S-phase checkpoint activation for 40 minutes, and then released into media containing cycloheximide (CHX) to stop protein synthesis during checkpoint inactivation. Protein samples were taken every half hour during CHX treatment to determine Mrc1 stability over time as cells progressed into the cell cycle. I found that the level of Mrc1 protein decreased during checkpoint recovery in wildtype

cells. Interestingly, the Mrc1 protein was partially stabilized in *dia2*Δ cells (Fig. 5A, upper panel). It appears that both phosphorylated and unmodified Mrc1 are stabilized in *dia2*Δ cells. These results are consistent with the hypothesis that Dia2 targets Mrc1 for degradation to facilitate checkpoint recovery.

Because *rad9*Δ, *tof1*Δ, and *csm3*Δ mutants also suppress *dia2*Δ MMS sensitivity and checkpoint recovery defects, I tested if these Rad53 mediators are also targeted for degradation during checkpoint recovery. Although Rad9 appeared to be slowly degraded during recovery, the turnover rate was indistinguishable for Rad9, Tof1, and Csm3 proteins in wildtype and *dia2*Δ cells (Fig. 5A, lower panel). These data suggest that Dia2 specifically targets Mrc1 among Rad53 mediators for degradation during checkpoint recovery.

Next, I investigated the mechanism of Dia2 targeting Mrc1 for degradation to facilitate checkpoint inactivation. I asked if Dia2 targets checkpoint-activated Mrc1 for degradation by recognizing specific sites on Mrc1 critical for its checkpoint function. One well-characterized mechanism of Mrc1 checkpoint activation is phosphorylation of S/TQ sites by Mec1 [64]. I tested if the S/TQ phosphosites are essential for Dia2-mediated Mrc1 degradation during checkpoint recovery. Wildtype, *mrc1*$_{AQ}$, *dia2*Δ, and *dia2*Δ *mrc1*$_{AQ}$ cells were treated as described for Fig. 5A. The amount of total Mrc1 protein was measured over time. I observed that the Mrc1$_{AQ}$ protein is partially stabilized compared to wildtype Mrc1 (Fig. 5B), suggesting that S/TQ phosphorylation contributes to Mrc1 degradation during checkpoint recovery. However, partial stabilization of the Mrc1$_{AQ}$ protein suggests that S/TQ phosphorylation is not the only determinant of Mrc1 degradation. Mrc1$_{AQ}$ protein stability is even slightly enhanced in *dia2*Δ cells (Fig. 5B),

suggesting that another mechanism in addition to Dia2 may mediate Mrc1 degradation during checkpoint recovery. These results do not rule out the possibility that S/TQ phosphorylation events are important for both Dia2-dependent and Dia2-independent degradation of Mrc1. Nevertheless, the results are consistent with a model in which Dia2 mediates degradation of Mrc1 during recovery from MMS-induced DNA damage.

If the change in the stability of Mrc1 was due to ubiquitin-mediated degradation, I would expect that the stability would be proteasome-dependent. To test this, wildtype cells were subjected to the same treatment as described for Fig. 5A, except that during checkpoint recovery one set of cells was released into CHX-containing media and the other set was released into media containing both CHX and the proteasome inhibitor MG-132. As shown in Fig. 5C, checkpoint-activated Mrc1 was strongly stabilized in the presence of MG-132. These data suggest that phosphorylated Mrc1 is targeted by Dia2 for proteasome-dependent degradation during checkpoint recovery.

A previous study identified two putative Dia2 binding regions in Mrc1 by yeast two-hybrid analysis using *mrc1* fragments [114]. These two regions span residues 380 - 557 and 701 - 800 of Mrc1 [114] (Fig. 6A). I constructed two deletion mutants with one lacking residues 380 - 430 and 701 - 800 and another lacking residues 461 - 557 and 701 - 800. In an attempt to avoid the complication of compromising Mrc1 checkpoint function, I did not delete the region between residues 431 and 460 because that region was reported to be important for Rad53 checkpoint activation [94] and smaller variations of the 380 - 557 region were shown to be insufficient for the two-hybrid interaction between Dia2 and Mrc1 [114]. I tested the functionality of these deletion mutants by examining their HU sensitivity. As shown in Fig. 6B, the two deletion mutants are both

checkpoint-defective. The *mrc1*$_{\Delta 380\text{-}430,701\text{-}800}$ mutant did not have a single S/TQ phosphosite deleted but was still modestly sensitive to HU. The *mrc1*$_{\Delta 461\text{-}557,701\text{-}800}$ mutant had two of the phosphosites deleted but was at least as sensitive to HU as *mrc1*$_{AQ}$. These results indicate that checkpoint activation is not solely dependent on the S/TQ phosphosites and are consistent with earlier results [94]. Although checkpoint function of these deletion mutants is compromised, I went ahead to test whether a Dia2-specific degron is contained within these regions.

If these putative Dia2-binding regions are important for degradation during recovery, I would expect that deleting these regions would stabilize Mrc1 proteins relative to full-length Mrc1, and that no difference in stability would be observed in *dia2*Δ cells. However, the deletion of these two-hybrid Dia2-binding regions in Mrc1 do not stabilize the protein in a wildtype background and these mutant proteins are still stabilized in *dia2*Δ cells (Fig. 6C). Consistent with the stability data, I did not observe any checkpoint recovery defect in these *mrc1* mutants (Fig. 6D). These data indicate that they are not required for Dia2-dependent turnover of Mrc1.

*S. cerevisiae* Mrc1 has two overlapping DSGxxS degron sequences that are analogous to residues important for Claspin degradation during G2/M recovery in humans [117, 119] (Fig. 6E, upper panel). I mutated the three serine residues within the degron to alanine (*mrc1*$_{3SA}$) and examined the mutant for Mrc1 stability and checkpoint recovery. I found that the stability of Mrc1 was indistinguishable between wildtype and *mrc1*$_{3SA}$ cells (Fig. 6E). Furthermore, wildtype and *mrc1*$_{3SA}$ cells completed DNA replication at the same rate during checkpoint recovery (Fig. 6F). These data suggest that the DSGxxS phosphodegrons are not required for S-phase checkpoint recovery or the

degradation of Mrc1 during recovery, and that Mrc1 degradation in S-phase is unlikely to be the analogous pathway to Claspin degradation in G2/M recovery.

*Degradation of checkpoint-functional Mrc1 is critical for Dia2-mediated S-phase checkpoint recovery*

My central hypothesis is that Dia2 mediates Mrc1 degradation for cell cycle re-entry during checkpoint recovery. If this hypothesis is valid, I would expect that the degradation of Mrc1 to be critical to the resumption of DNA replication in the *dia2*Δ strain after cells have been exposed to MMS. To test this directly, I utilized the auxin-inducible degron system [125, 126] to induce degradation of Mrc1 in wildtype and *dia2*Δ cells during S-phase checkpoint recovery. Cells were arrested in G1, released into media containing MMS for S-phase checkpoint activation, and then released into media without MMS to observe DNA replication recovery when the checkpoint was deactivated. During the time course in media without MMS, one set of cells was treated with auxin indole-3-acetic acid (IAA) to induce degradation of Mrc1 whereas another set of cells did not receive IAA. As a control, protein samples were taken 20 and 40 minutes after cells were released from MMS-induced checkpoint to observe degradation of Mrc1 (Fig. 7A). Mrc1 was rapidly degraded in the presence of IAA and was barely detectable after 40 minutes of treatment (Fig. 7A). As shown in Fig. 7B, the majority of cells (measured as 70% or more cells with 2C DNA content) had completed DNA replication by 80 minutes regardless whether the cells were treated with IAA. Induced degradation of Mrc1 does not cause any significant change to the kinetics of DNA replication recovery in wildtype cells post-MMS exposure. This result also suggests that ongoing degradation of Mrc1

does not noticeably slow down DNA replication during checkpoint recovery from MMS-induced DNA damage.

When I compared the DNA replication profiles of untreated and IAA-treated *dia2*Δ cells, I observed that induced degradation of Mrc1 accelerated checkpoint recovery to nearly wildtype kinetics in *dia2*Δ cells. At the 80-minute time point, 72% of IAA-treated *dia2*Δ cells had completed DNA replication, whereas only 58% of untreated *dia2*Δ cells had finished replication by that point. Indeed, only by the 120-minute time point did I find that about 71% of untreated *dia2*Δ cells reach 2C DNA content. Thus, induced degradation of Mrc1 rescues *dia2*Δ checkpoint recovery defects.

To test whether the acceleration of checkpoint recovery kinetics in *dia2*Δ cells is due to a down-regulation of Mrc1 checkpoint signaling or a modulation of its replication function, I repeated the same experiment to induce degradation of checkpoint-defective but replication-proficient $Mrc1_{1\text{-}971}$ protein in *dia2*Δ cells. Consistent with our earlier results, $mrc1_{1\text{-}971}$ suppressed *dia2*Δ checkpoint recovery defects to nearly wildtype kinetics. By 80 minutes, over 70% of untreated *dia2*Δ $mrc1_{1\text{-}971}$ cells had reached 2C DNA content. Interestingly, IAA-induced degradation of $Mrc1_{1\text{-}971}$ protein did not accelerate checkpoint recovery further in *dia2*Δ cells. These data are consistent with a model in which degradation of checkpoint-activated Mrc1 is critical to inactivate checkpoint signaling during Dia2-mediated recovery, rather than to modulate Mrc1 replication function. Altogether these results indicate that degradation of checkpoint-functional Mrc1 is key to Dia2-mediated checkpoint recovery. Fig. 8 depicts my proposed model of Dia2 role in S-phase checkpoint.

## Discussion

*Role of Dia2 in checkpoint recovery*

Results presented in this thesis support a model in which Dia2 mediates the degradation of Mrc1 so that cells can resume DNA replication during recovery from MMS-induced DNA damage in S-phase (Fig. 8). This model is supported by the following observations: 1) Dia2 is required for robust Rad53 deactivation and timely completion of DNA replication when cells are recovering from an MMS-induced S-phase checkpoint; 2) the checkpoint recovery defect in *dia2*Δ*F-box* cells is indistinguishable from that in *dia2*Δ cells; 3) checkpoint-defective alleles of *MRC1* suppress the checkpoint recovery defect of *dia2*Δ cells; 4) Dia2 is specifically required for proteasome-dependent degradation of Mrc1 among Rad53 mediators during S-phase checkpoint recovery; and 5) induced degradation of checkpoint-functional Mrc1 rescues *dia2*Δ checkpoint recovery defects. While this model is focused on the degradation of Mrc1 to down-regulate checkpoint signaling during recovery, I do not rule out any biological role for Dia2 in targeting Mrc1 for degradation in an unperturbed S-phase.

It is a bit puzzling that Mrc1 is degraded during S-phase checkpoint recovery since Mrc1 has been shown to be required for normal replication efficiency [71] and stabilization of the replisome complex [65]. However, those studies relied on an *mrc1* null strain. Our results only address degradation of Mrc1 when cells resume the cell cycle during checkpoint recovery. It is possible that the initial association of Mrc1 to the replisome complex allows stabilization of the replisome and efficient DNA replication. During checkpoint recovery, perhaps Mrc1 is degraded to decrease the checkpoint signal below a particular threshold without compromising the integrity of the replisome. Indeed,

I observed that the auxin-induced degradation of Mrc1 did not noticeably affect the replication kinetics in wildtype cells during checkpoint recovery.

My results put Dia2 in a parallel pathway to DNA repaid scaffold Slx4-Rtt107 and Rad53 phosphatases Pph3-Psy2 and Ptc2 in response to MMS. While it is currently unknown the downstream factor(s) that the Slx4-Rtt107 scaffold acts on in order to facilitate checkpoint recovery, Pph3-Psy2 and Ptc2 promote checkpoint recovery by dephosphorylating Rad53. Furthermore, Dia2 and Pph3 act in parallel pathways within the S-phase checkpoint recovery network. Similar to Pph3, Dia2 is required for DNA replication recovery from MMS but not from HU treatment (data not shown). In addition, the deactivation of Rad53 is also slower in *dia2*Δ cells during recovery from MMS-induced S-phase checkpoint activation. The two pathways may work together for a common goal; Pph3 removes phosphates from checkpoint-activated Rad53, while Mrc1 degradation prevents Rad53 from further activation. It is interesting that checkpoint-activated Mrc1 is degraded rather than dephosphorylated like Rad53 during checkpoint recovery. However, the human homolog of Mrc1, Claspin, is also degraded for checkpoint inactivation, albeit during G2/M recovery [117, 118]. Rad53 activation is also dependent on other checkpoint mediators such as Rad9, Tof1, Csm3 [64, 69, 77, 82, 85, 86]. I did not find mediators other than Mrc1 to be targeted by Dia2 for degradation during S-phase checkpoint recovery. Intriguingly, Rad9 is also degraded during recovery although the degradation is independent of Dia2. I speculate that diversity in the regulation of checkpoint recovery may be advantageous for cells in the event that one of these mechanisms fails.

In *pph3*Δ cells, replication forks fail to restart post-MMS exposure. Instead, cells appear to rely on late-firing origins to finish DNA replication passively [106]. I speculate that similar defects may contribute to the slow DNA replication in *dia2*Δ cells during checkpoint recovery. However, when checkpoint-defective *mrc1* alleles suppress the *dia2*Δ recovery defect, it may be because they allow more robust fork restart in *dia2*Δ cells as Rad53 deactivation is faster in these cells. This model is consistent with the report showing that Rad53 deactivation is sufficient for fork restart in *pph3*Δ cells during recovery [107]. Alternatively, late-firing origins may initiate prematurely in *dia2*Δ *mrc1*$_{1\text{-}971}$ *and dia2*Δ *mrc1*$_{AQ}$ cells, allowing the faster completion of DNA replication post-MMS exposure. This alternative is consistent with data showing that Rad53 checkpoint activation is critical to protect origins from firing inappropriately in the presence of MMS [95-97]. Future work will be needed to distinguish between these possibilities.

*Mechanism of Mrc1 degradation during checkpoint recovery*

My results suggest that stabilization of Dia2 by activation of the S-phase checkpoint [110] may serve as a mechanism to ready cells for Mrc1 degradation during checkpoint recovery. My data suggest that recognition of Mrc1 by Dia2 is complex. Both checkpoint-phosphorylated and unmodified Mrc1 are stabilized in *dia2*Δ cells. However, Mrc1$_{AQ}$ is only partially stabilized relative to wildtype Mrc1 and its stability is slightly enhanced in *dia2*Δ cells. One possible explanation is that a change of Mrc1 conformation and perhaps protein-protein interaction upon S/TQ phosphorylation triggers the degradation of phosphorylated Mrc1 protein during checkpoint recovery. This possibility

is consistent with a previously proposed model in which S/TQ phosphorylation of Mrc1 changes its conformation and association with replisome components [73]. It is possible that Dia2 maintains a basal level of association with Mrc1 in S-phase and their interaction is strengthened by the phosphorylation-dependent conformational change to Mrc1, leading to enhanced Mrc1 degradation during checkpoint recovery.

Alternatively, it is possible that Dia2 recognizes one or more of the identified domains [94] that lack S/TQ and yet are required for checkpoint activation in order to trigger Mrc1 degradation. Two pieces of data are consistent with the possibility that Dia2-mediated Mrc1 degradation is independent of S/TQ phosphorylation. First, I observed that both unmodified and phosphorylated forms of Mrc1 are stabilized in *dia2*Δ cells. Second, the $Mrc1_{AQ}$ protein is modestly stabilized in *dia2*Δ cells. This result is consistent with an additional S/TQ-dependent mechanism for Mrc1 degradation during checkpoint recovery. It will be interesting to elucidate the complex regulation of Mrc1 degradation in future studies.

A previous study identified two putative Dia2-interacting regions by yeast two-hybrid analysis with *mrc1* fragments of various lengths [114]. However, the study only used fragments of Mrc1 in a directed two-hybrid test, so the importance of each domain for Mrc1 protein integrity and function was not determined. Regardless, the two regions are unlikely to contain the Dia2-specific degron because deletion of the regions did not stabilize Mrc1. Rather, I found the two mutants to be checkpoint-defective. Although one of the mutants retained all the S/TQ phosphosites and the other mutant only had two of the sites deleted, I did not observe noticeable phosphorylation defects in these mutants.

These findings are consistent with the published study suggesting that checkpoint activation is not solely dependent on the S/TQ phosphosites [94].

When I mutated the DSGxxS phosphosites similar to those important for Claspin degradation during G2/M checkpoint recovery [117, 119], I did not see any defect in S-phase checkpoint recovery or stabilization of Mrc1. Furthermore, induced degradation of Mrc1 is critical to Dia2-mediated S-phase checkpoint recovery. My findings raise the question of whether Claspin degradation is also regulated for S-phase checkpoint recovery in mammalian cells.

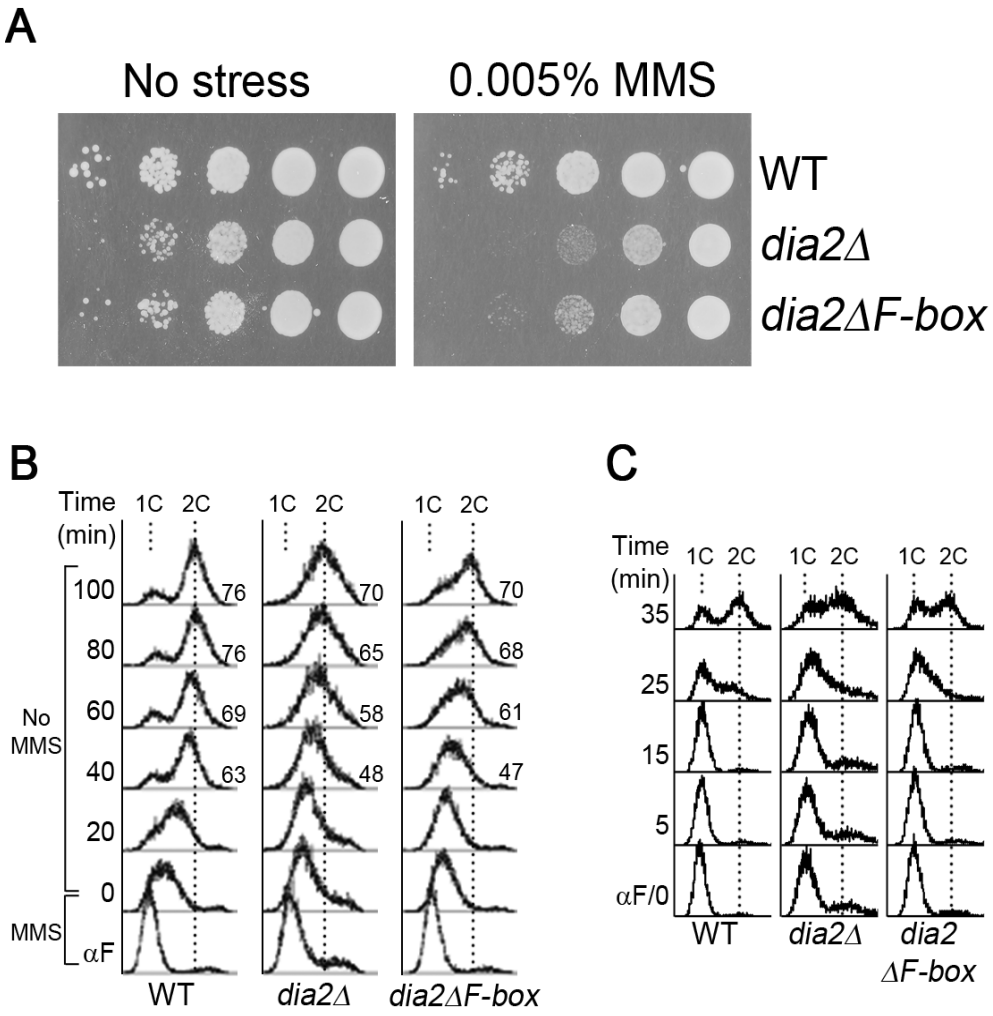


**Fig. 1.** Dia2 is required for checkpoint recovery from MMS-induced DNA damage in S-phase. (A) 10-fold serial dilutions of indicated strains were spotted onto rich media (YPD) with or without 0.005% MMS and incubated at 30ºC. (B) Cells were arrested in late G1 by α-factor (αF), released into rich media (YPD) + 0.033% MMS for 40 minutes, and then released into YPD. Samples were analyzed at the indicated time points by flow cytometry. 1C and 2C indicate DNA content. Percentage of cells with 2C DNA content is indicated on the right of selected profiles. (C) Cells were arrested in late G1 by αF and then released into YPD at 30ºC. Samples were analyzed at the indicated time points by flow cytometry. 1C and 2C indicate DNA content.

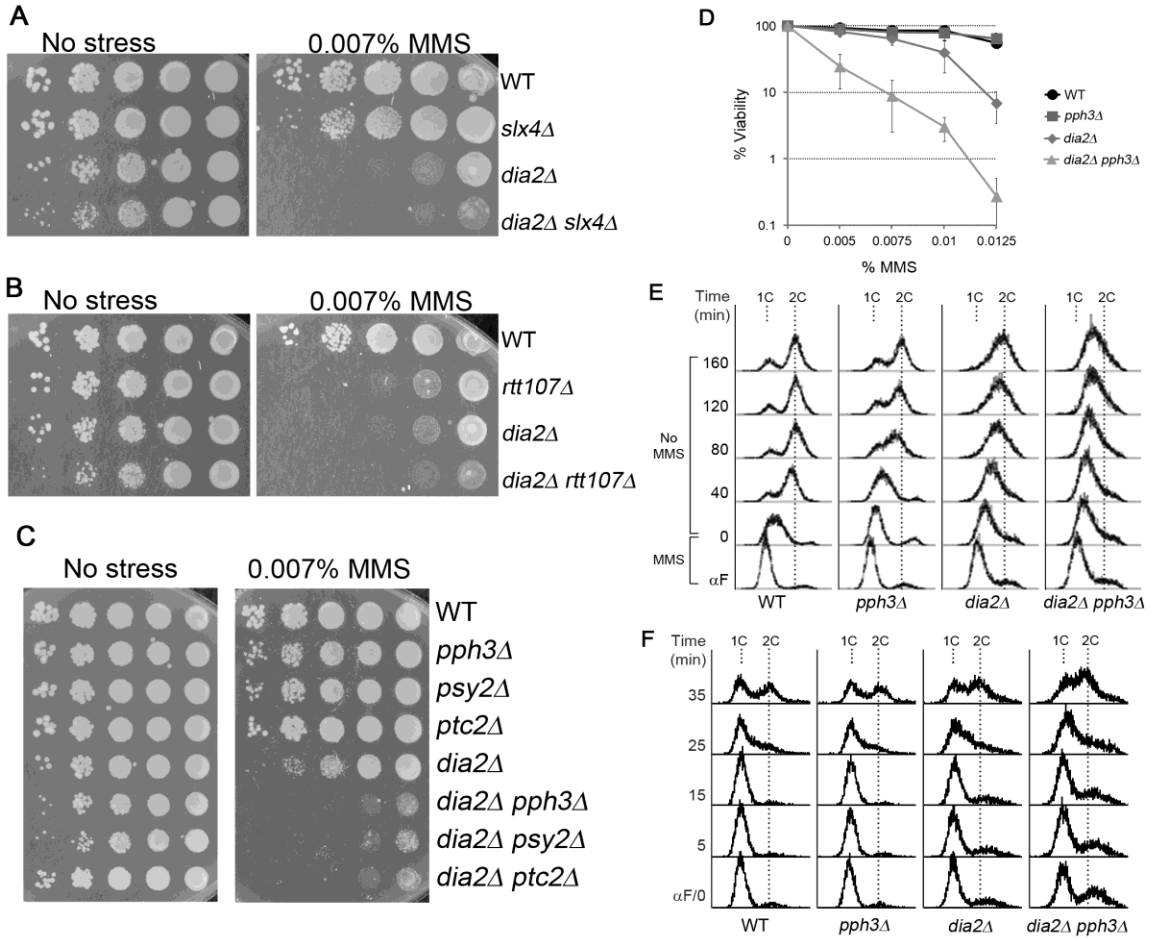


**Fig. 2.** Dia2 functions in a parallel pathway to DNA repair scaffold and Rad53 phosphatases in response to MMS. (A-B) *DIA2* genetically interacts with DNA repair scaffold components *SLX4* and *RTT107* in response to MMS. 10-fold serial dilutions of the indicated strains were spotted on YPD or YPD + 0.007% MMS and incubated at 30ºC. (C) *DIA2* genetically interacts with Rad53-phosphatases *PTC2* and *PPH3*, as well as *PPH3* co-factor *PSY2* in response to MMS. 10-fold serial dilutions of the indicated strains were spotted on YPD or YPD + 0.007% MMS and incubated at 30ºC. (D) Dia2 and Pph3 contribute to viability of cells in response to MMS. Equal numbers of cells were plated on media containing the indicated amounts of MMS, and colony forming units were counted after four days at 30ºC. Error bars represent standard deviations from

three independent experiments. (E and F) Dia2 functions in parallel to Pph3 for S-phase checkpoint recovery. (E) Cells were arrested in late G1 by α-factor (αF), released into rich media (YPD) + 0.033% MMS for 40 minutes, and then released into YPD. Samples were analyzed at the indicated time points by flow cytometry. 1C and 2C indicate DNA content. (F) Cells were arrested in late G1 by αF and then released into YPD at 30ºC. Samples were analyzed at the indicated time points by flow cytometry. 1C and 2C indicate DNA content.

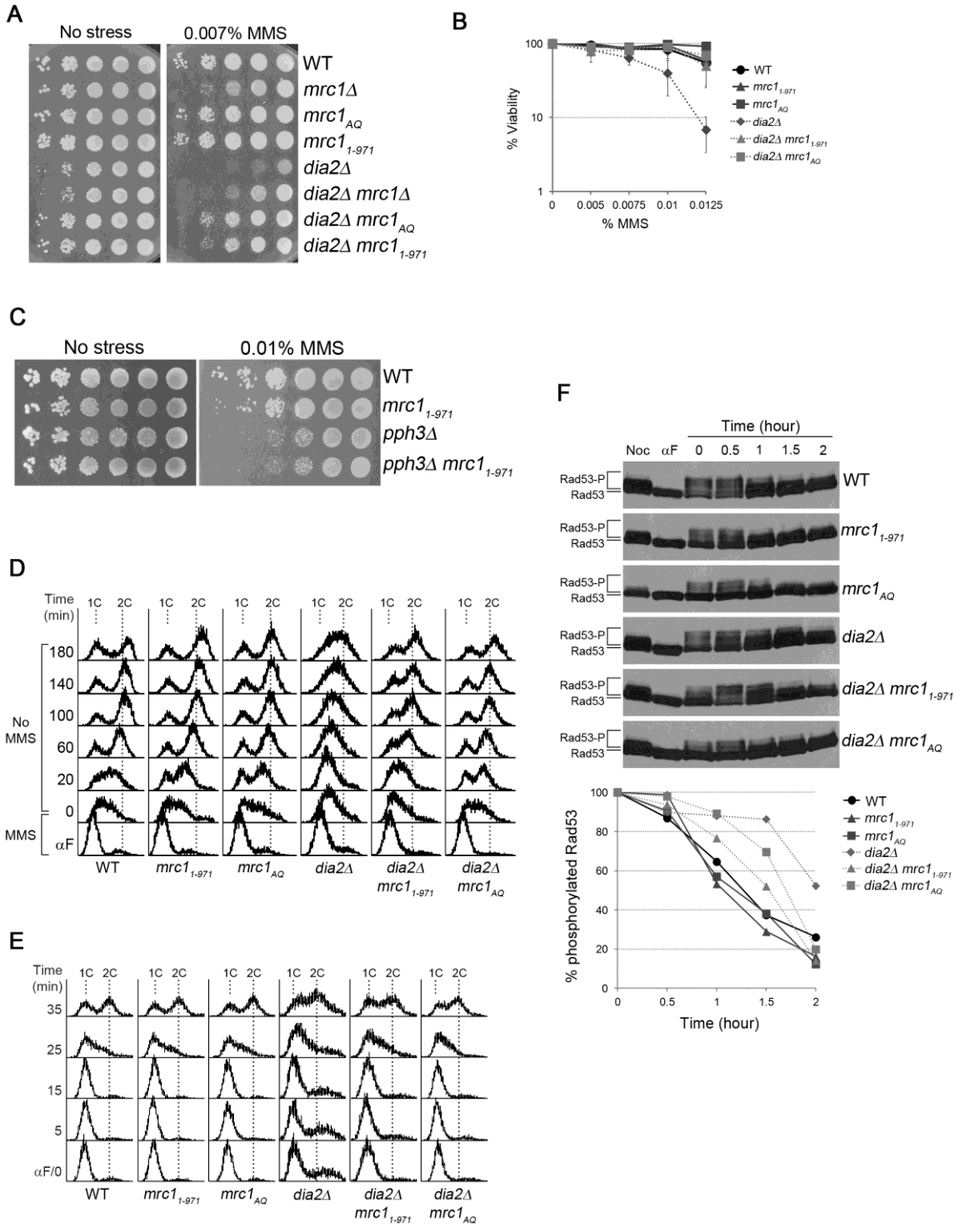


**Fig. 3.** Checkpoint-defective alleles of *mrc1* suppress *dia2*Δ MMS sensitivity and checkpoint recovery defects. (A) *mrc1* mutant alleles suppress *dia2*Δ MMS sensitivity.

10-fold serial dilutions of the indicated strains were spotted on YPD or YPD + 0.007% MMS and incubated at 30ºC. (B) Checkpoint-defective *mrc1* alleles enhance viability of *dia2*Δ in MMS. Equal numbers of cells were plated on media containing the indicated amounts of MMS, and colony forming units were counted after four days at 30ºC. Error bars represent standard deviations from three independent experiments. (C) $mrc1_{1\text{-}971}$ does not suppress *pph3*Δ MMS sensitivity. 10-fold serial dilutions of the indicated strains were spotted on YPD or YPD + 0.01% MMS and incubated at 30ºC. (D-E) Checkpoint-defective *mrc1* alleles accelerate *dia2*Δ checkpoint recovery. Cells were arrested in late G1 by α-factor, (D) released into YPD + 0.033% MMS for 40 minutes, and then released into YPD or, (E) released into YPD at 30ºC. 1C and 2C indicate DNA content. (F) $mrc1_{1\text{-}971}$ and $mrc1_{AQ}$ accelerate Rad53 deactivation of *dia2*Δ. Cells were arrested in G1 by α-factor, released in YPD + 0.009% MMS for one hour, and then released into YPD + 15μg/ml nocodazole. Protein samples were taken as indicated. Rad53-P and Rad53 represent phosphorylated and unphosphorylated Rad53 proteins, respectively. The very top modified band of Rad53 was quantified using ImageJ and the percentage of that in each time point relative to time zero is shown in the graph.

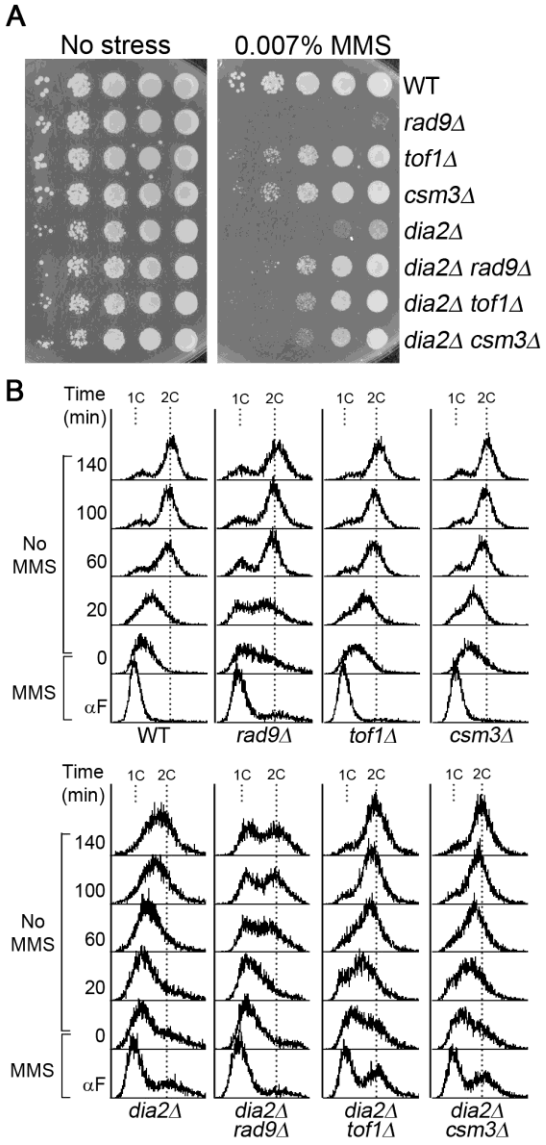


**Fig. 4.** Rad53 mediator mutants suppress *dia2*Δ MMS sensitivity and checkpoint recovery defects. (A) *rad9*Δ*, tof1*Δ*, and csm3*Δ mutants suppress *dia2*Δ MMS sensitivity. 10-fold serial dilutions of the indicated strains were spotted on YPD or YPD + 0.007% MMS and incubated at 30ºC. (B) *rad9*Δ*, tof1*Δ*, and csm3*Δ mutants accelerate *dia2*Δ checkpoint recovery. Cells were arrested in G1 by α-factor, released into YPD + 0.033% MMS for 40 minutes, and then released into YPD at 30ºC. 1C and 2C indicate DNA content.

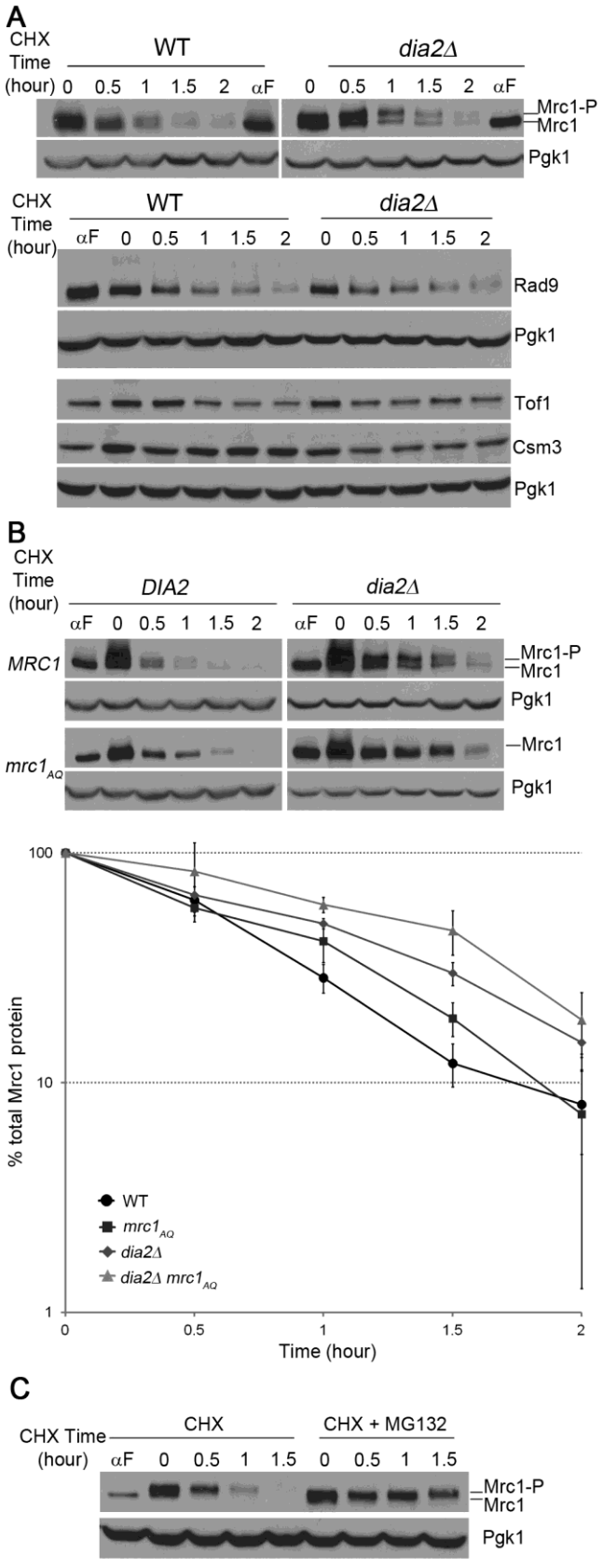
A
CHX Time (hour)
WT
dia2Δ
0 0.5 1 1.5 2 αF
Mrc1-P
Mrc1
Pgk1
Rad9
Tof1
Csm3
B
DIA2
MRC1
mrc1AQ
% total Mrc1 protein
Time (hour)
WT
mrc1AQ
dia2Δ
dia2Δ mrc1AQ
C
CHX
CHX + MG132

**Fig. 5.** Dia2 is required for the degradation of Mrc1 during checkpoint recovery. (A) Mrc1 is degraded in a Dia2-dependent manner whereas Rad9, Tof1, and Csm3 are not. Cells were arrested in G1 by α-factor, released into YPD + 0.033% MMS for 40 minutes, and then released into YPD + 200μg/ml CHX. Protein samples were taken at indicated times. Mrc1-P and Mrc1 represent phosphorylated and unphosphorylated Mrc1 proteins, respectively. Pgk1 serves as a loading control. (B) S/TQ phosphosites play a role in the degradation of Mrc1 during checkpoint recovery. Cells were treated as described in (A). Protein samples were taken as indicated. Pgk1 serves as a loading control. Stability of total Mrc1 protein was quantified from three independent experiments. Error bars were derived from standard deviations of the three experiments. (C) Degradation of phosphorylated Mrc1 is proteasome-dependent. Wildtype cells were subjected to the same arrest and release treatment as described in (A), except that during checkpoint recovery one set of cells were released into YPD + 200μg/ml CHX and another set into YPD + 200μg/ml CHX + 50μM MG-132.

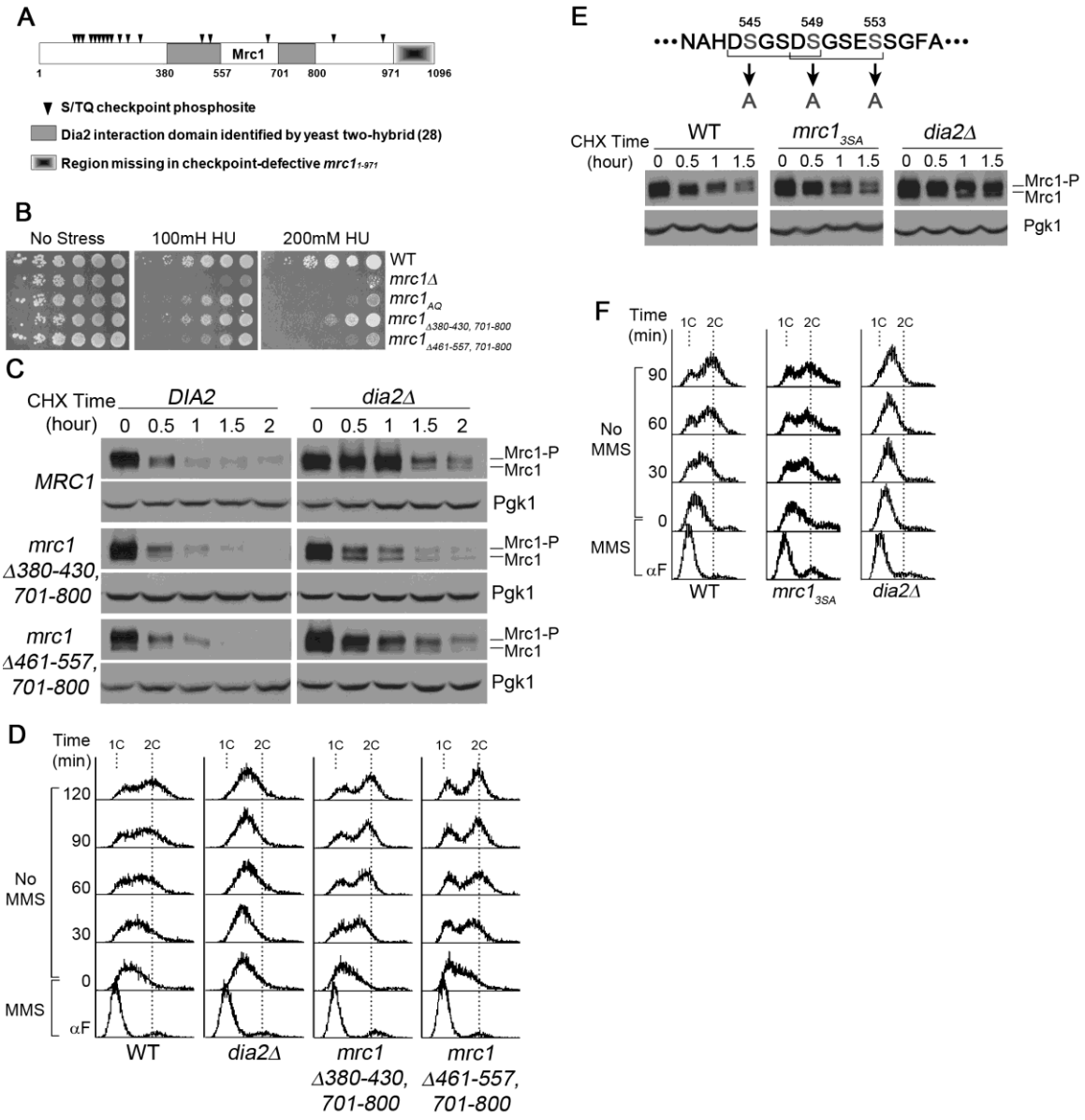


**Fig. 6.** Internal Mrc1 domains and DSGxxS sites do not affect protein stability. (A) Structural schematic of the Mrc1 protein. (B) Deletion of the putative Dia2-binding regions rendered *mrc1* checkpoint-defective. 10-fold serial dilutions of the indicated strains were spotted on YPD, YPD + 100mM HU, or YPD + 200mM HU and incubated at 30ºC. (C-D) Dia2-mediated degradation of Mrc1 is not dependent on the putative two-

hybrid Dia2-interacting regions spanning residues 380-557, 701-800 of Mrc1. (C) Cells were arrested in G1 by α-factor, released into YPD + 0.033% MMS for 40 minutes, and then released into YPD + 200μg/ml CHX. Protein samples were taken at indicated times. Pgk1 serves as a loading control. (D) Cells were treated as described in (C), except that CHX was not added. Samples from at the indicated time points were analyzed by flow cytometry. 1C and 2C indicate DNA content. (E-F) The DSGxxG phosphodegron does not play a role in the degradation of Mrc1 or S-phase checkpoint recovery. Samples were prepared as described in (C-D) and were analyzed for (E) Mrc1 stability and (F) DNA replication by flow cytometry.

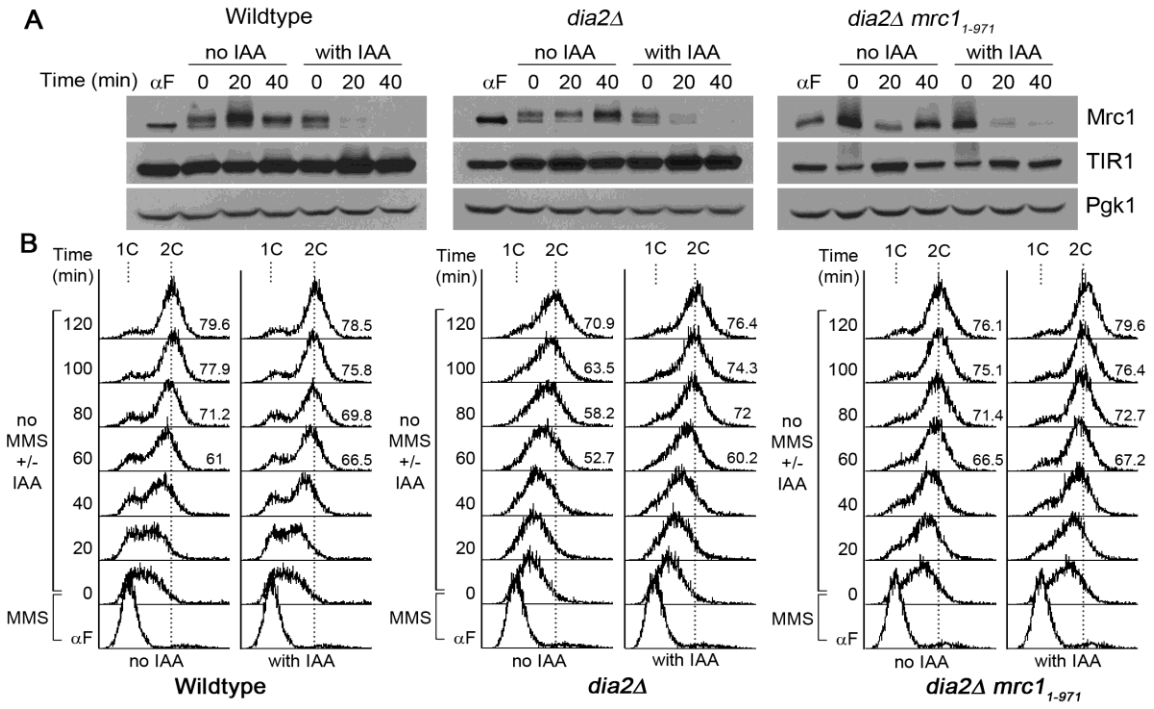


**Fig. 7.** Induced degradation of checkpoint-functional Mrc1 is critical to Dia2-mediated checkpoint recovery. (A-B) Cells were arrested in G1 by α-factor, released into YPD + 0.033% MMS for 1 hour, and then released into either YPD + ethanol (vehicle) or YPD + 1.5mM IAA (auxin) at 30ºC. (A) Mrc1 was rapidly degraded upon IAA treatment. Protein samples were taken at indicated times with or without IAA treatment. Pgk1 serves as a loading control. (B) Induced degradation of checkpoint-functional Mrc1 rescues *dia2*Δ checkpoint recovery defects. DNA replication of wildtype, *dia2*Δ, and *dia2*Δ *mrc1*$_{1\text{-}971}$ cells during checkpoint recovery with or without IAA treatment were analyzed by flow cytometry. 1C and 2C indicate DNA content. Percentage of cells with 2C DNA content was analyzed by the FlowJo software and indicated on the right of selected profiles.

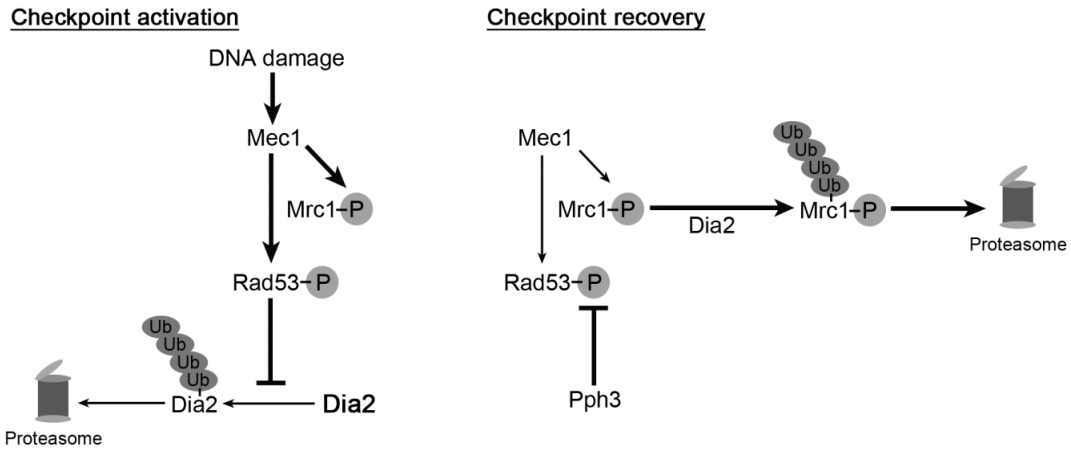


**Fig. 8.** Model for the role of Dia2 in S-phase checkpoint recovery. Dia2 is stabilized by the activation of the S-phase checkpoint [110]. During checkpoint recovery, Dia2 targets checkpoint-activated Mrc1 for degradation to down-regulate the checkpoint activation of Rad53. Rad53 phosphatase Pph3 removes phosphate groups from Rad53 to allow DNA replication to resume in S-phase [106, 107]. Ub = ubiquitin, P = phosphorylation.

# Chapter V

# Summary and discussion

*Summary*

Genome maintenance is critical to the survival and well-being of the cell. For higher eukaroytes, loss of genomic integrity often leads to pathogenic disorders or cancer development. To maintain genomic integrity, cells tightly regulate DNA replication due to inherent risks of mutations during the replication process. Environmental stress including agents that induce damage to the DNA structure puts extra pressure on the cell to stabilize DNA replication. The S-phase checkpoint is in place to help cells maintain the integrity of the replication machinery in the face of exogenous or endogenous stress. After stress is resolved, cells must find a way to inactivate the S-phase checkpoint in order for DNA replication to resume.

Dia2 has been proposed to have a role in the S-phase checkpoint since the F-box protein was found to regulate DNA replication and protein level was stabilized in response to MMS [110, 115]. I determined that Dia2 role in the S-phase checkpoint is to promote the resumption of DNA replication after cells have been exposed to MMS-induced DNA damage. Genetics analysis placed Dia2 in a parallel pathway to previously identified checkpoint recovery mechanisms that include DNA repair scaffold Slx4-Rtt107 and Rad53 phosphatases Pph3-Psy2 and Ptc2. Interestingly, both Dia2 and Rad53 phosphatases are not required for recovery from HU-induced replication stress. This suggests that cells use distinct recovery mechanisms to handle different replication stress.

To elucidate the downstream target of Dia2 in response to MMS, I performed a genetic suppressor screen and identified a novel *mrc1* allele that suppresses *dia2*Δ MMS sensitivity and is checkpoint defective. Mrc1 is a ubiquitin-mediated degradation substrate of $SCF^{Dia2}$ [114] and checkpoint defective *mrc1* alleles suppress the checkpoint recovery defect of *dia2*Δ cells, which raise the possibility that Dia2 targets Mrc1 for degradation to mediate checkpoint recovery. Consistent with this hypothesis, I found that Dia2 mediates Mrc1 degradation during recovery from MMS-induced DNA damage. Furthermore, induced degradation of Mrc1 promote resumption of DNA replication in *dia2*Δ cells to almost wildtype kinetics, whereas degradation of checkpoint-defective $Mrc1_{1\text{-}971}$ did not affect the kinetics of recovery. Altogether these results indicate that Dia2 mediates checkpoint-activated Mrc1 degradation to promote resumption of DNA replication during checkpoint recovery from MMS-induced DNA damage.

*Speculation and future direction*

DNA replication is thought to slow down in response to DNA damage in order to allow cells to repair the damage. This view is challenged by studies that suggest checkpoints are in place to activate the DNA repair pathway and DNA replication slowed down as a result [76]. Thus, it is unknown whether the ultimate function of the S-phase checkpoint is to slow down DNA replication enough to make sure all damage is resolved before cells enter the G2/M phase. During the course of my thesis study, I often found that when cells are treated with MMS in S-phase, wildtype cells would complete DNA replication, albeit at a slower rate, followed by the activation of the G2/M checkpoint. DNA replication in S-phase in the presence of MMS is extremely slow in *dia2*Δ cells. If

DNA replication is to be slowed down to buy cells some time to fix the DNA damage, I would not expect to see accumulation of DNA damage in *dia2*Δ cells or prolonged activation of the G2/M DNA damage checkpoint. However, after completion of DNA replication, recombination repair proteins are enriched on chromatin in *dia2*Δ cells and the cells are arrested in G2/M phase [112] presumably due to activation of the G2/M checkpoint, suggesting that slowing down DNA replication does not necessarily lead to better DNA repair. One caveat for this argument is that Dia2 may be involved in DNA repair and the accumulation of damage stems from a compromised DNA repair pathway in the absence of Dia2.

DNA replication is resumed as cells recover from DNA damage. However, it is unclear how cells defined an appropriate time to resume DNA replication in the face of DNA damage. Do cells coordinate the DNA repair and the recovery mechanisms so that replication fork progresses as cells actively repair the DNA damage? Interestingly, DNA repair scaffold Slx4-Rtt107 is phosphorylated by Mec1 and recruited to the replication fork as S-phase checkpoint is activated, and this association is important for the restart of the replication fork post-MMS exposure [102, 103, 105]. I found that Dia2 functions in a parallel pathway to Slx4-Rtt107, consistent with a role of Dia2 in checkpoint recovery, but it also raises the possibility that Dia2 may also have a role in the DNA repair pathway. Future studies will be required to determine if the genetic relationship is within the DNA repair and/or the checkpoint recovery network.

Replication forks fail to restart properly in the Rad53 phosphatase mutant *pph3*Δ due to sluggish Rad53 deactivation during checkpoint recovery [106, 107]. Indeed, already-initiated replication forks fail to restart and cells fire late origins to complete DNA

replication passively [106]. I found that Dia2 functions in parallel to Pph3 in the resumption of DNA replication post MMS-exposure. Because Rad53 deactivation is also slow in *dia2*Δ cells, I speculate that replication forks also fail to restart in *dia2*Δ cells as well. In that case, I would expect that *dia2*Δ cells would fire late origins to complete DNA replication passively. If this is the case, prolonged stalling of replication fork could lead to collapse of replication machinery, breaks in the DNA and incomplete replication. The exposed single-stranded DNA serves as hot spot for recombination and this may contribute to some of the genomic instabilities observed in *dia2*Δ cells.

I showed that Dia2 targets Mrc1 for degradation during checkpoint recovery and checkpoint defective *mrc1* alleles suppress *dia2*Δ checkpoint recovery and Rad53 deactivation defects. These results are consistent with a model that Dia2 mediates Mrc1 degradation to down-regulate Rad53 signaling during checkpoint recovery. Because Rad53 deactivation is sufficient for replication fork restart during recovery [107], the degradation of Mrc1 likely leads to restart of replication forks. Intriguingly, I found that induced degradation of Mrc1 promotes resumption of DNA replication in *dia2*Δ cells using flow cytometry. I look forward to future experiments to examine the effect of induced Mrc1 degradation on Rad53 deactivation and replication fork restart.

I proposed a model in which Dia2 mediates Mrc1 degradation to promote checkpoint recovery. Does Dia2 specifically recognize and target checkpoint-activated Mrc1 for degradation? Activation of the S-phase checkpoint leads to phosphorylation of Mrc1 at the S/TQ sites, however additional domains of Mrc1 are required to relay the checkpoint signal to Rad53 [64, 94]. Interestingly, I found that the $Mrc1_{AQ}$ protein, which has all the phosphosites mutated to alanine, is partially stabilized during checkpoint recovery. The

data suggest that the checkpoint phosphorylation events play a role in Mrc1 degradation, and potentially checkpoint recovery, in response to MMS. However, it is technically challenging to test whether the S/TQ-dependent degradation of Mrc1 would promote checkpoint recovery because the *mrc1*$_{AQ}$ allele is checkpoint defective and would suppress any checkpoint recovery defect. Because the Mrc1$_{AQ}$ protein is stabilized in *dia2*Δ cells, it is possible that 1) Dia2 recognizes domains in addition to S/TQ phosphorylation events to target Mrc1 for degradation or 2) another degradation mechanism recognizes the phosphorylation events and Dia2 recognition of Mrc1 is S/TQ independent. Extensive future studies will be required to examine these possibilities. One approach is to construct a series of Mrc1 deletion mutants and test for both Mrc1 stability and interaction with Dia2. However, I found that deleting regions of Mrc1 often compromises its checkpoint function. While it is important to identify the degron(s) on Mrc1 that Dia2 recognizes, once again it may be technically challenging to test whether it is important for checkpoint recovery if the degron overlaps with checkpoint activation domains. Regardless, this thesis work present evidence to support a model in which Dia2 targets Mrc1 for degradation to mediate recovery from MMS-induced DNA damage.

Does Dia2 have additional substrate targets that contribute to checkpoint recovery? Checkpoint defective *mrc1* alleles partially suppress *dia2*Δ MMS sensitivity, which would suggest that additional components may be required to fulfill Dia2 role in checkpoint recovery. However, checkpoint activation is compromised in these alleles to handle DNA damage, making it difficult to distinguish whether the incomplete rescue is due to a defective checkpoint response to DNA damage or a requirement of additional components to fulfill Dia2 role in checkpoint recovery. The checkpoint defective *mrc1*

alleles boost the viability of *dia2*Δ cells in response to MMS to wildtype level. In addition, induced degradation of Mrc1 promotes recovery in *dia2*Δ cells to almost wildtype kinetics, and it is important to note that the auxin-inducible degron system may not exactly mimic how Mrc1 degradation is regulated *in vivo*. These results suggest that Mrc1 is a major substrate of Dia2 in checkpoint recovery. It remains an open question whether Dia2 modifies additional substrates during recovery from DNA damage. The unidentified *dia2*Δ suppressors may provide insight to this question. However, I found that checkpoint mutants such as *rad9*Δ, *tof1*Δ, and *csm3*Δ, which are not substrates of Dia2 but mutating them results in a defective checkpoint activation, also suppress *dia2*Δ MMS sensitivity. Thus, the remaining suppressors may not necessarily be additional targets of Dia2 but simply mutants in the S-phase checkpoint pathway.

In humans, Claspin is targeted for degradation by $SCF^{\beta\text{-}TrCP}$ during recovery from the G2/M DNA damage checkpoint [117, 118]. In yeast, $SCF^{Dia2}$ mediates degradation of Mrc1 during recovery from the S-phase checkpoint. It is tempting to speculate whether the F-box protein β-TrCP is the human homologue of Dia2. First, these two F-box proteins differ in structure. While β-TrCP has WD40 repeats at the C-terminus, Dia2 has TPR and LRR repeats at the N- and C-terminus, respectively [16, 17]. Second, Dia2 and β-TrCP mediates Mrc1 degradation at different stages of the cell cycle. Third, recognition mechanism is different between the two F-box proteins; β-TrCP recognizes the DSGxxG phosphodegron [117, 119], whereas Dia2 does not. Interestingly, Claspin is also targeted for degradation in S-phase in response to DNA damage in mammalian cells and the degradation is not dependent on β-TrCP (R. Centore and L. Zou, personal communication). Thus, the work of this thesis may also be applicable to Claspin

degradation in humans, and determining the F-box that mediates Claspin degradation in S-phase may identify the human homologue of Dia2. The ATR pathway is a target of interest in drugs development for cancer treatment [120]. One approach is to inhibit checkpoint activation in order to sensitize cancer cells to genotoxic stress in order to promote cell death. Thus, a better understanding of checkpoint recovery may provide alternative target(s) to sensitize cancer cells.

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