Accumulation of unligated Okazaki fragments induces PCNA ubiquitination at lysine 107 and Rad59-dependent replication fork progression

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

Dr. Anja-Katrin Bielinsky

July 2012

Acknowledgements

Over the last 5.5 years, it has been a joy coming to work and discussing science with everyone in the Bielinsky laboratory. Anja, you have helped me in many ways to develop the skills I need to continue on this scientific endeavor to become an independent scientist. You have helped me develop my own questions, design experiments, and the opportunity to explore in my own quest of knowledge. Besides science, I also appreciate your continuous support in my artistic interests. It meant a lot for me to know that I was able to do both in graduate school.

To my family, especially my parents, you have invested so much in me to support my dreams in life. Mom, thank you for all the sacrifices you have made in life to allow me to dream big and giving me the opportunity to become who I am today. The day when you and I arrived at Woodstock School in India 16 years ago, I knew that my life has changed completely. Your support has been beyond words.

Lastly, I am grateful for all support and encouragements from Torsten during my time in graduate school. You have helped me find my balance in life and shared all the great time and stressful time in life. It has been great to know that you are always there when I needed it. I have made it this far because of you and all your distractions.

Dedication

This dissertation is dedicated to mom, who has sacrificed so much of her life to give me to opportunity to become who I am today.

Abstract

DNA ligase I, encoded by the *CDC9* gene in *Saccharomyces cerevisiae*, is an essential enzyme that catalyzes the ligation of newly synthesized DNA on the lagging strand called Okazaki fragments. In humans, approximately 30 million Okazaki fragments are synthesized during every S phase and require further processing prior to DNA ligation. An individual harboring DNA ligase I mutations exhibited growth retardation, sunlight sensitivity, severe immunosuppression and developed lymphoma, indicating a link between defects in Okazaki fragment maturation and cancer predisposition. How cells monitor and suppress such accumulation of DNA damage that arises due to defective Okazaki fragment processing is unclear. Using *S. cerevisiae* as a model system, we uncovered a novel and conserved ubiquitination pathway that targets proliferating cell nuclear antigen (PCNA) at lysine 107 when DNA ligase I activity is inhibited. The modification at K107 is catalyzed by the E2 variant Mms2 together with Ubc4 and the E3 ubiquitin ligase Rad5. Most importantly, this signal is crucial to activate the S phase checkpoint, which promotes cell cycle arrest. In support of this notion, a *pol30K107* mutation alleviated cell cycle arrest in *cdc9* mutants. To determine whether PCNA ubiquitination occurred in response to nicks or the lack of PCNA-DNA ligase interaction, we complemented *cdc9* cells either with wild-type DNA ligase I or *Chlorella* virus ligase, the latter of which fails to interact with PCNA. Both enzymes reversed PCNA ubiquitination, arguing that the modification is likely triggered directly by nicks. To further understand how cells cope with nicks during replication, we utilized *cdc9-1* in a genome-wide synthetic lethality screen and identified *RAD59* as a strong negative

interactor. *cdc9 rad59Δ* mutants did not alter PCNA ubiquitination but enhanced phosphorylation of the mediator of the replication checkpoint, Mrc1, indicative of increased replication fork stalling. Thus, Rad59 promotes fork progression when Okazaki fragment processing is compromised and counteracts PCNA-K107 mediated cell cycle arrest.

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List of abbreviations

8-oxoG	8-oxo-7,8-dihydroguanine
ACS	ARS consensus sequence
alt-NHEJ	alternative NHEJ
AMP	5'-adenosine monophosphate
AP	apurinic/apyrimidinic
APC/C	Anaphase promoting complex or cyclosome
ARS	autonomously replicating sequences
ATM	ataxia-telangiectasia
ATR	ataxia-telangiectasia mutated- and Rad3-related
BER	base excision repair
BLM	Bloom
BRCA1	breast cancer 1
BRCT	BRCA1 C-terminus
BS	Bloom's syndrome
cdc	cell division cycle
cdc9-td	*cdc9*-degron
Cdk1	Cyclin dependent kinase 1
Cdt1	chromatin licensing and DNA replication factor 1
Chk	checkpoint kinase
Clb	B-type cyclin
Cln	G1 or A-type cyclin
CMG	Cdc45-Mcm2-7-GINS
Csm3	chromosome segregation in meiosis 3
Ctf4	chromosome transmission fidelity
Ddc2	DNA damage checkpoint 2
DDK	Dbf4-dependent Cdc7 kinase
DDR	DNA damage response
DDT	DNA damage tolerance
DNA	Deoxyribonucleic acid
Dna2	DNA synthesis defective 2
DNA-PK	DNA-dependent protein kinase
dNTPs	di-deoxynucleotide
ds	double-stranded
DSB	double-stranded break
Dun	DNA damage induced
E1	ubiquitin activating
E2	ubiquitin conjugating
E2	ubiquitin ligase

EM	electron microscopy
Exo1	exonuclease 1
Fen1	flap endonuclease 1
G1	Gap phase 1
G2	Gap phase 2
GCR	Gross chromosomal rearrangement
GINS	Go, Ichi, Nii, and San 5-1-2-3 referred to number of the subunits in Japanese
Gy	Gray
HNPCC	hereditary nonpolysis colon cancer
HR	homologous recombination
HU	hydroxyurea
IDCL	inter-domain connector loop
IR	ionizing radiation
K	lysine
kDa	Kilodalton
LIG1	DNA ligase I
M	mitosis
Mcm	minichromosome maintenance
Mec1	mitotic entry checkpoint 1
MEF	mouse embryonic fibroblast
MLH1	MutL homolog 1
MMR	mismatch repair
MMS	methyl methansulfonate
Mrc1	mediator of replication checkpoint 1
MSH2	MutS homolog 2
NER	nucleotide excision repair
NHEJ	non-homologous end joining
nt	nucleotide
NTase	nucleotidyltransferase
OB-fold	oligonucleotide/oligosaccharide binding fold
OFs	Okazaki fragments
ORC	origin recognition complex
PARP	poly (ADP-ribose) polymerase
PCNA	proliferating cell nuclear antigen
Pif1	petite integration frequency 1
PIKK	phosphatidylinositol 3-kinase-like protein kinase
PIP	PCNA-interacting protein
pol	polymerase
pre-IC	pre-initiation complex

pre-LC	pre-loading complex
pre-RC	pre-replicative complex
PRR	post-replicative repair
rad	radiation sensitive
RFC	replication factor C
RNA	ribonucleic acid
RNR	ribonucleotide reductase
rNTPs	ribonucleotides
ROS	reacive oxygen species
RPA	replication protein A
S	Synthesis phase
S. cerevisiae	*Saccharomyces cerevisiae*
S-CDK	S phase dependent-kinase
SCF	SKP1-CUL1-F-Box
shRNA	short hairpin RNA
SIM	SUMO-interacting motif
Sld	synthetic lethality with Dpb11
Sml	suppressor of Mec1 lethality
Srs2	suppressor of rad six 2
ss	single stranded
SSA	single-stranded annealing
SSB	single-stranded break
ssDNA	single-stranded DNA
SUMO	small ubiquitin-like modifier
Tel1	telomere maintenance 1
TLS	translesion synthesis
Tof1	topoisomerase 1-interacting factor
Top1	topoisomerase 1
ts	temperature sensitive
Ubc	ubiquitin-conjugating
UV	Ultraviolet

CHAPTER 1

Introduction

Eukaryotic cell proliferation is regulated by a highly complex network of processes to ensure the preservation of genomic integrity and faithful transmission of genetic information during cell divisions. Error-free DNA replication is the key process to guarantee accurate duplication of chromosomes prior to their segregation and transmission from a mother to a daughter cell. This is a daunting task because the genome is most vulnerable during DNA replication. Cells must overcome many obstacles during DNA replication to prevent accumulation of mutations that arise from endogenous as well as exogenous DNA damaging events. Failure to promote efficient and error-free DNA replication can result in DNA double-stranded breaks (DSBs) and ultimately gross chromosomal rearrangements (GCR), a hallmark of many cancers [Kolodner *et al.*, 2002; Mitelman *et al.*, 2007]. Eukaryotic cells have developed a network of signaling pathways known as the DNA damage response (DDR) to monitor the presence of DNA damage and coordinate DNA replication with DNA repair, chromosome segregation, and cell cycle progression [Ciccia and Elledge, 2010]. Therefore, a better understanding of how cells integrate different cellular processes to ensure proper DNA replication and suppress genomic instability will provide more insight into how genetic mutations cause cancer and other pathological disorders.

Mechanism of DNA replication

Cell cycle regulation.

The cell cycle consists of two essential events, DNA replication of the genome (Synthesis or S phase) and segregation of the fully duplicated genome into two daughter

cells (mitosis or M phase) (Figure 1.1). To ensure that genome segregation occurs only after the completion of DNA synthesis, the two events are separated by a "gap" phase called G2. Similarly, another gap phase, G1, takes place before the start of every S phase to ensure that cells have the adequate size and all the necessary components required for DNA replication.

The progression through different phases of the cell cycle in budding yeast *Sacharomyces cerevisiae* is regulated solely by cyclin dependent kinase 1 (Cdk1) [Hartwell *et al.*, 1973; Reed *et al.*, 1985]. *S. cerevisiae* expresses nine cyclins that associate with Cdk1 throughout the cell cycle to regulate its kinase activity, three G1 or A-type cyclins (Cln1-3) [Reed *et al.*, 1989; Richardson *et al.*, 1989] and six B-type cyclins (Clb1-6) (Figure 1.1) [Epstein and Cross, 1992; Fitch *et al.*, 1992; Schwob and Nasmyth, 1993; Surana *et al.*, 1991]. Although Cln1-3 have redundant roles primarily in the G1-S phase transition [Richardson *et al.*, 1989], their molecular functions are different. Cln3/Cdk1 regulates transcriptional programs, including Cln1 and Cln2 [Cross and Tinkelenberg, 1991; Dirick *et al.*, 1995; Dirick and Nasmyth, 1991], whereas association of Cdk1 with either Cln1 or Cln2 regulates spindle pole duplication [Jaspersen *et al.*, 2004] and bud morphogenesis [Lew and Reed, 1993]. To promote the G1-S transition, Cdk1 must dissociate from Cln1-3 and bind to Clb1-6. In fact, Cln1-3 are phosphorylated by Cln/Cdk1 and targeted for proteasomal degradation by the SCF^{Grr1} (SKP1-CUL1-F-box containing E3 ubiquitin ligase Grr1) complex [Lanker *et al.*, 1996; Skowyra *et al.*, 1999; Tyers *et al.*, 1992; Yaglom *et al.*, 1995]. Additionally, Cln/Cdk1 is

required to phosphorylate Sic1, an inhibitor of Clb/Cdk1, to promote Sic1 proteolysis [Koivomagi *et al.*, 2011; Nash *et al.*, 2001; Schwob *et al.*, 1994; Verma *et al.*, 1997].

Following Cln1-3 function in G1, the B-type cyclins, Clb1-6, associate with Cdk1 at different times during S, G2, and M phase (Figure 1.1). In G1, Clb5 and Clb6 are both highly expressed to drive DNA replication [Schwob and Nasmyth, 1993]. Clb5,6/Cdk1, also known as S-CDK, is involved in the assembly of initiation factors for DNA replication while simultaneously preventing reinitiation events at origins of replication that have already "fired" [Dahmann *et al.*, 1995; Piatti *et al.*, 1996; Schwob and Nasmyth, 1993; Zou and Stillman, 1998]. Whereas Clb5 is stable until mitosis, Clb6 is ubiquitinated by the SCF^{CDC4} at the G1/S border and is rapidly degraded [Jackson *et al.*, 2006]. Clb3,4 are expressed in S phase until anaphase and are involved in DNA replication, spindle assembly, and G2/M transition [Richardson *et al.*, 1992]. Clb1,2 are expressed in G2/M phase to the end of M phase and are involved in mitotic events [Seufert *et al.*, 1995]. Clb/Cdk1 activate an E3 ubiquitin ligase called the anaphase promoting complex or cyclosome (APC/C), which is important for the progression through mitosis, mitotic exit, and in turn reduce Cdk1 kinase activity in M and early G1 phase [Alexandru *et al.*, 1999; Rahal and Amon, 2008]. The activation of APC/C is regulated by the interaction with two different proteins, Cdc20 or Cdh1, contributing to cyclin proteolysis [Alexandru *et al.*, 1999; Rahal and Amon, 2008; Shirayama *et al.*, 1999; Wasch and Cross, 2002]. Lastly, following mitosis, cells need to reset their cell cycle program in G1 to proceed through the next round of replication. This is

accomplished by the phosphatase Cdc14 to dephosphorylate Cdk1 targets [Bloom and Cross, 2007].

Origin licensing.

DNA replication initiates at multiple regions along chromosomes that are termed origins of replication. Since the first defined eukaryotic origin of replication in budding yeast was identified in 1979 [Stinchcomb *et al.*, 1979], ongoing efforts aim to determine the molecular mechanisms by which these sequences direct initiation events. Replication origins in yeast are known as autonomously replicating sequences (ARS), which contain an essential and conserved A-element called the ARS consensus sequence (ACS). The origins are recognized by the evolutionarily conserved six-subunit origin recognition complex (ORC) that is essential for the stepwise assembly of a replication initiation complex [Bell and Dutta, 2002]. Although replication initiation is dependent on ORC in metazoa, they do not have a stringent sequence-specific ARS requirement. Instead, they depend on other factors such as chromatin structure and epigenetics [Cadoret and Prioleau, 2010; Kuo *et al.*, 2012; Wyrick *et al.*, 2001].

Detailed molecular mechanisms of replication initiation events have been elucidated in budding yeast using cell-free systems. The initiation of DNA synthesis is a multi-step process that can be divided into two distinct temporal events, origin licensing (preparation of origins for initiation) and origin activation (initiation of DNA synthesis) [Bell and Dutta, 2002]. During origin licensing, ORC binds to origins in an ATP-dependent manner in late M/G1 phase of the cell cycle when Cdk1 activity is low

[Aparicio *et al.*, 1997; Bell and Stillman, 1992; Klemm *et al.*, 1997]. ORC recruits two additional replication factors Cdc6 (cell division control protein 6) [Coleman *et al.*, 1996; Liang *et al.*, 1995] and Cdt1 (chromatin licensing and DNA replication factor 1) [Maiorano *et al.*, 2000; Nishitani *et al.*, 2000; Whittaker *et al.*, 2000]. These factors are needed to load the head-to-head double-hexameric, replicative helicases Mcm2-7 (minichromosome maintenance 2-7) proteins around the dsDNA origin [Evrin *et al.*, 2009; Gambus *et al.*, 2011; Remus *et al.*, 2009], forming the pre-replicative complex (pre-RC) (Figure 1.2, step I) [Bowers *et al.*, 2004; Chen *et al.*, 2007; Diffley *et al.*, 1995; Klemm and Bell, 2001; Randell *et al.*, 2006]. The loading of Mcm2-7 helicase to origins complete the origin licensing event, however, they remain inactive because S-CDK activity is low [Piatti *et al.*, 1996].

Origin activation.

As cells transition from G1 to S phase, DNA replication initiation requires a step-wise recruitment of multiple factors that are coordinated by two S phase dependent kinases, S-CDK (Clb5,6/Cdk1) and Dbf4-dependent Cdc7 kinase (DDK) to form a complete replisome (Figure 1.2) [Bell and Dutta, 2002]. The activation of the Mcm2-7 helicase at pre-RCs requires the association of two helicase cofactors, Cdc45 and GINS (Go, Ichi, Nii, and San, 5-1-2-3 referred to number of the subunits in Japanese), forming the Cdc45-Mcm2-7-GINS (CMG) complex [Moyer *et al.*, 2006], which is also known as the pre-initiation complex (pre-IC) [Zou and Stillman, 1998]. How DDK and S-CDK regulate the pre-IC formation and subsequent initiation of DNA synthesis has been controversial

until a recent report dissected their functions using an *in vitro* replication initiation assay [Heller *et al.*, 2011]. The authors demonstrated a two-step mechanism leading to the formation of the pre-IC, first by DDK and subsequently by S-CDK. DDK phosphorylates the chromatin-bound Mcm2-7 complex [Francis *et al.*, 2009; Randell *et al.*, 2010; Sclafani, 2000; Sheu and Stillman, 2006], promoting the interdependent recruitment and stable association of the synthetic lethality with Dpb11 (Sld) 3/Sld7 complex and Cdc45 to the pre-RC complex [Aparicio *et al.*, 1999; Kanemaki and Labib, 2006; Masai *et al.*, 2006; Takayama *et al.*, 2003; Tanaka *et al.*, 2011] (Figure 1.2, step II).

Following DDK-dependent activity, S-CDK phosphorylates both Sld2 and Sld3, which are essential for replication initiation [Tanaka *et al.*, 2007; Zegerman and Diffley, 2007]. Because phospho-mimetic mutants of Sld2 and Sld3 can bypass the requirement of S-CDK activity, Sld2 and Sld3 phosphorylations are the "minimal set" of S-CDK substrates required for replication initiation. S-CDK phosphorylation of Sld2 and Sld3 stimulates their association to another essential replication factor, Dpb11 in order to recruit GINS to the origins [Kamimura *et al.*, 1998; Tanaka *et al.*, 2007; Zegerman and Diffley, 2007]. Dpb11 can simultaneously interact with both phosphorylated Sld2 and Sld3 through its C- and N-terminus BRCT (BRCA1 (breast cancer 1) C-terminus) domains, respectively [Tak *et al.*, 2006; Tanaka *et al.*, 2007; Zegerman and Diffley, 2007]. Additionally, S-CDK also stimulates an unstable formation of the preloading complex (pre-LC), which consists of Sld2, Dpb11, the replicative polymerase pol-ε, and GINS [Muramatsu *et al.*, 2010]. This formation of the pre-LC is independent of DDK

and DNA binding. Altogether, Dpb11 bridges the interaction between Sld3 (at the pre-RC) with Sld2 (in the pre-LC), thereby recruits GINS as well as pol-ε to the origins (Figure 1.2, step III). The formation of the CMG complex leads to the activation of the helicase and subsequent unwinding of the double-stranded (ds) DNA origin [Moyer *et al.*, 2006]. Upon unwinding, single-stranded (ss) DNA is coated with replication protein A (RPA), allowing for the recruitment of pol-α (Figure 1.2, step IV) [Tanaka and Nasmyth, 1998; Walter and Newport, 2000; Zou and Stillman, 2000]. Additionally, the stability and recruitment of pol-α to origins for *de novo* DNA synthesis is dependent on Mcm10 likely through two separate mechanisms, (i) stabilization of ssDNA and (ii) direct binding to the catalytic subunit of pol-α [Ricke and Bielinsky, 2004; 2006; Warren *et al.*, 2009; Warren *et al.*, 2008]. In higher eukaryotes, MCM10 interacts with CTF4 and recruits pol-α to replication origins [Zhu *et al.*, 2007]. In budding yeast, Ctf4 (chromosome transmission fidelity 4) is a non-essential gene and thus dispensable. Depletion of Mcm10 in *S. cerevisiae* results in a reduced recruitment of pol-α and subsequently pol-δ, but not pol-ε to origins [Heller *et al.*, 2011; Ricke and Bielinsky, 2004; 2006]. In addition to the CMG complex, additional factors such as Mrc1 (mediator of the replication checkpoint 1), Tof1 (topoisomerase 1-interacting factor 1), Csm3 (chromosome segregation in meiosis 3), Ctf4, components of the histone chaperones including FACT, topoisomerase 1 (Top1), and Mcm10 are subsequently recruited to form a complete replisome for DNA synthesis [Gambus *et al.*, 2006].

Prevention of re-replication.

In eukaryotic cells, DNA replication occurs once per cell cycle due to a small time window when the B-type cyclin-dependent Clb/Cdk1 (S-CDK) activity is low. The activation of Clb/Cdk1 ultimately prevents the reloading of the Mcm2-7 helicase at the origins by phosphorylating components of the pre-RC complex, ORC, Cdc6, and Mcm2-7 [Dahmann *et al.*, 1995; Detweiler and Li, 1998; Piatti *et al.*, 1996]. In budding yeast, deregulation of any two or all three components of the pre-RC leads to detectable DNA rereplication, contributing to DNA damage and ultimately genome instability [Green *et al.*, 2010; Green and Li, 2005; Green *et al.*, 2006; Tanny *et al.*, 2006]. S-CDK inhibits ORC function by steric hindrance via two distinct mechanisms, (i) phosphorylation of two ORC subunits, Orc2 and Orc6 and (ii) direct interaction with Orc6 [Nguyen *et al.*, 2001; Wilmes *et al.*, 2004]. The N-terminal regions of both Orc2 and Orc6 facilitate the initial recruitment of Cdt1 and Mcm2-7 complex to origins. S-CDK-dependent phosphorylation of these regions prevents the interaction between ORC and Cdt1, resulting in the complete loss of Mcm2-7 double-hexameric helicase loading [Chen and Bell, 2011]. Orc6 also contains an "RXL" motif at the N-terminus that interacts with Clb5 throughout S phase into mitosis, but only when origins have already fired [Wilmes *et al.*, 2004]. Eliminating the Clb5/Orc6 interaction does not affect origin initiation but instead results in DNA rereplication [Wilmes *et al.*, 2004]. Furthermore, Clb5-Orc6-RXL interaction also stimulates S-CDK phosphorylation of Orc6, suggesting a localized role of Clb5 in preventing reinitiation of replicated origins [Chen and Bell, 2011].

Another target of Cdk1 that prevents rereplication is Cdc6 [Piatti *et al.*, 1996]. At least three layers of regulation exist to ensure that Cdc6's function is inhibited. Cdk1 indirectly inhibits Cdc6 expression by phosphorylating a Cdc6-transcriptional activator Whi5, preventing its nuclear import [Moll *et al.*, 1991; Piatti *et al.*, 1995; Zwerschke *et al.*, 1994]. Alternatively, Cdc6 is phosphorylated by Cdk1 and subsequently targeted for degradation by the SCF^{CDC4} complex, resulting in reduced Cdc6 expression [Drury *et al.*, 1997; 2000; Sanchez *et al.*, 1999]. In addition, the association of Cdc6 with Clb2/Cdk1 also prevents Cdc6 binding to pre-RCs [Mimura *et al.*, 2004].

Finally, the last component of the pre-RC complex that is targeted by Cdk1 is Mcm2-7, where phosphorylation of Mcm2-7 helicase leads to its exclusion from the nucleus [Labib *et al.*, 1999; Nguyen *et al.*, 2000]. Under physiological conditions, Mcm2-7 enters the nucleus at the end of mitosis and G1 phase and becomes primarily cytoplasmic in later stages of the cell cycle. Expression of a stable Clb2/Cdk1 complex in G1 arrested cells is sufficient to exclude Mcm2-7 from the nucleus, suggesting a role of Clb2/Cdk1 in regulating Mcm2-7 localization. Although Cdt1 is constitutively expressed throughout the cell cycle, its localization is co-regulated with Mcm2-7 by Cdk1 [Tanaka and Diffley, 2002]. Following Mcm2-7 helicase complex loading onto chromatin by ORC and Cdt1, Cdt1 is released from pre-RCs and excluded from the nucleus [Tanaka and Diffley, 2002]. The prevention of rereplication is slightly different in metazoans where hCDKs regulate either hCDT1's degradation [Liu *et al.*, 2004a; Nishitani *et al.*, 2004; Nishitani *et al.*, 2006] or its DNA binding activity [Sugimoto *et al.*, 2004]. In addition to CDK-dependent phosphorylation in metazoans, geminin, a

competitive inhibitor of hCDT1, accumulates from S phase to the metaphase-anaphase transition in mitosis, binds directly to hCDT1, and inhibits origin licensing [McGarry and Kirschner, 1998; Wohlschlegel *et al.*, 2000]. In fact, overexpression of CDT1 alone or in combination with CDC6 in $p53^{-/-}$ human cancer cells is sufficient to induce rereplication [Vaziri *et al.*, 2003].

Activation of the Mcm2-7 helicase.

DNA unwinding by the activated CMG complex is an essential step for DNA replication initiation as well as elongation. The observation that the replisome consists of a single Mcm2-7 helicase [Gambus *et al.*, 2006] suggests that the double-hexameric helicase needs to be separated into two individual hexamers during or after its activation. It is unclear as to whether the CMG complex translocates along ss or ds DNA. Fu *et al.* utilized a converging replication fork systems in *Xenopus* egg extracts where a "roadblock" was added specifically to either the leading or lagging strands or both [Fu *et al.*, 2011]. The authors demonstrated that "roadblocks" specifically on the leading strand, but not the lagging strand, impeded the advancing CMG, suggesting that Mcm2-7 translocates along ssDNA in 3'-5' direction. This result suggests that Mcm2-7 needs to extrude the lagging strand from its central core in order to unwind dsDNA. In a different study, using single particle electron microscopy and 3D reconstruction, Costa *et. al.* provides structural information that may explain how the lagging strand is extruded [Costa *et al.*, 2011]. Purified Mcm2-7 complex existed in two different conformations, a planar, notched-ring and a spiral lock-washer with an opening between Mcm2 and 5

[Costa *et al.*, 2011], which has been hypothesized to be required for the loading of the helicase onto dsDNA origin [Bochman *et al.*, 2008]. In contrast, the formation of CMG exhibited a planar closed state where Cdc45 and GINS closed the gap between the Mcm2/Mcm5 interface. Additionally, the CMG complex constricted the central Mcm2-7 pore and generated a second, external channel that may interact with the lagging strand template DNA. The sequestration of the lagging strand template is hypothesized to be stabilized by ss binding proteins such as RPA, Mcm10, Sld2, or Sld3 [Bruck *et al.*, 2011; Kanter and Kaplan, 2011; Warren *et al.*, 2008; Zou and Stillman, 2000]. In line with this notion, recent studies suggest a role of Mcm10 in DNA unwinding of the origin prior to DNA synthesis, likely stabilizing the ssDNA rather than truly activating the CMG helicase [Kanke *et al.*, 2012; van Deursen *et al.*, 2012; Watase *et al.*, 2012]. Besides the role of Mcm10 in ssDNA stabilization, it has been shown to travel with the replication fork and interact with different components involved in DNA synthesis [Araki *et al.*, 2003; Chattopadhyay and Bielinsky, 2007; Das-Bradoo *et al.*, 2006; Hart *et al.*, 2002; Lee *et al.*, 2010; Merchant *et al.*, 1997; Pacek *et al.*, 2006; Ricke and Bielinsky, 2004; 2006; Yang *et al.*, 2005]. These properties of Mcm10 indicate its crucial role in coordinating DNA unwinding with DNA synthesis.

DNA replication and synthesis.

Upon origin activation, DNA synthesis proceeds bidirectionally [Prescott and Kuempel, 1972]. DNA synthesis depends on three major replicative polymerases, pol-α, δ, and ε, all of which synthesize DNA in the 5'-3' direction (Figure 1.3) [Garg and

Burgers, 2005a]. Due to the anti-parallel structure of DNA and the unidirectional polymerization activity of the DNA polymerases, synthesis occurs in a semi-discontinuous manner, generating a continuous leading strand and a discontinuous lagging strand also known as Okazaki fragment (Figure 1.3) [Sakabe and Okazaki, 1966]. Upon DNA unwinding, Mcm10 binds to ssDNA and, together with RPA, recruits the pol-α/primase complex, which is the only known enzyme that can catalyze *de novo* DNA synthesis on both template strands (Figure 1.2 and 1.3) [Ricke and Bielinsky, 2004; 2006; Tanaka and Nasmyth, 1998; Walter and Newport, 2000; Warren *et al.*, 2008; Zou and Stillman, 2000]. DNA primase catalyzes a short 8-11 nucleotides (nt) of RNA primer [Santocanale *et al.*, 1993], which can then be elongated for another ~20 nt of DNA by pol-α activity, thereby generating short RNA/DNA primers [Denis and Bullock, 1993]. Because pol-α is nonprocessive and lacks proofreading activity, the bulk of the genome DNA replication is synthesized by the processive pol-δ and -ε, which are responsible for lagging and leading strand synthesis, respectively [Garg and Burgers, 2005a; Nick McElhinny *et al.*, 2008; Pursell *et al.*, 2007].

The switch from pol-α to pol-δ and -ε is thought to be mediated by two additional proteins. After pol-α/primase recruitment to the replication fork by Mcm10, Mcm10 is mono-ubiquitinated at two lysine residues (hereforth referred to as di-ubiquitinated Mcm10). Di-ubiquitinated Mcm10 binds the DNA sliding clamp, proliferating cell nuclear antigen (PCNA), the processivity factor for pol-δ and -ε [Das-Bradoo *et al.*, 2006]. Interestingly, di-ubiquitinated Mcm10 only interacts with PCNA and not with

Cdc17, the catalytic subunit of pol-α [Das-Bradoo *et al.*, 2006; Ricke and Bielinsky, 2004; 2006]. This suggests an attractive model whereby Mcm10 di-ubiquitination releases pol-α from chromatin, resulting in Cdc17 degradation [Haworth *et al.*, 2010], and simultaneously recruits PCNA, which is loaded onto chromatin by replication factor C (RFC) [Lee *et al.*, 1991; Miyata *et al.*, 2004; Tsurimoto and Stillman, 1990; 1991; Waga *et al.*, 1994]. As the replication fork progresses to unwind dsDNA, pol-ε travels with the replisome through the interaction with Mrc1, a component of the replisome, to synthesize one continuous leading strand [Lou *et al.*, 2008]. However, lagging strand synthesis is more dynamic and requires additional components to join short Okazaki fragments together into one continuous strand, a process referred to as Okazaki fragment maturation.

Okazaki fragment maturation.

In order to ligate Okazaki fragments together, the RNA primers synthesized by primase need to be removed and replaced with DNA synthesized by pol-δ [Balakrishnan and Bambara, 2011]. The removal of the RNA primers involves the coordinated action of flap endonuclease 1 (Fen1, encoded by the *RAD27* gene in *S. cerevisiae*) [Garg *et al.*, 2004; Gary *et al.*, 1999; Liu *et al.*, 2004b; Reagan *et al.*, 1995] and pol-δ (Figure 1.3) [Burgers, 2009; Garg *et al.*, 2004; Jin *et al.*, 2003]. As pol-δ encounters the 5'-end of the downstream Okazaki fragment, it continues with synthesis while displacing the RNA primer, thereby generating a short 5'-flap [Garg *et al.*, 2004; Jin *et al.*, 2003]. This short 5'-flap is removed by Fen1 to generate two DNA-DNA ligatable ends [Garg *et al.*, 2004;

Jin *et al.*, 2003]. DNA ligase I is recruited by PCNA, encircles the DNA and seals the nick [Ellenberger and Tomkinson, 2008; Pascal *et al.*, 2004; Tomkinson *et al.*, 2006]. Since the homotrimeric PCNA can interact with pol-δ, Fen1, and DNA ligase I through a highly conserved PIP (PCNA-interacing protein) box motif, PCNA is thought to coordinate the recruitment of necessary factors and processing of lagging strands during Okazaki fragment maturation [Dionne *et al.*, 2003].

Although the short flap removal by Fen1 appears to be essential during Okazaki fragment maturation, deletion of Fen1 (*rad27Δ*) in *S. cerevisiae* retains viability, suggesting that different pathways for flap removal exist [Reagan *et al.*, 1995; Sommers *et al.*, 1995]. Indeed, both genetic and biochemical studies provide support for an alternative maturation pathway involving the DNA synthesis defective 2 (Dna2) protein [Ayyagari *et al.*, 2003; Bae and Seo, 2000; Budd and Campbell, 1995; 1997]. A temperature sensitive allele of *DNA2, dna2-1,* arrests in S phase, indicating a problem during replication [Budd and Campbell, 1995]. Moreover, Dna2 physically and genetically interacts with Fen1, suggesting that Fen1 and Dna2 may have complementary functions [Budd and Campbell, 1997]. In line with this notion, when Fen1 fails to cleave the short flap, excessive strand displacement generating longer 5'-flaps occurs by the combined activities of three components: (i) the 3'-exonuclease activity of the catalytic subunit of pol-δ, (ii) Pol32, a subunit of pol-δ, and (iii) the 5'-3' helicase activity of Pif1 (petite integration frequency 1) [Budd *et al.*, 2006; Burgers and Gerik, 1998; Jin *et al.*, 2003; Rossi *et al.*, 2008; Stith *et al.*, 2008]. The longer 30 nt 5'-flap is bound by RPA, which subsequently recruits and stimulates Dna2 activity but inhibits Fen1 action *in vitro*

[Bae *et al.*, 2001; Jin *et al.*, 2003; Kao *et al.*, 2004; Maga *et al.*, 2001]. Dna2 cleaves the RPA-coated flap, leaving a short 2-6 nt DNA flap that needs to be further processed by Fen1 [Bae and Seo, 2000; Kao *et al.*, 2004]. However, in the absence of Fen1, pol-δ or Exo1 (exonuclease 1) may play a role in generating a proper DNA nick for ligation by DNA ligase I [Burgers, 2009; Jin *et al.*, 2003; Tishkoff *et al.*, 1997]. Therefore, Dna2 can compensate for Fen1-mediated flap removal [Ayyagari *et al.*, 2003], explaining the non-essential characteristic of Fen1. However, an excess of long 5'-flaps can lead to cell death [Budd *et al.*, 2011; Budd and Campbell, 1997]. Conversely, suppressing the generation of long flaps by deleting *PIF1* gene can rescue the loss of viability of *dna2Δ* mutants [Budd *et al.*, 2006]. In summary, the DNA/RNA primer generated by pol-α/primase can be removed by two distinct mechanisms, either by Fen1- or Dna2-mediated short or long flap processing to generate a nick that can be ligated by DNA ligase I.

Structure and function of DNA ligase I.

DNA ligase I belongs to the nucleotidyltransferase (NTase) superfamily, which also contains RNA ligases and capping enzymes [Ellenberger and Tomkinson, 2008]. It catalyzes the sealing of nicks between adjacent 3'OH and 5'PO_4 termini and is crucial for DNA replication, repair and recombination. The DNA ligation mechanism involves three nucleotidyl transfer reactions (Figure 1.4) [Ellenberger and Tomkinson, 2008]. In the first step of the ligation reaction, DNA ligase reacts with either ATP or NAD^+ (in prokaryotes) to form a ligase-adenylate intermediate where 5'-adenosine monophosphate

(AMP) is linked by a phosphoamide bond with the lysine residue in the active site of the enzyme. In the second step, AMP is transferred to the 5'PO_4 terminus of the nick to form DNA-adenylate. Finally, DNA ligase catalyzes the nucleophilic attack of the 3'OH to the DNA-adenylate to covalently join the two ends of the DNA strand and release AMP.

The NTase and oligonucleotide/oligosaccharide binding fold (OB-fold) domains, containing both the DNA binding and active site residues, together form a minimal catalytic core that is conserved in all known NTase enzyme superfamily members (Figure 1.5) [Ellenberger and Tomkinson, 2008]. DNA ligase from C*hlorella virus*, the smallest known ATP-dependent DNA ligase I that contains only the NTase and OB-fold domains, can rescue the viability and DNA repair functions of DNA ligase I in both budding yeast and humans [Ho *et al.*, 1997; Simsek *et al.*, 2011; Sriskanda *et al.*, 1999]. This observation illustrates the high degree of conservation among this class of DNA ligases. Eukaryotic DNA ligases have additional N- and C-terminal domains that are crucial for their biological activities, either for protein-protein and/or protein-DNA interactions (Figure 1.5) [Ellenberger and Tomkinson, 2008]. For example, an additional DNA binding domain (DBD) in mammalian DNA ligase I is crucial for efficient ligation activity [Pascal *et al.*, 2004].

Molecular insights of human DNA ligase I (hLIG1) action can be extrapolated from the structural studies of the enzyme with DNA and PCNA from different species. Structural studies demonstrate that the DBD, OB-Fold, and NTase domains of hLIG1 are important to circularize and stabilize the nicked DNA substrate [Pascal *et al.*, 2004]. Another co-crystal structure of PCNA and DNA ligase from *Sulfolobus solfataricus*

further suggests that DNA ligase undergoes a dynamic conformational change, from an elongated form (in the absence of DNA) to a circularized form (in the presence of nicked DNA) [Pascal *et al.*, 2006]. Since DNA ligase I contains a PIP box motif, it is thought that PCNA may direct DNA ligase I to the nicked substrates [Refsland and Livingston, 2005; Subramanian *et al.*, 2005]. To better understand how DNA ligase I PIP-box motif interacts with PCNA, a structural study was carried out using the *S. cerevisiae* Cdc9 (homolog of hLIG1) PIP-box peptide crystallized with budding yeast PCNA [Vijayakumar *et al.*, 2007]. The authors demonstrated that Cdc9 PIP-box peptide interacted with both the inter-domain connector loop (IDCL), which connects the N- and C-terminus of each monomeric PCNA, and the hydrophobic pocket in the C-terminus of PCNA [Vijayakumar *et al.*, 2007]. In summary, DNA ligase I interacts with PCNA through its PIP-box and remains in an elongated conformation in the absence of DNA. When the nicked substrate is generated during Okazaki fragment maturation, DNA ligase I circularizes around the DNA, likely to make other contacts with PCNA, and ligates the two adjacent DNA fragments together.

S. cerevisiae encodes two different DNA ligases, Cdc9 and Dln4, which are homologs of human DNA ligases I and IV, respectively [Barnes *et al.*, 1990; Johnston and Nasmyth, 1978; Schar *et al.*, 1997; Teo and Jackson, 1997; Wei *et al.*, 1995; Wilson *et al.*, 1997]. The two proteins have different principal roles in DNA metabolism. While Dln4 functions in DSB repair via non-homologous end joining (NHEJ), Cdc9 participates in base excision repair (BER) and nucleotide excision repair (NER) pathways [Johnston and Nasmyth, 1978; Montelone *et al.*, 1981; Schar *et al.*, 1997; Teo and Jackson, 1997;

Wilson *et al.*, 1997; Wu *et al.*, 1999]. The *CDC9* gene also contains two alternative translational start sites that encode nuclear and mitrochondrial forms of DNA ligase I [Willer *et al.*, 1999]. In addition, Cdc9 is essential for the ligation of Okazaki fragments during lagging strand synthesis [Johnston and Nasmyth, 1978].

Different temperature sensitive (ts) alleles of *CDC9* were isolated from the original screen for cell division cycle (cdc) mutants [Culotti and Hartwell, 1971; Hartwell *et al.*, 1973]. At the non-permissive temperature, *cdc9* mutants arrest in late S/G2 phase of the cell cycle and accumulate small nascent DNA fragments similar to unligated Okazaki fragments [Johnston and Nasmyth, 1978; Schiestl *et al.*, 1989]. This suggests that cells replicate the whole genome leaving nicks in the DNA behind until G2/M. Furthermore, *cdc9* mutants are hyper-recombinogenic and synthetically lethal with homologous recombination (HR) repair protein genes, such as *RAD52* [Ireland *et al.*, 2000; Montelone *et al.*, 1981]. These phenotypes suggest that the increasing half-life of unligated DNA in S phase leads to DNA recombination and depends on HR for survival. Human *LIG1* was successfully cloned from a cDNA library screen for genes that were able to complements *cdc9ts* alleles in budding yeast [Barnes *et al.*, 1990]. Although Cdc9 and hLIG1 share only 37% sequence identity, the three core domains of hLIG1 were sufficient to rescue *cdc9ts* cell viability at the non-permissive temperature, again emphasizing the conserved nature of DNA ligase I among species.

Genomic instability and cancer.

The preservation of genomic integrity requires a variety of processes to ensure proper genome duplication during cell proliferation. Efficient and error-free DNA replication is crucial towards accurate genome duplication to prevent the accumulation of mutations. Such accumulation of mutations can either activate proto-oncogenes or inactivate tumor suppressor genes that are required to promote tumorigenesis. Fortunately, cells have evolved the DNA damage response (DDR) system to protect its DNA from damage induced by either exogenous environmental agents or endogenous DNA alterations during the cell's normal metabolic processes [Ciccia and Elledge, 2010]. DDR has the ability to sense and trigger appropriate cellular responses to counteract DNA damage. Not surprisingly, defects in genes involved in DDR have been linked to different human cancer susceptibility syndromes [Ciccia and Elledge, 2010]. Better understanding of the molecular mechanisms through which the DDR operates will provide therapeutic opportunities for many human diseases.

Sources of DNA damage.

Endogenous DNA damage is induced by spontaneous hydrolytic reactions of DNA bases, the cell's own metabolic processes, and DNA replication errors. First, during spontaneous hydrolytic reactions, DNA bases can either be deaminated or completely lost, which leaves an abasic site. Under physiological conditions, it is estimated that 2,000 - 10,000 DNA bases are hydrolyzed in a human cell a day owing to hydrolytic depurination [Lindahl, 1993; Lindahl and Nyberg, 1972]. If an

apurinic/apyrimidinic (AP) site is left unpaired, it can block replicative polymerases during DNA replication, forcing cells to depend on the function of the error-prone translesion synthesis (TLS) polymerases [Haracska *et al.*, 2001]. In the same vein, deamination of cytosine, adenine, guanine, or 5-methylcytosine, which converts these bases to uracil, hypoxanthine, xanthine, and thymine, respectively, are also highly mutagenic [Lindahl, 1993; Meira *et al.*, 2005]. The increase of mutations is due to the misincorporation of nucleotides opposite of the deaminated bases during DNA replication, resulting in permanent mutations in the genome in the next round of replication. Another type of endogenous DNA damage is caused by the exposure of DNA to reactive oxygen species (ROS) such as hydroxyl radicals [Lindahl, 1993; Meira *et al.*, 2005]. One of the most common DNA lesions caused by ROS is the highly mutagenic 8-oxo-7,8-dihydroguanine (8-oxoG), which can mismatch with an adenine, resulting in a G:C to A:T transition mutation [Cheng *et al.*, 1992]. Altogether, spontaneous hydrolysis of DNA bases causes approximately 20,000 potentially mutagenic lesions that arise in a human cell per day [Preston *et al.*, 2010].

Besides spontaneous nucleotide hydrolysis, mutations can be inserted in the genome naturally by the cell's own replicative pol-δ and pol-ε [Preston *et al.*, 2010]. Although pol-δ and pol-ε have intrinsic 3'-5' exonucleolytic proofreading activity, they occasionally make errors approximately once every $10^4 – 10^5$ nt polymerized [Fortune *et al.*, 2005; McCulloch and Kunkel, 2008; Shcherbakova *et al.*, 2003]. The majority of the errors are either base mismatches or ± 1 slippage events [Fortune *et al.*, 2005; Shcherbakova *et al.*, 2003]. Thus, at least $10^5 – 10^6$ polymerase errors occur for each

replicated human cell if left unrepaired [Preston *et al.*, 2010]. These replicative DNA polymerases are also capable of incorporating ribonucleotides (rNTPs) into DNA during replication *in vitro* and *in vivo* since the rNTP pools in cells are significantly higher than the dNTP (di-deoxynucleotide) pools [Nick McElhinny *et al.*, 2010a; Nick McElhinny *et al.*, 2010b; Nick McElhinny *et al.*, 2010c]. Incorporation of rNTPs into DNA results in single-base substitution and 2-5 base deletions [Nick McElhinny *et al.*, 2010b]. Besides DNA lesions that give rise to mutations, ROS can also damage DNA by attacking the sugar-backbone, generating ss and ds breaks (SSBs and DSBs, respectively) [Caldecott, 2008; Meira *et al.*, 2005]. Lastly, replication forks can collapse and give rise to DSBs either by prolonged stalled replication forks or forks that progress through a SSB that leads to the dissociation of replication fork components [Branzei and Foiani, 2008].

Besides coping with thousands of endogenous DNA lesions, a cell also needs to guard its genome against exogenous DNA damage such as ultraviolet (UV) or ionizing irradiation (IR), and other chemical sources [Ciccia and Elledge, 2010; Hoeijmakers, 2009]. UV-irradiation causes pyrimidine dimers and 6-4 cyclobutane pyrimidine dimers (CPD) that can induce up to 10^5 DNA lesions during a day-long exposure to sunlight [Hoeijmakers, 2009]. Ionizing irradiation can cause the combination of oxidative DNA bases, SSBs and DSBs [Ciccia and Elledge, 2010]. It has been estimated that mammalian cells exposed to 1 Gy (gray) of IR can elicit ~1000 SSBs and ~40 DSBs immediately after irradiation [Ciccia and Elledge, 2010]. Many chemical DNA damaging agents used in cancer chemotherapy also induce different types of lesions. For example, methyl methansulfonate (MMS) methylates N^7-deoxyguanine and N^3-deoxyadenine can cause

replication blocks and base mispairing [Beranek, 1990]. In conclusion, the combined numbers of DNA lesions caused by both endogenous and exogenous DNA damage throughout the genome can potentially lead to genomic instability. However, the fact that genome duplication occurs with high fidelity (~10^{-10} mutations per base pair per cell division) [Drake *et al.*, 1998; Preston *et al.*, 2010] indicates that cells have a remarkably efficient ability to repair DNA damage.

DNA defense mechanisms.

To counteract different types of lesions, cells have evolved several distinct repair mechanisms that are highly conserved between yeasts and humans (Figure 1.6a) [Hoeijmakers, 2001; Ulrich and Walden, 2010]. In BER, spontaneous DNA base hydrolysis and small chemical base alterations are excised by DNA glycosylase, generating AP sites [Hoeijmakers, 2001; Meira *et al.*, 2005]. The strand at the abasic site is excised and repaired by either short- or long patch BER. Second, the removal of misincorporated nucleotides caused by pol-δ and pol-ε relies heavily on the mismatch repair (MMR) pathway, which are active during DNA replication [Jiricny, 2006; Preston *et al.*, 2010]. Both BER and MMR pathways contribute significantly to the fidelity of genome duplication and mutation avoidance. The repair of intrastrand crosslinks such as lesions caused by UV-radiation or other damaging agents depends on NER, a repair mechanism in which a stretch of approximately 30 nucleotides encompassing the lesion is removed, leaving a ssDNA gap to be resynthesized by DNA polymerases and ligated together by DNA ligase [Hoeijmakers, 2001]. If damage is left unrepaired, these bulky

adducts can block DNA polymerases during DNA replication. In this case, however, lesions can be bypassed by the TLS polymerases to synthesize across the lesion. Alternatively, lesion bypass utilizes a template switch mechanism using newly synthesized nascent DNA as the template. These types of repair will be discussed in more detail in a separate section (See Postreplicative repair pathways section on page 36).

Lastly, HR and NHEJ primarily function to repair DSBs (Figure 1.6a) [Heyer *et al.*, 2010; Lieber, 2010]. HR is activated in S and G2 phases of the cell cycle where undamaged sister chromatids are available as a template to repair broken DNA ends [Branzei and Foiani, 2008]. If sister chromatids are not available for HR such as in G1 phase, NHEJ is the primary mode for DSB repair [Branzei and Foiani, 2008]. Besides these conserved repair pathways, there are other repair pathways that are only present in higher eukaryotes. For example, PARP (poly (ADP-ribose) polymerase) proteins, such as PARP1 and PARP2, are important for the activation of SSB repair pathway to recruit BER components [Caldecott, 2008; Schreiber *et al.*, 2006]. Furthermore, alternative non-homologous end joining (alt-NHEJ) is also present in mammalian cells where DSB ends are resected to expose a short microhomology region for DNA annealing of the ends [Lieber, 2010; Symington and Gautier, 2011].

DNA damage checkpoints.

To coordinate different repair mechanisms, the DDR network contains DNA damage checkpoint genes that regulate appropriate cellular responses to DNA damage. These checkpoint genes are important to coordinate DNA repair with other cellular

processes such as cell cycle progression, transcription and metabolism to provide sufficient time for DNA repair [Ciccia and Elledge, 2010]. Furthermore, they also regulate the recruitment and activation of different repair factors at DNA lesions that are most efficient for DNA repair [Symington and Gautier, 2011]. In humans, the DNA damage checkpoint is primarily mediated by the phosphatidylinositol 3-kinase-like protein kinases (PIKKs), ATM, ATR and DNA-PK (Figure 1.6b). ATM (ataxia-telangiectasia mutated) and DNA-PK (DNA-dependent protein kinase) are activated in response to DSBs such as IR [Ciccia and Elledge, 2010]. Notably, only ATR (ataxia-telangiectasia mutated- and Rad3-related) is essential for the viability of human and mouse cells, whereas ATM and DNA-PK are not [Brown and Baltimore, 2000; de Klein *et al.*, 2000; O'Driscoll, 2009; Ruis *et al.*, 2008]. While DNA-PK only phosphorylates proteins involved in NHEJ [Symington and Gautier, 2011], ATM regulates hundreds of substrates involved in many distinct pathways [Bensimon *et al.*, 2011; Matsuoka *et al.*, 2007]. Besides DSB recognition, ATM is a direct sensor of ROS in response to oxidative stress [Guo *et al.*, 2010a; Guo *et al.*, 2010b]. Moreover, a cytoplasmic fraction of ATM can also regulate metabolic processes in response to ROS [Cosentino *et al.*, 2011; Ditch and Paull, 2012].

ATR is activated by ssDNA structures that are generated by different types of DNA damage and replication interference such as DNA breaks and inhibition of DNA polymerases, respectively [Cimprich and Cortez, 2008]. The length of the ssDNA region and at a ssDNA-dsDNA junctions is crucial for effective activation of ATR [Cimprich and Cortez, 2008; Flynn and Zou, 2011; Shiotani and Zou, 2009a; b; Zou, 2007]. Upon

ATM/ATR activation, they phosphorylate serine or threonine followed by glutamine (SQ/TQ) motifs of different target proteins. For example, over 700 proteins with ATM/ATR-dependent SQ/TQ motifs associated with repair, checkpoint, cell cycle, and DNA replication processes are phosphorylated in response to IR [Matsuoka *et al.*, 2007]. How ATM- and ATR-dependent substrate phosphorylation in response to DNA damage affects their *in vivo* functions in still unknown and warrants further investigation in the future.

Genetic mutations and human diseases.

Since the DDR is vital for genome maintenance, defects in DDR result in an accumulation of mutations and DNA damage in a cell. The accumulation of mutations can predispose cells to cancer by activating oncogenes or deactivating tumor suppressor genes and/or caretaker genes such as ATM. The activation of oncogenes can promote premature entry into S phase, causing replication stress in the form of having too few replication forks that can lead to fork stalling and subsequence DNA damage [Bartkova *et al.*, 2005]. Such damage activates the ATM/ATR-mediated DDR signaling pathways. Consistent with this notion, activated DDR factors were found in early premalignant human tumors, likely to inhibit cancer progression [Bartkova *et al.*, 2005]. Therefore, DDR is thought to function as an early anti-cancer barrier against tumorigenesis. Perhaps, the strongest evidence for the role of the DDR in genome maintenance is that mutations in DDR genes were found in many human diseases and genetic disorders such as neurodegenerative disorders, immunodeficiencies, infertility, premature ageing and

cancer [Ciccia and Elledge, 2010]. Patients with inherited mutations in DDR genes have higher risks of cancer development. Mutations in ATR, ATM, and their downstream effector checkpoint kinases 1 and 2 (Chk1 and Chk2, respectively) have been reported in malignant human tumors [Meijers-Heijboer *et al.*, 2003; Menoyo *et al.*, 2001; Renwick *et al.*, 2006; Tanaka *et al.*, 2012; Wasielewski *et al.*, 2008]. Mutations in ATM are found in patients with ataxia telangiectasia (AT) syndrome, which exhibits immunodeficiency, hypersensitivity to x-rays, and most importantly, predisposes individuals to cancer [Biton *et al.*, 2008].

In addition to mutations in checkpoint genes, mutations in different DNA repair pathways have also been indicated in cancers [Ciccia and Elledge, 2010]. For example, mutations in *BRCA1* and *BRCA2* genes, which are involved in HR result in predisposition to breast cancer [Roy *et al.*, 2012]. Second, defects in DNA ligase IV, a component of NHEJ, was found in a patient with leukemia [Riballo *et al.*, 1999]. Third, mutations in *MSH2* (MutS homolog 2) and *MLH1* (MutL homolog 1) genes, which cause defective MMR, were found in hereditary nonpolyposis colon cancer (HNPCC) and in Lynch syndrome patients [Hoeijmakers, 2001; Jiricny, 2006]. The inability to reduce spontaneous mutation rates, repair DSBs, or trigger cell cycle arrest or apoptosis result in an increase of genome instability that fuels cancer development.

If DNA lesions are left unrepaired, cells face a bigger problem when the replisome encounters damage that blocks its progression in S phase. The replisome must be stabilized in order to resume replication after the removal of DNA damage or replication stress. Moreover, prolonged stalled replication forks can lead to fork collapse,

causing DSBs, and subsequently result in GCR, a hallmark of many cancers [Mitelman *et al.*, 2007]. Thus, maintaining fork stability is important for genome maintenance. In line with this notion, ATR is activated during S phase to regulate replication origin initiation, repair stalled forks and prevent entry into mitosis [Cimprich and Cortez, 2008], which may explain the essential function of ATR in humans and mice [Brown and Baltimore, 2000; de Klein *et al.*, 2000; O'Driscoll, 2009]. A germline mutation in *ATR* was recently identified in oropharyngeal cancer syndrome, which causes reduced activation of p53, a tumor suppressor protein, in response to stalled replication forks after depleting dNTP pools using hydroxyurea (HU) [Tanaka *et al.*, 2012]. Moreover, inhibition of DNA replication has been shown to induce breakage at specific regions of the chromosome, which are called fragile sites [Dereli-Oz *et al.*, 2011; Glover *et al.*, 1984; Hecht and Glover, 1984]. Chromosome breaks at fragile sites can lead to the deletion of common tumor suppressor genes, which paves the way for cancer progression [Bignell *et al.*, 2010].

In addition to mutations in DDR genes that stabilize stalled replication forks, impairment of DNA replication genes can also lead to genomic instability, GCR, and developmental defects [Aguilera and Gomez-Gonzalez, 2008]. Recently, mutations in *ORC1* and genes that encode other components of the pre-RC, *ORC4*, *ORC6*, *CDT1*, and *CDC6*, were found in Meier-Gorlin syndrome [Bicknell *et al.*, 2011a; Bicknell *et al.*, 2011b]. However, patients with Meier-Gorlin syndrome have yet to display either genomic instability or cancer predisposition. In contrast to Meier-Gorlin syndrome, Bloom's syndrome (BS) patients, who carry mutations in the *BLM* (Bloom) gene, display

developmental defects, premature aging and cancer predisposition [Ellis and German, 1996; Ellis *et al.*, 1995]. Although BLM is not a bona fide replication protein per se, BS cells exhibit slow rate in chain elongation, accumulate abnormal replication intermediates and may be impaired in the restart of stalled forks [Davies *et al.*, 2007; Hand and German, 1975; Lonn *et al.*, 1990]. Furthermore, a forward genetic screen searching for genes involved in maintaining genome stability identified a mutation in *MCM4* (*MCM4*$^{chaos3/chaos3}$) in mice that reduces MCM2-7 helicase stability on chromatin and results in abnormal chromosome segregation in mitosis with lagging chromosomes and acentric DNA fragments due to incomplete DNA synthesis, illustrating a direct link between defects in DNA replication and genomic instability [Kawabata *et al.*, 2011; Shima *et al.*, 2007]. Mutations in *FEN1*, an enzyme that is involved in the removal of RNA primers from Okazaki fragments, were identified in several different human tumors [Zheng *et al.*, 2007]. Additionally, mice harboring mutations in *FEN1* that abrogated its flap endonuclease activity developed cancers, suggesting that FEN1 is a tumor suppressor in mice [Larsen *et al.*, 2008; Zheng *et al.*, 2007]. Lastly, mutations in DNA ligase I, the only ligase that joins Okazaki fragments during lagging strand synthesis, were identified in a single individual, who developed lymphoma and died at a young age of pneumonia [Barnes *et al.*, 1992; Webster *et al.*, 1992]. In conclusion, knowledge gained from exploring the relationship between DNA replication and pathways that suppress genome instability to ensure proper genome duplication will be crucial to understand the mechanisms by which cancer and other pathological disorders arise.

DNA ligase I deficiency and human disease.

Only one individual, who was initially diagnosed with recurrent infections, has been identified with mutations in the *LIG1* gene (Figure 1.5) [Barnes *et al.*, 1992; Webster *et al.*, 1992]. The female patient exhibited sensitivity to sunlight, growth retardation, immunodeficiency, delays in development, lymphoma and died at the age of 19 from pneumonia [Webster *et al.*, 1992]. She carried an inherited maternal allele in which a conserved arginine residue in the OB-fold at position 771 was mutated to tryptophan (R771W) [Barnes *et al.*, 1992]. The inherited mutation was present in the mother and two brothers of the patient [Webster *et al.*, 1992]. However, all three heterozygotes were clinically normal. Although the R771W mutation caused accumulation of adenylated DNA intermediates in the second step of the ligation reaction, LIG1 R771W cells retained about 5-10% DNA ligase I activity [Barnes *et al.*, 1992; Prigent *et al.*, 1994]. A second, spontaneous mutation in the paternal allele affected the highly conserved glutamic acid residue at position 566 and replaced it with a lysine (E566K). E566 is two residues away from the active site lysine that catalyzes the first step of the ligation reaction [Barnes *et al.*, 1992; Ellenberger and Tomkinson, 2008]. The E566K mutation resulted in complete inactivation of LIG1 activity [Barnes *et al.*, 1992].

Because the *CDC9* gene in *S. cerevisiae* is essential for viability, it was presumed that inactivation of *LIG1* in human cells would result in cell lethality. In agreement with this hypothesis, homozygous deletion of *LIG1* gene in mouse embryonic stem cells was only viable when ectopic expression of LIG1 was introduced in these cells, suggesting

that DNA ligase I also is essential in human cells [Petrini *et al.*, 1995]. However, two different mouse embryos harboring a truncation at the 3' end of the *LIG1* gene developed normally until midgestation [Bentley *et al.*, 1996; Bentley *et al.*, 2002]. Although LIG1 protein levels were below detectable limits, it is possible that LIG1 function was not completely ablated in these embryos, thus resulting in normal embryo development at least in the early stages. Nevertheless, these presumed $LIG1^{-/-}$ mouse embryonic fibroblast (MEF) cell lines established from both of these animal models display defects in Okazaki fragment ligation and increased genome instability, suggesting a link between defects in Okazaki fragment maturation and cancer predisposition [Bentley *et al.*, 1996; Bentley *et al.*, 2002]. The link between DNA replication defects and cancer development was further supported by another *LIG1* defective mouse model, harboring homozygous R771W human mutation [Harrison *et al.*, 2002]. Similar to the human patient, the LIG1 R771W mice displayed a delayed growth phenotype. Notably, these mutant mice exhibited an increase in both genomic instability and incidence of spontaneous tumor formations. Therefore, the defective LIG1 function in Okazaki fragment maturation in mammals accumulates replication intermediates that promote genomic instability and increased incidence of cancer development.

S phase DNA damage response in *S. cerevisiae*

During S phase, cells rely on the S phase checkpoint to overcome many obstacles that arise during DNA replication. Failure to promote error-free DNA replication can result in the accumulation of DNA damage such as DSBs leading to the formation of

GCRs, which are commonly found in cancer cells [Kolodner *et al.*, 2002; Mitelman *et al.*, 2007]. In *S. cerevisiae*, mutations in the S phase checkpoint genes exhibited an increase in GCRs, suggesting that cells rely on the S phase checkpoint to suppress spontaneous genome rearrangements that arise from replication errors [Myung *et al.*, 2001; Myung and Kolodner, 2002].

As mentioned above, *cdc9ts* (DNA ligase I) mutants arrest in late S/G2 and delay the entry into mitosis at the non-permissive temperature, suggesting that cells have surveillance mechanisms to ensure the completion of DNA replication and removal of any remaining DNA damage before mitosis [Johnston and Nasmyth, 1978; Schiestl *et al.*, 1989]. This delay in G2 phase was later reported to be dependent on *S. cerevisiae RAD9* gene [Schiestl *et al.*, 1989; Weinert and Hartwell, 1989]. The loss of *RAD9* in *cdc9ts* mutants allows cells to enter mitosis and continue a couple of rounds of replication before losing viability. The Rad9-dependent checkpoint is also activated in response to X- and UV-irradiation [Weinert and Hartwell, 1989; Weinert and Hartwell, 1990]. These studies suggest that cells have different mechanisms to activate a "checkpoint" pathway that delays mitotic entry in response to DNA damage or defects in DNA synthesis [Weinert and Hartwell, 1989]. Over the past 20 years, identifying genes involved in these checkpoint pathways to understand how the cell recognizes different DNA structures and activates a checkpoint response have been the focus of many laboratories. Today, it is well appreciated that besides its role in regulating cell-cycle progression, the checkpoint pathway also plays a crucial role in response to replication stress during S phase. In particular, maintaining the stability of stalled forks is crucial to prevent them from

collapse, thereby facilitating fork restart after the stress is removed [Branzei and Foiani, 2010].

The activation of the S phase checkpoint.

A central response to replication stress is the activation of the checkpoint kinase, mitotic entry checkpoint 1 (Mec1, homolog of the human ATR) and its downstream effector, checkpoint kinase Rad53 (homolog of the human Chk2, but functions similar to human Chk1) (Figure 1.7) [Alcasabas *et al.*, 2001; Sanchez *et al.*, 1996; Sun *et al.*, 1996]. Evidence from different model organisms converge to a centralized theme that ssDNA, generated by inhibition of DNA polymerases, is the key structure that activates Mec1 [Cimprich and Cortez, 2008; Flynn and Zou, 2011]. Uncoupling of DNA synthesis and DNA unwinding caused by MMS- or UV-induced stalling of DNA polymerases on either strand leads to the accumulation of ssDNA at stalled forks. RPA binds to exposed ssDNA regions, and the ssDNA-RPA complex is critical for the recruitment of the Mec1/Ddc2 (DNA damage checkpoint 2) complex to stalled replication forks [Zou and Elledge, 2003]. Activation of Mec1 ultimately phosphorylates its downstream checkpoint kinase Rad53 through a mediator protein Mrc1, a component of the replisome [Alcasabas *et al.*, 2001]. Mrc1 plays a role during both unperturbed replication and replication stress. During normal replication, Mrc1 travels with the replisome and promotes efficient replication, but when the machinery encounters a replication block, Mrc1-Tof1-Csm3 form a stable pausing complex at the stalled fork [Katou *et al.*, 2003]. In addition, Mrc1 is phopshorylated in a Mec1- and Rad53-dependent manner [Alcasabas

et al., 2001]. Seventeen SQ and TQ consensus Mec1-dependent phosphorylation sites of Mrc1 were identified [Osborn and Elledge, 2003]. A recent study illustrates that phosphorylation of Mrc1 by Mec1, but not Rad53, is necessary to stabilize Mec1 and the replisome at stalled forks, establishing a positive feedback loop [Naylor *et al.*, 2009]. Cells carrying mutations of all 17 S/T-Q sites on Mrc1 ($Mrc1^{AQ}$) retain normal replication function but inhibit Mec1-dependent Rad53 phosphorylation as well as decrease Mec1 stability at stalled forks [Naylor *et al.*, 2009; Osborn and Elledge, 2003].

Although Rad9 functions as a mediator, similar to Mrc1, for Rad53 phosphorylation by Mec1 and Tel1 (telomere maintenance 1, homolog of human ATM) in response to DNA damage, it plays no role in response to replication stress in wild-type cells (Figure 1.7) [Alcasabas *et al.*, 2001; Emili, 1998; Gilbert *et al.*, 2001; Vialard *et al.*, 1998]. However, *mrc1Δ* mutants exhibit a delay in Rad53 phosphorylation when replication is blocked, suggesting that replication stress causes DNA damage in the absence of Mrc1 [Alcasabas *et al.*, 2001]. Thus, cells then activate Rad53 phosphorylation via a Rad9-dependent pathway. Phosphorylated Rad9 interacts with Rad53 and changes its conformation to activate autophosphorylation [Gilbert *et al.*, 2001; Sweeney *et al.*, 2005; Vialard *et al.*, 1998]. Consistent with this notion, *mrc1Δ rad9Δ* double mutants are synthetically lethal, underscoring the importance of Rad53 activation by these two independent branches of the S phase checkpoint [Alcasabas *et al.*, 2001; Kim and Weinert, 1997].

Role of the S phase checkpoint.

Although Rad53 is activated in response to replication stress, little is known about how its activation regulates replication and DNA damage checkpoint function. To date, only a couple of downstream targets of Rad53 have been identified and the functions of these targets range from affecting transcriptional regulation of repair genes and the pools of dNTPs, regulating late firing origins, and promoting fork stabilization (Figure 1.7) [Branzei and Foiani, 2006; Zegerman and Diffley, 2009]. Following replication stress, the level of dNTPs increase by 6-8 fold in budding yeast due to the upregulation of ribonucleotide reductase enzyme (RNR) [Chabes *et al.*, 2003]. This upregulation is controlled by the damaged-induced kinase Dun1 (DNA damage uninducible 1) [Zhao and Rothstein, 2002]. Dun1 phosphorylates Sml1 (suppressor of Mec1 lethality 1), which normally binds and inhibits RNR to limit the available dNTP pools during replication. Phosphorylation of Sml1 results in its degradation. This leads to RNR activation and ultimately increases dNTP pools that are necessary for efficient replication when cells are under replication stress. In line with this notion, deletion of *SML1* gene, similar to *RNR* upregulation, in *rad53Δ* and *mec1Δ* rescues their lethality even though these mutants are still sensitive to DNA damage [Zhao and Rothstein, 2002]. Another function of the S phase checkpoint is the Rad53-dependent suppression of late firing origins [Santocanale and Diffley, 1998]. Recent studies demonstrate that the replication initiation protein, Sld3, and Dbf4 subunit of DDK are the "minimal" targets of Rad53 activity to inhibit late firing origins [Lopez-Mosqueda *et al.*, 2010; Zegerman and Diffley, 2010]. Inhibition of

late firing origins by Rad53-mediated phosphorylation of Sld3 and Dbf4 allows S-CDK to remain active to prevent rereplication during S phase.

Perhaps the most crucial role of the S phase checkpoint in response to replication stress is the stabilization of replication forks. The notion that preserving replication fork stability, rather than inhibition of late firing origins, is more favorable for survival arises from a study comparing two different *mec1* mutants, *mec1Δ* and *mec1-100* [Tercero *et al.*, 2003]. The authors demonstrated that while both mutants failed to suppress late firing origins when treated with MMS, *mec1Δ* mutants are more sensitive to DNA damaging agents than *mec1-100* [Tercero et al., 2003]. The hypersensitivity to DNA damaging agents in *mec1Δ* mutants is likely due to much higher rate of replication fork collapse than in *mec1-100* cells. Replication checkpoint-deficient cells, such as *mec1* or *rad53* mutants, are also unable to resume replication after the removal of replication stress or complete DNA replication in the presence of MMS, suggesting that the replisome dissociates from stalled forks [Lopes *et al.*, 2001; Tercero *et al.*, 2003]. These data provide strong evidence that stabilization of replication forks by the S phase checkpoint is more beneficial for cell survival rather than regulating late firing origins.

Postreplicative repair pathways.

During replication of damaged DNA caused by UV-irradiation, large amounts of internal gaps and ssDNA at stalled forks are observed in NER-deficient cells by EM [Lopes *et al.*, 2006; Rupp and Howard-Flanders, 1968; Rupp *et al.*, 1971; Sogo *et al.*, 2002]. Internal gaps are most likely generated by repriming events downstream of the

lesions on both leading and lagging strands to bypass the damage [Heller and Marians, 2006]. These gaps can be filled-in by two different mechanisms, error-prone and error-free repair pathways, which together are known as postreplicative repair (PRR) (Figure 1.8) [Branzei and Foiani, 2010]. The error-prone repair pathway relies on *RAD6* and *RAD18* to recruit specialized low-fidelity polymerases that are capable of synthesizing across damaged template DNA known as translesion synthesis (TLS) (Figure 1.8) [Lehmann *et al.*, 2007; Prakash *et al.*, 2005]. In yeast, three TLS polymerases exist, pol η (pol-eta encoded by *RAD30*), Rev1, and pol ζ (pol-zeta encoded by *REV3* and *REV7*) [Ohmori *et al.*, 2001; Shcherbakova and Fijalkowska, 2006]. Because TLS polymerases lack proofreading activity and contain larger active sites to accommodate bulky lesions, TLS-mediated synthesis is generally mutagenic [Shcherbakova and Fijalkowska, 2006]. Alternatively, cells activate the error-free repair pathway that relies on the template switch mechanism, using nascent DNA from undamaged sister chromatid as a template for lesion bypass (Figure 1.8). Genetic evidence indicates that the template switch mechanism can be further categorized into two pathways, mediated either by *RAD51*/*RAD52* family or *RAD5*/*MMS2*/*UBC13*-mediated PRR [Gangavarapu *et al.*, 2007; Prakash, 1981; Torres-Ramos *et al.*, 2002; Zhang and Lawrence, 2005]. Whereas both *RAD5*/*MMS2*/*UBC13*-mediated template switch and TLS activities are dependent on *RAD6* and *RAD18*, *RAD51*/*RAD52*-mediated template switch is *RAD6*/*RAD18*-independent [Schiestl *et al.*, 1990; Torres-Ramos *et al.*, 2002]. This model is supported by the synergistic genetic interaction between *rad6* and *rad52* mutants, where the double mutants are much more sensitive to DNA damage than either single mutant [Schiestl *et*

al., 1990]. Altogether, DNA damage tolerance consists of three independent pathways, TLS (*RAD6*, *RAD18*), *RAD5*-mediated template switch (*RAD6*/*RAD18*/*RAD5*/*MMS2*/*UBC13*), and *RAD51*/*RAD52*-mediated template switch.

Linking postreplicative repair pathways to PCNA ubiquitination.

Although accumulating genetic evidence had shown the requirement of the *RAD6* pathway in DNA repair for almost 20 years, it was not until 2002 that PRR was linked to the ubiquitination of PCNA in a *RAD6*-dependent manner (Figure 1.9) [Hoege *et al.*, 2002]. Ubiquitination involves a chain reaction whereby an 8 kDa (kilodalton) ubiquitin peptide is transferred from an ubiquitin activating (E1) enzyme to an ubiquitin conjugating (E2) enzyme, then to an ubiquitin ligase (E3) and finally to a lysine (K) residue on the target protein [Hershko and Ciechanover, 1998]. This process can be repeated to form a chain of ubiquitin (poly-ubiquitin) linked through K residues of ubiquitin. While a poly-ubiquitin chain linked through K48 is known for its function in protein degradation, other ubiquitin chain linkages are not well characterized [Hochstrasser, 1996]. In yeast, all ubiquitination pathways occur through the E1 Uba1 enzyme [McGrath *et al.*, 1991]. Rad6, which is an E2 enzyme, interacts with the E3 ubiquitin ligase Rad18 to catalyze mono-ubiquitination at a highly conserved K164 of PCNA (Figure 1.9) [Bailly *et al.*, 1997a; Bailly *et al.*, 1997b; Hoege *et al.*, 2002]. The mono-ubiquitinated PCNA is extended to a poly-ubiquitin chain linked through an unconventional, non-proteolytic K63 linkage by another E2-E3 complex, Mms2/Ubc13 dimer and Rad5 [Hoege *et al.*, 2002; Hofmann and Pickart, 1999; Spence *et al.*, 1995].

This step-wise PCNA mono- and poly-ubiquitination chain elongation by the two distinct complexes was recently confirmed by *in vitro* reconstitution experiments [Parker and Ulrich, 2009].

Although Rad5/Mms2/Ubc13-mediated PCNA poly-ubiquitination promotes error-free template switching, the molecular mechanism is still unclear [Hoege *et al.*, 2002; Papouli *et al.*, 2005; Pfander *et al.*, 2005]. The Rad6/Rad18-mediated PCNA mono-ubiquitination is much better understood. Specifically, PCNA mono-ubiquitination enhances the binding or activity to TLS polymerases to PCNA, suggesting a model whereby PCNA mono-ubiquitination promotes the switch between replicative and TLS polymerases for lesion bypass by the error-prone repair pathway [Acharya *et al.*, 2007; Acharya *et al.*, 2008; Garg and Burgers, 2005b; Haracska *et al.*, 2006; Stelter and Ulrich, 2003; Zhuang *et al.*, 2008]. This is consistent with the genetic requirement for Rad6/Rad18-mediated TLS. As mentioned earlier, EM studies of cells exposed to UV-irradiation revealed the presence of ssDNA gaps behind replication forks, suggesting that DNA damage tolerance mechanisms can be active during S and G2/M phases of the cell cycle [Lopes *et al.*, 2006; Rupp and Howard-Flanders, 1968; Rupp *et al.*, 1971; Sogo *et al.*, 2002]. The molecular mechanisms of how PRR repairs lesions in G2/M were recently demonstrated by expressing genes involved in Rad6-mediated PCNA ubiquitination solely in G2. Induction of PCNA ubiquitination in G2 is still capable of activating lesion bypass mechanisms for cell survival [Daigaku *et al.*, 2010; Karras and Jentsch, 2010]. Furthermore, deletion of the *POL32* gene, a subunit of pol-δ, results in accumulation of ssDNA on the lagging strand and triggers PCNA ubiquitination in G2,

even in the absence of exogenous DNA damage [Karras and Jentsch, 2010]. These data suggest that DNA damage tolerance mechanisms can be active at stalled forks and at internal ss gaps behind replication forks in G2/M phase [Daigaku *et al.*, 2010; Karras and Jentsch, 2010; Vanoli *et al.*, 2010].

The activation of DNA damage tolerance pathways requires ssDNA-RPA regions. Biochemical studies showed that such ssDNA-RPA regions facilitate the recruitment of the Rad6/Rad18 complex, where Rad18 interacts directly with two RPA subunits, Rfa1 and Rfa2 [Davies *et al.*, 2008]. However, PPR activation requires only ssDNA-RPA structures that arise at stalled replication forks and internal ss gaps, but not at resected DSBs. Consistent with this notion, DNA damaging agents that stall replication fork progression induce PCNA ubiquitination, whereas DSB-induced damaging agents do not [Davies *et al.*, 2008]. Moreover, although the RPA-ssDNA structure is also crucial for the recruitment and activation of the S phase checkpoint kinase Mec1 [Zou and Elledge, 2003], an RPA mutant that is defective in checkpoint activation is still sufficient to trigger PCNA ubiquitination [Davies *et al.*, 2008]. This suggests that PCNA ubiquitination and the S phase checkpoint are independent pathways in *S. cerevisiae* [Davies *et al.*, 2008].

Besides ubiquitination, PCNA is also modified by the ubiquitin-like protein SUMO (small ubiquitin-like modifier) [Hoege *et al.*, 2002]. However, this modification is limited to S phase during which PCNA is sumoylated at K164 and to a lesser extent, at K127 (Figure 1.9) [Hoege *et al.*, 2002]. PCNA sumoylation recruits to the anti-recombinogenic helicase Srs2 (suppressor of rad six 2) to the fork during unperturbed

replication [Pfander *et al.*, 2005; Stelter and Ulrich, 2003]. Srs2 contains a PIP-like and a SUMO-interacting motif (SIM) that interacts specifically with sumoylated PCNA and is capable of dissociating Rad51 filaments on ssDNA [Armstrong *et al.*, 2012; Krejci *et al.*, 2003; Pfander *et al.*, 2005]. Thus, Srs2 helicase is thought to inhibit unscheduled homologous recombination events during normal replication. Importantly, a recent report demonstrates that PCNA sumoylation also facilitates the Rad5/Mms2/Ubc13-mediated template switch mechanism where PCNA is simultaneously sumoylated and ubiquitinated [Branzei *et al.*, 2008]. While sumoylated PCNA recruits Srs2 to inhibit template switch by a Rad51/Rad52-dependent pathway, poly-ubiquitin promotes Rad5/Mms2/Ubc13-mediated template switch [Branzei *et al.*, 2008]. Only in the absence of PCNA sumoylation or Srs2, undesired Rad51/Rad52-mediated HR becomes more active during S phase [Papouli *et al.*, 2005; Pfander *et al.*, 2005; Schiestl *et al.*, 1990]. A recent report of sumoylation in human cells suggests that these mechanims are evolutionarily conserved [Moldovan *et al.*, 2012].

Rationale

Induction of replication stress by DNA damaging agents such as MMS or UV-irradiation ultimately disrupts the coordination between DNA unwinding and DNA synthesis by inhibiting the progression of DNA polymerases during S phase. Such damage generates ssDNA-RPA structures that are crucial for both checkpoint activation and PCNA ubiquitination pathways [Cimprich and Cortez, 2008; Flynn and Zou, 2011]. However, extended ssDNA regions are likely not the only structures that are generated during S phase when replication goes awry and may cause genomic instability. In fact, mutations in genes involved in lagging strand synthesis such as *FEN1* and *LIG1* have been identified in cancer patients, suggesting that flapped and nicked DNA structures arise from defects in Okazaki fragment maturation processes also cause DNA damage that can lead to genomic instability [Barnes *et al.*, 1992; Webster *et al.*, 1992; Zheng *et al.*, 2007]. In humans, half of the replicated genome is dependent on the synthesis and maturation of 30,000,000 Okazaki fragments during every S phase. Furthermore, because normal cells can replicate their genomes efficiently and with high fidelity, this argues for the presence of DDR pathways to monitor and repair DNA damage that arises from Okazaki fragment processing defects.

The goal of the thesis presented here was to investigate whether cells could sense DNA damage that arose from deficient lagging strand synthesis. More specifically, we were curious as to whether cells can recognize damage "behind" the fork that does not disturb the replisome progression by inhibiting the replicative polymerases. Taking advantage of the fact that neither Fen1 nor DNA ligase I are stable parts of the replisome,

depletion of either protein should not disrupt the progression of DNA synthesis, leaving DNA damage "behind" the moving fork. Defects in DNA ligase I should result in the accumulation of nicked DNA intermediates, since Cdc9 is the only DNA ligase that functions in Okazaki fragment maturation [Johnston and Nasmyth, 1978]. Therefore, this thesis focuses on determining how cells sense and repair the accumulation of nicks behind the fork in the absence of Cdc9 and ssDNA. Human cells have PARP1 (poly (ADP-ribose) polymerase) that functions as a nick sensor and activates SSB repair pathway to cope with the accumulation of nicks [Menissier-de Murcia *et al.*, 1989]. However, no known homologue of PARP1 has been identified in *S. cerevisiae*, arguing for the presence of PARP1-independent repair pathways that may be conserved in humans. The greater goal was to gain a better understanding of how eukaryotic cells deal with nicks during DNA replication. This should not only lead to more insight into the DDR network, but may ultimately help in devising novel cancer diagnostics and treatments.

Figure 1.1

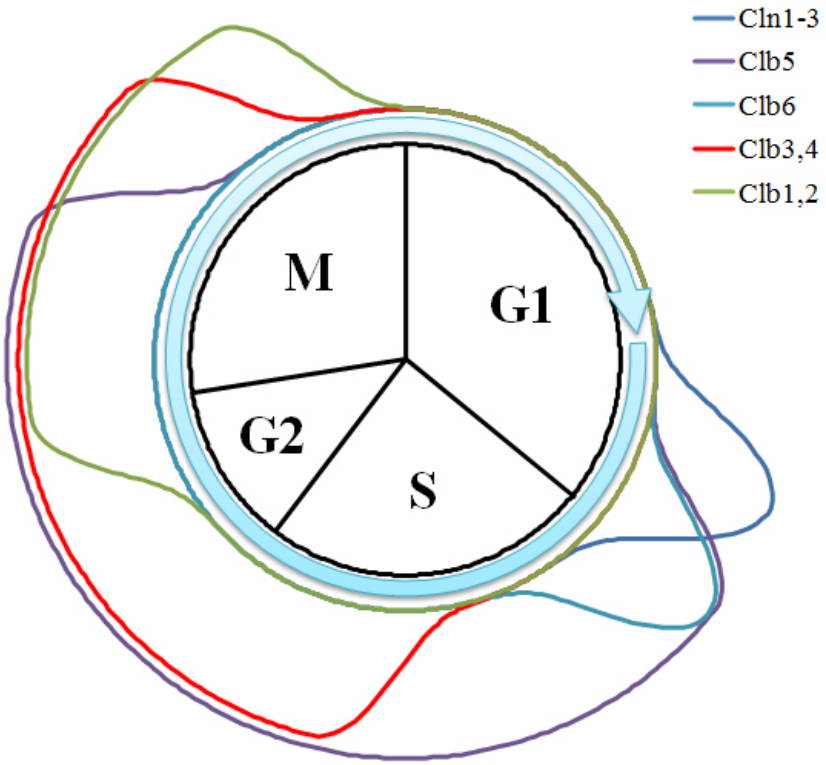

Figure 1.1 Cell cycle regulation by cyclins.

The cell cycle consists of four different phases, G1, S, G2, and M. The duration of individual cell cycle phases differs in various organisms and is not drawn to scale. Progression through the cell cycle is regulated by the activation of the cyclin dependent kinase 1, Cdk1 in *S. cerevisiae* with different cyclins. There are three G1 or A-type cyclins (Cln1-3) and six B-type cyclins (Clb1-6). Colored lines indicate expression levels of different cyclins at different phases of the cell cycle. Expression of all cyclins is low at the end of M phase and G1, resulting in low Cdk1 activity during origin licensing.

Figure 1.2

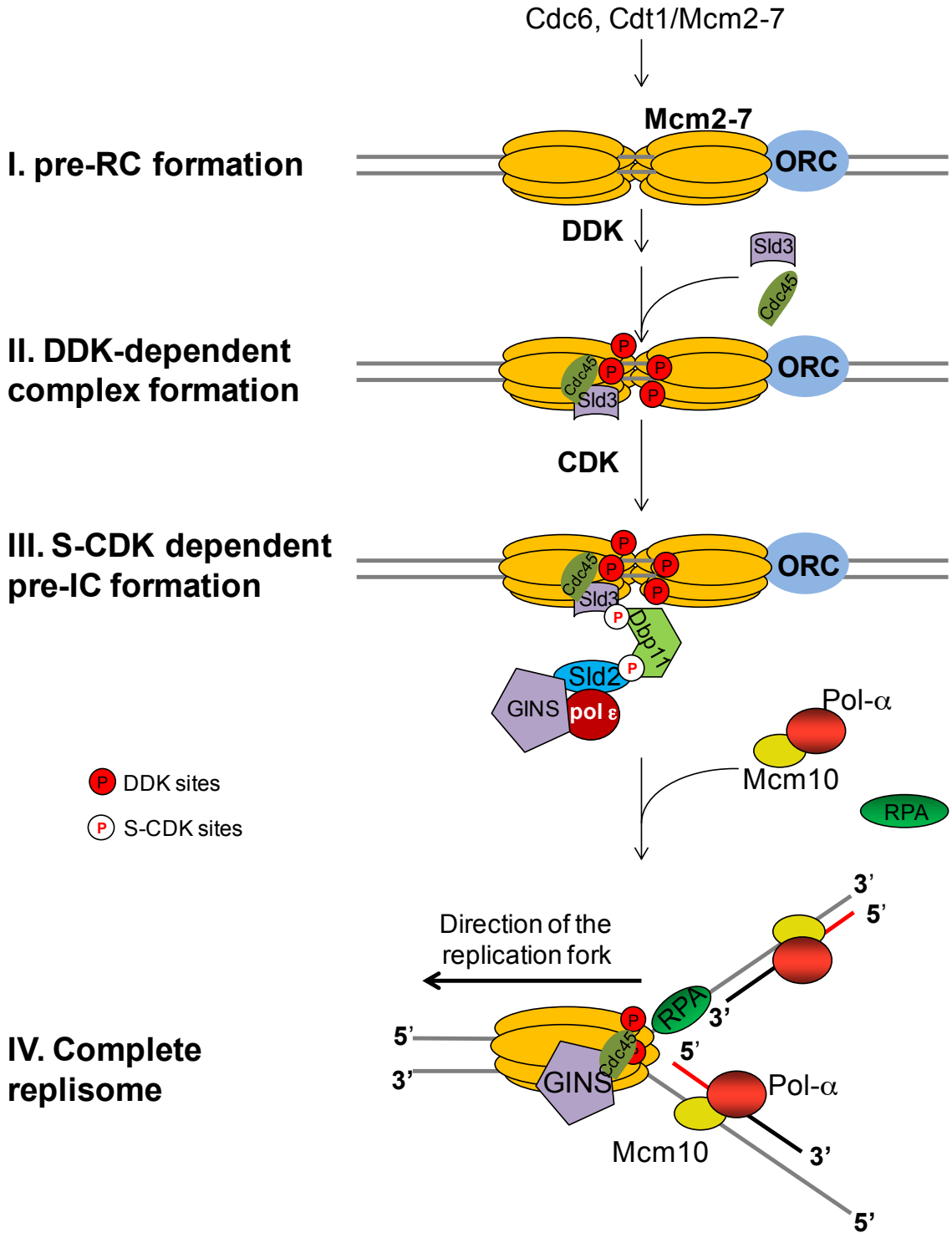
Cdc6, Cdt1/Mcm2-7
I. pre-RC formation
Mcm2-7
ORC
DDK
Sld3
Cdc45
II. DDK-dependent complex formation
ORC
CDK
III. S-CDK dependent pre-IC formation
ORC
Dbp11
Sld2
GINS
pol ε
Pol-α
Mcm10
RPA
DDK sites
S-CDK sites
IV. Complete replisome
Direction of the replication fork
3'
5'
RPA
GINS
Pol-α
Mcm10

Figure 1.2 Mechanism of DNA initiation.

Eukaryotic DNA initiation involves multiple, coordinated steps that ultimately lead to DNA unwinding and synthesis. The formation of the pre-replicative complex, pre-RC, involves the loading of the double-hexameric Mcm2-7 complex onto origins of replication. This is dependent on ORC, Cdc6, and Cdt1 (Step I). Replication initiation is activated by two S phase dependent kinases, first by DDK (Step II) and then by S-CDK (Step III). DDK phosphorylates the chromatin-bound Mcm2-7 helicase, which is a prerequisite for the loading of Sld3 and Cdc45 (Step II). Following DDK-dependent complex formation, S-CDK phosphorylates Sld2 and Sld3 to form a pre-initiation complex or pre-IC with Dpb11, GINS and pol-ε (Step III). Finally, Mcm10, together with RPA, recruits pol-α to origins to catalyze *de novo* DNA synthesis (Step IV).

Figure 1.3

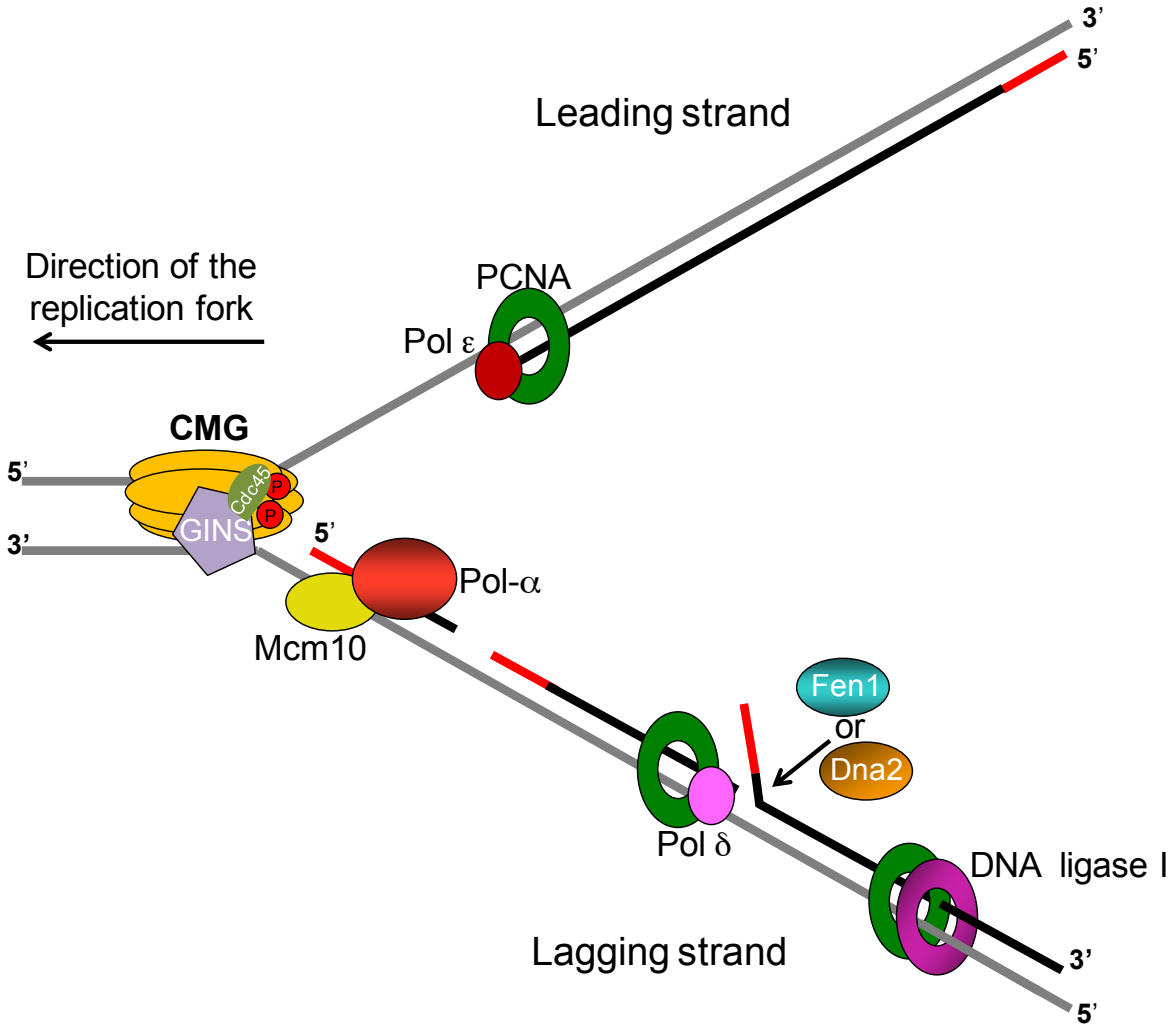
3'
5'
Leading strand
Direction of the
replication fork
PCNA
Pol ε
CMG
5'
3'
Cdc45
P
P
GINS
5'
Pol-α
Mcm10
Fen1
or
Dna2
Pol δ
DNA ligase I
Lagging strand
3'
5'

Figure 1.3 Eukaryotic DNA replication mechanism.

DNA replication occurs in an anti-parallel manner, synthesizing one continuous strand (leading strand) and multiple short DNA fragments (lagging strand or Okazaki fragments). First, Mcm10 recruits pol-α to synthesize *de novo* RNA/DNA primer on both leading and lagging strands. These structures serve as a substrate for PCNA (proliferating cell nuclear antigen) loading. PCNA increases the processivity of pol-ε and pol-δ. While pol-ε can synthesize continuously along the leading strand template, lagging strand synthesis requires additional steps. First, pol-δ synthesizes DNA and displaces the preceding RNA/DNA primer, generating a short 5'-flap that can be cleaved by the flap endonuclease, Fen1. In the absence of Fen1, the short flap extends in length and becomes a substrate for Dna2. Both Fen1 and Dna2 ultimately generate a DNA nick that can be sealed by DNA ligase I.

Figure 1.4

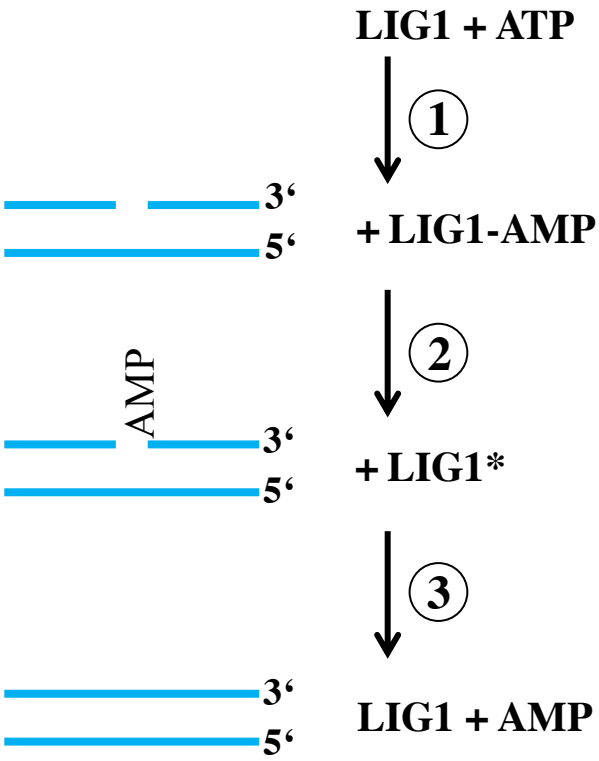

Figure 1.4 DNA ligation mechanism of DNA ligase I.

DNA ligation by DNA ligase I involves three nucleotidyl transfer reactions. First, DNA ligase reacts with ATP to form a ligase-adenylate intermediate, where the 5’AMP is covalently bound to a lysine in the DNA ligase I active site (Step 1). AMP is then transferred to the 5’-phosphate terminus of the nick, forming a DNA-adenylate intermediate (Step 2). Finally, DNA ligase catalyzes the nucleophillic attack of the 3’OH to the DNA-adenylate to join the ends of the DNA strands and release AMP. The figure is adapted from [Ellenberger and Tomkinson, 2008].

Figure 1.5

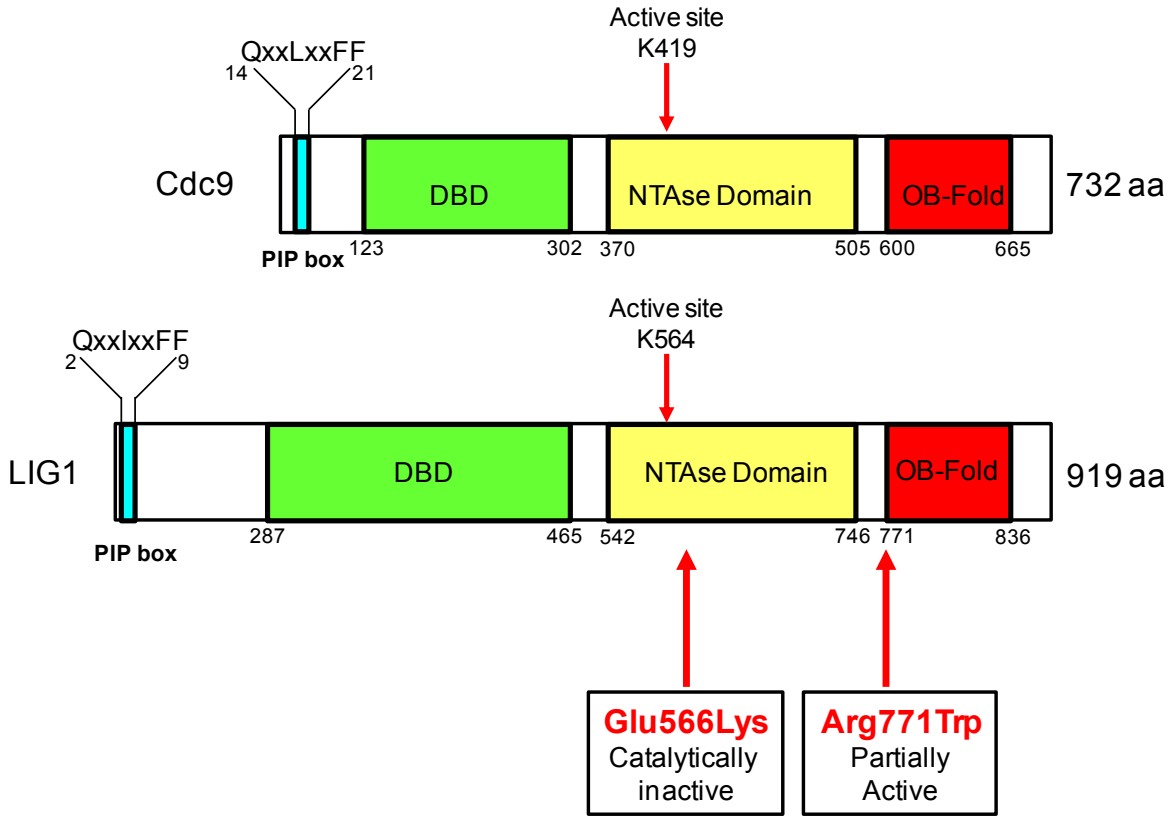

Figure 1.5 Domains of DNA ligase I.

DNA ligase I from *S. cerevisiae* (Cdc9) and humans (hLIG1) has a DNA binding domain (DBD, green), a nucleotidyltransferase (NTase) domain, which contains a lysine in the active site, and an oligonucleotide/oligosaccharide binding fold (OB-fold, red). A PCNA-interacting protein motif (PIP box) is located in the N-terminus of both Cdc9 and LIG1. Mutations in the *hLIG1* gene, Glu556Lys and Arg771Trp, were identified in a human patient [Webster *et al.*, 1992]. Whereas the Glu556Lys mutation inactivates LIG1 on one allele, the Arg771Trp carrying allele retains approximately 5-10% of hLIG1 activity. The figure is adapted from [Ellenberger and Tomkinson, 2008].

Figure 1.6

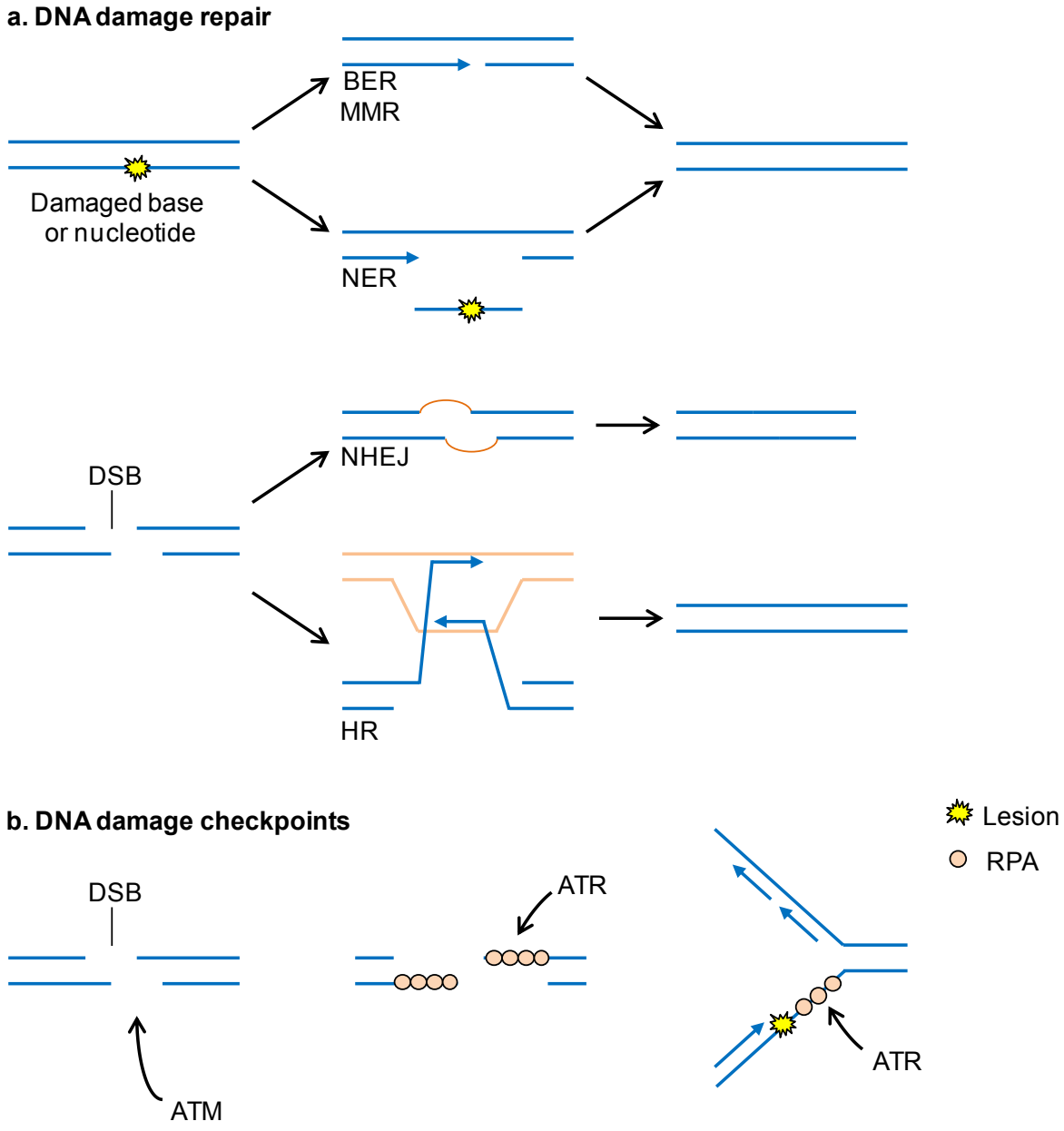
a. DNA damage repair
BER
MMR
Damaged base
or nucleotide
NER
DSB
NHEJ
HR
b. DNA damage checkpoints
Lesion
RPA
DSB
ATR
ATM
ATR

Figure 1.6 DNA damage response to different types of DNA damage.

(a) DNA damage that causes a damaged base or nucleotide in the genome can either be removed by base excision repair (BER) or mismatch repair (MMR). In nucleotide excision repair (NER), which removes pyrimidine dimers, a stretch of ~30 nucleotides flanking the lesion is removed, leaving a single-stranded DNA gap. This gap is filled-in by DNA polymerases and ligated together by DNA ligase I. When DNA damage induces a double-strand break (DSB), this can be repaired either by non-homologous end joining (NHEJ) or homologous recombination (HR). Whereas NHEJ is error-prone, HR is an error-free repair pathway because cells utilize undamaged sister chromatid as a template to resynthesize the missing DNA bases. **(b)** In higher eukaryotes, cells have two DNA checkpoint proteins that are activated depending on the type of lesions. ATM is activated primarily in response to double-stranded ends (left). When the DSB is resected and ssDNA is coated with RPA, ATR is recruited (middle). In addition, RPA-ssDNA structures are also generated at stalled replication forks and are responsible for recruiting ATR (right). This figure is adapted from [Ulrich and Walden, 2010].

Figure 1.7

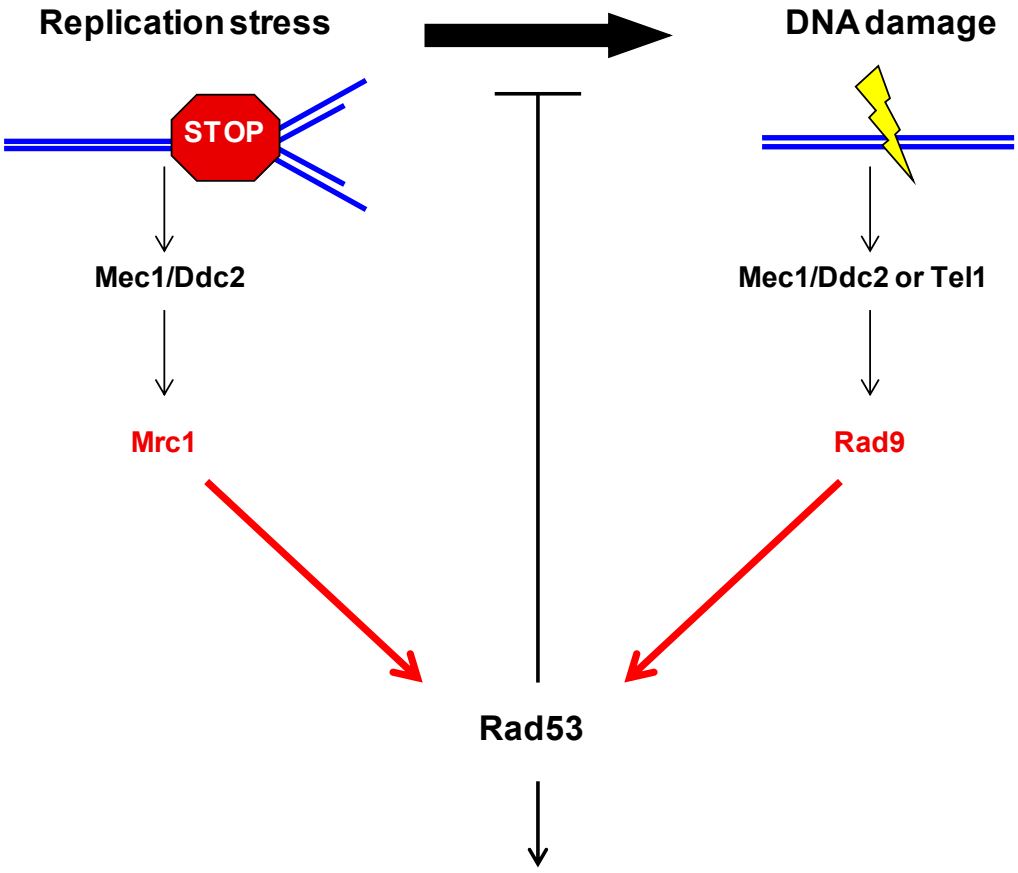
Replication stress
DNA damage
STOP
Mec1/Ddc2
Mec1/Ddc2 or Tel1
Mrc1
Rad9
Rad53
• Upregulates dNTP pools
• Inhibits late firing origins
• Stabilizes stalled replication forks

Figure 1.7 The S phase checkpoint signaling pathway in *S. cerevisiae*.

The S phase checkpoint kinase, Rad53, is activated in response to replication stress and physical DNA damage such as double-stranded breaks, DSBs. In response to replication stress (left), Mec1 (yeast homolog of ATR), together with Ddc2 (yeast homolog of ATRIP), phosphorylates Rad53, which is mediated through Mrc1. Rad53 phosphorylation regulates several different pathways ranging from upregulation of dNTPs pools, inhibition of late firing origins, and most importantly, stabilization of replication forks to prevent fork collapse. If fork stabilization cannot be established, replication forks can give rise to DSBs (right). In response to DSBs, Rad53 is activated through a second pathway. Either the Mec1 or Tel1 (yeast homologue of ATM) checkpoint kinase phosphorylates Rad53, which is mediated through "the Rad53-adaptor" Rad9 [Sweeney *et al.*, 2005].

Figure 1.8

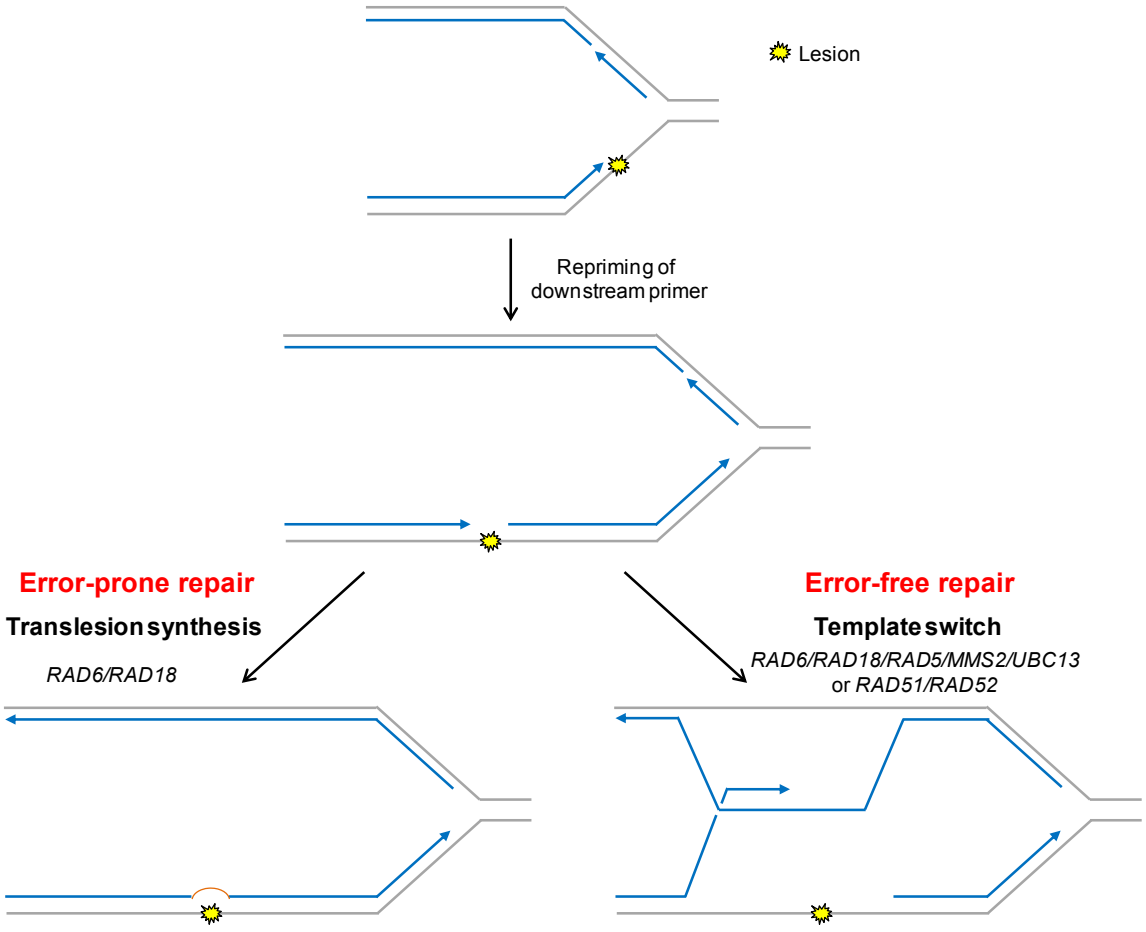
Lesion
Repriming of
downstream primer
Error-prone repair
Translesion synthesis
RAD6/RAD18
Error-free repair
Template switch
RAD6/RAD18/RAD5/MMS2/UBC13
or RAD51/RAD52

Figure 1.8 Postreplicative repair pathways.

Lesions on the template DNA such as bulky adducts caused by UV-irradiation inhibit the progression of the replicative polymerases. The lesion depicted here is on the leading strand, but such lesions can also occur on the lagging strand. Cells can re-initiate DNA synthesis by repriming downstream of the lesion, which leaves a small single-stranded DNA (ssDNA) gap. The lesion can be repaired by either error-prone or error-free repair pathways. In error-prone repair pathway, which is mediated by *RAD6* and *RAD18*, cells recruit special translesion polymerases to synthesize across the lesion. Alternatively, cells promote error-free repair by a template switch mechanism using the nascent, undamaged sister chromatid as a template to bypass the lesion. Template switch mechanisms are mediated through two distinct pathways, one by *RAD6/RAD18/RAD5/MMS2/UBC13* and the other by *RAD51/RAD52*. This figure was adapted from [Branzei and Foiani, 2007].

Figure 1.9

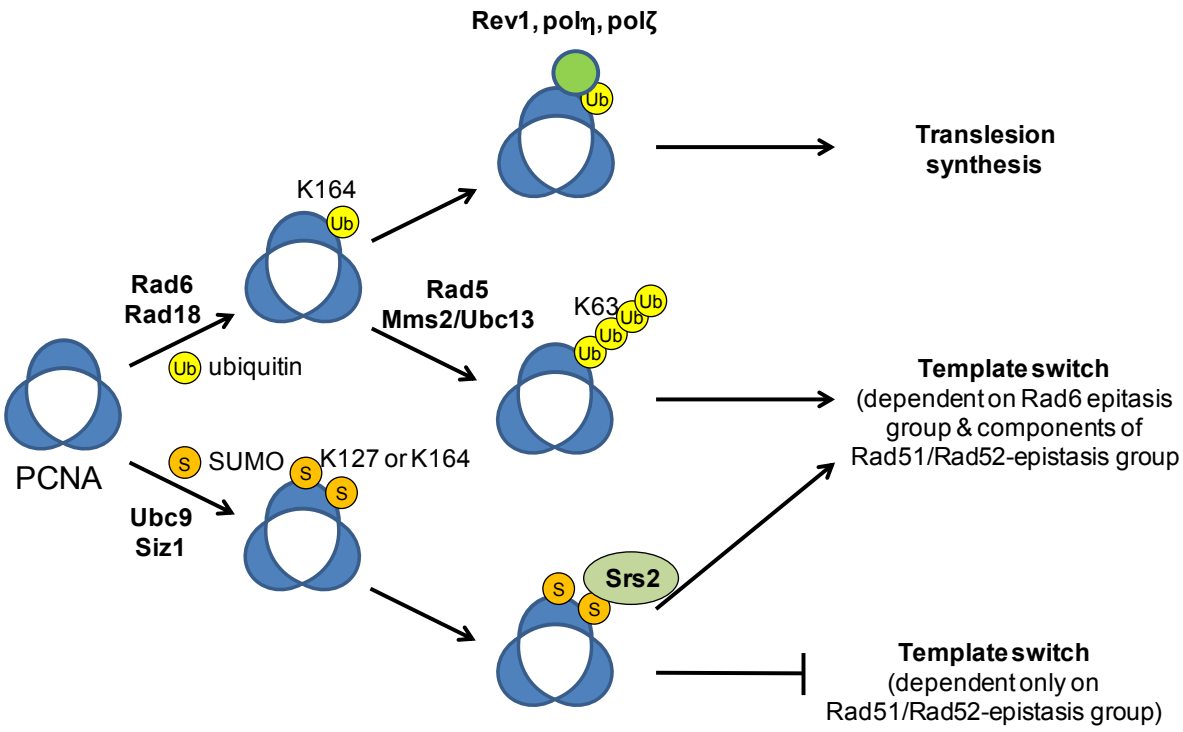
Rev1, polη, polζ
Ub
Translesion synthesis
K164
Ub
Rad6
Rad18
Rad5
Mms2/Ubc13
K63
Ub
Ub
Ub
Ub
ubiquitin
Template switch
(dependent on Rad6 epitasis group & components of Rad51/Rad52-epistasis group
PCNA
S
SUMO
K127 or K164
S
S
Ubc9
Siz1
Srs2
S
S
Template switch
(dependent only on Rad51/Rad52-epistasis group)

Figure 1.9 Functions of PCNA modification by ubiquitin and SUMO.

The trimeric PCNA clamp can be modified by both ubiquitin and SUMO at lysine (K) 164 (sumoylation can occur at K127 to a lesser extent). For simplicity, modification is illustrated only on one subunit, but all modifications can theoretically occur on all three subunits of PCNA simultaneously or in any combination. PCNA mono-ubiquitination at K164 is catalyzed by Rad6 and Rad18, whereas SUMO is conjugated by Ubc9 (the SUMO E2) and Siz1 (the SUMO E3). Mono-ubiquitination facilitates the recruitment of Y-family polymerases (Rev1, polη, and polζ) to promote translesion synthesis. Alternatively, mono-ubiquitin on PCNA can be extended to a poly-ubiquitin chain linked through lysine (K) 63. This is catalyzed by the E2/E3 complex Mms2/Ubc13/Rad5 and promotes template switching by an unknown mechanism. Sumoylation of PCNA at K164, and to a lesser extent at K127, recruits the antirecombinogenic helicase Srs2 to displace Rad51 from single-stranded DNA and prevent template switch mediated by the Rad51/Rad52-epistasis group. A recent study demonstrates that disassembling Rad51 filaments by sumoylated PCNA is crucial to promote Mms2/Ubc13/Rad5-mediated template switch by PCNA ubiquitination of the same trimeric PCNA molecule. The figure is adapted from [Sale, 2012].

CHAPTER 2

Defects in DNA ligase I trigger PCNA ubiquitination at lys 107

(The work in this chapter was published Das-Bradoo*, S., Nguyen, H.D.*, Wood, J.L.*, Ricke, R.M., Haworth, J.C., Bielinsky, A.K. (2010) Nat. Cell Biol. **12**(1):74-9.
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Authors contributions:

S.D.-B and H.D.N conducted all PCNA ubiquitination experiments in yeast, helped with data analysis and helped write the manuscript. J.L.W conducted all experiments with human cells (Figure 2.16), helped with data analysis and helped write the manuscript. R.M.R conducted cell-cycle arrest and RIP mapping experiments (Figures 2.1-2.4), and helped with data analysis. J.C.H helped with the construction of yeast mutants. A.-K.B planned and supervised the project, and wrote the manuscript.

In all eukaryotes, the ligation of newly synthesized DNA, also known as Okazaki fragments, is catalyzed by DNA ligase I [Ellenberger and Tomkinson, 2008]. An individual with a DNA ligase I deficiency exhibited growth retardation, sunlight sensitivity and severe immunosuppression [Webster *et al.*, 1992], likely due to accumulation of DNA damage. Surprisingly, not much is known about the DNA damage response (DDR) in DNA ligase I-deficient cells. Because DNA replication and DDR pathways are highly conserved in eukaryotes, we utilized *Saccharomyces cerevisiae* as a model system to address this question. We uncovered a novel pathway, which facilitates ubiquitination of lysine 107 of proliferating cell nuclear antigen (PCNA). Unlike ubiquitination at lysine 164 of PCNA in response to UV irradiation, which triggers translesion synthesis [Hoege *et al.*, 2002], modification of lysine 107 is not dependent on the ubiquitin conjugating enzyme (E2) Rad6 [Jentsch *et al.*, 1987] nor the ubiquitin ligase (E3) Rad18 [Bailly *et al.*, 1997a], but requires the E2 variant Mms2 [Broomfield *et al.*, 1998] in conjunction with Ubc4 [Seufert and Jentsch, 1990] and the E3 Rad5 [Torres-Ramos *et al.*, 2002; Ulrich and Jentsch, 2000]. Surprisingly, DNA ligase I-deficient *cdc9-1* cells that carry a $PCNA^{K107R}$ mutation are inviable, because they cannot activate a robust DDR. Furthermore, we show that ubiquitination of PCNA in response to DNA ligase I-deficiency is conserved in humans, yet the lysine that mediates this modification remains to be determined. We propose that PCNA ubiquitination provides a "DNA damage code" that allows cells to categorize different types of defects that arise during DNA replication.

Materials and methods

Strains and plasmids.

All strains are isogenic derivatives of W303-1a or SSL204. A complete list of strains can be found in Table 2.1. The W303-1a strain carries the *rad5-535* mutation (http://wiki.yeastgenome.org/index.php/CommunityW303.html), whereas SSL204 derivatives (*CDC9*, *cdc9-1*, *cdc9-2*) harbor wild type *RAD5*, which was confirmed by sequencing. SSL204 exhibits severe temperature sensitivity at 37°C and therefore the temperature shift experiments were performed at 35°C. To construct the *cdc9-td* strain, the first 510 base pairs of *CDC9* were inserted into pPW66R (a gift from J. F. X. Diffley) using *Hind*III restriction sites [Dohmen *et al.*, 1994]. The resulting pPW66R-*CDC9* plasmid was linearized with *Bcl*I and transformed into YKL83, which contains *UBR1* under the control of the *GAL1* promoter at the endogenous *UBR1* locus [Labib *et al.*, 2000]. To delete various genes (*MRC1, RAD9, UBC4, UBC5, RAD6, RAD18, MMS2, UBC13, RAD5*) in SSL204 and SSL612, standard one-step PCR gene replacement was used [Lorenz *et al.*, 1995] and correct integration was confirmed by sequencing. Two independent colonies of each strain were isolated.

The *UCB4*-NES-3HA fragment containing a nuclear export sequence (NES) [Wen *et al.*, 1995] and three hemagglutinin (HA) tags was generated by PCR. This was inserted into the pRS404 plasmid (a gift from D. Koepp, University of Minnesota) using *Kpn*I and *Sac*I restriction sites. The resulting integration plasmid was linearized using *Bgl*II and transformed into two independent *cdc9-1 ubc5Δ*strains. Correct integration was

confirmed by sequencing and immunoblotting with an anti-HA (16B12, Covance) antibody.

For the *MRC1* complementation experiments, pAO138 and p*MRC1* (gifts from S. J. Elledge, Harvard University) were transformed into ABy287 (*CDC9 mrc1Δ*) and ABy293 (*cdc9-1 mrc1Δ*). pAO138 contains wild-type *MRC1* expressed from its endogenous promoter and p*MRC1* expresses a *mrc1* mutant that has 17 putative SQ or TQ phosphorylation sites switched to AQ ($mrc1^{AQ}$ mutant) and is also expressed from its endogenous promoter [Osborn and Elledge, 2003]. pRS316 served as the empty vector control.

mrc1Δ rad9Δ cells were derived from a strain containing a high copy number plasmid expressing *RNR1* from its endogenous promoter (a gift from D. J. Clarke, University of Minnesota) to maintain cell viability.

PCNA lysine mutants were generated using plasmid YCplac22-*POL30* (a gift from S. Jentsch) [Hoege *et al.*, 2002]. YCplac22-*POL30* expresses full length PCNA from its endogenous promoter. Lysine to arginine substitutions in PCNA were introduced at positions 107, 108, 117, 168, 183, 253 by QuikChange mutagenesis (Stratagene). Four other lysine to arginine mutations (K127R, K164R, K127/164R, K242R) used in this study were a gift from the Jentsch laboratory [Hoege *et al.*, 2002]. As described previously [Lorenz *et al.*, 1995], the endogenous *POL30* gene was subsequently deleted by replacement with either a *URA3* or *LEU2* marker in SSL204 and YKL83 strains, respectively.

To determine the cell viability of the double mutant *cdc9-1 pol30-K107R*, a plasmid expressing *POL30* from its endogenous promoter (*pRS316-POL30*, a gift from D. M. Livingston, University of Minnesota) was first transformed into SSL204 (*CDC9*) and *cdc9-1*. PCNA lysine K107R mutant was generated using plasmid pCH1572 (a gift from L. Prakash, University of Texas Medical Branch, Galveston TX). The resulting Leu2.PCNA-K107R PCR product was subsequently transformed into strains containing *pRS316-POL30* plasmid. PCNA-K107R mutation at the endogenous locus was confirmed by sequencing. Strains were streaked out onto SC-Ura plate and 5'-FOA (2 mg/ml) plates. All plates were incubated at 25°C for 2-3 days.

For immunoprecipitation of ubiquitinated PCNA, YEp105 plasmid, expressing a synthetic Myc-tagged ubiquitin gene from a copper-inducible promoter (a gift from M. Hochstrasser, Yale University) [Ellison and Hochstrasser, 1991] was transformed into SSL204 (*CDC9*), SSL612 (*cdc9-1*), SSL613 (*cdc9-2*). For the expression of ubiquitin mutants carrying lysine to arginine substitutions at positions 6, 11, 27, 29, 33, 48, 63, YEp105 was altered by QuikChange mutagenesis. The C-terminal glycine to arginine mutations in ubiquitin at positions 75 and 76 were constructed in YEp105 by QuikChange mutagenesis.

Yeast culture conditions.

Temperature shift experiments were carried out in YPD, unless stated otherwise. For a typical temperature shift experiment, cells were grown to mid-log phase (OD_{600} =

0.6) at 25°C and then shifted to the restrictive temperature of 35°C for 3 hours or as indicated.

The degron strain, *cdc9-td*, was grown overnight at 28°C in YP plus 2% raffinose and supplemented with 10 μM $CuSO_4$ to induce *CDC9* gene expression. Copper sulfate was omitted when cells were shifted to 37°C. To increase the efficiency of Cdc9-td degradation, Ubr1 was overexpressed from a galactose-inducible promoter in the presence of 2% galactose.

For all experiments in which PCNA or ubiquitin were expressed from a plasmid, two independent colonies of each strain were grown in synthetic complete medium lacking tryptophan. In ubiquitin overexpressing strains, ubiquitin was induced over the time period of three generations by addition of 0.1 mM $CuSO_4$ at a low cell density (OD_{600} = 0.1) as described earlier [Ellison and Hochstrasser, 1991].

Co-Immunoprecipitation.

Whole cell extracts from exponentially growing cells were prepared using glass beads as described earlier [Ricke and Bielinsky, 2004]. A cocktail of freshly prepared protease inhibitors (pepstatin, leupeptin, benzamidine and phenylmethyl sulfonyl fluoride) and N-ethylmaleimide were used to preserve ubiquitinated proteins by inhibiting deubiquitinating enzymes [Das-Bradoo *et al.*, 2006]. For immunoprecipitation, 4 μg of anti-Myc antibody (9E11, Thermo scientific) was added to the extract for 2 hours at 4°C. PCNA was detected by Western blotting with an anti-PCNA antibody (clone S871, a gift from Z. Zhang) [Zhang *et al.*, 2000].

Protein Preparation and Western Blot Analysis.

Total protein extracts were prepared from cycling yeast cultures using TCA precipitation [Ricke and Bielinsky, 2006] and proteins were detected by Western blot analysis. Cdc9-td-HA was detected with an anti-HA antibody (16B12, Covance), histone H3 was detected with an anti-histone H3 antibody (Abcam), endogenous Cdc9 was detected with an anti-Cdc9 antibody (gift from A. E. Tomkinson) and endogenous Rad53 was detected with an anti-Rad53 antibody (gift from J. F. X. Diffley). α-tubulin served as a loading control. In some experiments shown in the Supplementary Information, Ponceau S staining of the nitrocellulose membrane prior to blotting served as a loading control.

Detection of PCNA ubiquitination.

Unmodified and mono-ubiquitinated PCNA were analyzed by Western blotting with an anti-yeast PCNA antibody (clone S871, a gift from Z. Zhang, Mayo Clinic MN) [Zhang *et al.*, 2000]. To detect mono-ubiquitination with the polyclonal S871 antibody, we diluted WCEs 10-fold. Poly-ubiquitinated PCNA was detected with the same antibody in undiluted, TCA-precipitated whole cell extracts. Please note that this antibody was not able to detect the 76 kDa poly-ubiquitinated form of PCNA in immunoprecipitated samples (Figure 2.6b).

Cell Synchrony, FACS Analysis, and Microscopy.

To arrest cells in G1, alpha-factor was added to a final concentration of either 50 ng/ml (*bar1Δ* cells) or 15 μg/ml (*BAR1* cells). Cells were blocked in S phase with the addition of 200 mM hydroxyurea (HU). For G2/M arrest, nocodazole was added to a final concentration of 10 μg/ml. Cell cycle progression was monitored using flow cytometry as described earlier [Tanaka and Diffley, 2002]. DNA was stained with propidium iodide in case of YKL83 strains, whereas Sytox Green was used for all other strains. All FACS samples were analyzed using a Becton Dickinson FACSCalibur.

MMS sensitivity.

Cells were grown to mid-log phase (OD_{600} = 0.5-0.6) at 25°C for *cdc9* strains and at 28°C for YKL83 derived strains. Cells were then incubated with 0.02% MMS [Hoege *et al.*, 2002] at 30°C for *cdc9-1* strains and at 37°C for YKL83 strains for the indicated time period. For YKL83 strains, 2% galactose was added for 30 minutes prior to shifting the cells to 37°C. After each time point, sodium thiosulfate (10%) was added to the treated cells to inactivate the MMS. Yeast cells were pelleted, washed with distilled water and total protein was TCA-precipitated.

Generation of stable shRNA expressing cell lines.

U2OS cells were maintained in RPMI 1640 supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin at 37°C in 5% CO_2 (v/v). To generate

stable DNA ligase I knockdown cell lines, DNA ligase I specific lentiviral shRNA plasmids and control shRNA were purchased from Open Biosystems. The sequences are shLIG#1 TGCTGTTGACAGTGAGCGCGCTTTCACCTGCGAATACAAATAGTGAA GCCACAGATGTATTTGTATTCGCAGGTGAAAGCTTGCCTACTGCCTCGGA; shLIG#2 TGCTGTTGACAGTGAGCGACCTGTTTGTACCGGAAGCAAATAGTGA AGCCACAGATGTATTTGCTTCCGGTACAAACAGGCTGCCTACTGCCTCGGA. We also used a non-silencing control shRNA TGCTGTTGACAGTGAGCGATCTCGC TTGGGCGAGAGTAAGTAGTGAAGCCACAGATGTACTTACTCTCGCCCAAGCG AGAGTGCCTACTGCCTCGGA. These vectors have been described previously [Silva *et al.*, 2005]. Stable cell lines were constructed as described [Hannon and Conklin, 2004]. Briefly, U2OS cells were transfected with 10 μg of shRNA plasmid (control or ligase I) with Fugene (Roche) per manufacturer's instructions. 48 h later cells were trypsinized and plated in media containing 2 μg /ml puromycin at different dilutions for colony formation. 12 days later drug-resistant colonies were picked and expanded and screened by Western blot for DNA ligase I expression.

Chromatin fractionation for human cells.

To obtain the chromatin fraction, one 10 cm plate for each cell line was harvested and chromatin fractions were prepared exactly as described [Motegi *et al.*, 2008]. Briefly, cells were harvested, extracted to release soluble proteins and nuclear (insoluble) proteins pelleted. The nuclear pellet was then sonicated to release chromatin bound proteins. Proteins were fractionated on SDS polyacrylamide gels and analyzed by Western blots.

Anti-DNA ligase I- (Santa Cruz), anti-histone H3- (Abcam), anti-PCNA- (Labvision), anti-ubiquitin- (Millipore), and anti-tubulin antibodies (Sigma) were used in this study.

Mammalian Cell Lysate and Western blotting.

Cells were lysed with NETN (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 % Nonidet P-40 plus protease inhibitors) on ice and then rocked for 10 min at 4°C. Crude cell lysates were then centrifuged at 14,000 rpm for 10 min and cleared lysates were collected. Samples were boiled in 2X Laemmli buffer and fractionated on SDS-PAGE. Membranes were blocked in 5% milk-TBST and then probed with antibodies as indicated.

Table 2.1. **List of yeast strains used in this study.**

Strain Name	Relevant Genotype	Source
	W303-derived strains	
YKL83	*GAL-UBR1 (HIS3)*	[Labib *et al.*, 2000]
ABy778	*GAL-UBR1 (HIS3) pol30::LEU2 [YCplac22-POL30-K107R]*	This Study
ABy010	*GAL-UBR1 (HIS3) bar1::LEU2*	This Study
ABy317	*GAL-UBR1 (HIS3) cdc9::cdc9-td (URA3)*	This Study
ABy780	*GAL-UBR1 (HIS3) cdc9::cdc9-td (URA3) pol30::LEU2 [YCplac22-POL30-K107R]*	This Study
ABy008	*GAL-UBR1 (HIS3) bar1::LEU2 cdc9::cdc9-td (URA3)*	This Study
	SSL204-derived strains	
SSL204	*MATa ade2 his3D200 trp1 leu2 ura3-52*	[Dornfeld and Livingston, 1991]
ABy287	*mrc1::TRP1*	This Study
ABy296	*rad9::TRP1*	This Study
ABy343	*mrc1::TRP1 [pRS316]*	This Study
ABy344	*mrc1::TRP1 [pAO138]*	This Study
ABy345	*mrc1::TRP1 [pMRC1]*	This Study
ABy349	*[YEp105]*	This Study
ABy352	*[pRNR1]*	This Study
ABy356	*mrc1::TRP1 rad9::URA3 [pRNR1]*	This Study
ABy458	*bar1::URA3*	This Study
ABy517	*pol30::URA3 [YCplac22-POL30]*	This Study
ABy669	*[pRS316-POL30]*	This Study

Table 2.1. List of yeast strains used in this study.

ABy685	*pol30::pol30-K107R (LEU2)*	This Study
ABy704	*pol30::pol30-K107R (LEU2) [pRS316-POL30]*	This Study
ABy366	*dna2-1 [YEp105]*	This Study
ABy365	*rad27Δ [YEp105]*	This Study
SSL612	*cdc9-1*	[Ireland *et al.*, 2000]
ABy293	*cdc9-1 mrc1::TRP1*	This Study
ABy297	*cdc9-1 rad9::TRP1*	This Study
ABy346	*cdc9-1 mrc1::TRP1 [pRS316]*	This Study
ABy347	*cdc9-1 mrc1::TRP1 [pAO138]*	This Study
ABy348	*cdc9-1 mrc1::TRP1 [pMRC1]*	This Study
ABy350	*cdc9-1 [YEp105]*	This Study
ABy353	*cdc9-1 [pRNR1]*	This Study
ABy357	*cdc9-1 mrc1::TRP1 rad9::URA3 [pRNR1]*	This Study
ABy375	*cdc9-1 mms2::URA3*	This Study
ABy388	*cdc9-1 ubc13::URA3*	This Study
ABy392	*cdc9-1 rad18::URA3*	This Study
ABy410	*cdc9-1 rad5::URA3*	This Study
ABy520	*cdc9-1 rad6::URA3*	This Study
ABy538	*cdc9-1 ubc4::URA3*	This Study
ABy539	*cdc9-1 ubc5::URA3*	This Study
ABy579	*cdc9-1 ubc5::URA3 ubc4::UBC4-NES-3HA (TRP1)*	This Study

Table 2.1. List of yeast strains used in this study.

ABy439	*cdc9-1 pol30::URA3 [YCplac22-POL30]*	This Study
ABy440	*cdc9-1 pol30::URA3 [YCplac22-POL30-K164R]*	This Study
ABy441	*cdc9-1 pol30::URA3 [YCplac22-POL30-K127R/164R]*	This Study
ABy442	*cdc9-1 pol30::URA3 [YCplac22-POL30-K242R]*	This Study
ABy459	*cdc9-1 pol30::URA3 [YCplac22-POL30-K107R]*	This Study
ABy461	*cdc9-1 pol30::URA3 [YCplac22-POL30-K108R]*	This Study
ABy463	*cdc9-1 pol30::URA3 [YCplac22-POL30-K183R]*	This Study
ABy465	*cdc9-1 POL30::URA3 [YCplac22-POL30-K253R]*	This Study
ABy467	*cdc9-1 POL30::URA3 [YCplac22-POL30-K117R]*	This Study
ABy469	*cdc9-1 POL30::URA3 [YCplac22-POL30-K168R]*	This Study
ABy471	*cdc9-1 POL30::URA3 [YCplac22-POL30-K127R]*	This Study
ABy479	*cdc9-1 [YEp105-Ub-K48,63R]*	This Study
ABy485	*cdc9-1 [YEp105-Ub-K29R]*	This Study
ABy489	*cdc9-1 [YEp105-Ub-KO]*	This Study
ABy497	*cdc9-1 [YEp105-Ub-G75,76A]*	This Study
ABy453	*cdc9-1 bar1::URA3*	This Study
ABy712	*cdc9-1 [pRS316-POL30]*	This Study
ABy777	*cdc9-1 pol30::pol30-K107R (LEU2) [pRS316-POL30]*	This Study
ABY782	*cdc9-1* pol30::pol30-K107R* from tetrad dissection	This Study
SSL613	*cdc9-2*	[Ireland *et al.*, 2000]
ABy351	*cdc9-2 [YEp105]*	This Study

Results

Defects in DNA ligase I trigger mid-S phase arrest.

Previous studies have suggested that DNA ligase I-deficient *cdc9* mutants arrest in G2 phase after completing DNA synthesis [Schiestl *et al.*, 1989; Weinert and Hartwell, 1993] because of the accumulation of nicked DNA [Johnston and Nasmyth, 1978]. These observations implied that S phase proceeds normally despite single-stranded breaks in nascent DNA, suggesting that cells may not be able to efficiently sense this type of DNA damage during S phase. To reinvestigate whether DNA ligase I is required for S phase progression, we have analyzed three different temperature sensitive alleles of *CDC9* (Figure 2.1a and b and Figure 2.2 a and b). One of these alleles is a thermo labile degron mutant [Dohmen *et al.*, 1994] (*cdc9-td* in Figure 2.3a). To ensure that ligase activity was sufficiently inactivated in these cells, we performed replication initiation point mapping and did not detect any measurable ligation of Okazaki fragments over the yeast origin *ARS1* (Figure 2.2c) as previously demonstrated for the *cdc9-1* allele [Bielinsky and Gerbi, 1999]. Whereas DNA ligase I was not required for entry into S phase (Figure 2.3b), we found it to be necessary to release from a hydroxyurea (HU) block (Figure 2.3c). This was also true for two additional temperature sensitive alleles, *cdc9-1* and *cdc9-2* [Ireland *et al.*, 2000] (Figure 2.3d). This result was surprising as earlier reports suggested that DNA ligase I-deficient cells could complete DNA synthesis without joining Okazaki fragments to each other [Schiestl *et al.*, 1989; Weinert and Hartwell, 1993]. As expected, cell cycle delay in S phase was dependent on the DDR gene *RAD9* [Schiestl *et al.*, 1989; Weinert and Hartwell, 1993] because *cdc9-1 rad9Δ*

mutants progressed farther than *cdc9-1* cells (Figure 2.4a). Importantly, however, the mediator of the replication checkpoint (Mrc) 1 appeared to contribute equally to Rad9 (Figure 2.4a and b).

Mrc1 has been shown to have two roles, one in DNA replication and one in activating Rad53 after replication fork stalling, which results in exposure of single-stranded DNA [Osborn and Elledge, 2003]. Rad53 is a downstream target of the mitotic entry checkpoint gene *MEC1*, a homolog of the ATM/ATR checkpoint kinases in humans [Sanchez *et al.*, 1996]. Complementation of *cdc9-1 mrc1Δ* double mutants with the S phase checkpoint-deficient $mrc1^{AQ}$ allele [Osborn and Elledge, 2003] failed to induce cell cycle arrest (Figure 2.4c), suggesting that the S phase checkpoint and not the replication function of Mrc1 is important to delay S phase progression. Furthermore, the finding that both Mrc1 and Rad9 are activated in *cdc9-1* cells at the non-permissive temperature implies that the DNA substrate recognized contains single stranded DNA at stalled replication forks as well as physical damage, which may have arisen from the lack of Okazaki fragment ligation [Johnston and Nasmyth, 1978].

PCNA is ubiquitinated in *S. cerevisiae cdc9* mutants.

Besides triggering a checkpoint response, certain types of DNA damage at replication forks have also been shown to cause ubiquitination of PCNA [Moldovan *et al.*, 2007]. PCNA can be either mono-ubiquitinated or poly-ubiquitinated. Mono-ubiquitination of PCNA triggers the error-prone repair pathway through translesion polymerases, whereas PCNA poly-ubiquitination is needed for error-free repair

[Haracska *et al.*, 2004; Hoege *et al.*, 2002; Stelter and Ulrich, 2003]. Mono-ubiquitination depends on Rad6 and Rad18 and is a pre-requisite for poly-ubiquitination, which in turn is mediated by the ubiquitin conjugating complex Ubc13/Mms2 and Rad5 [Hoege *et al.*, 2002]. Interestingly, ubiquitin is linked through lysine 63 in these poly-ubiquitin chains [Hoege *et al.*, 2002; Hofmann and Pickart, 1999].

To explore whether loss of DNA ligase I leads to PCNA ubiquitination, we examined the status of PCNA in whole cell extracts. We utilized an antibody specific for yeast PCNA [Zhang *et al.*, 2000], which displays multiple non-specific bands in undiluted extracts (Figure 2.5), but produces clean immunoblots with diluted extracts (Figure 2.6a). Both *cdc9-1* and *cdc9-2* mutants exhibited a modified form of PCNA of approximately 39 kDa when shifted to the non-permissive temperature (Figure 2.6a). Co-immunoprecipitation (Co-IP) experiments with strains that expressed Myc-tagged ubiquitin [Das-Bradoo *et al.*, 2006; Ellison and Hochstrasser, 1991] identified this 39 kDa band as ubiquitinated PCNA, which we did not observe when we mixed cell extracts only with beads (Figure 2.6b). Curiously, we observed a non-specific band slightly above the 49 kDa marker, which was especially obvious in extracts from *cdc9-1* cells. Unfortunately, our co-IP studies did not allow us to draw any conclusions about the nature of this band, although we cannot exclude that it represents poly-ubiquitinated PCNA sticking non-specifically to the beads. Therefore, we overexpressed different ubiquitin mutants, including a G75,76A double mutant, specifically designed to interfere with mono-ubiquitination. Another mutant carried substitutions in all seven lysines (Ub-KO) and was thus defective in forming poly-ubiquitin chains. Analysis of trichloroacetic

acid-precipitated whole cell extracts confirmed that the 39 kDa form of PCNA represented mono-ubiquitinated protein (Figure 2.6c). Furthermore, two forms of PCNA that represented poly-ubiquitinated protein (at ~52- and 76 kDa), because they disappeared in cells overexpressing the Ub-KO mutant, were visible (Figure 2.6d). A distinct ladder of ubiquitinated PCNA with similar molecular weight distribution has also been reported by van der Kemp et al. [van der Kemp *et al.*, 2009]. Surprisingly, poly-ubiquitin chains on PCNA were not linked through K63 [Hofmann and Pickart, 1999], as they are in response to other forms of replication stress [Hoege *et al.*, 2002], but rather through K29 (Figure 2.6d).

PCNA ubiquitination in *cdc9* mutants requires Ubc4, Mms2 and Rad5.

Based on this result, we predicted that at least some of the genes that are known to play a role in K63 linked poly-ubiquitination (namely *RAD6*, *RAD18*, *MMS2*, *UBC13*, *RAD5*) [Hoege *et al.*, 2002] are dispensable for the ubiquitination of PCNA in *cdc9-1* mutants. Indeed, whereas *MMS2* and *RAD5* were required for mono- as well as poly-ubiquitination, *UBC13* and *RAD18* were not (Figure 2.7a and b). Moreover, deletion of *RAD6* did not have any effect (Figure 2.7c), suggesting that PCNA ubiquitination in response to Okazaki fragment ligation defects differs from that triggered by other types of DNA damage. Our results also indicated that Mms2 likely cooperates with other ubiquitin conjugating enzymes besides Ubc13 [Hofmann and Pickart, 1999].

To determine Mms2's potential partner in this reaction, we took advantage of the fact that lysine 29 linkages are catalyzed by UbcH5A in mammalian cells [Wang and

Pickart, 2005]. UbcH5A's homolog in yeast is Ubc4, which is 93% identical to Ubc5 [Seufert and Jentsch, 1990]. Unlike Ubc4, which is highly expressed in cycling cells, steady state levels of Ubc5 are low and are upregulated in stationary phase [Seufert and Jentsch, 1990]. Ubc4 and 5 have previously been implicated in the degradation of unfolded proteins in yeast [Seufert and Jentsch, 1990], but not in PCNA ubiquitination. When we deleted *UBC4* from *cdc9-1* mutants and performed a temperature shift experiment, PCNA ubiquitination was drastically reduced, whereas deletion of *UBC5* had no effect (Figure 2.8a). Because we were unable to generate an *ubc4Δubc5Δ* double mutant in the *cdc9-1* background, we attached a nuclear export sequence onto *UBC4* in a *cdc9-1 ubc5Δ* strain. Again, this resulted in a 95% reduction of PCNA mono-ubiquitination (Figure 2.8b). These results suggested to us that the nuclear fraction of Ubc4 cooperates with Mms2 and Rad5 to ubiquitinate PCNA in DNA ligase I-deficient cells.

Mms2, but not Ubc13, is required for S phase checkpoint activation in *cdc9* mutants.

To further discern the function of PCNA ubiquitination in DNA ligase I-deficient yeast, we monitored cell cycle progression upon release from G1. Specifically, we compared *cdc9-1* cells to *cdc9-1 mms2Δ* and *cdc9-1 ubc13Δ* double mutants. Consistent with our finding that *UBC13* is not involved in the ubiquitination of PCNA (Figure 2.7a), *cdc9-1 ubc13Δ* cells arrested in S phase similar to the *cdc9-1* strain (Figure 2.9a). In contrast, *cdc9-1 mms2Δ* mutants that fail to ubiquitinate PCNA at the non-permissive

temperature (Figure 2.7a), readily progressed through S phase (Figure 2.9a). The observed lack of PCNA ubiquitination and S phase arrest went hand-in-hand with the inability to phosphorylate Rad53 (Figure 2.9b). Importantly, this was not due to a general defect in checkpoint activation, as we detected Rad53 phosphorylation in all three strains after exposure to MMS (Figure 2.9c).

To address whether mono-ubiquitination is sufficient for Rad53 activation, we disrupted poly-ubiquitination by overexpressing a ubiquitin mutant in which lysine 29 was substituted with arginine. This did not significantly affect Rad53 activation (Figure 2.10a and b). These results argue that mono- rather than poly-ubiquitination of PCNA is a prerequisite for checkpoint activation of DNA ligase I-deficient yeast cells.

PCNA ubiquitination occurs at Lysine 107 and is a prerequisite for Rad53 activation in *cdc9* mutants.

Next, we set out to determine which lysine in PCNA was ubiquitinated. PCNA has a total of 18 lysines, nine of which are exposed and easily accessible [Pettersen *et al.*, 2004]. We mutated these nine lysines at positions 107, 108, 117, 127, 164, 168, 183, 242 and 253. Wild-type or mutated forms of PCNA were introduced on a plasmid before the endogenous copy was deleted. When we monitored mono-ubiquitination of PCNA in *cdc9-1* cells after they were shifted to the non-permissive temperature, the only mutant that lacked the characteristic 39 kDa band was *pol30K107R* (Figure 2.11a). However, further examination revealed that this particular mutant had a second site suppressor mutation, which resulted in elevated DNA ligase I protein levels that were comparable to

those in wild-type cells (Figure 2.11b). Moreover, the strain was no longer temperature sensitive (Figure 2.11c). We attempted several times to make the double mutant but these efforts remained unsuccessful, suggesting that the mutations were synthetically lethal.

To prove this point, we performed a plasmid shuffle assay. We introduced *pol30K107R* into the chromosomal copy of PCNA in wild-type and *cdc9-1* mutants, and expressed *POL30* from a plasmid. Cells were forced to abandon the plasmid by re-streaking them onto 5-fluoroorotic acid-containing medium. Whereas *CDC9 pol30K107R* cells were viable and formed colonies, *cdc9-1 pol30K107R* mutants did not, indicating that K107 of PCNA becomes essential in this background (Figure 2.12). This was further confirmed by tetrad analysis (data not shown), which seemingly yielded viable double mutants, but all of these strains had elevated DNA ligase I levels due to a second site suppressor mutation (data not shown). However, we were able to isolate a single double mutant in which Cdc9 levels were only slightly increased compared to *cdc9-1* cells (Figure 2.13a). We designated this mutant *cdc9-1* pol30K107R*. In fact, the double mutant retained its temperature sensitivity (Figure 2.13b) and failed to ubiquitinate PCNA under non-permissive conditions (Figure 2.14a). Importantly, PCNA ubiquitination in response to MMS was still functional (Figure 2.14b).

At this point, it is worthwhile to point out that we detected ubiquitinated and unmodified PCNA at an apparent ratio of approximately 1:1. However, it is not clear that the antibody binds these two forms of PCNA with equal affinity, and therefore it is difficult to make quantitative assessments. In addition, we take the fact that we could

readily generate *cdc9-1 mms2Δ* and *cdc9-1 rad5Δ*, but not *cdc9-1 pol30K107R* double mutants as an indication that a redundant ubiquitination pathway might exist that targets K107 with low efficiency, thereby ensuring cell survival.

To confirm that K107 is the target of PCNA ubiquitination in DNA ligase I-deficient cells, we introduced the *pol30K107* mutation into *cdc9-td* cells. As expected, *cdc9-td pol30K107R* double mutants retained viability, because *CDC9* is expressed from an inducible copper promoter [Dohmen *et al.*, 1994]. Under non-permissive conditions, the ratio of mono-ubiquitinated to unmodified PCNA in *cdc9-td pol30K107R* cells resembled that of the DNA ligase I-proficient control strain, which carries the same mutation in *POL30* (Figure 2.14c). The same was true for poly-ubiquitinated PCNA (Figure 2.14d). Furthermore, *cdc9-td pol30K107R* cells failed to exhibit robust Rad53 phosphorylation in the absence of DNA ligase I, whereas they displayed Rad53 activation in response to MMS (Figure 2.14e). From these results we conclude that ubiquitination of PCNA at lysine 107 is a prerequisite for checkpoint activation of DNA ligase I-deficient yeast cells. This is in stark contrast to the previously described ubiquitination of PCNA at lysine 164 in response to UV irradiation or DNA alkylating agents [Hoege *et al.*, 2002]. Although exposure to UV light or DNA alkylating agents triggers both Rad53 phosphorylation and ubiquitination of PCNA at lysine 164, these processes are thought to occur independently and belong to separate genetic pathways [Frampton *et al.*, 2006]. We demonstrate that this cannot be generalized, but that PCNA ubiquitination in response to DNA ligase I deficiency must occur before Rad53 can be fully activated.

PCNA is ubiquitinated in DNA ligase I-deficient human cells.

It is worth noting that K107 is conserved in *S. pombe* and *C. elegans*, but higher eukaryotes have a conserved lysine residue at position 110, raising the possibility that this PCNA ubiquitination pathway is not only specific for *S.cerevisiae* (Figure 2.15). To test whether human cells depleted for DNA ligase I exhibit PCNA modifications, we generated several U2OS cell lines expressing short hairpin RNAs (shRNAs) that interfere with DNA ligase I expression (Figure 2.16a). In the chromatin-bound protein fractions of these cells, we detected a slower migrating form of PCNA with the same mobility of mono-ubiquitinated PCNA induced by UV irradiation [Kannouche *et al.*, 2004] (Figure 2.16b and c, upper pannel). In contrast, cells expressing control shRNA behaved like untreated U2OS cells (Figure 2.16b). Importantly, when we analyzed one representative DNA ligase I-specific shRNA clone side by side with a PCNA- or an ubiquitin-specific antibody, both antibodies recognized bands at the exact same position, consistent with the notion that PCNA ubiquitination in response to DNA ligase I-deficiency is conserved in humans, although the lysine residue of PCNA that is ubiquitinated in these cells is still under investigation (Figure 2.16b, upper and middle panel).

PCNA ubiquitination at Lys107 in *cdc9* mutants may be specific to DNA ligase I deficiency.

In the course of our studies, we also explored *rad27Δ* and *dna2-1* mutants in *S. cerevisiae*, because both Rad27 and Dna2 have been implicated in lagging strand synthesis [Kao *et al.*, 2004]. However, these mutants did not ubiquitinate PCNA (Figure

2.17). This suggested that PCNA ubiquitination at K107 might be specific for DNA ligase I-deficiency. In summary, whereas ubiquitination at lysine 164 is responsible for activation of translesion synthesis, and is thus a result of DNA damage in the template strand, ubiquitination of lysine 107 in DNA ligase I-deficient cells might provide a signal for DNA damage residing in freshly synthesized, nascent DNA. Most importantly, our study demonstrates that PCNA provides a DNA damage-specific code via the ubiquitination of different lysines.

Figure 2.1

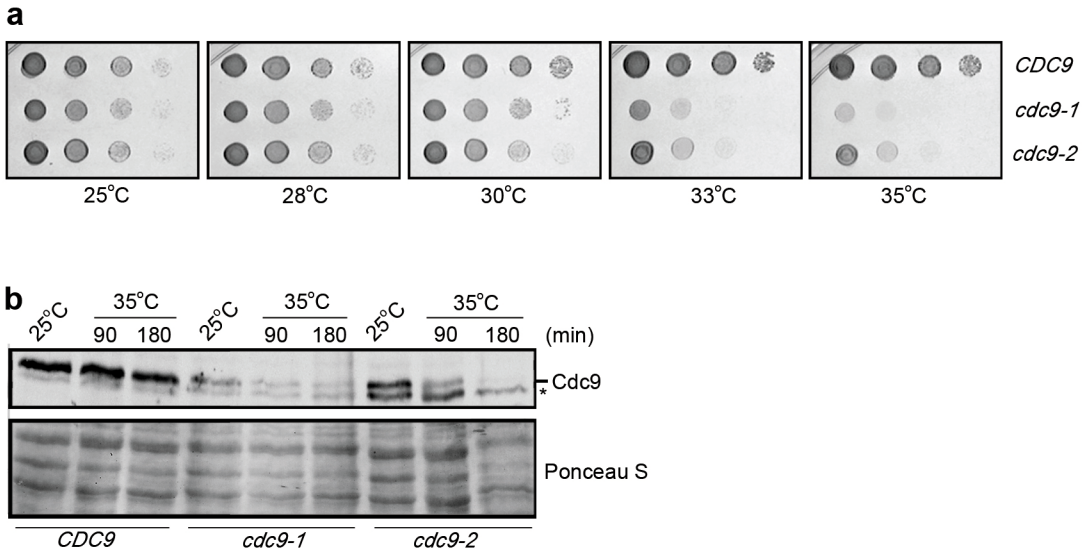


Figure 2.1 Temperature sensitivity of DNA ligase I alleles.

(a) SSL204 (*CDC9*), SSL612 (*cdc9-1*) and SSL613 (*cdc9-2*) cells were spotted in ten-fold serial dilutions on YPD plates and incubated at the indicated temperatures for 2 days. **(b)** Asynchronous cultures of SSL204 (*CDC9*), SSL612 (*cdc9-1*) and SSL613 (*cdc9-2*) were grown at 25°C and shifted to the restrictive temperature of 35°C for 90 or 180 min. Total protein was TCA precipitated [Ricke and Bielinsky, 2006]. Cdc9 was detected with an anti-Cdc9 antibody. Ponceau S staining of the membrane prior to blotting served as a loading control. The asterisk denotes a degradation product.

Figure 2.2

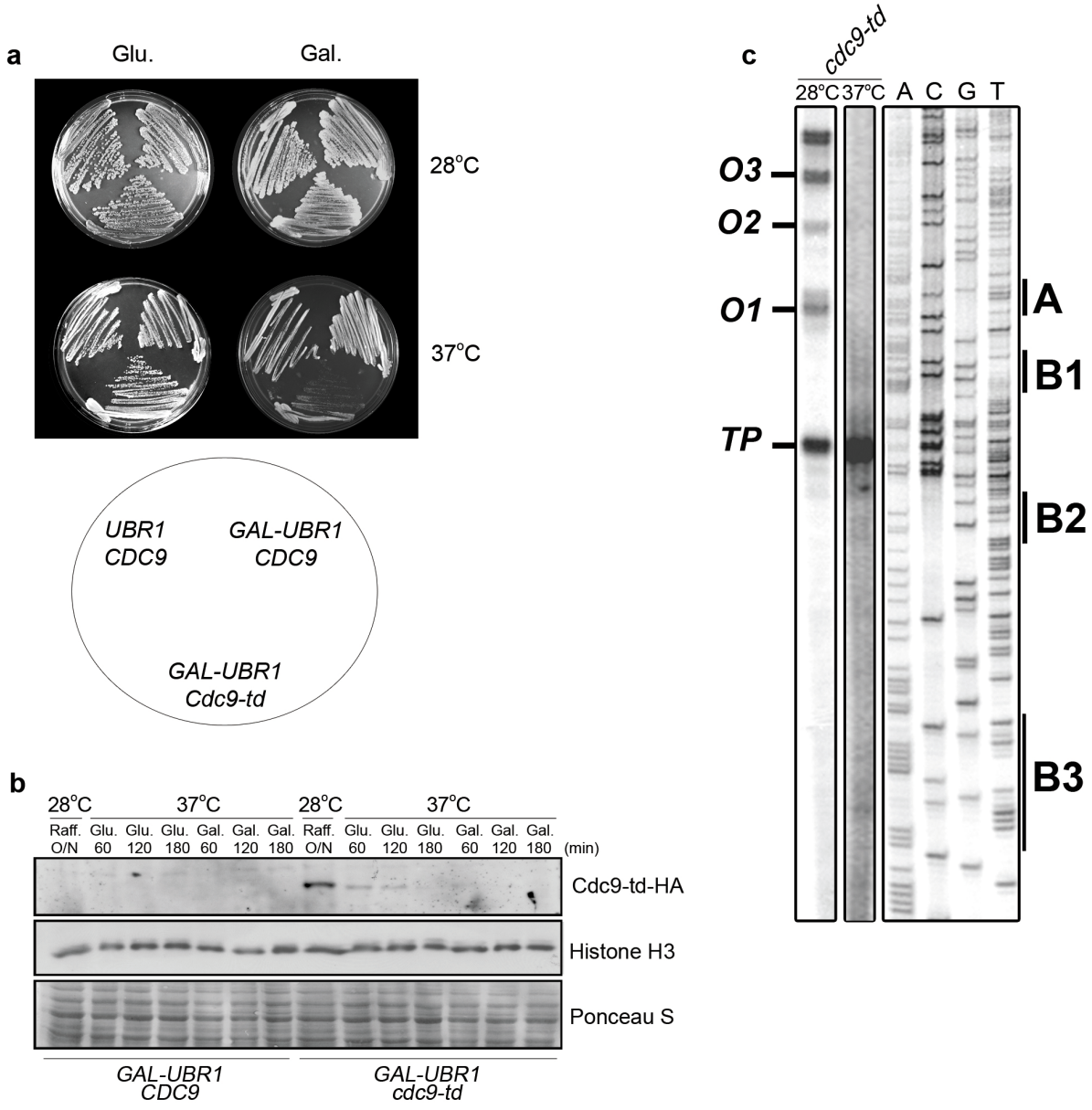

a
Glu.
Gal.
28°C
37°C
UBR1
CDC9
GAL-UBR1
CDC9
GAL-UBR1
Cdc9-td
b
28°C
37°C
28°C
37°C
Raff. O/N
Glu. 60
Glu. 120
Glu. 180
Gal. 60
Gal. 120
Gal. 180
Raff. O/N
Glu. 60
Glu. 120
Glu. 180
Gal. 60
Gal. 120
Gal. 180
(min)
Cdc9-td-HA
Histone H3
Ponceau S
GAL-UBR1
CDC9
GAL-UBR1
cdc9-td
c
cdc9-td
28°C
37°C
A
C
G
T
O3
O2
O1
TP
A
B1
B2
B3

Figure 2.2 *cdc9-td* cells lack DNA ligase I at elevated temperatures.

(a) ABy021 (*UBR1 CDC9),* ABy010 (*GAL-UBR1 CDC9)*, and ABy008 (*GAL-UBR1 cdc9-td*) cells were streaked onto complete medium containing either 2% glucose (Glu.) or 2% galactose (Gal.) as the sole carbon source. The plates were incubated for two days at 28°C or 37°C as noted. **(b)** Asynchronous cultures of ABy010 (*GAL-UBR1 CDC9)* and ABy008 (*GAL-UBR1 cdc9-td*) were grown overnight at 28°C in YP plus raffinose medium supplemented with 10 μM $CuSO_4$. At OD_{600} = 0.6, the culture was split, centrifuged and resuspended in either YP (Glu.) or YP plus galactose (Gal.) and incubated at 37°C. Samples were collected at 1, 2 or 3 hours. Cdc9-td-HA and histone H3 were detected by immunoblotting with anti-HA (16B12, Covance) and anti-histone H3 (Abcam) antibodies, respectively. Ponceau S staining served as an additional loading control. **(c)** DNA was isolated from ABy317 cells (*GAL-UBR1 cdc9-td*) grown asynchronously at 28°C. In parallel, cells were arrested in G1 with alpha-factor. Following arrest, cultures were released and shifted to 37°C in the presence of galactose. RIP mapping was performed at the replication origin *ARS1* using the primer ARS1/630 as described previously [Bielinsky and Gerbi, 1999]. The transition point (TP) maps between elements B1 and B2. Okazaki fragment initiation sites visible at 28°C but not at 37°C (in the absence of DNA ligase I) are indicated by O1, O2 and O3. Sequencing reactions were fractionated adjacent to the primer extension reactions on a 6% denaturing polyacrylamide gel.

Figure 2.3

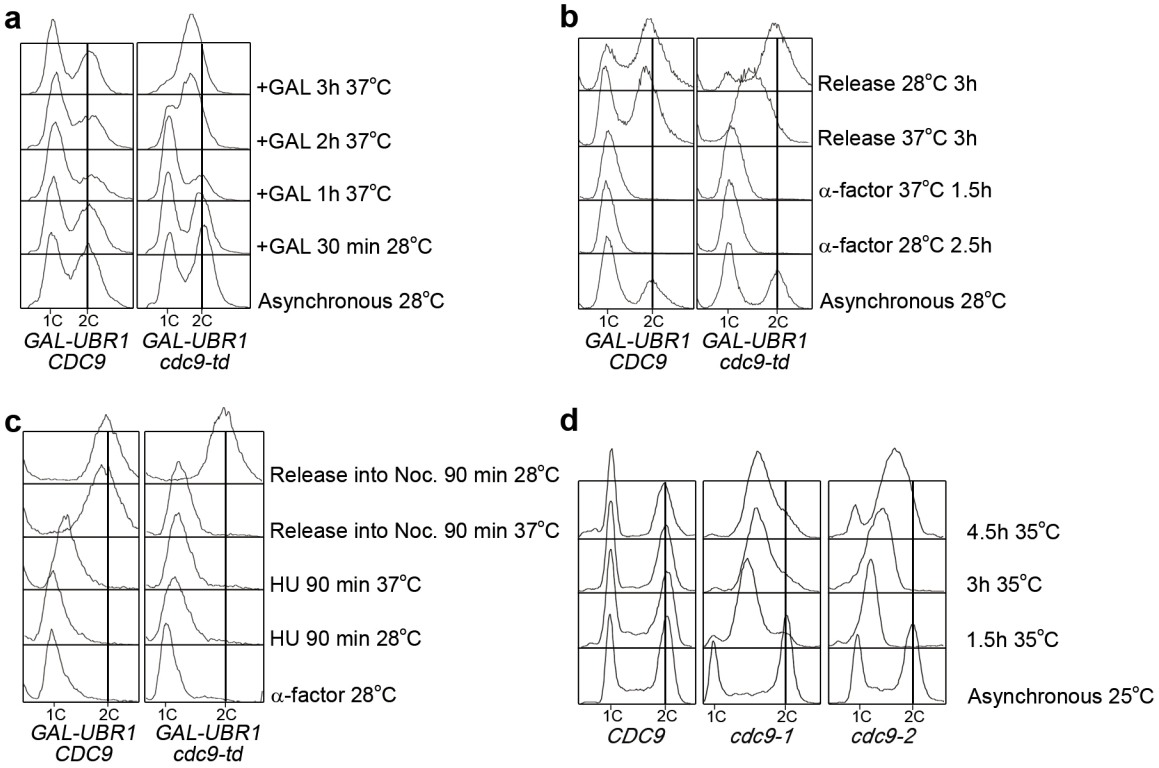

a
+GAL 3h 37°C
+GAL 2h 37°C
+GAL 1h 37°C
+GAL 30 min 28°C
Asynchronous 28°C
1C 2C
GAL-UBR1 CDC9
GAL-UBR1 cdc9-td
b
Release 28°C 3h
Release 37°C 3h
α-factor 37°C 1.5h
α-factor 28°C 2.5h
Asynchronous 28°C
1C 2C
GAL-UBR1 CDC9
GAL-UBR1 cdc9-td
c
Release into Noc. 90 min 28°C
Release into Noc. 90 min 37°C
HU 90 min 37°C
HU 90 min 28°C
α-factor 28°C
1C 2C
GAL-UBR1 CDC9
GAL-UBR1 cdc9-td
d
4.5h 35°C
3h 35°C
1.5h 35°C
Asynchronous 25°C
1C 2C
CDC9
cdc9-1
cdc9-2

Figure 2.3 DNA ligase I is required for S phase progression.

(a) Asynchronous cultures of ABy010 (*GAL-UBR1 CDC9*) and ABy008 (*GAL-UBR1 cdc9-td*) were induced with galactose at 28°C for 30 min and subsequently shifted to 37°C. **(b)** Strains were arrested in G1 phase at 28°C and shifted to 37°C in 2% galactose and α-factor. After 90 min, cells were released from G1 phase either at 37°C in galactose or at 28°C in glucose. **(c)** Strains were arrested in G1 and released into S phase at 28°C in the presence of HU. Once arrested in S phase, cultures were shifted to 37°C in the presence of 2% galactose and HU. After 90 min, cells were transferred into nocodazole either at 37°C or 28°C. **(d)** Asynchronous cultures of SSL204 (*CDC9*), SSL612 (*cdc9-1*), and SSL613 (*cdc9-2*) were shifted to 35°C. In **a-d**, DNA content was monitored by flow cytometry and the vertical line indicates a 2C DNA content.

Figure 2.4

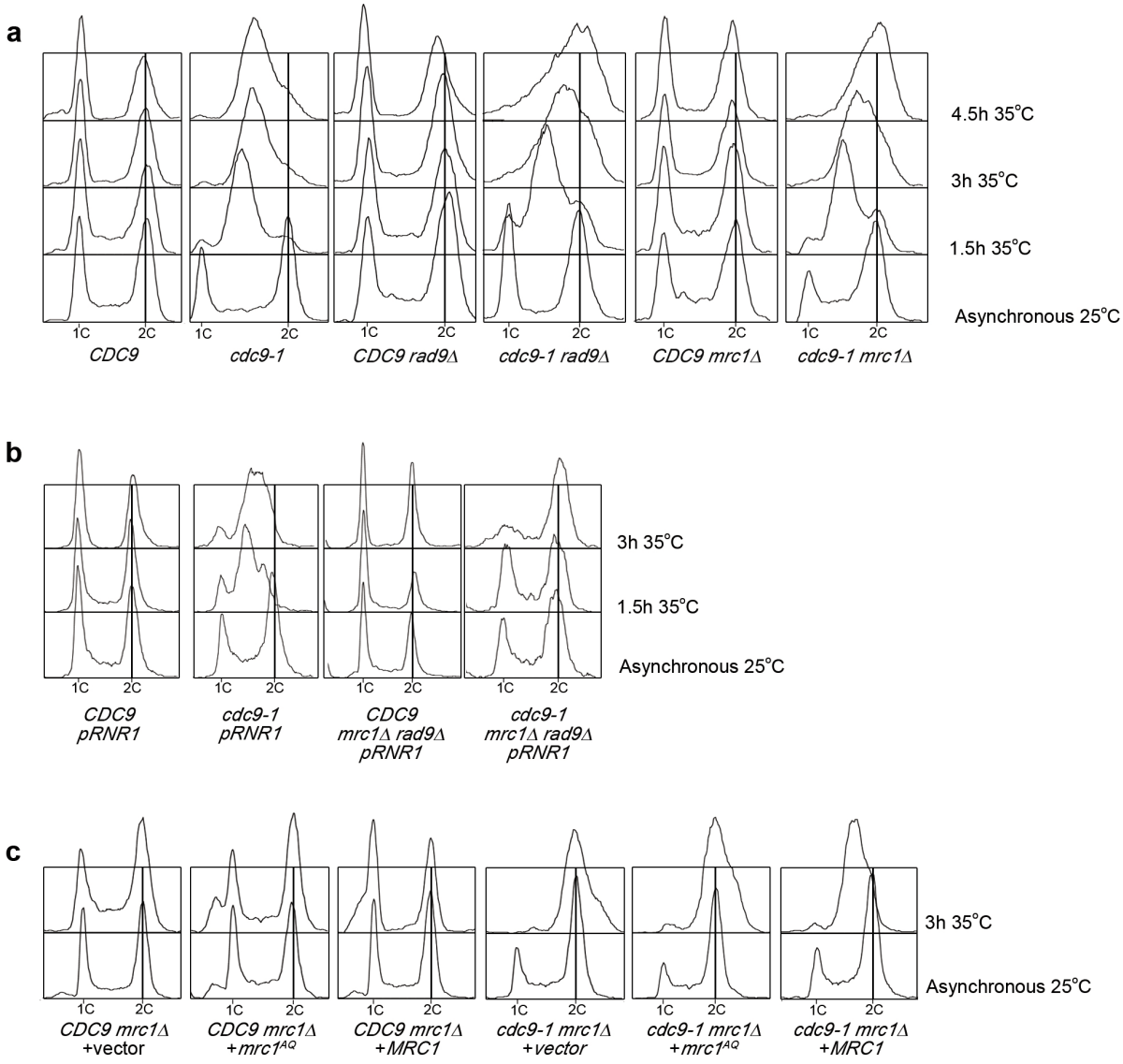

a
4.5h 35°C
3h 35°C
1.5h 35°C
Asynchronous 25°C
1C
2C
CDC9
cdc9-1
CDC9 rad9Δ
cdc9-1 rad9Δ
CDC9 mrc1Δ
cdc9-1 mrc1Δ
b
3h 35°C
1.5h 35°C
Asynchronous 25°C
CDC9 pRNR1
cdc9-1 pRNR1
CDC9 mrc1Δ rad9Δ pRNR1
cdc9-1 mrc1Δ rad9Δ pRNR1
c
3h 35°C
Asynchronous 25°C
CDC9 mrc1Δ +vector
CDC9 mrc1Δ +mrc1AQ
CDC9 mrc1Δ +MRC1
cdc9-1 mrc1Δ +vector
cdc9-1 mrc1Δ +mrc1AQ
cdc9-1 mrc1Δ +MRC1

Figure 2.4 Deletion of *MRC1* or *RAD9* abrogates the S phase arrest in *cdc9* mutants.

(a) Asynchronous cultures of SSL204 (*CDC9*), SSL612 (*cdc9-1*), ABy296 (*CDC9 rad9Δ*), ABy297 (*cdc9-1 rad9Δ*), ABy287 (*CDC9 mrc1Δ*) and ABy293 (*cdc9-1 mrc1Δ*) were grown at 25°C and shifted to the restrictive temperature of 35°C for 1.5, 3 or 4.5 h. DNA was stained with Sytox Green and monitored using flow cytometry. **(b)** Asynchronous cultures of ABy352 (*CDC9 pRNR1*), ABy353 (*cdc9-1 pRNR1*), ABy356 (*CDC9 mrc1Δ rad9Δ pRNR1*) and ABy357 (*cdc9-1 mrc1Δ rad9Δ pRNR1*) were grown at 25°C and shifted to the restrictive temperature of 35°C for the indicated times. DNA was stained with Sytox Green and monitored using flow cytometry. **(c)** Asynchronous cultures of ABy343 (*CDC9 mrc1Δ* +vector), ABy344 (*CDC9 mrc1Δ* +*mrc1*^{AQ}), ABy345 (*CDC9 mrc1Δ* +*MRC1*), ABy346 (*cdc9-1 mrc1Δ* +vector), ABy347 (*cdc9-1 mrc1Δ* +*mrc1*^{AQ}), and ABy348 (*cdc9-1 mrc1Δ* +*MRC1)* were grown at 25°C and shifted to the restrictive temperature of 35°C for 3 h. DNA was stained with Sytox Green and monitored using flow cytometry. In **a-c**, the vertical line indicates a 2C DNA content.

Figure 2.5

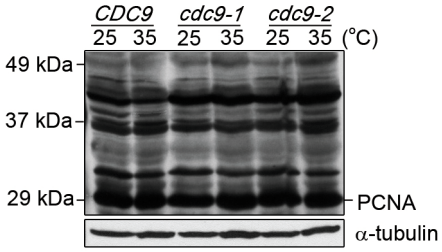


Figure 2.5 Detection of robust PCNA signal in *cdc9* mutants using a yeast specific PCNA antibody.

Asynchronous cultures were grown at 25°C and shifted to 35°C for 3 h. Total protein was TCA-precipitated and PCNA was detected using a yeast specific PCNA antibody (S871) [Zhang *et al.*, 2000]. α-tubulin served as a loading control.

Figure 2.6

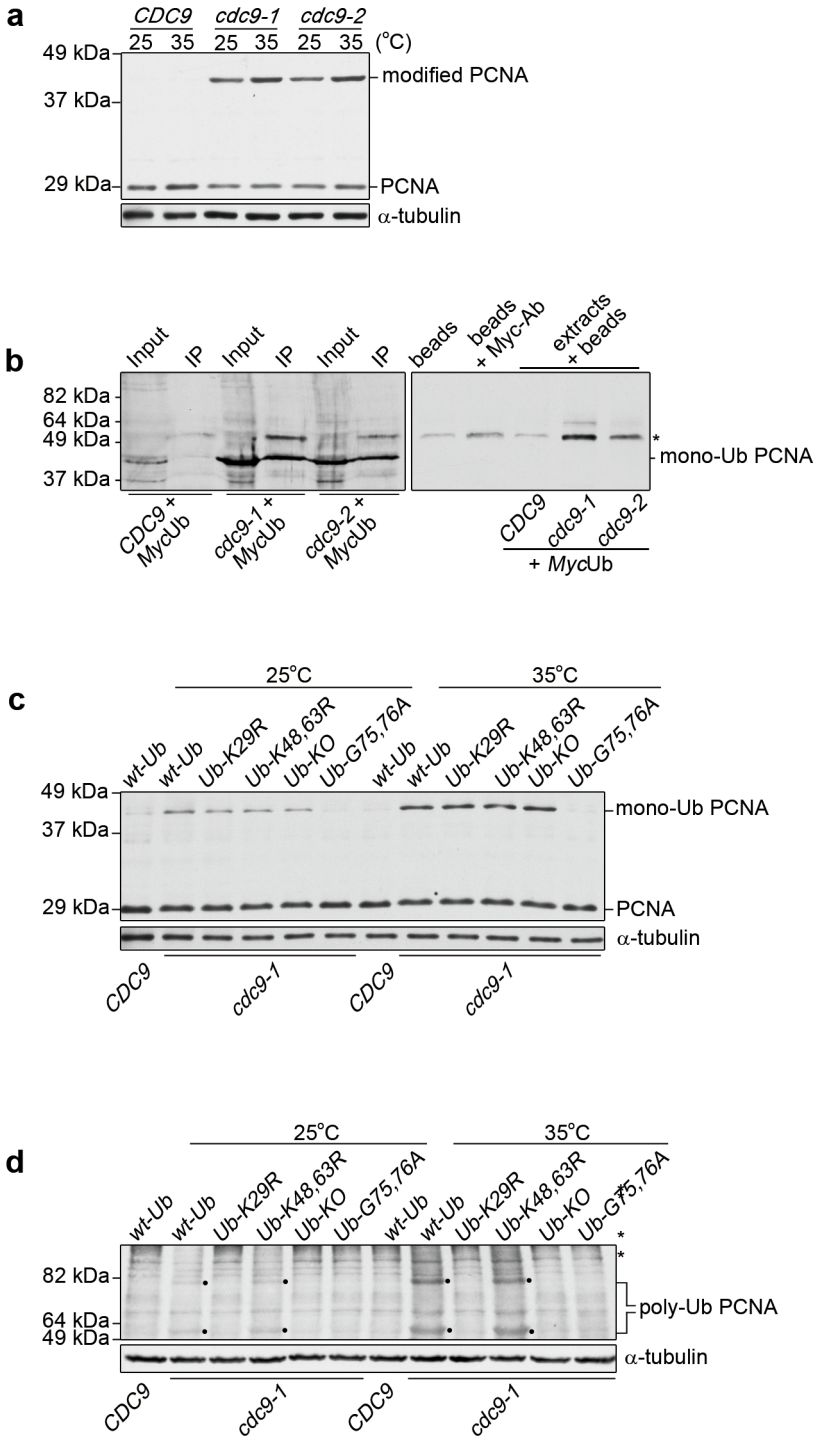

a
CDC9
cdc9-1
cdc9-2
25 35 25 35 25 35 (°C)
49 kDa
37 kDa
29 kDa
modified PCNA
PCNA
α-tubulin
b
Input
IP
Input
IP
Input
IP
beads
beads + Myc-Ab
extracts + beads
82 kDa
64 kDa
49 kDa
37 kDa
*
mono-Ub PCNA
CDC9 + MycUb
cdc9-1 + MycUb
cdc9-2 + MycUb
CDC9
cdc9-1
cdc9-2
+ MycUb
c
25°C
35°C
wt-Ub
wt-Ub
Ub-K29R
Ub-K48,63R
Ub-KO
Ub-G75,76A
wt-Ub
wt-Ub
Ub-K29R
Ub-K48,63R
Ub-KO
Ub-G75,76A
49 kDa
37 kDa
29 kDa
mono-Ub PCNA
PCNA
α-tubulin
CDC9
cdc9-1
CDC9
cdc9-1
d
25°C
35°C
wt-Ub
wt-Ub
Ub-K29R
Ub-K48,63R
Ub-KO
Ub-G75,76A
wt-Ub
wt-Ub
Ub-K29R
Ub-K48,63R
Ub-KO
Ub-G75,76A
*
*
82 kDa
64 kDa
49 kDa
poly-Ub PCNA
α-tubulin
CDC9
cdc9-1
CDC9
cdc9-1

Figure 2.6 S. *cerevisiae* PCNA is mono-ubiquitinated in *cdc9* mutants.

(a) Asynchronous cultures of SSL204 (*CDC9*), SSL612 (*cdc9-1*), and SSL613 (*cdc9-2*) were shifted to 35°C for 3 h. Total protein was TCA-precipitated, diluted and probed with a yeast specific PCNA antibody (S871). **(b)** Cells were shifted to 35°C for 3 h in the presence of copper sulfate to induce expression of Myc-tagged ubiquitin. Whole cell extracts were immunoprecipitated with an anti-Myc antibody and PCNA was detected with anti-PCNA antibody (S871). The right panel shows the beads control with or without the cell lysate. 5% of the input was loaded. **(c)** PCNA is mono-ubiquitinated in *cdc9* mutants. Wild-type or mutant ubiquitin expression was induced in asynchronously growing cultures. Total protein was TCA-precipitated and diluted. Unmodified and mono-ubiquitinated PCNA was detected by PCNA antibody (S871). **(d)** PCNA poly-ubiquitination is linked through lysine 29 in *cdc9* mutants. Undiluted TCA-precipitated protein samples from **c** were probed with anti-PCNA antibody (S871) to detect poly-ubiquitinated PCNA. In **b, d,** asterisks indicate non-specific bands. In **d,** filled circles (right side of the band) indicate poly-ubiquitinated forms of PCNA. In **a, c, d,** α-tubulin served as a loading control.

Figure 2.7

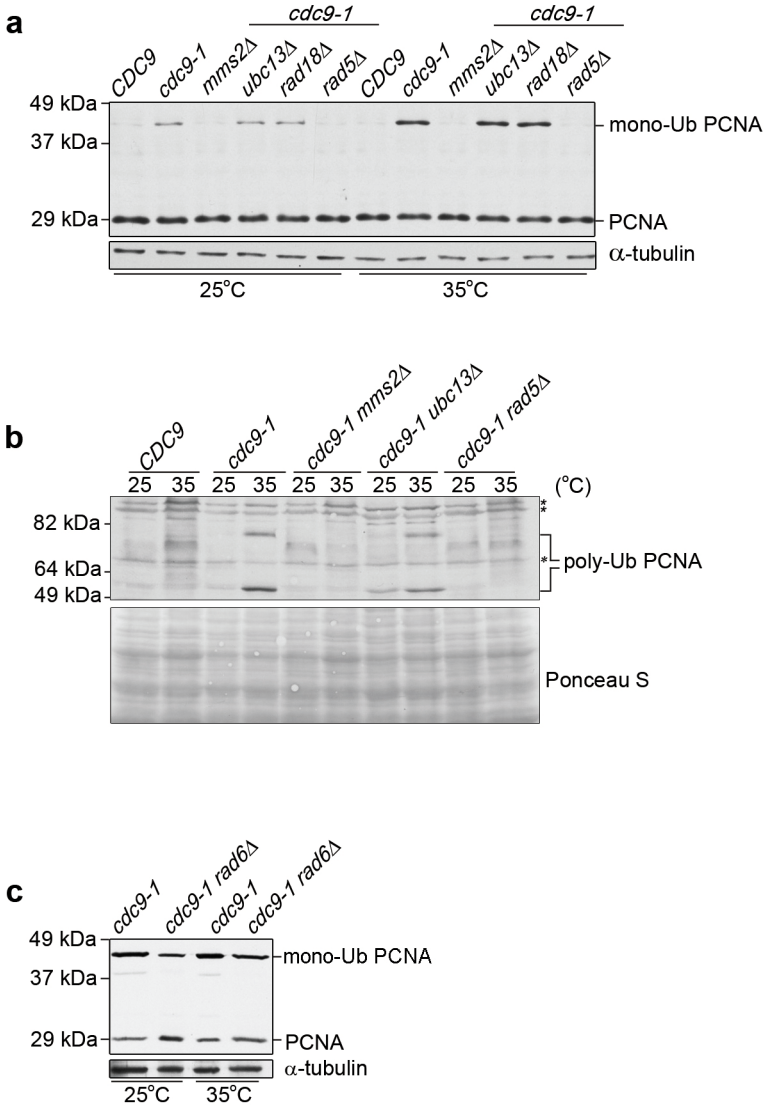

a
cdc9-1
cdc9-1
CDC9
cdc9-1
mms2Δ
ubc13Δ
rad18Δ
rad5Δ
CDC9
cdc9-1
mms2Δ
ubc13Δ
rad18Δ
rad5Δ
49 kDa
37 kDa
29 kDa
mono-Ub PCNA
PCNA
α-tubulin
25°C
35°C
b
CDC9
cdc9-1
cdc9-1 mms2Δ
cdc9-1 ubc13Δ
cdc9-1 rad5Δ
25 35 25 35 25 35 25 35 25 35 (°C)
82 kDa
64 kDa
49 kDa
**
*
poly-Ub PCNA
Ponceau S
c
cdc9-1
cdc9-1 rad6Δ
cdc9-1
cdc9-1 rad6Δ
49 kDa
37 kDa
29 kDa
mono-Ub PCNA
PCNA
α-tubulin
25°C
35°C

Figure 2.7 PCNA ubiquitination in *cdc9* mutants is mediated by Mms2, Rad5 and Ubc4 but not Ubc13.

(a, b) SSL204 (*CDC9*), SSL612 (*cdc9-1*), ABy375 (*cdc9-1 mms2Δ*), ABy388 (*cdc9-1 ubc13Δ*), ABy410 (*cdc9-1 rad5Δ*) were grown asynchronously and shifted to non-permissive temperature of 35°C for 3 h. **(c)** Asynchronous cultures of SSL612 (*cdc9-1*) and ABy520 (*cdc9-1 rad6Δ*) were grown overnight at 25°C to a density of 0.6 at 600 nm. Cultures were shifted to the non-permissive temperature of 35°C for 3 h. In **a-c,** total protein was TCA-precipitated. Samples were fractionated on SDS-PAGE gels with either diluted extracts (in **a**, **c**) or undiluted extracts (in **b**). Unmodified and mono-ubiquitinated PCNA was detected in **a**, **c** whereas poly-ubiquitinated PCNA was detected in **b** using PCNA antibody (S871). The asterisks indicate nonspecific bands. α-tubulin and Ponceau S served as a loading control.

Figure 2.8

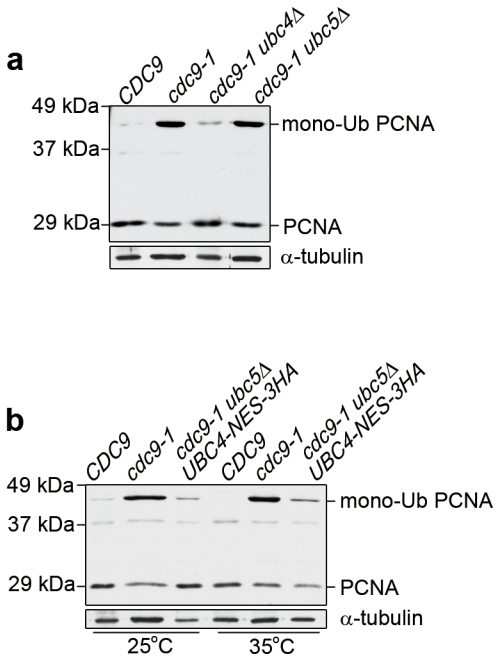


Figure 2.8 PCNA mono-ubiquittination in *cdc9* mutants is mediated by E2 ubiquitin conjugating enzyme, Ubc4.

(a) Asynchronous cultures of SSL204 (*CDC9*), SSL612 (*cdc9-1*), ABy538 (*cdc9-1 ubc4Δ*), and ABy539 (*cdc9-1 ubc5Δ*) were shifted to 35°C for 3 h. **(b)** Asynchronous cultures of SSL204 *(CDC9)*, SSL612 (*cdc9-1*) and ABy579 (*cdc9-1 ubc5Δ UBC4-NES-3HA*) were grown at 25°C and shifted to 35°C for 3 h. In **a** and **b**, total protein was TCA-precipitated, diluted and PCNA was detected by anti-PCNA antibody (S871) [Zhang *et al.*, 2000]. α-tubulin served as a loading control.

Figure 2.9

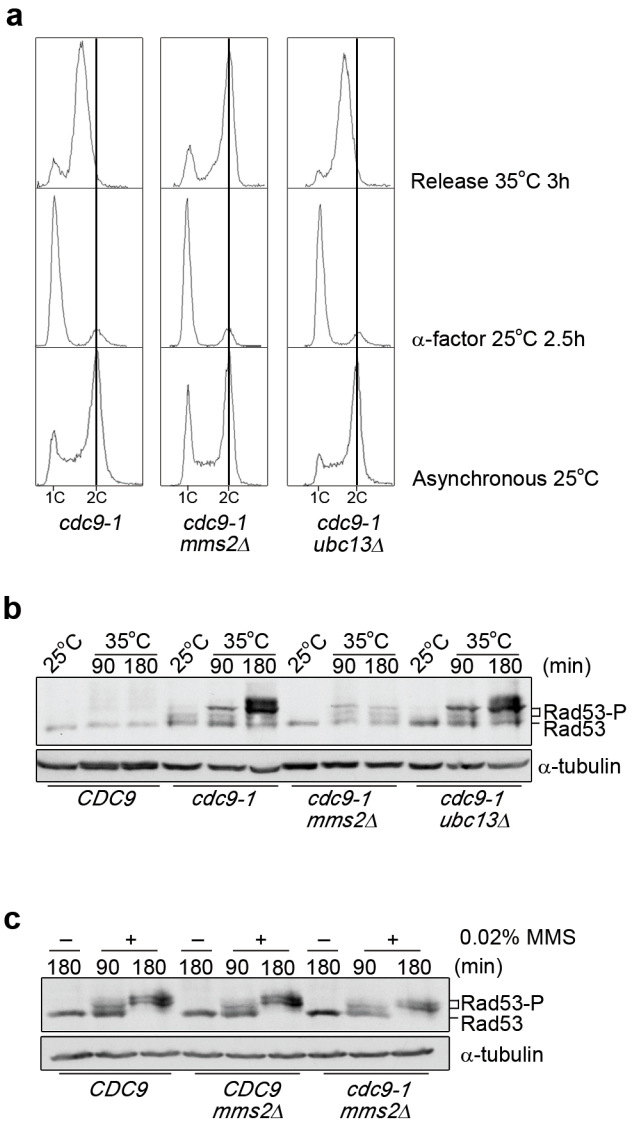

a
Release 35°C 3h
α-factor 25°C 2.5h
Asynchronous 25°C
1C 2C
cdc9-1
cdc9-1 mms2Δ
cdc9-1 ubc13Δ
b
25°C 35°C 25°C 35°C 25°C 35°C 25°C 35°C
90 180 90 180 90 180 90 180 (min)
Rad53-P
Rad53
α-tubulin
CDC9
cdc9-1
cdc9-1 mms2Δ
cdc9-1 ubc13Δ
c
– + – + – + 0.02% MMS
180 90 180 180 90 180 180 90 180 (min)
Rad53-P
Rad53
α-tubulin
CDC9
CDC9 mms2Δ
cdc9-1 mms2Δ

Figure 2.9 *MMS2* but not *UBC13* is required for S phase checkpoint activation in *cdc9* mutants.

(a) Cells were arrested in G1 phase at 25°C and released at 35°C for 3 h. DNA content was monitored by flow cytometry. The vertical line indicates a 2C DNA content. **(b)** Cells were grown asynchronously at 25°C and then shifted to 35°C for the indicated time period. Total protein was TCA-precipitated and Rad53 was detected by an anti-Rad53 antibody. **(c)** Asynchronous cultures were split and shifted to the non-permissive temperature of 35°C in the presence of methyl methanesulfonate (MMS) for 90 and 180 min or left untreated for 180 min. Rad53 was detected as described in **b**. In **b-c**, α-tubulin served as a loading control.

Figure 2.10

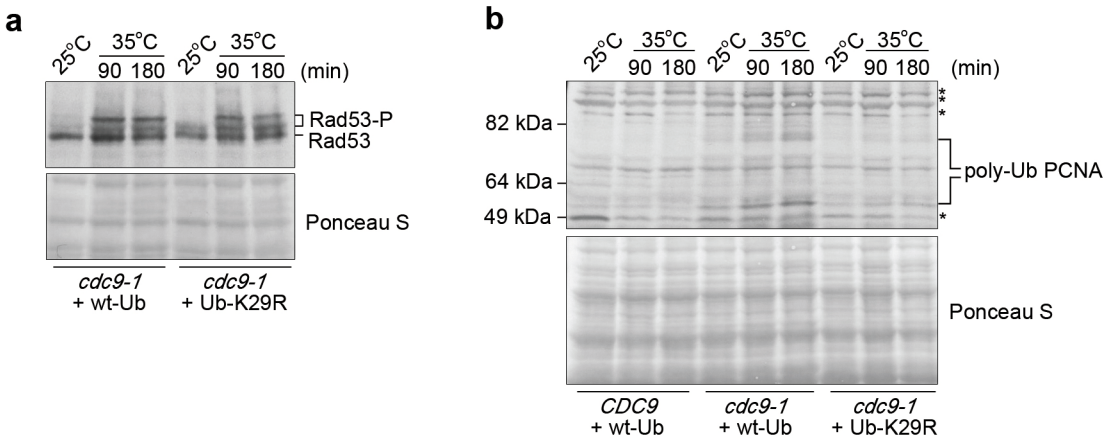
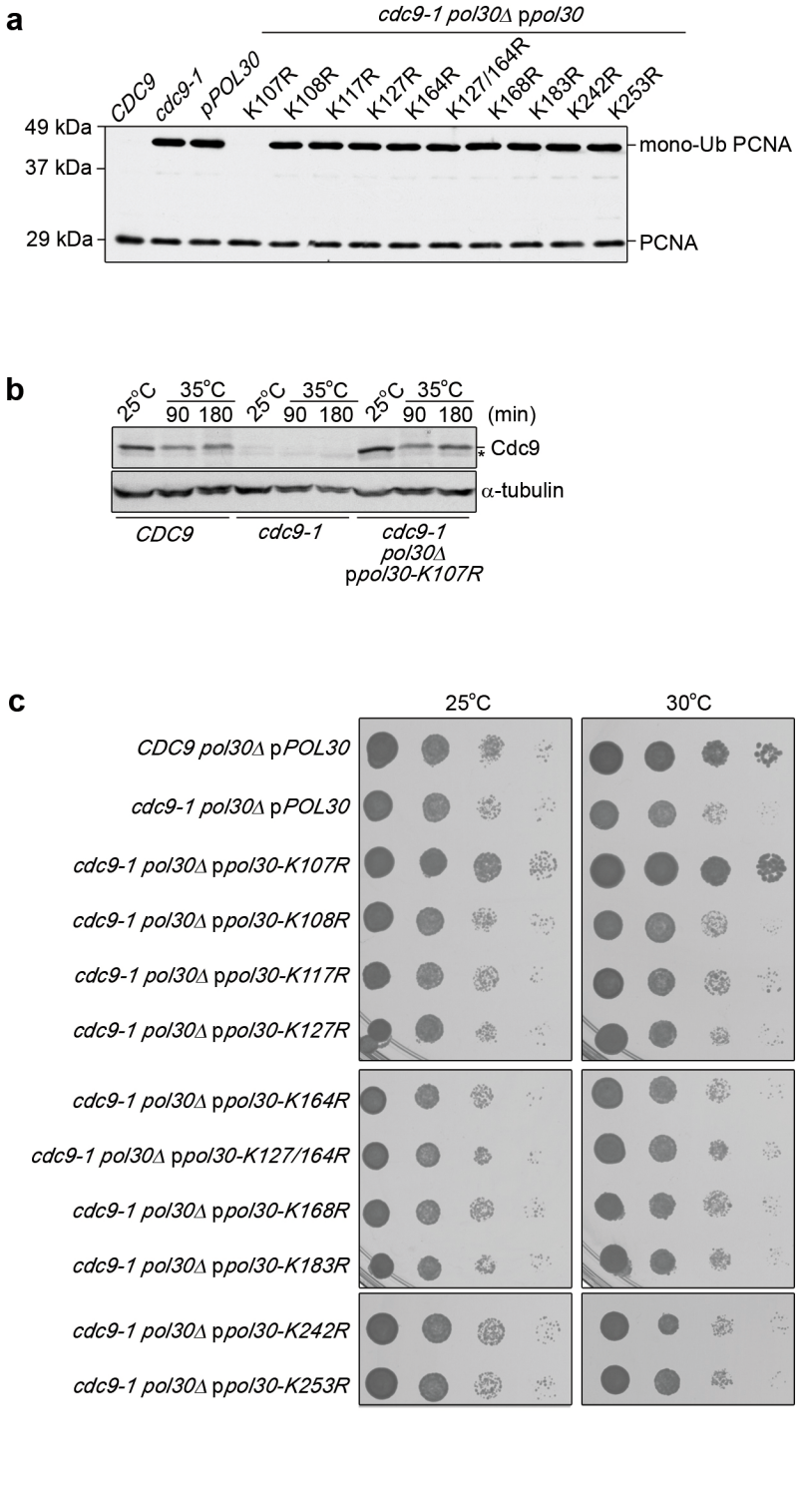


Figure 2.10 Mono-ubiquitination of PCNA in *cdc9* mutants is sufficient for Rad53 activation.

(a) Asynchronous cultures of ABy350 (*cdc9-1 Cu-Myc-Ub*) and ABy485 (*cdc9-1 Cu-Myc-Ub-K29R*) were grown overnight at 25°C to a density of 0.6 at 600 nm. Cells were shifted to 35°C for 3 h in the presence of copper sulfate to induce expression of Myc-tagged ubiquitin. Samples were taken at the indicated times and total protein was TCA-precipitated. Rad53 was detected by Western blotting using an anti-Rad53 antibody. Ponceau S served as a loading control. **(b)** Asynchronous cultures of ABy349 (*CDC9 Cu-Myc-Ub*), ABy350 (*cdc9-1 Cu-Myc-Ub*) and ABy485 (*cdc9-1 Cu-Myc-Ub-K29R*) were grown as described in **a**. Total protein was TCA-precipitated. Poly-ubiquitinated PCNA was detected by Western blot with a yeast specific anti-PCNA antibody (S871) [Zhang *et al.*, 2000]. The asterisks indicate nonspecific bands. Ponceau S staining served as a loading control.

Figure 2.11


a
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CDC9
cdc9-1
pPOL30
K107R
K108R
K117R
K127R
K164R
K127/164R
K168R
K183R
K242R
K253R
49 kDa
37 kDa
29 kDa
mono-Ub PCNA
PCNA
b
25°C
35°C
90 180
(min)
Cdc9
α-tubulin
CDC9
cdc9-1
cdc9-1 pol30Δ ppol30-K107R
c
25°C
30°C
CDC9 pol30Δ pPOL30
cdc9-1 pol30Δ pPOL30
cdc9-1 pol30Δ ppol30-K107R
cdc9-1 pol30Δ ppol30-K108R
cdc9-1 pol30Δ ppol30-K117R
cdc9-1 pol30Δ ppol30-K127R
cdc9-1 pol30Δ ppol30-K164R
cdc9-1 pol30Δ ppol30-K127/164R
cdc9-1 pol30Δ ppol30-K168R
cdc9-1 pol30Δ ppol30-K183R
cdc9-1 pol30Δ ppol30-K242R
cdc9-1 pol30Δ ppol30-K253R

Figure 2.11 A second site suppressor in ABy459 (*cdc9-1 pol30Δ* p*pol30-K107R)* results in elevated DNA ligase I levels and loss of temperature sensitivity.

(a) PCNA lysine mutants in DNA ligase I deficient cells (*cdc9-1*) were grown asynchronously and then shifted to 35°C for 3 h. TCA-precipitated protein samples were diluted and PCNA was detected with anti-PCNA antibody (S871). **(b)** Asynchronous cultures of SSL204 (*CDC9*), SSL612 (*cdc9-1*), ABy459 (*cdc9-1 pol30Δ* p*pol30-K107R*) were shifted to 35°C for 90 and 180 min. DNA ligase I was detected in TCA-precipitated protein with an anti-Cdc9 antibody and α-tubulin served as a loading control. The asterisk denotes a degradation product. **b,** Cells were spotted in ten-fold serial dilutions on SC-trp plates and incubated at the indicated temperatures for 2 days.

Figure 2.12

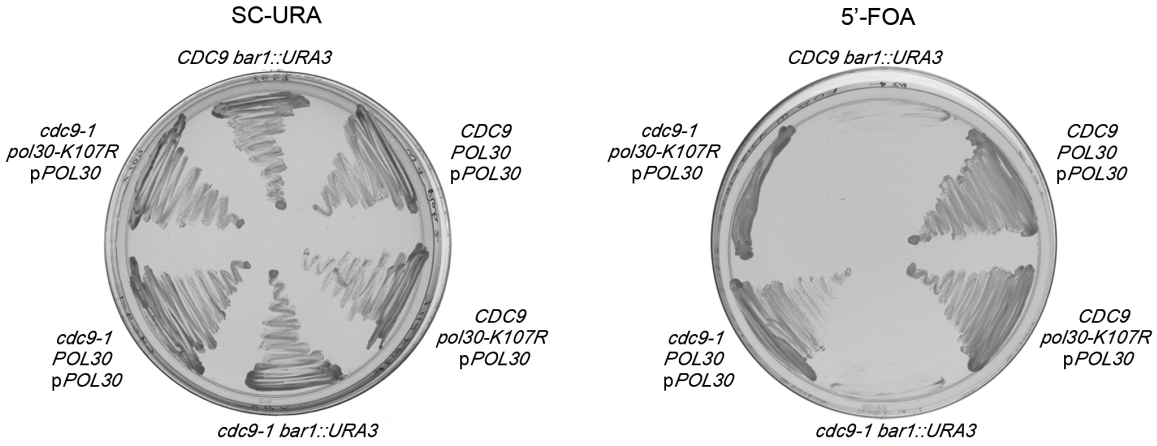

Figure 2.12 A PCNA mutation at K107 in DNA ligase I deficient cells inhibits cell proliferation.

Wild-type (*CDC9*) and DNA ligase I deficient cells (*cdc9-1*) either with or without *pol30-K107R* integrated at the endogenous PCNA locus, all expressing *POL30* from a low copy number plasmid, were grown on SC-ura and FOA plates for 2-3 days at 25°C. Wild-type (*CDC9*) and DNA ligase I deficient cells (*cdc9-1*) carrying a *URA3* disruption of the *BAR1* gene served as controls.

Figure 2.13

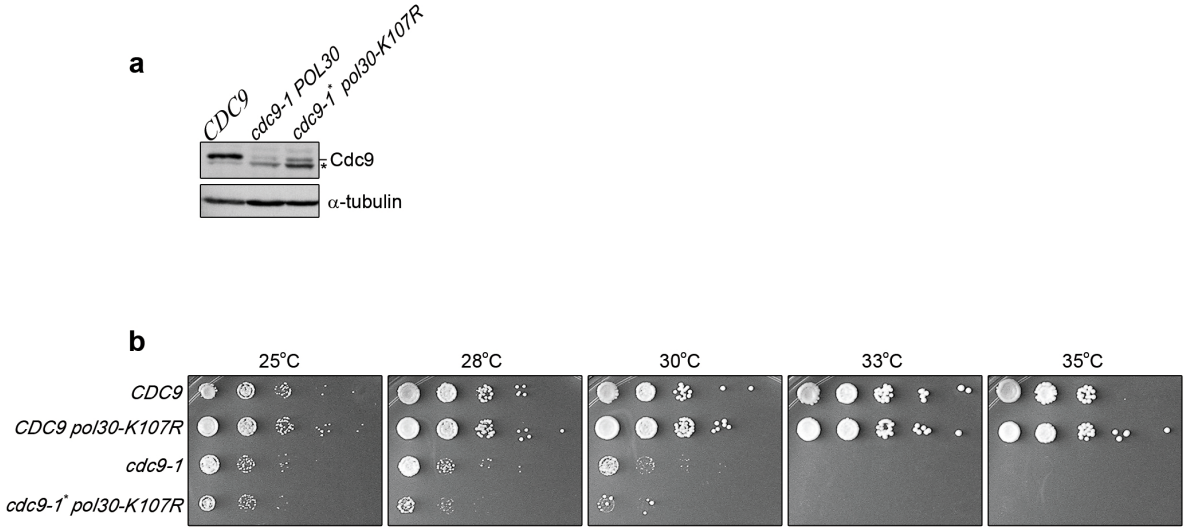

Figure 2.13 *cdc9-1* pol30-K107R* has slightly elevated DNA ligase I levels but still retains its temperature sensitivity.

(a) Cultures of SSL204 (*CDC9*), SSL612 (*cdc9-1*) and ABy782 (*cdc9-1* pol30-K107R*) were grown asynchronously at 25°C and total protein was TCA-precipitated. DNA ligase I was detected using anti-Cdc9 antibody and α-tubulin served as a loading control. The asterisk denotes a degradation product. **(b)** Cultures of SSL204 (*CDC9*), ABy685 (*CDC9 pol30-K107R*), SSL612 (*cdc9-1*), ABy782 (*cdc9-1* pol30-K107R*) were spotted in ten-fold serial dilutions on YPD plates and incubated at the indicated temperatures for 2-3 days.

Figure 2.14

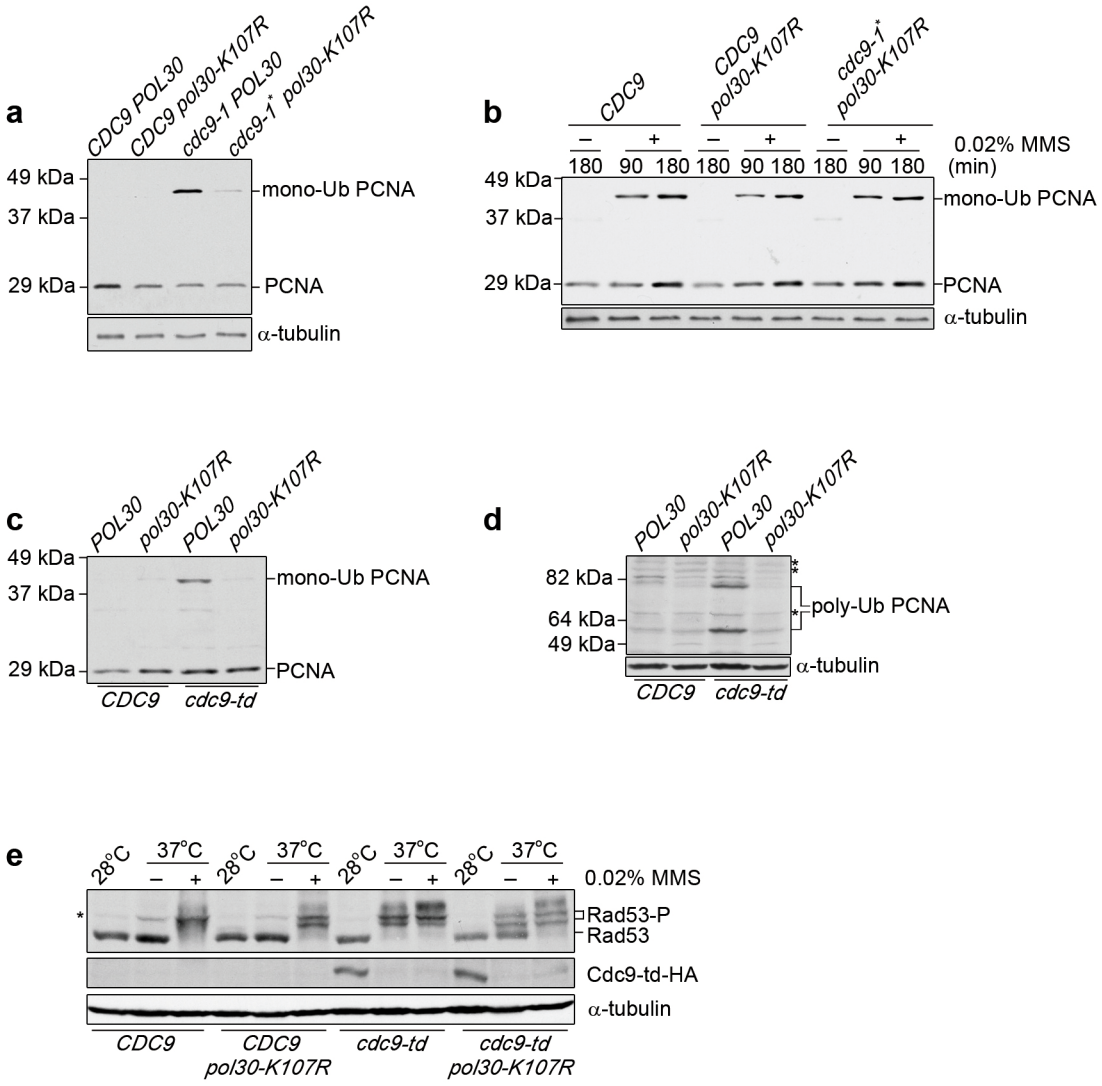
a
CDC9 POL30
CDC9 pol30-K107R
cdc9-1 POL30
cdc9-1* pol30-K107R
49 kDa
37 kDa
29 kDa
mono-Ub PCNA
PCNA
α-tubulin
b
CDC9
CDC9 pol30-K107R
cdc9-1* pol30-K107R
– + – + – +
0.02% MMS
180 90 180 180 90 180 180 90 180
(min)
49 kDa
37 kDa
29 kDa
mono-Ub PCNA
PCNA
α-tubulin
c
POL30
pol30-K107R
POL30
pol30-K107R
49 kDa
37 kDa
29 kDa
mono-Ub PCNA
PCNA
CDC9
cdc9-td
d
POL30
pol30-K107R
POL30
pol30-K107R
82 kDa
64 kDa
49 kDa
poly-Ub PCNA
α-tubulin
CDC9
cdc9-td
e
28°C 37°C 28°C 37°C 28°C 37°C 28°C 37°C
– + – + – + – +
0.02% MMS
Rad53-P
Rad53
Cdc9-td-HA
α-tubulin
CDC9
CDC9 pol30-K107R
cdc9-td
cdc9-td pol30-K107R

Figure 2.14 PCNA mono-ubiquitination occurs at lysine 107 in DNA ligase I mutants and is required for Rad53 activation.

(a) Cultures of SSL204 (*CDC9*), ABy685 (*CDC9 pol30-K107R*), SSL612 (*cdc9-1*), ABy782 (*cdc9-1* pol30-K107R*) were grown asynchronously and then shifted to 30°C for 3 h. **(b)** Asynchronous cultures were grown at 25°C and shifted to 30°C in the presence of MMS for the indicated time. Cultures not treated with MMS served as negative controls. **(c)** Cultures were induced with galactose at 28°C for 30 min and subsequently shifted to 37°C for 3 h. In **a-c,** TCA-precipitated protein samples were diluted and PCNA was detected with anti-PCNA antibody (S871). **(d)** Undiluted TCA-precipitated samples from **c** were fractionated on SDS-PAGE and poly-ubiquitinated PCNA was detected with anti-PCNA antibody (S871). **(e)** Cells were grown asynchronously at 28°C and then shifted to 37°C in the presence or absence of MMS for 3 h. Rad53 and Cdc9-td-HA were detected with anti-Rad53 and anti-HA antibodies, respectively. In **a**, **b, d, e,** α-tubulin served as a loading control. In **d, e,** the asterisks indicate non-specific bands.

Figure 2.15

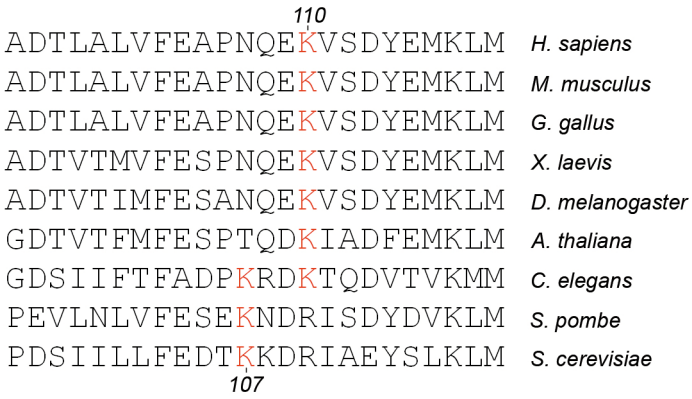

Figure 2.15 Evolutionary conservation of lysines 107 and 110 in selected eukaryotes.

Lysine 107 in PCNA is conserved in *S. pombe* and *C. elegans* but higher eukaryotes have a conserved lysine at position 110.

Figure 2.16

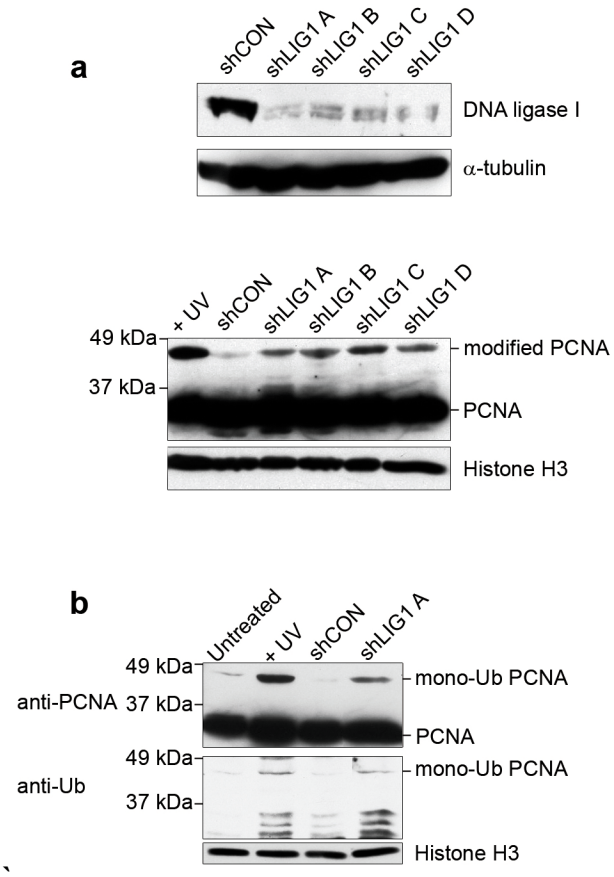
a
shCON
shLIG1 A
shLIG1 B
shLIG1 C
shLIG1 D
DNA ligase I
α-tubulin
+ UV
shCON
shLIG1 A
shLIG1 B
shLIG1 C
shLIG1 D
49 kDa
37 kDa
modified PCNA
PCNA
Histone H3
b
Untreated
+ UV
shCON
shLIG1 A
49 kDa
anti-PCNA
37 kDa
mono-Ub PCNA
PCNA
49 kDa
anti-Ub
37 kDa
mono-Ub PCNA
Histone H3

Figure 2.16 PCNA mono-ubiquitination is conserved in human DNA ligase I-deficient cells.

(a) Generation of LIG1 shRNA stable cell lines. U20S cells stably expressing either control or DNA ligase I shRNA were harvested and subjected to Western blot analysis with the indicated antibodies. DNA ligase I expression was determined in whole cell extracts, whereas PCNA expression was examined in chromatin-bound protein fractions. α-tubulin and histone H3 served as loading controls. **(b)** U2OS cells were either untreated or treated with 60 J/m^2 UV and harvested 2 h later along with control shRNA (shCON) and DNA Ligase I shRNA (shLIG1 A) cell lines. Chromatin fractions were prepared, fractionated on SDS-PAGE and analyzed with the indicated antibodies. Samples on the PCNA and ubiquitin blots were analyzed side by side. Unmodified PCNA served as a loading control for the PCNA blot and histone H3 served as a loading control for the ubiquitin blot.

Figure 2.17

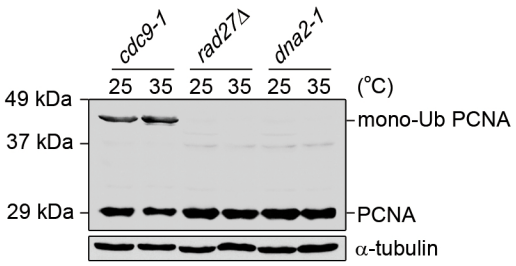

Figure 2.17 PCNA ubiquitination does not occur in *rad27Δ* and *dna2-1* mutants.

Asynchronous cultures were grown at 25°C and shifted to the restrictive temperature of 35°C for 3 h. Total protein was TCA-precipitated and PCNA was detected by a yeast specific anti-PCNA antibody (S871) [Zhang *et al.*, 2000]. α-tubulin served as a loading control.

CHAPTER 3

Damage-specific modification of PCNA

(The work in this chapter was published in Das-Bradoo, S.*, Nguyen, H.D.*, Bielinsky, A.K. (2010) Cell Cycle **9**(18):3674-9. * indicates co-authors.)

Authors contributions:

S.D.-B. conducted experiment in Figure 3.2. H.D.N. conducted experiment in Figure 3.1 and generated Figure 3.3. S.D.-B. and H.D.N. helped with data analysis and write the manuscript. A.-K.B. planned and supervised the project, and wrote the manuscript.

Okazaki fragment processing is an integral part of DNA replication. For a long time, we assumed that the maturation of these small RNA-primed DNA fragments did not necessarily have to occur during S phase, but could be postponed to late in S phase after the bulk of DNA synthesis had been completed. This view was primarily based on the arrest phenotype of temperature-sensitive DNA ligase I mutants in yeast, which accumulated with an almost fully duplicated set of chromosomes. However, many temperature-sensitive alleles can be leaky, and the re-evaluation of DNA ligase I-deficient cells has offered new and unexpected insights into how cells keep track of lagging strand synthesis. It turns out that if Okazaki fragment joining goes awry, cells have their own alarm system in the form of ubiquitin that is conjugated to the replication clamp PCNA. Although this modification results in mono- and poly-ubiquitination of PCNA, it is genetically distinct from the known post-replicative repair mark at lysine 164. In this Extra View, we discuss the possibility that eukaryotic cells utilize different enzymatic pathways and ubiquitin attachment sites on PCNA to alert the replication machinery to the accumulation of single-stranded gaps or nicks behind the fork.

Introduction

In humans, more than 30,000,000 Okazaki fragments (OFs) have to be initiated (assuming an average size of ~200 nt/OF), processed and ligated during a single round of DNA replication [Hubscher and Seo, 2001]. It is easy to imagine that failure to properly join OFs would cause a large number of single-strand breaks (SSBs) – at a frequency that would likely overwhelm the SSB repair machinery and thus be lethal. It is therefore not surprising that to date only one individual with a severe defect in DNA ligase I, the enzyme that joins OFs during DNA replication, has been identified and described [Barnes *et al.*, 1992; Webster *et al.*, 1992]. The individual carried compound heterozygous mutations in the gene and retained approximately 5% ligase activity [Mackenney *et al.*, 1997; Prigent *et al.*, 1994]. Importantly, this reduction caused growth retardation, immunodeficiency, UV sensitivity and lymphoma [Barnes *et al.*, 1992; Webster *et al.*, 1992]. Most of these phenotypes are compatible with the known function of DNA ligase I in DNA replication and nucleotide excision repair [Ellenberger and Tomkinson, 2008]. The only exception is the immunodeficiency, which went hand-in-hand with a profound lack of immunoglobulin class switching [Barnes *et al.*, 1992; Webster *et al.*, 1992]. Although this latter defect was not recapitulated in a mouse model carrying a homozygous mutation of the viable missense allele, DNA ligase I deficient animals displayed growth retardation, abnormal erythropoiesis and increased genome instability [Bentley *et al.*, 2002; Harrison *et al.*, 2002]. Moreover, they developed an unexpectedly wide range of spontaneous tumors, such as lymphomas, adeno- and other carcinomas. The importance of proper OF maturation is further underscored by the fact that mutations

in other enzymes that participate in OF processing, such as the flap endonuclease Fen1 (*e.g.*, a E160D mutation), which retains partial endonuclease activity [Frank *et al.*, 1998], have been directly linked to cancer in humans [Zheng *et al.*, 2007]. Interestingly, recent evidence suggests that the E160D Fen1 mutation, similar to the DNA ligase I deficiency, causes early-onset lymphoma in mice [Larsen *et al.*, 2008]. This raises the question of whether cells harbor a system capable of monitoring perturbations during OF processing. In addition, it remains unclear if abnormalities in lagging strand maturation affect replication fork progression.

Post-replicative repair and DNA damage induction by Okazaki fragment processing defects.

Today we have a relatively good understanding of the post-replication repair (PRR) pathways involved in sensing DNA lesions, like ultraviolet (UV) irradiation-induced thymine dimers [Setlow *et al.*, 1963], in the leading or lagging strand template [Bergink and Jentsch, 2009; Lee and Myung, 2008; Ulrich, 2009]. PRR seemed for many years somewhat of a misnomer, as most studies analyzed the pathway in the context of an active replication fork [Frampton *et al.*, 2006; Hoege *et al.*, 2002; Kannouche *et al.*, 2004; Moldovan *et al.*, 2007; Stelter and Ulrich, 2003]. However, recent studies have come full circle demonstrating that PRR can occur outside of S phase, after replication has been largely completed [Daigaku *et al.*, 2010; Karras and Jentsch, 2010]. The first description of PRR dates back to 1968 when Rupp and Howard-Flanders described discontinuities in the newly synthesized DNA of nucleotide excision repair (NER)

defective *E. coli* [Rupp and Howard-Flanders, 1968]. Shortly thereafter, the concept was extended to mammalian cells [Lehmann, 1972]. During the past 42 years much progress has been made toward understanding error-prone and error-free PRR, respectively [Bridges and Walker, 2004]. Nevertheless, most studies published to date utilize UV or other mutagens like methyl methanesulfonate (MMS) to create large adducts in the DNA that prompt collisions with the replicative polymerases, thereby causing transient replication fork arrest [Kannouche *et al.*, 2004; Niimi *et al.*, 2008; Stokes and Michael, 2003; Tercero and Diffley, 2001]. This is usually accompanied by formation of long stretches of replication protein A (RPA)-coated, single-stranded (ss) DNA [Zou and Elledge, 2003]. These RPA-coated regions facilitate PRR activation by ubiquitination of PCNA at lysine 164 [Davies *et al.*, 2008; Hoege *et al.*, 2002] and also trigger the S phase checkpoint [Zou and Elledge, 2003]. Both events are genetically separable and are thought to occur in parallel [Frampton *et al.*, 2006; Yang and Zou, 2009]. Although the above-mentioned experimental approach utilizing UV-irradiation or MMS has provided a powerful model to dissect the molecular steps of these pathways, it has obvious limitations, because forks that accumulate ssDNA are probably not the only structures that arise in replication-defective mutants. Aberrant Okazaki fragment processing, for example, can cause nicks – in a wide range of varieties: "clean" nicks due to ligation deficiency, "dirty" nicks that exhibit adenylated 5' ends if the ligation reaction is aborted prematurely or "flapped" nicks in the case of improper removal of RNA primers [Rossi and Bambara, 2006; Tomkinson *et al.*, 2006]. This latter reaction requires the complicated interplay of multiple activities. Limited displacement synthesis by DNA

polymerase (pol)-δ is required to initially create a short flap that is deleted one nucleotide at a time by Fen1 [Garg *et al.*, 2004]. Excessive strand displacement is counteracted by the intrinsic 3'-exonuclease activity of the catalytic subunit of pol-δ and its non-essential binding partner, Pol32 [Jin *et al.*, 2005; Stith *et al.*, 2008]. Cells defective in Fen1, appear to create larger flaps (in a Pol32-dependent manner) that can be processed by a second endonuclease, Dna2 [Ayyagari *et al.*, 2003; Budd *et al.*, 2000; Lee *et al.*, 2000]. Recent studies have also implicated other enzymes, such as exonuclease 1 and the helicase Pif1 in this backup system [Rossi *et al.*, 2008; Stith *et al.*, 2008]. Thus, the first two steps (strand displacement and flap removal) of OF processing (OFP) already involve an elaborate network of partially redundant pathways to assure proper production of a ligatable nick that can be sealed by DNA ligase I. Importantly, primer removal by Fen1 and OF ligation by DNA ligase I are coordinated by PCNA [Karanja and Livingston, 2009]. Both enzymes bind PCNA through a PCNA interacting peptide (PIP) box [Maga and Hubscher, 2003; Sakurai *et al.*, 2005; Vijayakumar *et al.*, 2007].

Results

Okazaki fragment processing defects trigger PCNA ubiquitination.

The fact that multiple OFP pathways exist suggests that cells have the ability to actively monitor maturation events during lagging strand synthesis. To address this question, we analyzed DNA ligase I-deficient cells [Bielinsky and Gerbi, 1999]. In budding yeast, temperature-sensitive *cdc9* mutants were among the first strains identified to have a cell cycle progression defect under non-permissive conditions as they arrested in late S/G2 phase [Culotti and Hartwell, 1971; Johnston and Nasmyth, 1978]. The arrest was dependent on the DNA damage response [Weinert *et al.*, 1994] and cell viability required functional homologous recombination [Montelone *et al.*, 1981], indicating that the nicks might be – at least partially – converted into double strand breaks (DSBs). These early studies suggested that OF ligation was uncoupled from replication fork progression and could occur very late in S phase after the bulk of the DNA had been replicated. This was also consistent with observations made much later that PCNA remained on chromatin after passage of the replication fork, providing anchor points for DNA ligase I on chromatin [Shibahara and Stillman, 1999]. In fact, experiments with *cdc9-1* cells that had two consecutive shifts, to the non-permissive temperature first and then back to the permissive temperature demonstrated that OF synthesis could be temporally separated from OF ligation [Bielinsky and Gerbi, 1999]. All of these findings were consistent with the notion that DNA ligase I was not required until the end of S phase. However, our recent analysis of the *cdc9-1* mutation in a different strain background suggested that the cells do not move through S phase with normal kinetics,

but rather appeared to accumulate in mid-S phase (Figure 2.3) [Das-Bradoo *et al.*, 2010b]. Further exploration of a temperature-sensitive degron mutant (*cdc9-td*) verified that DNA replication was extensively delayed in the absence of DNA ligase I (Figure 3.1). Although it still remains unclear how this delay was mediated (*e.g.*, through reduced fork progression or inhibition of late-firing origins), it was dependent on the activation of the S phase checkpoint kinase Rad53 [Branzei and Foiani, 2007]. Rad53 phosphorylation was not only Rad9- (and thus DNA damage) dependent [Sweeney *et al.*, 2005], but also required the mediator of the replication checkpoint, Mrc1 [Naylor *et al.*, 2009; Osborn and Elledge, 2003], the yeast homolog of Claspin [Kumagai and Dunphy, 2000]. This finding implied that replication fork stalling was occurring [Alcasabas *et al.*, 2001; Tanaka and Russell, 2001]. How this happened in a scenario in which none of the DNA polymerases were actively inhibited was puzzling. Even more surprising was the observation that the phosphorylation of Rad53 was amplified by a novel PRR-related pathway that triggered mono- and poly- ubiquitination of PCNA (Figure 2.14) [Das-Bradoo *et al.*, 2010b]. Mutation of lysine 164 of PCNA in *cdc9* mutants had no effect on cell viability, arguing that neither error-prone translesion synthesis [Kannouche *et al.*, 2004; Stelter and Ulrich, 2003; Terai *et al.*, 2010; Watanabe *et al.*, 2004; Zhuang *et al.*, 2008] nor error-free replication by template switch [Blastyak *et al.*, 2007; Branzei *et al.*, 2008; Minca and Kowalski, 2010; Torres-Ramos *et al.*, 2002] played a role in this process. Instead, we observed synthetic lethality between *cdc9-1* and a mutation in lysine 107 of PCNA (Figure 2.11-2.13), leading us to propose that ubiquitin is conjugated to lysine 107 in DNA ligase I-deficient cells. One aspect that we did not address in our

recent study is whether PCNA is also modified by the ubiquitin-related SUMO peptide, which is attached to PCNA during S phase at lysine 164 and to a lesser extent at lysine 127 [Hoege *et al.*, 2002]. In Figure 3.2, we demonstrate that PCNA is indeed sumoylated in wild-type cells and *cdc9* mutants. Because the mutants accumulated in S phase, they displayed stronger sumoylation signals. In addition, we observed higher molecular weight forms of sumoylated PCNA. This is consistent with the finding that PCNA carries SUMO chains in the face of profound replication stress [Windecker and Ulrich, 2008]. Alternatively, it is also possible that a single PCNA monomer is ubiquitinated (at lysine 107) and sumoylated (at lysine 164) in *cdc9* mutants. Sumoylation of PCNA is thought to repress homologous recombination [Branzei *et al.*, 2006; Haracska *et al.*, 2004; Papouli *et al.*, 2005] at stalled replication forks. This raises an interesting point because homologous recombination is required for the viability of *cdc9* mutants and therefore its activity needs to be highly regulated. Future experiments are needed to clarify these issues. However, regardless of their outcome, it is important to mention that ubiquitination of PCNA is conserved in human cells that are depleted for DNA ligase I, even though lysine 107 of PCNA is not evolutionarily conserved (Figure 2.16) [Das-Bradoo *et al.*, 2010b]. This underscores the significance of our findings.

Consistent with the observation that a DNA ligase I deficiency triggers ubiquitin conjugation at a non-canonical lysine residue of PCNA in budding yeast, we have also uncovered genetic requirements for this process that clearly distinguish it from the classical DNA damage tolerance (DDT) pathways. For example, whereas DDT is dependent on Rad6 and Rad18, both proteins are dispensable for PCNA ubiquitination in

cdc9 mutants. This makes sense in as far as Rad6 and Rad18 mediate predominantly translesion synthesis [Daigaku *et al.*, 2010], which would help little in facilitating the repair of nicks. Moreover, the poly-ubiquitin chains observed in *cdc9* mutants are linked through lysine 29 and not lysine 63 as in PRR [Hoege *et al.*, 2002]. In the light of this result, it is not surprising that Ubc13, which together with Mms2 catalyzes lysine 63 linked ubiquitin chains [Eddins *et al.*, 2006; Hofmann and Pickart, 1999; VanDemark *et al.*, 2001], did not appear to play a role in this pathway (Figure 2.7 and 2.9) [Das-Bradoo *et al.*, 2010b]. What was puzzling, however, was the genetic requirement for *MMS2* and *UBC4* (Figure 2.8). The human homolog of Ubc4, UbcH5A, synthesizes lysine 29 linked ubiquitin chains *in vitro* [Mastrandrea *et al.*, 1999]. Although Ubc13 has been reported to function independently of Mms2 [Stewart *et al.*, 2009], no other studies have demonstrated a genetic function for Mms2 that is separable from Ubc13. How Mms2 fits into the PCNA ubiquitination pathway is not at all clear and will require additional genetic validation. Furthermore, biochemical studies are currently underway to dissect whether Mms2, Ubc4 and Rad5 cooperate in one physical complex – possibly together with other proteins – or not. At this point, we have no evidence to suggest a direct interaction between Mms2 and Ubc4. Moreover, we would like to emphasize that our data does not dispute nor contradict any of the established rules for the DDT and PRR pathways [Ulrich and Walden, 2010]. Rather, we interpret them to mean that both the repertoires of damaged DNA structures that cause PCNA ubiquitination as well as the pathways catalyzing these reactions are likely larger and more complex than anticipated. This is also underscored by recent observations that described the Rad6-independent

ubiquitination of PCNA in response to chronic HU and MMS treatment at lysine 164 [Kats *et al.*, 2009]. Although we are still missing many pieces of the puzzle, it appears that different types of DNA damage may trigger different PCNA ubiquitination pathways that result in distinct attachment sites and/or differently linked ubiquitin chains (*i.e.*, a PCNA "DNA damage code"; Figure 3.3 and Chapter 2) [Das-Bradoo *et al.*, 2010b].

Taken together, our results suggest that lagging strand synthesis is actively monitored and that persistent nicks in newly replicated DNA (and maybe also those that arise during abortive repair or result from oxidative damage) can indeed be detected during S phase. Whether earlier steps in Okazaki fragment processing are surveyed in a similar manner remains obscure. Although we tested *rad27* and *dna2* mutants, and neither displayed PCNA ubiquitination under non-permissive conditions (Figure 2.17) [Das-Bradoo *et al.*, 2010b], it is highly likely that the respective enzymes substitute for one another in these particular mutants. Therefore, the lack of PCNA ubiquitination does not necessarily exclude the possibility that other OFP intermediates are recognized by the cell and trigger modification(s) of the PCNA clamp. Indeed, *pol32* mutants cause lagging strand defects, which result in ss gaps [Karras and Jentsch, 2010]. Although it is not clear that these gaps are generated during OFP (*e.g.*, they might occur during actual synthesis), they cause PCNA ubiquitination. The most burning question for us concerns the biological function of ubiquitinated PCNA in *cdc9* mutants. What is the connection between the modified clamp, the slow replication dynamics and Rad53 activation? Replication fork slowdown would certainly aid in extending the time frame of an individual ligation reaction. In the same vein, modified PCNA may help to retain the

enzyme on site until Okazaki fragments have been successfully joined and might counteract abortive ligation reactions due to the limited amounts of DNA ligase I present in the cell. The exciting new technical developments in producing ubiquitinated PCNA *in vitro* [Chen *et al.*, 2010; Freudenthal *et al.*, 2010] certainly provide invaluable tools toward finding answers to these questions.

Figure 3.1

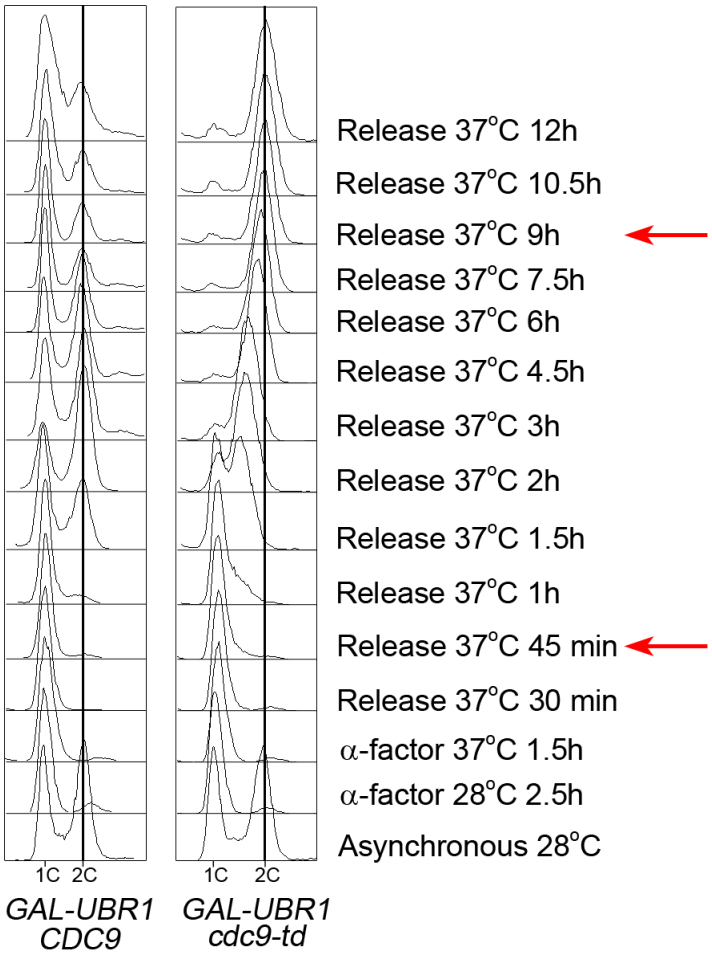
Release 37°C 12h
Release 37°C 10.5h
Release 37°C 9h
Release 37°C 7.5h
Release 37°C 6h
Release 37°C 4.5h
Release 37°C 3h
Release 37°C 2h
Release 37°C 1.5h
Release 37°C 1h
Release 37°C 45 min
Release 37°C 30 min
α-factor 37°C 1.5h
α-factor 28°C 2.5h
Asynchronous 28°C
1C 2C
1C 2C
GAL-UBR1 CDC9
GAL-UBR1 cdc9-td

Figure 3.1 DNA ligase I is required for S phase progression.

Asynchronous cultures of ABy010 (*GAL-UBR1 CDC9)* and ABy008 (*GAL-UBR1 cdc9-td*) were grown at 28°C and arrested in G_1 with α-factor. After 2 hours, Ubr1 expression was induced with 2% galactose for 30 min. Subsequently, cultures were shifted to 37°C in medium containing α-factor and 2% galactose. After 90 min, cells were released from G_1 for 12 hours. DNA content at indicated time was monitored by flow cytometry and the vertical line indicates a 2C DNA content. The red arrows mark entry into S phase and completion of DNA replication in *cdc9-td* mutants, respectively.

Figure 3.2

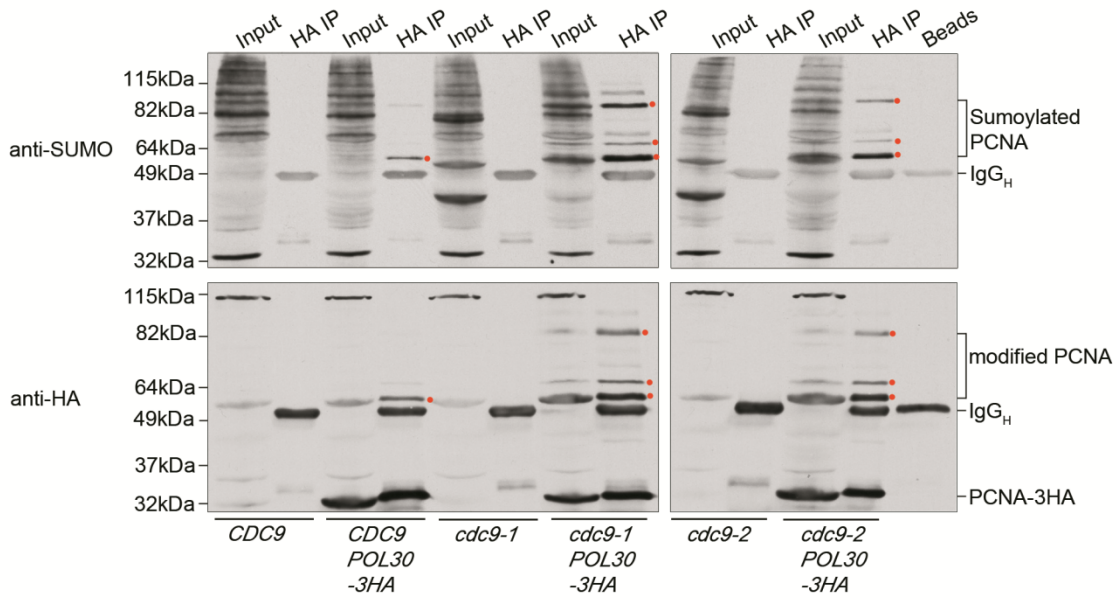

Figure 3.2 PCNA is sumoylated in *cdc9* mutants.

Wild-type (*CDC9*) and *cdc9* mutants (*cdc9-1* and *cdc9-2*) expressing PCNA-3HA were grown asynchronously at 25°C and shifted to 35°C for 3 hours. Whole-cell extracts were immunoprecipitated with anti-HA conjugated agarose beads (Sigma). Both unmodified and modified PCNA was detected with an anti-HA antibody (Covance, 16B12). Sumoylated PCNA was detected with an anti-SUMO antibody (a gift from Xiaolan Zhao, Memorial Sloan-Kettering Cancer Center). The red dots mark prominent sumoylated PCNA bands. IgG_H indicates the immunoglobulin heavy chain. Mono-ubiquitinated PCNA is visible in darker exposures around 42kDa in *cdc9* mutants with HA-tagged PCNA (only visible as a faint band in this exposure). *cdc9-1* is a more stringent allele than *cdc9-2* (Figure 2.1) [Das-Bradoo *et al.*, 2010b], and thus sumoylation of PCNA is more prominent in this mutant.

Figure 3.3

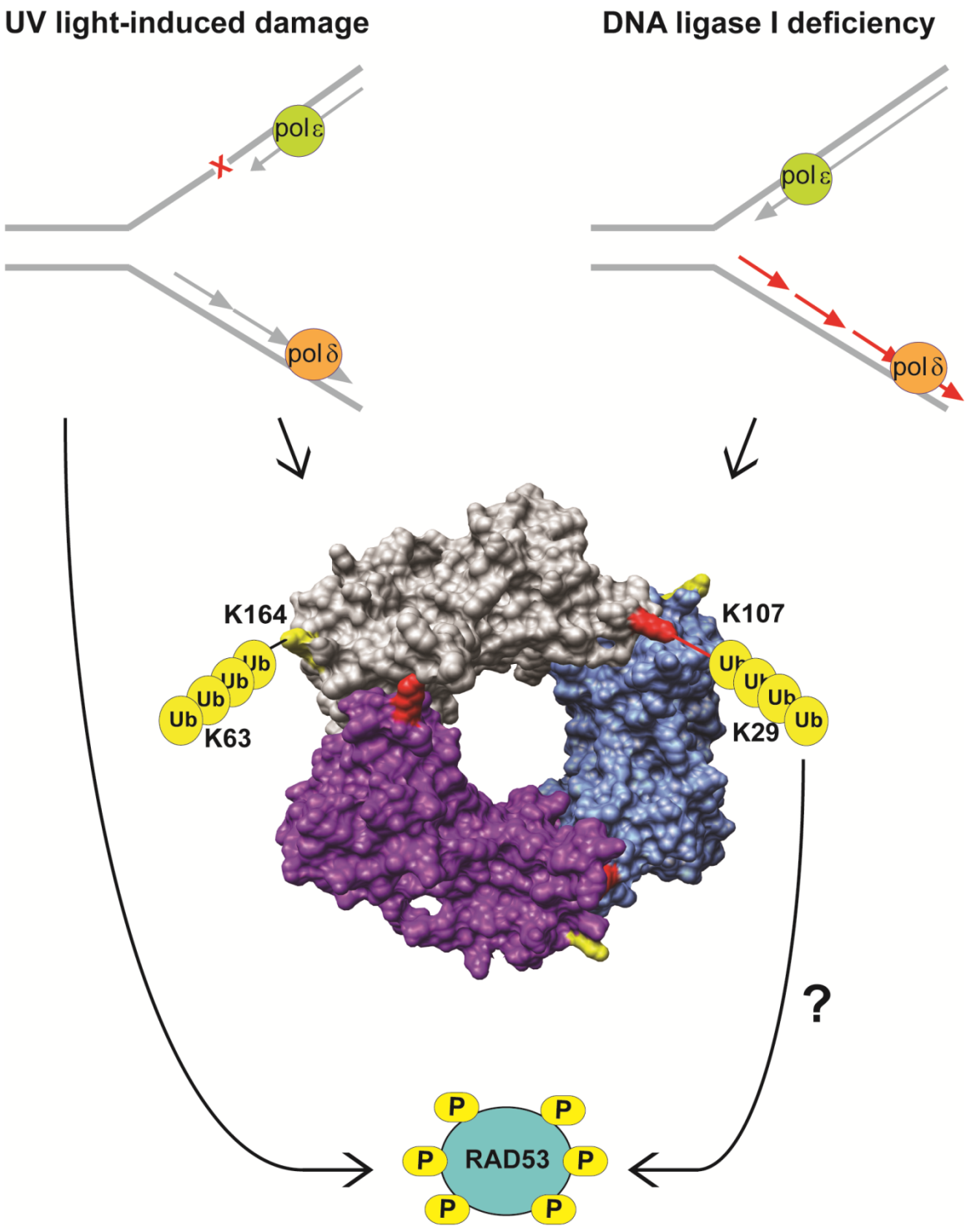
UV light-induced damage
DNA ligase I deficiency
pol ε
pol δ
pol ε
pol δ
K164
Ub
Ub
Ub
Ub
K63
K107
Ub
Ub
Ub
Ub
K29
?
P
P
P
RAD53
P
P
P

Figure 3.3 Damage-dependent PCNA ubiquitination in budding yeast.

DNA synthesis occurs in a semi-discontinuous manner. Nascent DNA on the leading strand is synthesized by pol ε, whereas pol δ is required to synthesize Okazaki fragments (short arrows) on the lagging strand. In response to UV light-induced damage, which causes pyrimidine dimers on the template DNA (red X, left), PCNA is ubiquitinated at lysine (K) 164 and the poly-ubiquitin chain is linked through K63. Activation of the S phase checkpoint by UV damage is independent of PCNA ubiquitination at K164. In contrast, PCNA is ubiquitinated at K107 and the poly-ubiquitin chain is linked through K29 in response to DNA ligase I deficiency, which accumulates persistent nicks due to unligated Okazaki fragments (red arrows, right). PCNA ubiquitination at K107 appears to be a prerequisite for activation of the S phase checkpoint, illustrated by hyperphosphorylation of Rad53. The importance of the K29-linked poly-ubiquitin chain and how PCNA ubiquitination results in activation of the S phase checkpoint is still unknown. The *S. cerevisiae* homotrimeric PCNA structure (PDB ID 2OD8) was generated using Chimera software program [Pettersen *et al.*, 2004]. Ub denotes ubiquitin. K164 and K107 on each of the subunits are indicated as yellow and red surfaces, respectively.

CHAPTER 4

Accumulation of unligated Okazaki fragments induces PCNA ubiquitination and Rad59-dependent replication fork progression

(The work in this chapter is manuscript in preparation: Nguyen, H.D., Costanzo, M., Koch, E.N., Smith, S., Myung, K, Myers, C.L., Boone, C., and Bielinsky, A.K.)

Authors contribution:

M.C and C.B designed and performed all SGA experiments. E.N.K and C.L.M carried out analysis of SGA results for Figure 4.7a, Table 4.2 and Supplementary Table S1. S.S and K.M carried out GCR and DNA damage sensitivity in Figures 4.4 and 4.5. H.D.N and A.-K.B designed experiments, analyzed data for all experiments except for data mentioned above.

A deficiency in DNA ligase I/Cdc9, which catalyzes the joining of Okazaki fragments, triggers PCNA ubiquitination at lysine 107 in *S. cerevisiae*. This signal is crucial to activate the S phase checkpoint, which promotes cell cycle arrest. We report here that a *pol30K107* mutation alleviated cell cycle arrest in *cdc9* mutants. To determine whether PCNA ubiquitination occurred in response to nicks or the lack of a PCNA-DNA ligase interaction, we complemented *cdc9* cells either with wild-type DNA ligase I or *Chlorella* virus ligase, the latter of which fails to interact with PCNA. Both enzymes reversed PCNA ubiquitination, arguing that the modification is likely triggered directly by nicks. To further understand how cells cope with nicks during replication, we utilized *cdc9-1* in a genome-wide synthetic lethality screen and identified *RAD59* as a strong negative interactor. *cdc9 rad59Δ* mutants did not alter PCNA ubiquitination but enhanced phosphorylation of the mediator of the replication checkpoint, Mrc1, indicative of increased replication fork stalling. Thus, Rad59 promotes fork progression when Okazaki fragment processing is compromised and counteracts PCNA-K107 mediated cell cycle arrest.

Supplementary Table S1. A complete SGA results for all query strains used in this study. See Materials and Methods for more description of the SGA screen. Table is available online.

Introduction

Replication fork arrest in response to DNA lesions, such as UV-induced thymine dimers that physically block DNA synthesis and lead to exposure of unreplicated, single-stranded (ss) DNA has been studied extensively in multiple different model organisms [Branzei and Foiani, 2007]. However, how cells monitor the integrity of replication intermediates that undergo Okazaki fragment processing is less well understood. Given that human cells produce on the order of 30 million Okazaki fragments that need to be processed and ligated during a single round of replication, a tracking system should be in place to account for possible errors that could lead to the accumulation of nicked DNA. The importance of such a surveillance system is underscored by mutations impinging on proper Okazaki fragment processing that have been identified in human cancer patients and whose cancer-causing effect has been recapitulated in animal studies [Barnes *et al.*, 1992; Larsen *et al.*, 2008; Webster *et al.*, 1992; Zheng *et al.*, 2007]. In particular, a DNA ligase I-deficiency causes not only growth retardation similar to other replication-associated genetic syndromes but lymphoma as well [Barnes *et al.*, 1992; Webster *et al.*, 1992].

DNA ligase I catalyzes the sealing of nicks between adjacent 3'-OH and 5'-PO_4 termini and is crucial for DNA replication, repair and recombination. The DNA ligation mechanism involves three nucleotidyl transfer reactions [Ellenberger and Tomkinson, 2008]. In the first step of the ligation reaction, DNA ligase reacts with either ATP or NAD^+ (in prokaryotes) to form a ligase-adenylate intermediate where 5'-adenosine monophosphate (AMP) is linked by a phosphoamide bond with the lysine residue in the

active site. In the second step, AMP is transferred to the 5'-PO_4 terminus of the nick to form a DNA-adenylate. Finally, DNA ligase catalyzes the nucleophilic attack of the 3'-OH to the DNA-adenylate to covalently join the two ends of the DNA strands and release AMP.

The budding yeast *S. cerevisiae* encodes two different DNA ligases, Cdc9 and Dln4, which are homologs of human DNA ligases I and IV, respectively [Barnes *et al.*, 1990; Johnston and Nasmyth, 1978; Schar *et al.*, 1997; Teo and Jackson, 1997; Wei *et al.*, 1995; Wilson *et al.*, 1997]. Given their different substrate specificities, the two proteins have clearly distinct roles in DNA metabolism. Whereas Dln4 primarily functions in DSB repair via NHEJ, Cdc9 participates in BER and NER [Johnston and Nasmyth, 1978; Montelone *et al.*, 1981; Schar *et al.*, 1997; Teo and Jackson, 1997; Wilson *et al.*, 1997; Wu *et al.*, 1999]. Additionally, Cdc9 is essential for the ligation of Okazaki fragments and interacts genetically and physically with many proteins involved in Okazaki fragment maturation [Ireland *et al.*, 2000; Karanja and Livingston, 2009; Refsland and Livingston, 2005; Vijayakumar *et al.*, 2007]. One such interacting protein is PCNA [Montecucco *et al.*, 1998]. The N-terminus of Cdc9 contains a conserved PCNA interacting peptide (PIP) box motif, QxxLxxFF, which facilitates its interaction with PCNA [Refsland and Livingston, 2005; Subramanian *et al.*, 2005; Vijayakumar *et al.*, 2007]. However, deletion of the PIP-box in Cdc9 does not affect *S. cerevisiae* mitotic growth, suggesting that the interaction is not essential for Okazaki fragment maturation [Sriskanda *et al.*, 1999]. In agreement with these data, the smallest form of *Chlorella* virus DNA ligase, containing only the minimal core domain of DNA ligase but not a PIP-

box, complements *cdc9Δ* cells [Sriskanda *et al.*, 1999], and must therefore have a PCNA-independent nick-sensing activity [Nair *et al.*, 2007].

Temperature sensitive (ts) alleles of *CDC9* were isolated from the original screen for cell division cycle (cdc) mutants [Culotti and Hartwell, 1971; Hartwell *et al.*, 1973]. At the restrictive temperature, *cdc9* mutants arrest in late S/G2 phase of the cell cycle and accumulate unligated Okazaki fragments that are joined upon shifting to permissive conditions [Bielinsky and Gerbi, 1999]. This suggested that cells replicate the whole genome leaving nicks behind for repair in G2 prior to entry into mitosis [Johnston and Nasmyth, 1978; Schiestl *et al.*, 1989]. The G2 arrest of *cdc9ts* mutants is controlled by Rad9-dependent phosphorylation of the S phase checkpoint kinase Rad53, which is triggered when nicks are converted to double strand breaks (DSBs) [Emili, 1998; Naiki *et al.*, 2004; Schiestl *et al.*, 1989; Scott and Plon, 2003; Sweeney *et al.*, 2005; Weinert and Hartwell, 1993]. However, we reported recently that the absence of DNA ligase I in more stringent *cdc9ts* alleles caused a delay in S phase progression and activation of the S phase checkpoint kinase Rad53, which was mediated through both Mrc1, the mediator of the replication checkpoint [Alcasabas *et al.*, 2001] and Rad9 (Chapter 2) [Das-Bradoo *et al.*, 2010b]. This indicated that not only were DSBs present in *cdc9ts* mutants, but also stalled replication forks [Alcasabas *et al.*, 2001; Emili, 1998; Naylor *et al.*, 2009; Osborn and Elledge, 2003]. Importantly, robust activation of Rad53 in *cdc9ts* mutants required PCNA ubiquitination at a novel residue, K107 rather than K164, the well-known conserved site, which is ubiquitinated in response to DNA damaging agents such as UV-irradiation or methyl methansulfonate (MMS) that induce stalled forks (Chapter 2) [Das-

Bradoo *et al.*, 2010b; Hoege *et al.*, 2002]. We hypothesized that cells can distinguish the DNA structures arising from nicked DNA due to *cdc9ts* versus extended ssDNA regions caused by UV-irradiation or MMS exposure (Figure 3.3) [Das-Bradoo *et al.*, 2010a]. How cells cope with the accumulation of such putative nicked replication intermediates and promote S phase progression is unknown. Several studies demonstrated that the inhibition of DNA ligase I activity in both yeasts and humans result in a higher incidence of DNA recombination [Barnes *et al.*, 1992; Game *et al.*, 1979; Henderson *et al.*, 1985; Prigent *et al.*, 1994]. In budding yeast, this notion is supported by the synthetic lethality between a recombination deficient mutant, *rad52*, and *cdc9* mutants [Montelone *et al.*, 1981]. These results are consistent with the assumption that DSBs are formed in *cdc9ts* mutants that require repair by homologous recombination (HR).

RAD52 is essential to promote DSB repair by HR, which is divided into two sub-categories, *RAD51*-dependent and *RAD51*-independent pathways [Krogh and Symington, 2004; Paques and Haber, 1999; Symington, 2002]. *RAD51*-dependent HR repairs most DSBs in mitotic cells by initiating strand invasion of a 3'-ssDNA tail following the formation of a Rad51 filament [Benson *et al.*, 1998; Sugawara *et al.*, 2000; Sugawara *et al.*, 1995]. The alternative *RAD51*-independent pathway involves a single strand annealing (SSA) step, mediated by *RAD52* and *RAD59* for DSB repair between direct repeat sequences and in break-induced replication [Bai *et al.*, 1999; Bai and Symington, 1996; Fishman-Lobell and Haber, 1992; Ira and Haber, 2002; Ivanov *et al.*, 1996; Jablonovich *et al.*, 1999; Signon *et al.*, 2001; Sugawara *et al.*, 2000]. Additionally, genes involved in both the *RAD51*- and *RAD59*-pathways can promote recombination between

sister chromatids independently of DSBs via a template switching mechanism, which bypasses DNA lesions that inhibit the progression of the replicative DNA polymerases [Gangavarapu *et al.*, 2007; Mott and Symington, 2011; Vanoli *et al.*, 2010]. While genes involved in the *RAD51*-dependent pathway, but not *RAD59*, promote template switching in response to UV-light and MMS exposure [Gangavarapu *et al.*, 2007; Vanoli *et al.*, 2010], *RAD59* facilitates spontaneous mitotic recombination by template switching between inverted repeats [Mott and Symington, 2011]. Furthermore, biochemical studies demonstrating that the Rad52/Rad59 complex is distinct from the Rad51/Rad52 complex support the role of both *RAD51*- and *RAD59*-dependent pathways in DNA recombination events [Davis and Symington, 2001; 2003].

In this study, we report that the accumulation of either "clean" (3'-OH and 5'-PO_4 termini) or "dirty" (DNA-adenylate; 3'-OH and 5'-AMP ends) nicks in *cdc9-1* mutants triggered PCNA mono- and poly-ubiquitination at K107. Moreover, K107 ubiquitination was responsible for causing a severe delay in S phase progression. To identify pathways involved in nick resolution, we performed a synthetic genetic array (SGA) screen with *cdc9-1* mutants and verified results by selected manual tetrad dissections. Besides the known requirement for genes involved in DSB repair via *RAD51/RAD52*-mediated HR, we uncovered strong genetic interactions with components of the *RAD51*-independent SSA pathway, comprising *RAD59*, *RAD1*, and *RAD10*. Surprisingly however, deletion of *SLX4* (synthetically lethal with *sgs1*), a crucial component of SSA-mediated DSB repair [Flott *et al.*, 2007; Toh *et al.*, 2010], did not affect *cdc9-1* mitotic growth. These results suggested that SSA might be dispensable for DSB repair in *cdc9-1* cells. This was further

corroborated by the fact that the genetic requirement for *RAD59* was much stronger than that for *RAD1* and *RAD10*, arguing that the three genes did not necessarily act in the same pathway. Targeted analysis of *RAD59* revealed that its deletion in *cdc9-td* mutants resulted in enhanced Mrc1 phosphorylation. We concluded that stalled replication forks accumulated more frequently in *cdc9-td rad59Δ* double mutants than *cdc9-td* cells. Together, these results uncover a role for non-canonical PCNA ubiquitination in facilitating S phase delay and for *RAD59* in promoting slow fork progression when DNA ligase I is limiting.

Materials and methods

Yeast strains.

Yeast strains used in this study are isogenic derivatives of SSL204, YKL83, or the RDKY3615 background. The genotypes of all the strains used in this study can be found in Table 4.1. Strains were constructed using standard genetic methods [Brachmann *et al.*, 1998]. PCNA lysine mutants were generated as described in Chapter 2 [Das-Bradoo *et al.*, 2010b].

To construct the N-terminally tagged hemagglutinin (HA) *MRC1* (*3HA-MRC1*) in the endogenous locus, two step PCR-mediated integration was performed as described elsewhere [Tong and Boone, 2006]. Briefly, two pairs of oligonucleotides were synthesized to amplify *3HA-MRC1* and the *KanMX4* marker separately. The *3HA-MRC1*, which includes 39 bp upstream and 288 bp downstream of its start and stop codons, respectively, was amplified from pRS405-*3HA-MRC1* (a gift from D. Koepp, University of Minnesota) using 5'-CGTTATTCGCT TTTGAACT TATCACC-3' and 5'-*GGGATCCGTCGACCTGCAGCGTACG*GCAAGATGCTTTGAATACAGAACTG-3'. The resulting PCR product contains a 25 bp overlapping segment with the *kanMX4* cassette at the 5' end (italicized sequence). The *KanMX4* gene was amplified using 5'-CGTACGCTGCAGGTCGACGGATCCC-3' and 5'-<u>AGCTTCTGGAGTTCAATCAAC TTCTTCGGAAAAGATAAAAAACCAATCGAT</u>GAATTCGAGCTCGTTTTCG-3' to create a fragment that overlapped with 40 bp immediately downstream of the endogenous *MRC1* locus (underlined sequence). PCR products were combined, denatured at 94°C for 3 to 4 min, cooled to room temperature and transformed into the desired yeast strains.

Synthetic genetic array (SGA) analysis.

A genome-wide screen for *CDC9* genetic interactions was conducted as described [Baryshnikova *et al.*, 2010a]. Briefly, a *cdc9-1* mutant strain marked with a nourseothricin (*NatMX4*) resistance cassette and harboring the SGA haploid specific markers and reporter [Baryshnikova *et al.*, 2010a] was mated the array of 4000 viable *S. cerevisiae* deletion mutants [Winzeler *et al.*, 1999]. Nourseothricin- and geneticin-resistant heterozygous diploid mutants were selected and sporulated and *MAT**a** cdc9-1* double mutants were subsequently selected as described [Baryshnikova *et al.*, 2010a]. In Figure 4.7a, and Supplementary Table S1, genetic interactions involving the *pol32Δ*, *rad27Δ*, and *rad6Δ* mutants were obtained from [Costanzo *et al.*, 2010]. Genetic interactions involving all other deletion mutants and temperature-sensitive mutants were obtained from the most recent SGA dataset (C. Boone, unpublished data, 2 January 2012). Both of these sources use SGA technology to compare query mutants to a collection of 4000 deletion mutants. PH designates alleles that came from Phil Hieter [Ben-Aroya *et al.*, 2008; Ben-Aroya *et al.*, 2010]. All genetic interactions were scored as described [Baryshnikova *et al.*, 2010b].

To confirm the SGA results, all gene deletions were constructed in SSL204 *MAT**a*** strain and crossed with an isogenic *cdc9-1 MAT**α*** strain (a gift from D. M. Livingston, University of Minnesota). Diploid cells were sporulated at 25°C and dissected. Plates were incubated for 3 to 5 days at either 25°C or 30°C. All spore genotypes were determined by temperature sensitivity at 35°C and growth on minimal medium lacking leucine (for *rad52Δ*) and uracil (for all other deleted mutants).

Plasmids.

The *CDC9* gene with its endogenous promoter was initially cloned into the vector pRS313 using the *BamH*I restriction site (*pCDC9*, a gift from D. M. Livingston, University of Minnesota). p*cdc9-NΔ60*, p*cdc9-K419A*, and p*cdc9-K598A* plasmids were derived from the plasmid p*CDC9* using Quikchange Lightning Site-Directed Mutagenesis (Agilent, Santa Clara, CA). p*ChVLig-3HA* was constructed by cloning two different PCR fragments into the vector pRS313. Between the *BamH*I-*Xho*I sites of the p*ChVLig-3HA* plasmid is the *CDC9* promoter (*CDC9pro*, 449-bp) driving the expression of the *Chlorella* virus DNA ligase coding sequence (*ChVLig*, a gift from S. Shuman, Memorial Sloan Kettering Institute) that is followed by three HA tags at its C-terminus (*BamH*I-*CDC9pro*-*Cla*I-*ChVLig-3HA*-*Xho*I) [Sriskanda *et al.*, 1999]. To overexpress *Chlorella* virus DNA ligase, the *ChVLig-3HA* fragment was subcloned from the p*ChVLig-3HA* plasmid into the pRS423gal vector (pgal) under the control of the *GAL10* promoter using *Cla*I-*Xho*I sites (pgal-*ChVLig-3HA*). All constructs were confirmed by DNA sequence analysis.

Complementation of *cdc9-1* temperature sensitivity.

All exogenous Cdc9 plasmids and the pRS313-*ChVLig-3HA* plasmids were transformed into wild type, *cdc9-1* and *cdc9-1 pol30-K164R* strains. 10-fold serial dilutions of cells were spotted on appropriate medium. Plates were incubated for 2 to 3 days at indicated temperatures. Temperature shift experiments were carried out as described (Chapter 2) [Das-Bradoo *et al.*, 2010b]. Briefly, cells were grown overnight to

mid-log phase (OD_{600} = 0.6) at 25°C and shifted to the restrictive temperature of 35°C for 3 hours. For degron strains, cells were grown overnight at 28°C in YP plus 2% raffinose and supplemented with 10 uM $CuSO_4$ to induce gene expression. Once cells have grown to mid-log phase, cells were switched to YP plus 2% galactose without $CuSO_4$ for an addition 30 min at 28°C and subsequently shifted to 37°C.

For the overexpression experiments of the ChVLig-3HA, both pgal and pgal-*ChVLIG-3HA* plasmids were transformed into either wild type or *cdc9-1* strains. 10-fold serial dilutions of cells were spotted on minimal medium lacking histidine (Sc-His), but containing either 2% glucose or 2% galactose. Plates were incubated for 4 to 5 days at either 25°C or 35°C. For temperature shift experiments, asynchronous cultures were grown overnight to mid-log phase at 25°C in Sc-His medium containing 2% raffinose. Cultures were split and shifted to 35°C for 3 hours in the presence of either 2% glucose or galactose.

Characterization of GCR and CAN1 forward mutation rates.

GCR rates and CAN1 forward mutation rates were determined as described [Chen *et al.*, 1999; Motegi and Myung, 2007]. Briefly, the GCR or mutation rates from two independent isolates were determined by fluctuation analyses twice using the method of the median [Lea and Coulson, 1949]. Each experiment was performed using 11 cultures and the average value from two different clones is reported.

Sensitivity to UV, MMS and γ-irradiation.

Overnight cultures of the indicated strains were serially diluted and spotted onto YPD plates or YPD plates containing MMS. For UV- or γ-irradiation, strains were spotted onto YPD plates and irradiated as indicated. Pictures were taken after 2 days incubation at 30°C.

Protein preparation and western blot analysis.

Total protein extracts were prepared from cycling cells using TCA precipitation and protein expression was analyzed by immunoblotting [Ricke and Bielinsky, 2006]. The endogenous Cdc9 and Rad53 proteins were detected using anti-Cdc9 (1:12000, a gift from A. E. Tomkinson, University of New Mexico) and anti-Rad53 (1:1000, a gift from J. F. Diffley, Cancer Research UK London Research Institute) antibodies, respectively. All HA-tagged proteins were detected using either an anti-HA (1:3000, 16B12, Covance) or anti-HA-HRP conjugated (1:250, 3F10, Roche) antibodies, respectively. Both unmodified and ubiquitinated forms of PCNA were detected using an anti-yeast PCNA antibody as described (1:4000, clone S871, a gift from Z. Zhang, Mayo Clinic MN and B. W. Stillman, Cold Spring Harbor Laboratory, NY) (Chapter 2) [Das-Bradoo *et al.*, 2010b]. For detection of mono-ubiquitinated PCNA, protein extracts were diluted prior to fractionating by SDS-PAGE gel electrophoresis as described (Chapter 2) [Das-Bradoo *et al.*, 2010b]. a-tubulin served as a loading control.

Cell cycle and FACS Analysis.

Cell cycle progression was monitored using flow cytometry as described in Chapter 2 [Das-Bradoo *et al.*, 2010b]. DNA was stained Sytox Green was used and all FACS samples were analyzed using a Becton Dickinson FACS Calibur.

Table 4.1 **List of yeast strains used in this study.**

Strain Name	Relevant Genotype	Source
	***W303*-derived strains**	
YKL83	*GAL-UBR1 (HIS3)*	[Labib *et al.*, 2000]
ABy010	*GAL-UBR1 (HIS3) bar1::LEU2*	This Study
ABy1643	*GAL-UBR1 (HIS3) bar1::LEU2 MRC1::3HA-MRC1(KanMX4)*	This Study
ABy1596	*GAL-UBR1 (HIS3) rad59::TRP1* Cl.1	This Study
ABy1597	*GAL-UBR1 (HIS3) rad59::TRP1* Cl.2	This Study
ABy1654	*GAL-UBR1 (HIS3) bar1::LEU2 MRC1::3HA-MRC1(KanMX4) rad59::TRP1*	This Study
ABy008	*GAL-UBR1 (HIS3) bar1::LEU2 cdc9::cdc9-td (URA3)*	This Study
ABy1541	*GAL-UBR1 (HIS3) bar1::LEU2 cdc9::cdc9-td (URA3) MRC1::3HA-MRC1(KanMX4)*	This Study
ABy1598	*GAL-UBR1 (HIS3) bar1::LEU2 cdc9::cdc9-td (URA3) rad59::TRP1* Cl.1	This Study
ABy1599	*GAL-UBR1 (HIS3) bar1::LEU2 cdc9::cdc9-td (URA3) rad59::TRP1* Cl.2	This Study
ABy1656	*GAL-UBR1 (HIS3) bar1::LEU2 cdc9::cdc9-td (URA3) MRC1::3HA-MRC1(KanMX4) rad59::TRP1*	This Study
	***SSL204*-derived strains**	
SSL204	*MATa ade2 his3Δ200 trp1 leu2 ura3-52*	[Dornfeld and Livingston, 1991]
ABy1321	*rad1::URA3*	This study

Table 4.1 List of yeast strains used in this study (continued)

ABy1323	*rad10::URA3*	This study
ABy1325	*rad14::URA3*	This study
ABy1451	*slx4::URA3*	This study
ABy1407	*rad59::URA3*	This study
ABy1430	*rad51::URA3*	This study
ABy1537	*exo1::URA3* Cl.1	This study
ABy1538	*exo1::URA3* Cl.2	This study
ABy805	*hxt13::URA3*	This study
SSL212A	*rad52Δ HS::LEU2*	[Dornfeld and Livingston, 1991]
ABy1055	pRS313	This study
ABy1056	pRS313-*CDC9*	This study
ABy1127	pRS313-*cdc9-K419A*	This study
ABy1177	pRS313-*cdc9-K598A*	This study
ABy1086	pRS313-*cdc9-NΔ60*	This study
ABy1185	pRS313-*ChVLig-3HA*	This study
ABy1277	pRS423gal	This study
ABy1278	pRS423gal-*ChVLig-3HA*	This study
ABy1146	pgal	This study
ABy1146	pgal-*rad53-K221A/D339A (KD)*	This study
ABy685	*pol30::pol30K107R (LEU2)*	[Das-Bradoo *et al.*, 2010b]

Table 4.1 List of yeast strains used in this study (continued)

SSL612α	*cdc9-1 MATα ade2 his3Δ200 trp1 leu2 ura3-52*	[Ireland *et al.*, 2000]
	SSL612a *(cdc9-1)*-derived strains	
SSL612a	*cdc9-1 MATa ade2 his3Δ200 trp1 leu2 ura3-52*	[Ireland *et al.*, 2000]
ABy1539	*exo1::URA3* Cl.1	This study
ABy1540	*exo1::URA3* Cl.2	This study
ABy807	*hxt13::URA3*	This study
ABy1057	*cdc9-1,* pRS313	This study
ABy1058	*cdc9-1,* pRS313-*CDC9*	This study
ABy1128	*cdc9-1,* pRS313-*cdc9-K419A*	This study
ABy1087	*cdc9-1,* pRS313-*cdc9-K598A*	This study
ABy1178	*cdc9-1,* pRS313- *cdc9-NΔ60*	This study
ABy1186	*cdc9-1,* pRS313-*ChVLig-3HA*	This study
ABy1279	*cdc9-1,* pRS423gal	This study
ABy1280	*cdc9-1,* pRS423gal-*ChVLig-3HA*	This study
ABy1148	pgal	This study
ABy1149	pgal-*rad53-K221A/D339A (KD)*	This study
ABy1439-4a	*cdc9-1 rad59::URA3 Cl.1*	This study
ABy1439-7c	*cdc9-1 rad59::URA3 Cl.2*	This study
ABy1439-13a	*cdc9-1 rad59::URA3 Cl.3*	This study
ABy1605	*cdc9-1 pol30::pol30K164R (LEU2),* pRS313	This study

Table 4.1 List of yeast strains used in this study (continued)

ABy1606	*cdc9-1 pol30::pol30K164R (LEU2),* pRS313-*CDC9*	This study
ABy1607	*cdc9-1 pol30::pol30K164R (LEU2),* pRS313-*cdc9-K419A*	This study
ABy1608	*cdc9-1 pol30::pol30K164R (LEU2),* pRS313-*cdc9-K598A*	This study
ABy1609	*cdc9-1 pol30::pol30K164R (LEU2),* pRS313- *cdc9-NΔ60*	This study
ABy872	*cdc9-1* pol30::pol30K107R (LEU2)*	[Das-Bradoo *et al.*, 2010b]
	***RDKY3615*-derived strains**	
RDKY3615	*ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, htx13::URA3*	[Chen and Kolodner, 1999]
RDKY3735	*sml1::KanMX, mec1::HIS3*	[Myung *et al.*, 2001]
YKJM5789	*pol30::HIS3,* YCPlac22-*POL30*	This study
YKJM5799	*pol30::HIS3,* YCPlac22-*pol30-K183R*	This study
YKJM5985	*pol30::HIS3,* YCPlac22-*pol30-K107R*	This study
YKJM5986	*pol30::HIS3,* YCPlac22-*pol30-K117R*	This study
YKJM5988	*pol30::HIS3,* YCPlac22-*pol30-K127R*	This study
YKJM5989	*pol30::HIS3,* YCPlac22-*pol30-K127/164R*	This study
YKJM5991	*pol30::HIS3,* YCPlac22-*pol30-K164R*	This study

Results

Accumulation of nicked DNA due to DNA ligase I deficiency triggers PCNA ubiquitination independently of lysine 164.

The depletion of Cdc9 in *S. cerevisiae* drastically slows down S phase progression (Figure 3.1) [Das-Bradoo *et al.*, 2010a]. Specifically, the loss of DNA ligase I triggers a novel ubiquitination pathway that targets PCNA at a lysine residue distinct from K164 and acts upstream of S phase checkpoint activation (Figure 2.14) [Das-Bradoo *et al.*, 2010b]. However, it was still unclear what molecular defect caused PCNA ubiquitination in *cdc9* mutants. We envisioned three possible scenarios that could arise when DNA ligase I is limiting. First, cells could directly sense the accumulation of nicks; second, cells might recognize the absence of the PCNA-Cdc9 interaction or third, because *cdc9* mutants are known to be highly mutagenic [Montelone *et al.*, 1981], secondary defects unrelated to the generation of nicks could cause the ubiquitination of PCNA.

To distinguish between these different scenarios, we complemented *cdc9-1*, and *cdc9-1 pol30-K164R* (PCNA-K164R) cells with plasmids expressing either wild-type or mutant Cdc9 under its endogenous promoter. As a control, these plasmids were also introduced into wild-type cells (Figure 4.1). As expected, expression of wild-type *CDC9* rescued the temperature sensitivity of *cdc9-1* mutants at 35°C (Figure 4.1a). In addition, we introduced two plasmids carrying DNA ligase I mutations in the active center of the enzyme, p*cdc9-K419A* and p*cdc9-K598A*. Cdc9-K419 is critical for the covalent binding of AMP in the first step of ligation and mutation of this residue results in the accumulation of "clean" nicks, whereas Cdc9-K598 is important for the final DNA de-

adenylation step, and substitution of this residue with alanine results in the accumulation of "dirty" nicks [Sriskanda *et al.*, 1999; Subramanian *et al.*, 2005; Tomkinson *et al.*, 1991]. Neither of the two catalytically inactive Cdc9 mutants allowed *cdc9-1* cells to grow at the restrictive temperature (Figure 4.1a). In contrast, deletion of the PIP-box motif in Cdc9 (p*cdc9-NΔ60*), which abolishes the interaction with PCNA [Subramanian *et al.*, 2005], had no effect on the ability to rescue *cdc9-1* at 35°C. Lastly, *cdc9-1* mutants expressing PCNA-K164R (*cdc9-1 pol30-K164R*), which renders cells sensitive to UV-irradiation and MMS [Hoege *et al.*, 2002], did not exhibit any additional temperature sensitivity as compared to *cdc9-1* single mutants (Figure 4.1a). These results indicated that the survival of *cdc9-1* mutants did not depend on K164 of PCNA, but rather solely on the reconstitution of DNA ligase I activity.

In parallel to testing cell viability, we also monitored DNA ligase I expression levels and the status of PCNA modification at 35°C (Figures 4.1b and c). PCNA is ubiquitinated, likely at K107, and sumoylated at either K127 or K164 in *cdc9-1* mutants (Figures 2.14 and 3.2) [Das-Bradoo *et al.*, 2010a; Das-Bradoo *et al.*, 2010b]. Consistent with an earlier report (Chapter 2) [Das-Bradoo *et al.*, 2010b], we detected four different PCNA species in *cdc9-1* cells that carried an empty vector (Figure 4.1b): unmodified PCNA (29 kDa), mono-ubiquitinated PCNA (39 kDa), and putatively poly-ubiquitinated PCNA (at ~52 and 76 kDa). However, it was possible that a single PCNA monomer could be simultaneously sumoylated (at K164) and ubiquitinated (at K107). Indeed, a K164R substitution in PCNA diminished the 76 kDa band in *cdc9-1* cells (Figure 4.1c, compare lanes 2 and 3). Thus, the 76 kDa band most likely represented PCNA that was

di-ubiquitinated at K107 and sumoylated at K164 (marked as Ub_2/S^{164}-PCNA in Figures 4.1b and c). In contrast, the 39 kDa and ~52 kDa bands represented solely mono- and di-ubiquitinated PCNA species, respectively (marked as Ub_1-PCNA and Ub_2-PCNA in Figures 4.1b and c), as reported (Figure 2.6b) [Das-Bradoo *et al.*, 2010b].

Importantly, when we complemented *cdc9-1* cells with wild-type Cdc9 (*cdc9-1* + p*CDC9*), both PCNA mono- and poly-ubiquitination were abolished (Figure 4.1b). Because PCNA ubiquitination is readily detectable at 25°C (Figure 2.6a) [Das-Bradoo *et al.*, 2010b], the disappearance of the ubiquitinated PCNA molecules presented a true reversal of the ubiquitination response. This result allowed us to exclude nonspecific, secondary effects as a trigger of PCNA ubiquitination. Moreover, expression of the Cdc9 PIP-box mutant (*cdc9-1* + p*cdc9-NΔ60*) also reversed PCNA mono- and poly-ubiquitination, making it highly unlikely that cells recognized the absence of PCNA-Cdc9 interaction (Figure 4.1b). To further corroborate this notion, we examined the ability of *Chlorella* virus DNA ligase (ChVLig) to complement the DNA ligase I deficiency in yeast. ChVLig is the smallest known ATP-dependent ligase [Ho *et al.*, 1997], containing only a conserved catalytic core that consists of a nucleotidyltransferase (NTase) and an oligonucleotide/oligosaccharide binding (OB)-fold domain [Nair *et al.*, 2007]. Since ChVLig has no additional domains beyond its NTase-OB core, it does not interact with PCNA. When we expressed *ChVLig-3HA* under the control of the *CDC9* promoter, it only partially rescued the temperature sensitivity of *cdc9-1* mutants (*cdc9-1* + p*ChVLig-3HA*) at 33°C, but not at 35°C at which temperature PCNA mono-ubiquitination remained visible (Figures 4.2a and b). However, upon overexpression of

ChVLig-3HA from a galactose-inducible promoter we rescued *cdc9-1* temperature sensitivity and reversed PCNA mono-ubiquitination (Figures 4.2c and d). Thus, the ligation of nicks appeared to eliminate PCNA ubiquitination in the complete absence of a PCNA-DNA ligase interaction. This was further substantiated by the observation that PCNA ubiquitination remained unchanged in *cdc9-1* cells that were complemented with either p*cdc9-K419A* or p*cdc9-K598A*, encoding two different catalytically inactive forms of DNA ligase I, which retain PCNA binding activity (Figure 4.1b). Importantly, similar alterations to the ubiquitination pattern of PCNA (Ub_1-PCNA and Ub_2-PCNA) were observed in *cdc9-1 pol30-K164R* mutants after complementation with different DNA ligase I constructs (Figure 4.1c). These data suggest that cells induce PCNA mono- and poly-ubiquitination independently of K164 in response to nicked DNA. Because we have demonstrated that PCNA ubiquitination disappears in a viable *cdc9-1 pol30-K107R* double mutant (Figure 2.14a) [Das-Bradoo *et al.*, 2010b], we postulate that PCNA ubiquitinated at K107 ($PCNA^{K107\text{-}Ub}$) functions as a nick sensor at replication forks in budding yeast.

PCNA ubiquitination at K107 is important for the S phase delay in *cdc9-1* cells.

$PCNA^{K107\text{-}Ub}$ is a prerequisite to activate the S phase checkpoint kinase Rad53 in *cdc9ts* mutants (Figure 2.14e) [Das-Bradoo *et al.*, 2010b]. Although most *cdc9-1 pol30-K107R* double mutants were synthetically lethal after tetrad dissection, we were able to isolate a viable double mutant with slightly increased DNA ligase I levels, designated as *cdc9-1* pol30-K107R* (Figures 2.13 and 2.14) [Das-Bradoo *et al.*, 2010b]. Because

robust Rad53 activation was drastically reduced in *pol30-K107R* mutants upon DNA ligase I depletion, we predicted that *cdc9-1* pol30-K107R* mutants would readily progress through S phase. Therefore, we monitored cell cycle progression of *cdc9-1*, *cdc9-1* pol30-K107R*, and their respective parental strains (Figure 4.3a). Because *cdc9-1* pol30-K107R* mutants are severely more temperature sensitive than *cdc9-1* cells (Figure 2.13b) [Das-Bradoo *et al.*, 2010b], we performed the temperature shift experiments at 30°C instead of 35°C. Neither *CDC9* nor *CDC9 pol30-K107R* strains exhibited any cell cycle defects at 30°C (Figure 4.3a). However, *cdc9-1* cells accumulated in, and progressed through, S phase very slowly after 1.5 and 3h temperature shifts. In contrast, *cdc9-1* pol30-K107R* mutants did not exhibit a delay in S phase and accumulated in G2, likely due to segregation defects (Figure 4.3a). These results support the notion that PCNA ubiquitination at K107 facilitates robust activation of Rad53, which is responsible for the observed S phase delay. In line with this notion, galactose-induced overexpression of a dominant negative Rad53 kinase-dead mutant (Rad53-K221A/D339A) [Pellicioli *et al.*, 1999; Szyjka *et al.*, 2008] also completely suppressed the accumulation of *cdc9-1* cells in S phase (compare galactose on the left vs. glucose on the right in Figure 4.3b).

Because K107 of PCNA appeared to have an important role in S phase checkpoint activation in *cdc9* mutants, we asked whether a K107R substitution exhibited any effect on the growth or DNA damage signaling in DNA ligase I-proficient cells. We tested UV- and gamma irradiation as well as MMS, but did not detect any growth sensitivity (Figure 4.4a). In parallel, we also determined the rate of spontaneous mutations by testing

resistance to canavanine. In this assay, the K107R mutant displayed a slightly elevated mutation frequency comparable to that of K164R and K183R mutants (Figure 4.4b). These results are consistent with the idea that PCNA ubiquitination at K107 is specific to nicked replication intermediates that persist during lagging strand synthesis.

Identification of the DNA repair network in *cdc9-1* mutants.

To elucidate the pathways that are crucial to cope with the accumulation of nicked DNA during DNA replication, we conducted a SGA screen using the *cdc9-1* mutation as the query strain, which was mated with approximately 4000 array strains carrying single deletions of non-essential genes [Baryshnikova *et al.*, 2010a]. The fitness of the double mutants was scored quantitatively by colony size at the semi-permissive temperature of 30°C [Baryshnikova *et al.*, 2010b]. We observed synthetic sickness between *cdc9-1* and *rad9Δ* (Table 4.2), in accordance with the documented role of *RAD9* in response to DNA damage [Das-Bradoo *et al.*, 2010b; Schiestl *et al.*, 1989; Scott and Plon, 2003; Weinert and Hartwell, 1993]. Since Rad9 functions primarily in response to DSBs [Naiki *et al.*, 2004], the synthetic sickness of the *cdc9-1 rad9Δ* double mutants indicates that some of the nicks in *cdc9-1* cells are converted to DSBs. Indeed, the rate for gross chromosomal rearrangements (GCRs) in *cdc9-1* mutants was 30-fold elevated over wild-type (Figure 4.5), indicative of the presence of spontaneous DSBs.

The MRX (Mre11-Rad50-Xrs2) complex is the primary sensor of DSBs and crucial to initiate HR [Symington, 2002]. Consistent with the presence of DSBs in *cdc9-1* cells, we also identified synthetic sickness between *cdc9-1* and *mre11Δ* in our screen

(Table 4.2), in line with the reported negative genetic interaction between *cdc9* and *rad50Δ* [Davierwala *et al.*, 2005; Symington, 2002]. Curiously, *RAD52*, a major HR component was not synthetically lethal with the *cdc9-1* allele in the SGA screen, although *cdc9 rad52* double mutants have been reported to be synthetically lethal [Ireland *et al.*, 2000; Montelone *et al.*, 1981]. Because *cdc9-1* mutants are highly mutagenic [Montelone *et al.*, 1981], we postulate that the *cdc9-1 rad52Δ* double mutants on the array likely accumulated a second site suppressor that allowed these mutants to grow at a similar rate as *cdc9-1* cells. To ensure that our *cdc9-1* strain exhibited the same genetic properties as described in the literature, we manually crossed it with *rad51Δ* and *rad52Δ* cells, respectively. As expected, we were not able to isolate any viable *cdc9-1 rad51Δ* or *cdc9-1 rad52Δ* double mutants, indicating that *RAD51*/*RAD52*-mediated HR is essential for survival (Figures 4.6a, b and g). Because our primary interest was the identification of novel factors that facilitate nick resolution and promote replication fork progression in *cdc9-1* cells, we focused on genes participating in pathways different from HR.

The single strand annealing proteins, Rad59, Rad1, and Rad10 but not Slx4 are important for *cdc9-1* survival.

Besides genes involved in DSB repair, we observed that *cdc9-1* has genetic interactions with genes involved in the nucleotide excision repair (NER) pathway, *RAD1* and *RAD14* (Figures 4.7a and g). Strikingly, deletions of *RAD1* and *RAD14* showed drastically opposite effects (Figures 4.7a and g). Whereas the loss of *RAD1* decreased viability, the deletion of *RAD14* promoted cell growth, suggesting that *RAD1* and *RAD14*

have independent roles in *cdc9-1* mutants. Rad1 interacts with Rad10 to form a structure-specific endonuclease, a homolog of the XPF/ERCC1 (for Xeroderma pigmentosum group F/Excision repair cross-complementing rodent repair deficiency, complementation group 1) complex in humans [Bailly *et al.*, 1992; Sung *et al.*, 1993]. During NER, Rad14 recruits the Rad1-Rad10 endonuclease to the site of DNA damage to incise 5' of the lesion [Guzder *et al.*, 2006]. Besides NER, the Rad1-Rad10 endonuclease has been implicated in the SSA pathway, a form of Rad51-independent repair that acts at DSBs and stalled replication forks between small repeat regions [Ivanov and Haber, 1995; Mott and Symington, 2011; Schiestl and Prakash, 1988; 1990]. Importantly, the role of Rad1-Rad10 in SSA is independent of Rad14 [Guzder *et al.*, 2006], which offered an explanation for the contrasting interactions with *cdc9-1* (Figures 4.7a and g) and prompted us to direct our attention to *RAD59*, another known component of the SSA pathway [Ivanov *et al.*, 1996; Jablonovich *et al.*, 1999]. Indeed, we also observed a negative genetic interaction between *RAD59* and *cdc9-1* (Table 4.2). Based on these results, we hypothesized that *RAD1* and *RAD59* could possibly function in the same pathway in *cdc9-1* mutants and independently of *RAD14*.

To validate the genetic interactions identified in the SGA screen, we constructed *cdc9-1 rad1Δ, cdc9-1 rad14* and *cdc9-1 rad59Δ* double mutants. Curiously, since *RAD1* interacts genetically and physically with *RAD10* in both NER and the SSA pathway [Schiestl and Prakash, 1988; 1990; Sung *et al.*, 1993], we were surprised that *Rad10* did not elicit any genetic interaction in our SGA screen. Thus, we also generated *cdc9-1 rad10Δ* double mutants to perform tetrad analysis. We were able to validate the

separation of function between *RAD1* and *RAD14* in *cdc9-1* cells, as we observed synthetic sickness in *cdc9-1 rad1Δ* mutants, but improved fitness in *cdc9-1 rad14Δ* mutants at 30°C (Figure 4.7b and d). The two different double mutants grew comparably to *cdc9-1* cells at 25°C, indicating that the differential growth phenotype at 30°C was due specifically to the loss of DNA ligase I. Similar to *cdc9-1 rad1Δ* double mutants, deletion of *RAD10* also reduced *cdc9-1* viability at 30°C but not at 25°C (Figure 4.7c), suggesting a role for the Rad1-Rad10 endonuclease in maintaining cell growth in *cdc9-1* cells. Surprisingly, in a manner more severe than *rad1Δ* and *rad10Δ*, we did not observe any viable *cdc9-1 rad59Δ* spores at 30°C, suggesting that *RAD59* was crucial for the survival of *cdc9-1* cells at the semi-permissive temperature (Figure 4.7e). At 25°C, however, *cdc9-1 rad59Δ* mutants were viable although the spores grew significantly slower than *cdc9-1* cells (Figure 4.7e). Since *RAD1, RAD10* and *RAD59* have been indicated to function in both DSB repair as well as template switching at stalled replication forks [Bai *et al.*, 1999; Bai and Symington, 1996; Fishman-Lobell and Haber, 1992; Flott *et al.*, 2007; Mott and Symington, 2011], we examined the genetic interaction between *cdc9-1* and *SLX4*, an essential gene required for DSB repair by SSA between direct repeats [Flott *et al.*, 2007]. We did not observe any differences in the growth phenotypes of *cdc9-1* single and *cdc9-1 slx4Δ* double mutants at 25°C or 30°C (Figure 4.7f). This accurately reflected the result of our SGA screen in which we did not observe a genetic interaction between *cdc9-1* and *slx4Δ*. Altogether, these newly uncovered genetic interactions indicated that *RAD59* and *RAD1/RAD10* were irrelevant for DSB

repair per se in *cdc9-1* cells. This was supported by the differential requirement for Rad59 and the Rad1-Rad10 complex, which is inconsistent with all three proteins acting in concert to perform SSA.

Deletion of *RAD59* resulted in an increase of stalled forks in *cdc9* mutants.

To better understand the role of Rad59 in *cdc9* mutants, we deleted *RAD59* in a *cdc9-td* strain, which expresses DNA ligase I when copper is added to the medium and grows similar to wild-type cells at the permissive temperature. *cdc9-td rad59Δ* mutants grew similar to wild-type and *rad59Δ* mutants on glucose at 28°C (Figure 4.8a). In contrast, when *cdc9-td rad59Δ* mutants were spotted on 2% galactose, cell growth was inhibited, even when additional copper was added to express Cdc9. The phenotype was further exacerbated at 37°C, which facilitated degradation of Cdc9-td. The growth phenotype of *cdc9-td rad59Δ* mutants was thus very similar to that of *cdc9-1 rad59Δ* cells (Figures 4.7e and 4.8a).

Besides its well-documented role in break-induced replication [Mizuno *et al.*, 2009; Paek *et al.*, 2009], Rad59 has recently been implicated to function at stalled replication forks to promote spontaneous recombination between inverted repeats [Mott and Symington, 2011]. Furthermore, a different study in *S. pombe* provided evidence that Rad52 could associate with stalled replication forks independently of Rad51 [Irmisch *et al.*, 2009]. Since Rad52 forms distinct complexes with either Rad51 or Rad59 [Davis and Symington, 2001; 2003], we postulated that Rad52/Rad59 might be active at stalled replication forks in *cdc9* mutants. Because $PCNA^{K107\text{-}Ub}$ likely resides at stalled forks

(Chapter 2) [Das-Bradoo *et al.*, 2010b], we first determined whether deletion of *RAD59* had any effect on the status of PCNA ubiquitination. When we shifted both *cdc9-1 rad59Δ* and *cdc9-td rad59Δ* to their non-permissive temperatures, $PCNA^{K107\text{-}Ub}$ remained intact in the double mutants compared to the single mutants (Figure 4.8b). These data suggested that *RAD59* functions either downstream of $PCNA^{K107\text{-}Ub}$ or in a parallel pathway. If *RAD59* were to function downstream of PCNA ubiquitination to promote Rad53 checkpoint activation, we predicted that *cdc9-td rad59Δ* double mutants should exhibit reduced Rad53 activation. To determine whether Mrc1-mediated activation of Rad53 is reduced in these mutants, we examined the phosphorylation status of both Mrc1 and Rad53 [Alcasabas *et al.*, 2001; Osborn and Elledge, 2003]. To our surprise, we observed an increase in Mrc1 and Rad53 hyper-phosphorylation in *cdc9-td rad59Δ* as compared to *cdc9-td* cells (Figure 4.9). These findings are consistent with the notion that Rad59 plays a role to promote replication fork progression through S phase in the absence of DNA ligase I and is required to relieve Rad53 activation.

Figure 4.1

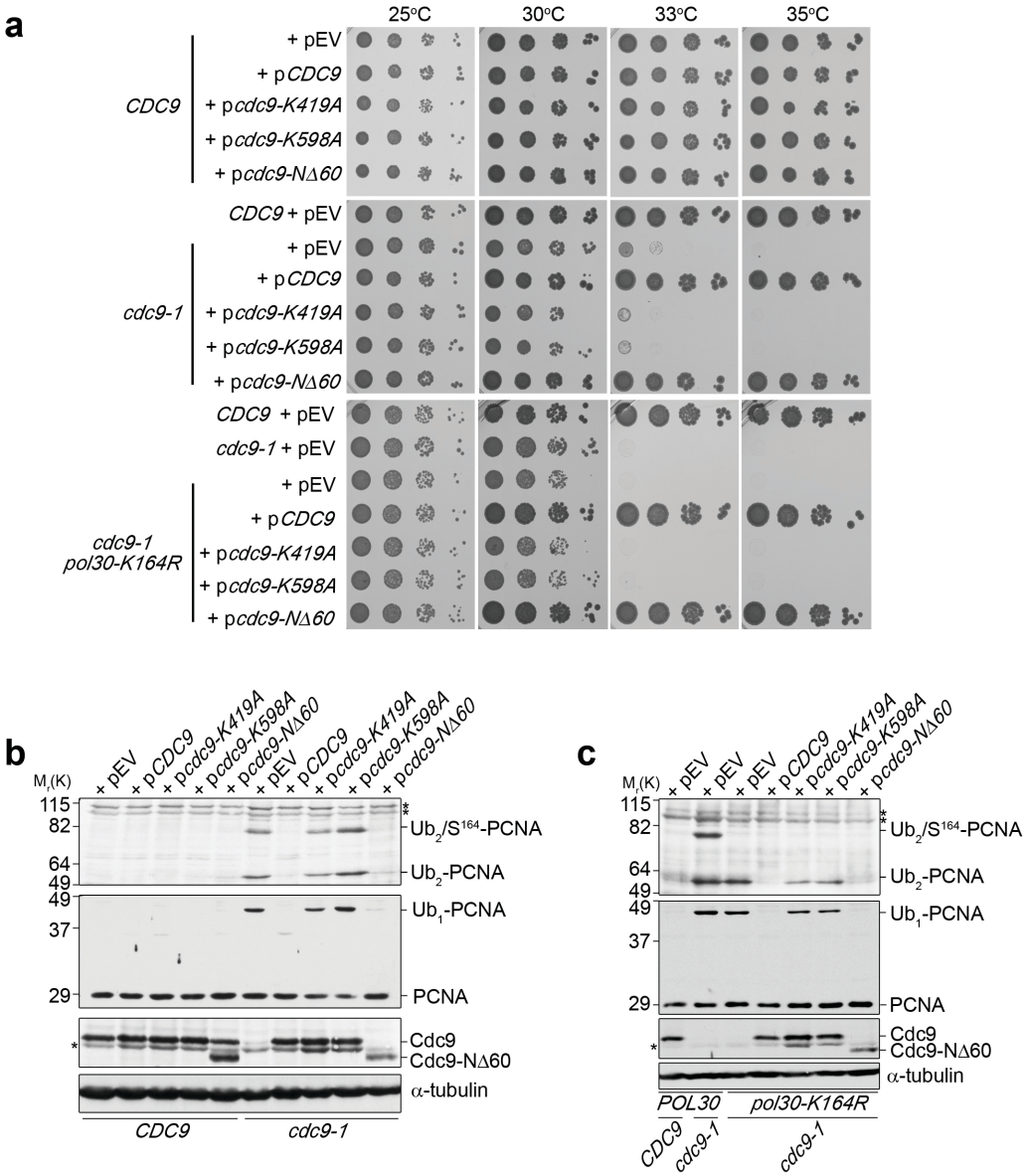
a
25°C
30°C
33°C
35°C
CDC9
+ pEV
+ pCDC9
+ pcdc9-K419A
+ pcdc9-K598A
+ pcdc9-NΔ60
cdc9-1
CDC9 + pEV
cdc9-1 + pEV
cdc9-1 pol30-K164R
b
c
Mr(K)
115
82
64
49
37
29
Ub2/S164-PCNA
Ub2-PCNA
Ub1-PCNA
PCNA
Cdc9
Cdc9-NΔ60
α-tubulin
POL30
pol30-K164R

Figure 4.1 Defects in DNA ligase I trigger PCNA mono- and poly-ubiquitination independently of lysine 164.

(a) Successive 10-fold dilutions of ABy1055 (*CDC9* + pEV), ABy1056 (*CDC9* + p*CDC9*), ABy1127 (*CDC9* + p*cdc9-K419A*), ABy1086 (*CDC9* + p*cdc9-K598A*), ABy1177 (*CDC9* + p*cdc9-NΔ60*), ABy1057 (*cdc9-1* + pEV), ABy1058 (*cdc9-1* + p*CDC9*), ABy1128 (*cdc9-1* + p*cdc9-K419A*), ABy1087 (*cdc9-1* + p*cdc9-K598A*), ABy1178 (*cdc9-1* + p*cdc9-NΔ60*), ABy1605 (*cdc9-1 pol30-K164R* + pEV), ABy1606 (*cdc9-1 pol30-K164R* + p*CDC9*), ABy1607 (*cdc9-1 pol30-K164R* + p*cdc9-K419A*), ABy1608 (*cdc9-1 pol30-K164R* + p*cdc9-K598A*), and ABy1609 (*cdc9-1 pol30-K164R* + p*cdc9-NΔ60*) were grown on Sc-His plates for 3 days at the indicated temperatures. **(b, c)** All strains in **a** were grown asynchronously to mid-log phase at 25°C. Subsequently, cultures were shifted to the restrictive temperature of 35°C for 3 hours. PCNA and its ubiquitinated forms, and Cdc9 were detected with anti-PCNA (S871), anti-Cdc9, respectively. α-tubulin served as a loading control. Asterisks indicate non-specific bands. Ub, ubiquitin and S^{164}, SUMO at K164.

Figure 4.2

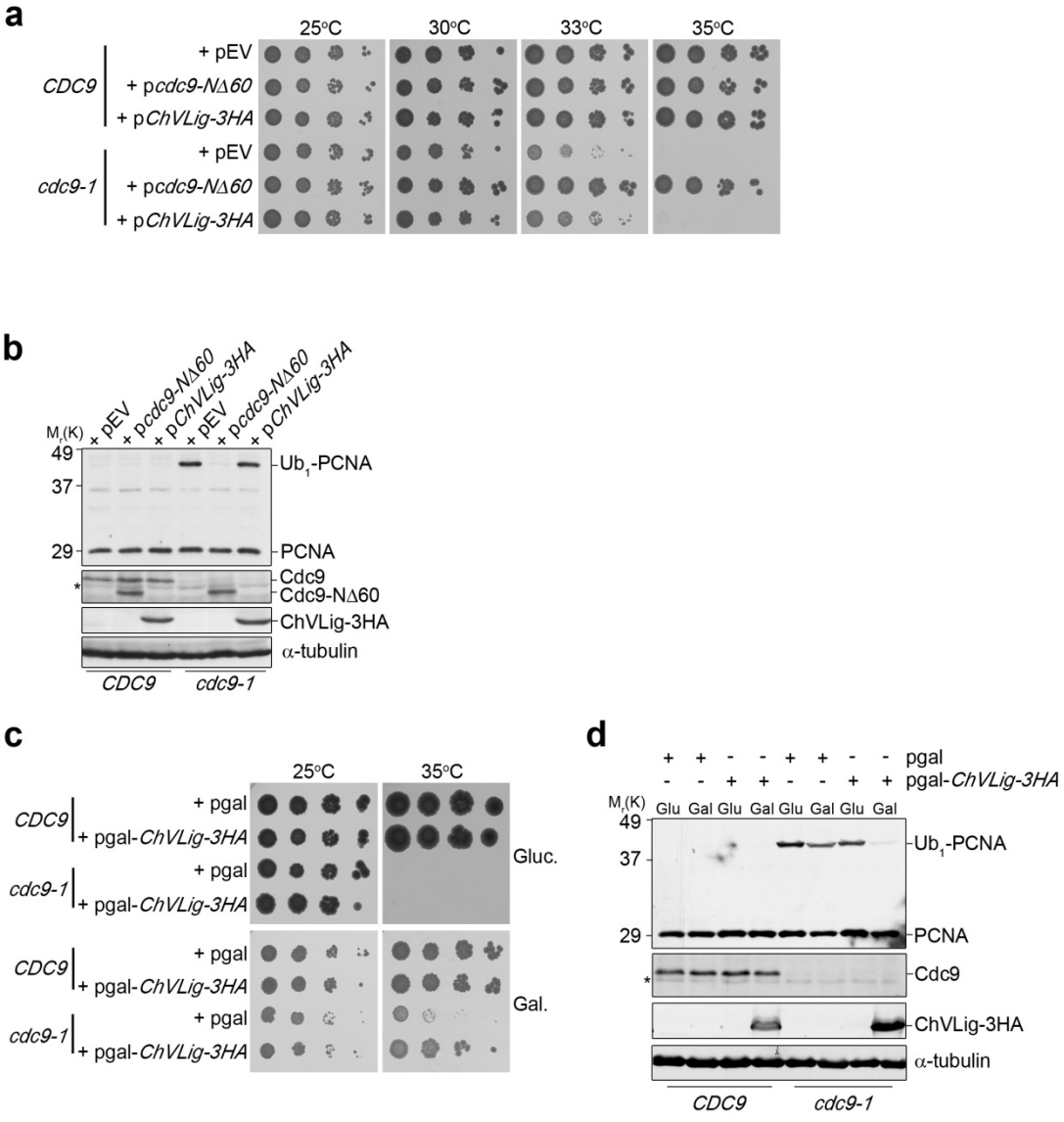
a
25°C
30°C
33°C
35°C
CDC9
+ pEV
+ pcdc9-NΔ60
+ pChVLig-3HA
cdc9-1
+ pEV
+ pcdc9-NΔ60
+ pChVLig-3HA
b
Mr(K)
+ pEV
+ pcdc9-NΔ60
+ pChVLig-3HA
+ pEV
+ pcdc9-NΔ60
+ pChVLig-3HA
49
37
29
Ub1-PCNA
PCNA
Cdc9
Cdc9-NΔ60
ChVLig-3HA
α-tubulin
CDC9
cdc9-1
c
25°C
35°C
CDC9
+ pgal
+ pgal-ChVLig-3HA
cdc9-1
+ pgal
+ pgal-ChVLig-3HA
Gluc.
CDC9
+ pgal
+ pgal-ChVLig-3HA
cdc9-1
+ pgal
+ pgal-ChVLig-3HA
Gal.
d
pgal
pgal-ChVLig-3HA
Mr(K)
Glu Gal Glu Gal Glu Gal Glu Gal
49
37
29
Ub1-PCNA
PCNA
Cdc9
ChVLig-3HA
α-tubulin
CDC9
cdc9-1

Figure 4.2 Overexpression of *Chlorella* virus DNA ligase fully complements *cdc9-1* temperature sensitivity and PCNA ubiquitination.

(a) Successive 10-fold dilutions of ABy1055 (*CDC9* + pRS313), ABy1177 (*CDC9* + p*cdc9-NΔ60*), ABy1185 (*CDC9* + p*ChVLig-3HA*), ABy1057 (*cdc9-1* + pRS313), ABy1178 (*cdc9-1* + p*cdc9-NΔ60*), and ABy1186 (*cdc9-1* + p*ChVLig-3HA*) were grown on Sc-His plates for 3 days at the indicated temperatures. Note: The expression of *Chlorella* virus DNA ligase in p*ChVLig-3HA* plasmid is under the *CDC9* promoter. **(b)** All strains in **a** were grown asynchronously to mid-log phase at 25°C. Subsequently, cultures were shifted to the restrictive temperature of 35°C for 3 hours. PCNA and its ubiquitinated forms, Cdc9 and ChVLig-3HA were detected with anti-PCNA (S871), anti-Cdc9, and anti-HA antibodies, respectively. **(c)** Successive 10-fold dilutions of ABy1277 (*CDC9* + pRS423gal), ABy1278 (*CDC9* + pRS423gal-*ChVLig-3HA*), ABy1279 (*cdc9-1* + pRS423gal), and ABy1280 (*cdc9-1* + pRS423gal-*ChVLig-3HA*) were grown on Sc-His plates containing either 2% glucose or 2% galactose for 5 days at 25°C and 35°C. Note: The expression of *Chlorella* virus DNA ligase in pRS423gal-*ChVLig-3HA* plasmid is under the *GAL10* promoter. **(d)** Yeast trains in **c** were grown asynchronously to mid-log phase in medium containing 2% raffinose at 25°C. Cultures were split and grown in the presence of either 2% glucose or 2% galactose at the restrictive temperature of 35°C for 3 hours. PCNA and its ubiquitinated forms, Cdc9 and ChVLig-3HA were detected with anti-PCNA (S871), anti-Cdc9, and anti-HA antibodies, respectively. In **b** and **c**, α-tubulin served as a loading control. Asterisks indicate non-specific bands.

Figure 4.3

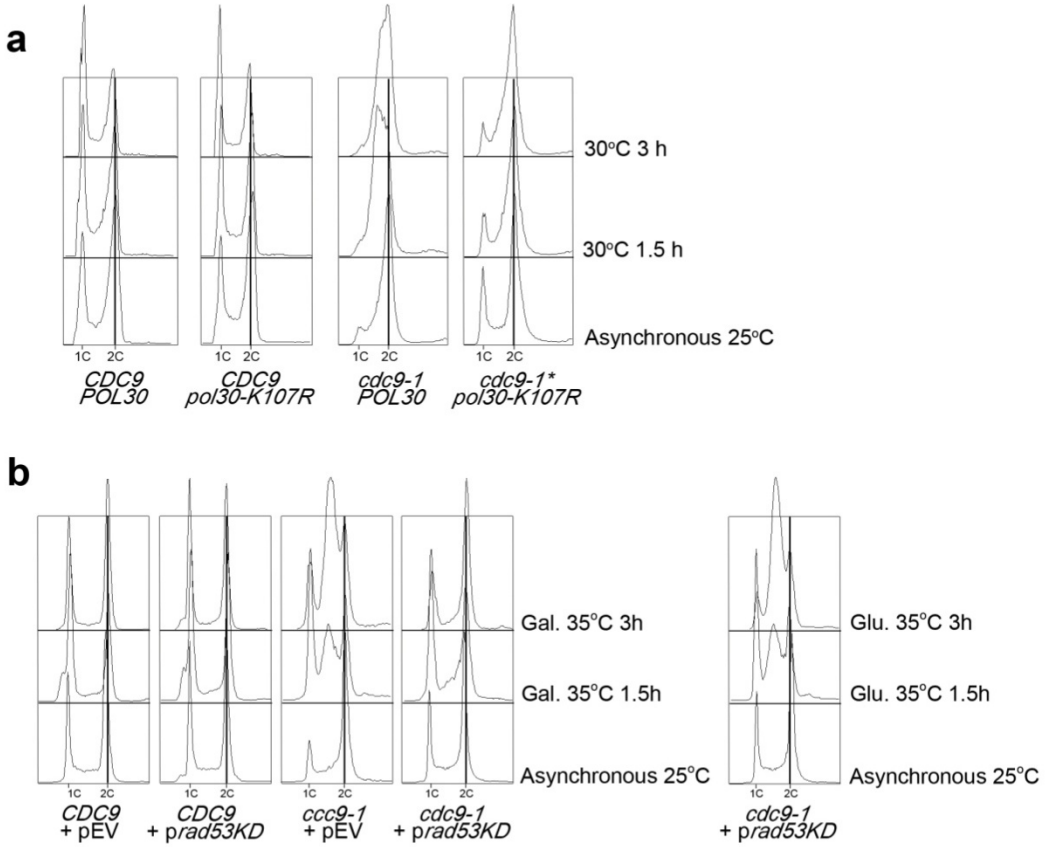
a
30°C 3 h
30°C 1.5 h
Asynchronous 25°C
1C 2C
CDC9 POL30
CDC9 pol30-K107R
cdc9-1 POL30
cdc9-1* pol30-K107R
b
Gal. 35°C 3h
Gal. 35°C 1.5h
Asynchronous 25°C
Glu. 35°C 3h
Glu. 35°C 1.5h
Asynchronous 25°C
CDC9 + pEV
CDC9 + prad53KD
ccc9-1 + pEV
cdc9-1 + prad53KD
cdc9-1 + prad53KD

Figure 4.3 PCNA ubiquitination at K107 is crucial for the S phase arrest in *cdc9-1* mutants.

(a) Asynchronous cultures of SSL204 (*CDC9*), ABy685 (*CDC9 pol30-K107R*), SSL612 (*cdc9-1*), ABy782 (*cdc9-1* pol30-K107R*) were grown at 25°C and shifted to 30°C for 1.5h and 3h. **(b)** Asynchronous cultures of ABy1146 (*CDC9* + pEV), ABy1147 (*CDC9* + p*rad53-KD*), ABy1148 (*cdc9-1* + pEV), ABy1149 (*cdc9-1* + p*rad53-KD*) were grown at 25°C and shifted to 35°C for 1.5h and 3h in the presence of 2% galactose. On the right, the same asynchronous ABy1149 culture at 25°C was split and grown in the presence of 2% glucose for 1.5h and 3h. In **a** and **b**, DNA was strained with Sytox Green and was monitored by flow cytometry. The vertical line indicates a 2C DNA content.

Figure 4.4

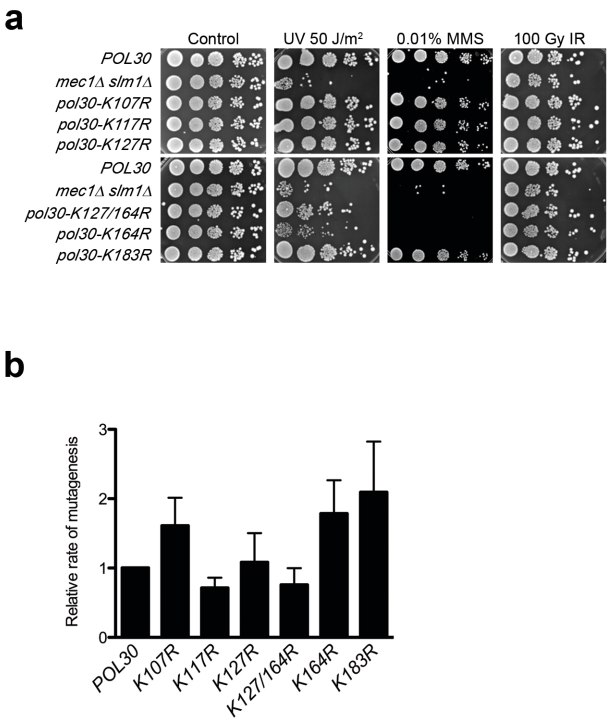

Figure 4.4 Differential DNA damage sensitivity and canavanine of various PCNA mutants.

(a) Successive 10-fold dilutions of either wild-type or different PCNA lysine to arginine mutants were grown on rich medium plates and treated with different DNA damaging agents as indicated. *mec1Δ sml1Δ* strain was used as a negative control. **(b)** CAN1 forward mutation analysis of two independent isolates of different PCNA mutants was analyzed. Briefly, mutation rates from two independent isolates were determined by fluctuation analyses twice using the method of the median [Lea and Coulson, 1949]. Each experiment was performed using 11 cultures and the average value from two different clones is reported.

Figure 4.5

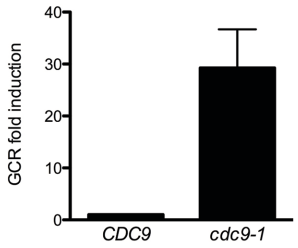

Figure 4.5 *cdc9-1* mutants exhibit enhanced gross chromosomal rearrangements (GCR).

GRC rates of wild-type and cdc9-1 cells were analyzed as described [Chen and Kolodner, 1999; Motegi and Myung, 2007]. Briefly, GCR rates from two independent isolates were determined by fluctuation analyses twice using the method of the median [Lea and Coulson, 1949]. Each experiment was performed using 11 cultures and the average value from two different clones is reported.

Figure 4.6

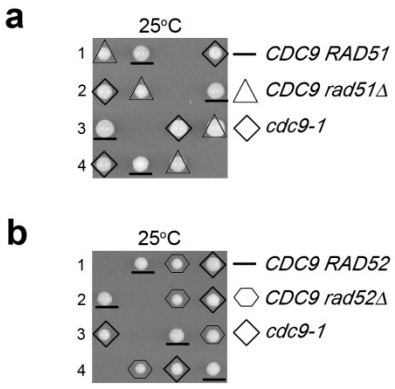

Figure 4.6 ***RAD51/RAD52*-mediated homologous recombination is required for *cdc9-1* survival.**

Diploid strains were dissected and incubated at either 25°C. All haploid genotypes are as indicated. Four independent tetrads (1-4) are laid out horizontally. **(a)** Segregates from *CDC9/cdc9-1 rad51Δ/RAD51* diploid. **(b)** Segregates from *CDC9/cdc9-1 rad52Δ/RAD52* diploid.

Figure 4.7

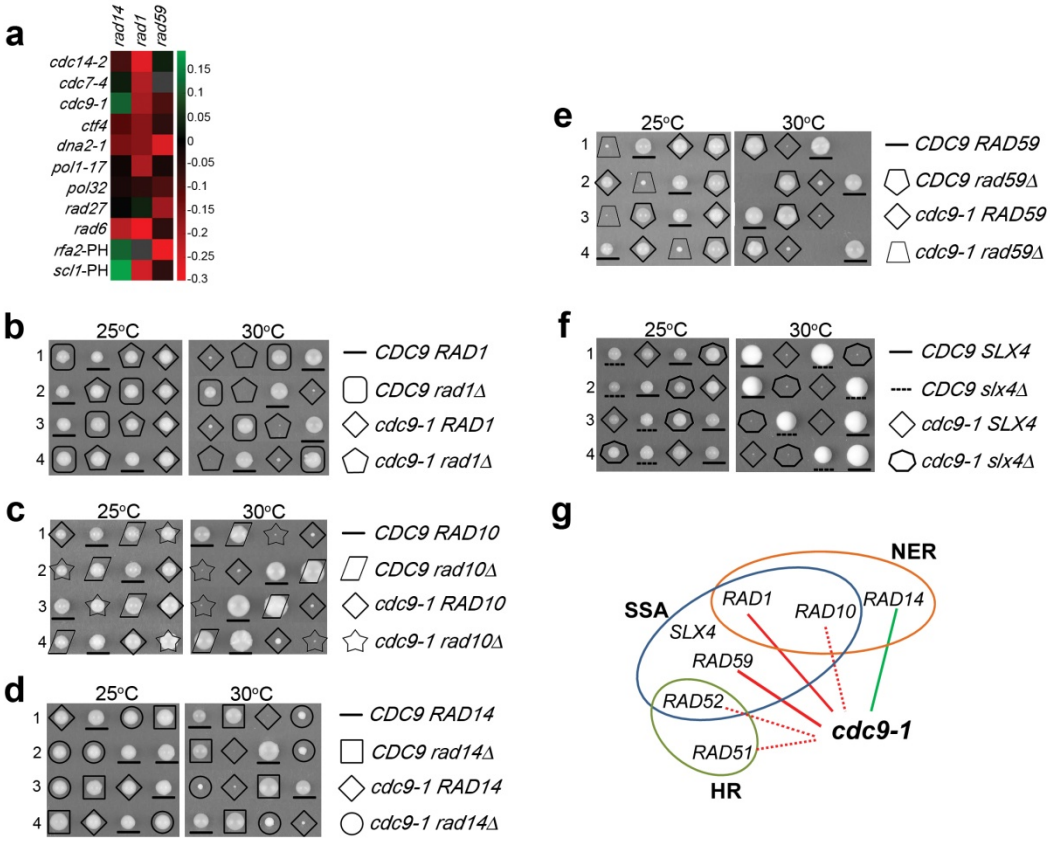

Figure 4.7 Genetic interactions of *RAD1*, *RAD10*, *RAD14*, and *RAD59* with *cdc9-1* mutants.

(a) Heatmap indicating strong positive (green) or negative (red) interactions between *cdc9-1* and *rad14Δ*, *rad1Δ* or *rad59Δ*, respectively. Black indicates no genetic interaction. Gray indicates that the interaction could not be scored. Among a collection of approximately 1800 SGA queries, only *scl1Δ* mutants displayed a genetic interaction signature similar to that of *cdc9-1* with respect to these three mutants. Other query mutants that exhibited similar genetic interactions with two out of the three genes are

shown as well as *rad27Δ*, which exhibited synthetic sickness with *rad59Δ*. PH indicates strains received from Phil Hieter. The heatmap was generated based on previously published [Costanzo *et al.*, 2010] and new SGA screens in Supplementary Table S1. **(b-f)** Selected diploid strains were dissected and incubated at either 25°C or 30°C. All haploid genotypes are as indicated on the right. Four independent tetrads (1-4) are laid out horizontally. The diploid strain genotypes are as followed: (**b)** *CDC9/cdc9-1 rad1Δ/RAD1*, **(c)** *CDC9/cdc9-1 rad14Δ/RAD14*, **(d)** *CDC9/cdc9-1 rad10Δ/RAD10*, **(e)** *CDC9/cdc9-1 rad59Δ/RAD59,* **(f)** *CDC9/cdc9-1 slx4Δ/SLX4*. **(g)** A Venn diagram summarizes some of the pertinent genetic interactions with *cdc9-1* mutants identified in this study. Genes are grouped into their respective repair pathways, homologous recombination (HR), HR-mediated single-strand annealing (SSA), and nucleotide excision repair (NER). Negative and positive genetic interactions with *cdc9-1* mutants identified in our SGA screen are illustrated as red and green solid lines, respectively. Red dotted lines indicate negative interactions with *cdc9-1* mutants that were only observed from manual tetrad analysis. No genetic interaction was observed between *SLX4* and *cdc9-1*.

Figure 4.8

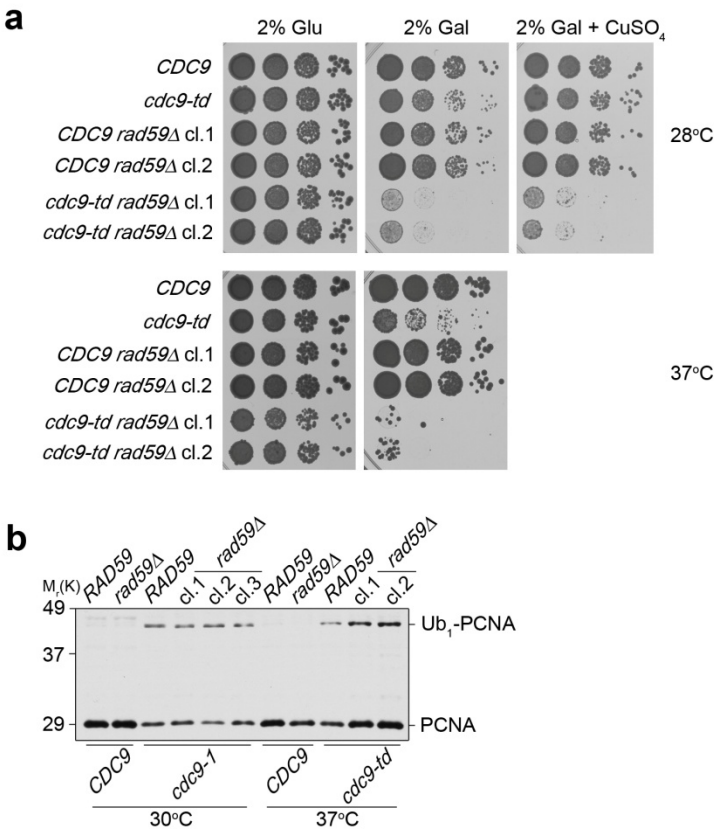

Figure 4.8 Deletion of *RAD59* in *cdc9* mutants does not affect PCNA mono-ubiquitination.

(a) Successive 10-fold dilutions of the indicated strains were grown on YP plates containing either 2% glucose or 2% galactose at 28°C and 37°C. Cells were also spotted on YP + 2% gal plates containing extra copper at 28°C to maintain wild-type Cdc9 expression in *cdc9-td* strains. **(b)** *cdc9 rad59Δ* double mutants were grown at the permissive temperature and subsequently shifted to the indicated temperatures for 3hr. PCNA and its mono-ubiquitinated form were detected with anti-PCNA antibody (S871).

Figure 4.9

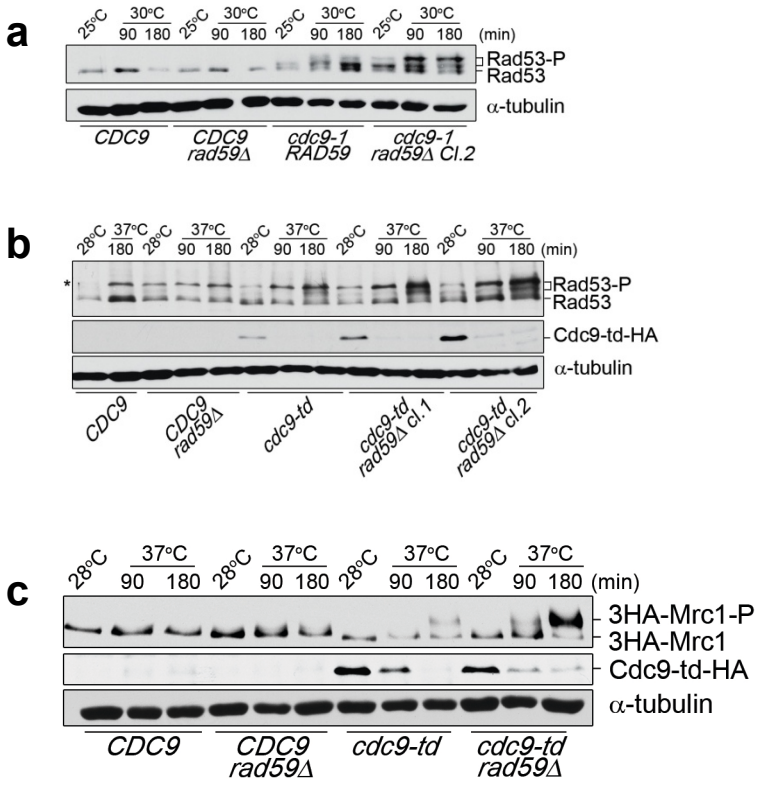

Figure 4.9 Deletion of *RAD59* in *cdc9* mutants displayed an increase in Mrc1 and Rad53 phosphorylation.

(a) Asynchronous cultures of the indicated strains were grown at 25°C and subsequently shifted to 30°C for 90 or 180 min. Unmodified and phosphorylated Rad53 was detected using an anti-Rad53 antibody. **(b and c)** Asynchronous cultures were grown at 28°C and subsequently shifted to 37°C for the indicated time. Cdc9-td-HA and unmodified and phosphorylated 3HA-Mrc1 and Rad53 levels were monitored using an anti-HA and anti-Rad53 antibody, respectively. α-tubulin was used as a loading control.

Table 4.2 Genetic interactions with *cdc9-1* mutants from SGA.

Genetic interactions were scored according to [Baryshnikova *et al.*, 2010b] and only those with the epsilon scores either ε < -0.09 or ε > 0.09 and p-values < 0.15 were included. Genetic interactions are listed from the smallest to largest ε-scores.

Query systematic name	Query standard name	Array systematic name	Array standard name	Epsilon score	p-value
YDL164C	CDC9-1	YKR049C	FMP46	-0.6638	3.70E-20
YDL164C	CDC9-1	YOL002C	IZH2	-0.5145	2.34E-12
YDL164C	CDC9-1	YMR031W-A	YMR031W-A	-0.4117	1.45E-02
YDL164C	CDC9-1	YJR100C	AIM25	-0.3881	1.38E-04
YDL164C	CDC9-1	YGL235W	YGL235W	-0.355	6.29E-04
YDL164C	CDC9-1	YJR011C	YJR011C	-0.3445	2.77E-04
YDL164C	CDC9-1	YDR156W	RPA14	-0.3395	9.72E-08
YDL164C	CDC9-1	YLR184W	YLR184W	-0.3257	7.11E-04
YDL164C	CDC9-1	YMR026C	PEX12	-0.3203	1.21E-04
YDL164C	CDC9-1	YBR215W	HPC2	-0.311	9.95E-04
YDL164C	CDC9-1	YKL168C	KKQ8	-0.286	6.48E-03
YDL164C	CDC9-1	YJL101C	GSH1	-0.2726	2.95E-04
YDL164C	CDC9-1	YKL044W	YKL044W	-0.266	7.95E-03
YDL164C	CDC9-1	YKL171W	NNK1	-0.2613	1.93E-03
YDL164C	CDC9-1	YKL188C	PXA2	-0.2491	1.85E-02
YDL164C	CDC9-1	YML047C	PRM6	-0.2486	4.66E-07
YDL164C	CDC9-1	YIL161W	YIL161W	-0.2479	5.16E-05
YDL164C	CDC9-1	YLR227C	ADY4	-0.2436	1.15E-03
YDL164C	CDC9-1	YMR244C-A	YMR244C-A	-0.2435	2.49E-03
YDL164C	CDC9-1	YFR040W	SAP155	-0.2391	1.97E-03
YDL164C	CDC9-1	YNL013C	YNL013C	-0.2309	5.50E-03
YDL164C	CDC9-1	YGR270W	YTA7	-0.2281	4.79E-04
YDL164C	CDC9-1	YML041C	VPS71	-0.2076	1.58E-02
YDL164C	CDC9-1	YHR180W	YHR180W	-0.2066	0.00E+00

Table 4.2 **Genetic interactions with *cdc9-1* mutants from SGA (continued).**

Query systematic name	Query standard name	Array systematic name	Array standard name	Epsilon score	p-value
YDL164C	CDC9-1	YKL184W	SPE1	-0.2058	1.59E-02
YDL164C	CDC9-1	YMR029C	FAR8	-0.2015	1.37E-02
YDL164C	CDC9-1	YPL022W	RAD1	-0.1954	1.31E-01
YDL164C	CDC9-1	YML090W	YML090W	-0.1902	3.34E-03
YDL164C	CDC9-1	YGL045W	RIM8	-0.1897	3.27E-02
YDL164C	CDC9-1	YCR102C	YCR102C	-0.1881	5.83E-32
YDL164C	CDC9-1	YML042W	CAT2	-0.1856	2.57E-02
YDL164C	CDC9-1	YIR001C	SGN1	-0.1809	5.65E-02
YDL164C	CDC9-1	YLR122C	YLR122C	-0.1744	1.45E-02
YDL164C	CDC9-1	YLL043W	FPS1	-0.1705	7.09E-02
YDL164C	CDC9-1	YMR241W	YHM2	-0.1704	2.63E-02
YDL164C	CDC9-1	YGR281W	YOR1	-0.169	2.23E-03
YDL164C	CDC9-1	YML051W	GAL80	-0.1679	1.92E-02
YDL164C	CDC9-1	YGR231C	PHB2	-0.1643	1.30E-02
YDL164C	CDC9-1	YKR035W-A	DID2	-0.1598	5.49E-02
YDL164C	CDC9-1	YDL013W	HEX3	-0.1593	5.20E-02
YDL164C	CDC9-1	YNL111C	CYB5	-0.1582	7.53E-03
YDL164C	CDC9-1	YDR532C	KRE28	-0.1573	4.68E-05
YDL164C	CDC9-1	YMR030W	RSF1	-0.1548	1.40E-01
YDL164C	CDC9-1	YJL110C	GZF3	-0.1545	8.38E-02
YDL164C	CDC9-1	YKL064W	MNR2	-0.1545	3.68E-02
YDL164C	CDC9-1	YJR099W	YUH1	-0.1527	9.80E-02
YDL164C	CDC9-1	YPR151C	SUE1	-0.1476	5.33E-02
YDL164C	CDC9-1	YMR016C	SOK2	-0.1469	5.38E-02
YDL164C	CDC9-1	YDR274C	YDR274C	-0.1451	3.08E-04
YDL164C	CDC9-1	YBR231C	SWC5	-0.145	2.69E-05
YDL164C	CDC9-1	YIL168W	YIL168W	-0.1434	6.00E-02

Table 4.2 **Genetic interactions with *cdc9-1* mutants from SGA (continued).**

Query systematic name	Query standard name	Array systematic name	Array standard name	Epsilon score	p-value
YDL164C	CDC9-1	YER162C	RAD4	-0.1431	1.30E-01
YDL164C	CDC9-1	YLR056W	ERG3	-0.1429	6.74E-02
YDL164C	CDC9-1	YHR081W	LRP1	-0.1419	9.55E-03
YDL164C	CDC9-1	YBL051C	PIN4	-0.1417	2.45E-04
YDL164C	CDC9-1	YJL112W	MDV1	-0.1406	1.18E-01
YDL164C	CDC9-1	YAR029W	YAR029W	-0.1401	8.09E-02
YDL164C	CDC9-1	YJL216C	IMA5	-0.1388	6.79E-02
YDL164C	CDC9-1	YIR003W	AIM21	-0.1385	1.06E-01
YDL164C	CDC9-1	YJL188C	YJL188C	-0.1381	1.32E-01
YDL164C	CDC9-1	YAR027W	UIP3	-0.138	2.68E-03
YDL164C	CDC9-1	YCL002C	YCL002C	-0.1358	1.78E-02
YDL164C	CDC9-1	YDR290W	YDR290W	-0.1342	3.41E-02
YDL164C	CDC9-1	YOL044W	PEX15	-0.1313	1.85E-02
YDL164C	CDC9-1	YJL198W	PHO90	-0.1305	1.26E-01
YDL164C	CDC9-1	YMR224C	MRE11	-0.1301	9.19E-02
YDL164C	CDC9-1	YDR359C	VID21	-0.129	1.17E-04
YDL164C	CDC9-1	YKL190W	CNB1	-0.129	9.23E-02
YDL164C	CDC9-1	YNL071W	LAT1	-0.1287	3.50E-02
YDL164C	CDC9-1	YGL031C	RPL24A	-0.1286	1.52E-04
YDL164C	CDC9-1	YLR415C	YLR415C	-0.1281	4.06E-02
YDL164C	CDC9-1	YMR158W-A	YMR158W-A	-0.1242	9.18E-02
YDL164C	CDC9-1	YLR287C-A	RPS30A	-0.1236	3.03E-03
YDL164C	CDC9-1	YHR143W	DSE2	-0.1224	4.11E-03
YDL164C	CDC9-1	YGR143W	SKN1	-0.1223	1.11E-03
YDL164C	CDC9-1	YNL032W	SIW14	-0.1202	3.92E-02
YDL164C	CDC9-1	YLR061W	RPL22A	-0.1191	4.73E-02
YDL164C	CDC9-1	YNL119W	NCS2	-0.119	5.17E-02

Table 4.2 **Genetic interactions with *cdc9-1* mutants from SGA (continued).**

Query systematic name	Query standard name	Array systematic name	Array standard name	Epsilon score	p-value
YDL164C	CDC9-1	YDR395W	SXM1	-0.1174	4.52E-02
YDL164C	CDC9-1	YBR103W	SIF2	-0.1145	1.09E-04
YDL164C	CDC9-1	YMR048W	CSM3	-0.113	6.84E-02
YDL164C	CDC9-1	YGL125W	MET13	-0.1127	4.35E-12
YDL164C	CDC9-1	YHR189W	PTH1	-0.1126	1.65E-05
YDL164C	CDC9-1	YJL093C	TOK1	-0.1117	5.20E-03
YDL164C	CDC9-1	YNL098C	RAS2	-0.1112	1.32E-01
YDL164C	CDC9-1	YGL046W	YGL046W	-0.1101	8.15E-02
YDL164C	CDC9-1	YKL053C-A	MDM35	-0.1101	9.67E-02
YDL164C	CDC9-1	YOR196C	LIP5	-0.109	1.19E-01
YDL164C	CDC9-1	YOL006C	TOP1	-0.1073	1.72E-02
YDL164C	CDC9-1	YPL008W	CHL1	-0.1072	3.69E-03
YDL164C	CDC9-1	YMR132C	JLP2	-0.1062	6.40E-02
YDL164C	CDC9-1	YCR033W	SNT1	-0.1058	2.10E-06
YDL164C	CDC9-1	YPL239W	YAR1	-0.1039	2.72E-04
YDL164C	CDC9-1	YLR143W	YLR143W	-0.1032	1.34E-01
YDL164C	CDC9-1	YDR056C	YDR056C	-0.1029	1.04E-01
YDL164C	CDC9-1	YPL069C	BTS1	-0.1021	1.20E-12
YDL164C	CDC9-1	YDR217C	RAD9	-0.1015	9.81E-02
YDL164C	CDC9-1	YNL037C	IDH1	-0.1006	9.70E-04
YDL164C	CDC9-1	YOR183W	FYV12	-0.1005	2.54E-02
YDL164C	CDC9-1	YOR043W	WHI2	-0.1004	1.94E-03
YDL164C	CDC9-1	YPR065W	ROX1	-0.1003	6.27E-03
YDL164C	CDC9-1	YMR044W	IOC4	-0.0996	8.91E-02
YDL164C	CDC9-1	YDR024W	YDR024W	-0.0978	1.39E-02
YDL164C	CDC9-1	YPR078C	YPR078C	-0.0976	1.46E-01
YDL164C	CDC9-1	YMR126C	DLT1	-0.0973	4.81E-03

Table 4.2 **Genetic interactions with *cdc9-1* mutants from SGA (continued).**

Query systematic name	Query standard name	Array systematic name	Array standard name	Epsilon score	p-value
YDL164C	CDC9-1	YPL152W	RRD2	-0.096	1.16E-01
YDL164C	CDC9-1	YLR205C	HMX1	-0.0958	1.35E-05
YDL164C	CDC9-1	YKL047W	YKL047W	-0.0957	1.42E-01
YDL164C	CDC9-1	YPR141C	KAR3	-0.0948	1.20E-01
YDL164C	CDC9-1	YDR493W	MZM1	-0.0947	9.12E-02
YDL164C	CDC9-1	YCL036W	GFD2	-0.0946	1.17E-03
YDL164C	CDC9-1	YDL059C	RAD59	-0.0939	1.39E-21
YDL164C	CDC9-1	YOR291W	YPK9	-0.0938	9.18E-02
YDL164C	CDC9-1	YMR065W	KAR5	-0.0931	9.92E-03
YDL164C	CDC9-1	YKR048C	NAP1	-0.093	2.74E-02
YDL164C	CDC9-1	YOR221C	MCT1	-0.093	7.73E-03
YDL164C	CDC9-1	YNL091W	NST1	-0.0909	1.06E-06
YDL164C	CDC9-1	YPR024W	YME1	-0.0904	8.07E-02
YDL164C	CDC9-1	YJR040W	GEF1	0.0906	1.77E-02
YDL164C	CDC9-1	YGR232W	NAS6	0.0909	1.71E-52
YDL164C	CDC9-1	YGL057C	GEP7	0.0911	1.25E-01
YDL164C	CDC9-1	YLL002W	RTT109	0.0913	9.04E-02
YDL164C	CDC9-1	YLR165C	PUS5	0.0916	1.27E-02
YDL164C	CDC9-1	YLR174W	IDP2	0.092	3.18E-02
YDL164C	CDC9-1	YLR283W	YLR283W	0.0924	1.19E-01
YDL164C	CDC9-1	YDR159W	SAC3	0.0925	1.28E-01
YDL164C	CDC9-1	YFR010W	UBP6	0.0928	6.54E-02
YDL164C	CDC9-1	YLR239C	LIP2	0.0929	5.26E-02
YDL164C	CDC9-1	YER178W	PDA1	0.0936	6.75E-02
YDL164C	CDC9-1	YLR332W	MID2	0.0937	1.61E-04
YDL164C	CDC9-1	YAL054C	ACS1	0.094	3.68E-04
YDL164C	CDC9-1	YDR276C	PMP3	0.0945	7.68E-04

Table 4.2 **Genetic interactions with *cdc9-1* mutants from SGA (continued).**

Query systematic name	Query standard name	Array systematic name	Array standard name	Epsilon score	p-value
YDL164C	CDC9-1	YHR012W	VPS29	0.0951	1.17E-01
YDL164C	CDC9-1	YPR018W	RLF2	0.0955	1.99E-02
YDL164C	CDC9-1	YGL152C	YGL152C	0.0958	5.19E-04
YDL164C	CDC9-1	YLR072W	YLR072W	0.096	8.21E-02
YDL164C	CDC9-1	YPL247C	YPL247C	0.0983	2.92E-02
YDL164C	CDC9-1	YKL127W	PGM1	0.0991	1.44E-03
YDL164C	CDC9-1	YNL147W	LSM7	0.1001	1.08E-01
YDL164C	CDC9-1	YFR008W	FAR7	0.1003	2.91E-02
YDL164C	CDC9-1	YLR200W	YKE2	0.1006	1.28E-01
YDL164C	CDC9-1	YIL122W	POG1	0.1007	4.27E-06
YDL164C	CDC9-1	YKL069W	YKL069W	0.1008	7.41E-02
YDL164C	CDC9-1	YNR049C	MSO1	0.101	9.88E-02
YDL164C	CDC9-1	YGL019W	CKB1	0.1011	7.40E-26
YDL164C	CDC9-1	YDR265W	PEX10	0.1012	3.81E-02
YDL164C	CDC9-1	YDR447C	RPS17B	0.1031	2.29E-03
YDL164C	CDC9-1	YJR014W	TMA22	0.1041	8.23E-13
YDL164C	CDC9-1	YGL149W	YGL149W	0.1047	7.81E-04
YDL164C	CDC9-1	YHR111W	UBA4	0.1084	1.21E-03
YDL164C	CDC9-1	YBR138C	YBR138C	0.1089	6.75E-02
YDL164C	CDC9-1	YBR195C	MSI1	0.1098	2.76E-54
YDL164C	CDC9-1	YJL120W	YJL120W	0.1099	3.14E-02
YDL164C	CDC9-1	YGL153W	PEX14	0.1102	7.56E-19
YDL164C	CDC9-1	YMR201C	RAD14	0.1123	5.55E-02
YDL164C	CDC9-1	YKR020W	VPS51	0.1124	1.24E-02
YDL164C	CDC9-1	YIL036W	CST6	0.1129	8.52E-03
YDL164C	CDC9-1	YHL034C	SBP1	0.113	1.20E-04
YDL164C	CDC9-1	YGL236C	MTO1	0.1143	6.05E-02

Table 4.2 **Genetic interactions with *cdc9-1* mutants from SGA (continued).**

Query systematic name	Query standard name	Array systematic name	Array standard name	Epsilon score	p-value
YDL164C	CDC9-1	YJR033C	RAV1	0.1148	1.29E-01
YDL164C	CDC9-1	YNL097C	PHO23	0.1153	1.21E-02
YDL164C	CDC9-1	YGR133W	PEX4	0.1156	1.31E-02
YDL164C	CDC9-1	YNL056W	OCA2	0.116	2.22E-02
YDL164C	CDC9-1	YOL053W	AIM39	0.117	1.01E-01
YDL164C	CDC9-1	YMR015C	ERG5	0.1179	4.64E-09
YDL164C	CDC9-1	YKR046C	PET10	0.1182	6.46E-02
YDL164C	CDC9-1	YFR049W	YMR31	0.1189	2.24E-04
YDL164C	CDC9-1	YHR041C	SRB2	0.1189	5.42E-02
YDL164C	CDC9-1	YHR132C	ECM14	0.1196	3.54E-03
YDL164C	CDC9-1	YGL180W	ATG1	0.1203	1.86E-06
YDL164C	CDC9-1	YLR289W	GUF1	0.1205	7.99E-02
YDL164C	CDC9-1	YKL050C	YKL050C	0.1248	2.46E-02
YDL164C	CDC9-1	YLR124W	YLR124W	0.1251	5.86E-02
YDL164C	CDC9-1	YML033W	YML033W	0.128	3.86E-03
YDL164C	CDC9-1	YLR386W	VAC14	0.1285	5.19E-02
YDL164C	CDC9-1	YGR200C	ELP2	0.1287	2.09E-03
YDL164C	CDC9-1	YBR118W	TEF2	0.1292	6.94E-05
YDL164C	CDC9-1	YHR031C	RRM3	0.1297	2.20E-04
YDL164C	CDC9-1	YHR067W	HTD2	0.1298	2.36E-02
YDL164C	CDC9-1	YGL168W	HUR1	0.1302	5.89E-02
YDL164C	CDC9-1	YKL009W	MRT4	0.1308	1.20E-02
YDL164C	CDC9-1	YGL056C	SDS23	0.1326	6.07E-02
YDL164C	CDC9-1	YNL127W	FAR11	0.1367	1.20E-03
YDL164C	CDC9-1	YGL054C	ERV14	0.1387	2.59E-02
YDL164C	CDC9-1	YBL104C	SEA4	0.139	1.45E-03
YDL164C	CDC9-1	YDR393W	SHE9	0.139	1.24E-03

Table 4.2 **Genetic interactions with *cdc9-1* mutants from SGA (continued).**

Query systematic name	Query standard name	Array systematic name	Array standard name	Epsilon score	p-value
YDL164C	CDC9-1	YIL017C	VID28	0.1392	2.28E-10
YDL164C	CDC9-1	YGL237C	HAP2	0.1407	2.89E-02
YDL164C	CDC9-1	YGL173C	KEM1	0.1417	3.66E-02
YDL164C	CDC9-1	YJL060W	BNA3	0.145	2.39E-02
YDL164C	CDC9-1	YDR414C	ERD1	0.1479	1.69E-04
YDL164C	CDC9-1	YLR391W	YLR391W	0.1488	2.07E-05
YDL164C	CDC9-1	YGL133W	ITC1	0.1497	5.07E-04
YDL164C	CDC9-1	YLR190W	MMR1	0.1516	3.34E-02
YDL164C	CDC9-1	YKR060W	UTP30	0.1552	2.16E-02
YDL164C	CDC9-1	YGL024W	YGL024W	0.1559	2.99E-05
YDL164C	CDC9-1	YLR290C	YLR290C	0.1602	5.68E-02
YDL164C	CDC9-1	YNR041C	COQ2	0.1625	4.44E-03
YDL164C	CDC9-1	YGL043W	DST1	0.1642	5.97E-04
YDL164C	CDC9-1	YBR095C	RXT2	0.1651	2.29E-03
YDL164C	CDC9-1	YER072W	VTC1	0.1707	2.51E-09
YDL164C	CDC9-1	YLR182W	SWI6	0.1728	1.20E-01
YDL164C	CDC9-1	YLR410W	VIP1	0.1835	9.97E-03
YDL164C	CDC9-1	YBR105C	VID24	0.1902	4.79E-03
YDL164C	CDC9-1	YJL177W	RPL17B	0.1919	4.11E-02
YDL164C	CDC9-1	YHR193C	EGD2	0.1924	1.64E-02
YDL164C	CDC9-1	YAR002W	NUP60	0.2052	1.67E-44
YDL164C	CDC9-1	YHR008C	SOD2	0.2174	4.11E-15
YDL164C	CDC9-1	YLR191W	PEX13	0.221	9.14E-20
YDL164C	CDC9-1	YDR148C	KGD2	0.2613	8.11E-28
YDL164C	CDC9-1	YPR066W	UBA3	0.2859	1.19E-10

CHAPTER 5

Discussion and future studies

Discussion

Defects in DNA ligase I trigger the S phase checkpoint.

The focus of this thesis was to investigate whether cells can sense and deal with the accumulation of DNA damage that arises "behind" replication forks. Here, we establish that in the absence of Cdc9, cells do indeed recognize the accumulation of nicked DNA and arrest in S phase (Figures 2.3 and 3.1). The difference between our results and the previously described G2 arrest phenotype [Culotti and Hartwell, 1971] is likely due to the stringency of *cdc9* alleles. We constructed a *cdc9-td* strain (Figure 2.2) in which DNA ligase I is rapidly degraded by the Ubr1-mediated N-end-rule degradation [Sanchez-Diaz *et al.*, 2004]. *cdc9-td* cells progressed much slower through S phase at the restrictive temperature than wild-type cells (Figure 3.1). Two other *cdc9* alleles, *cdc9-1* and *cdc9-2*, also exhibited a similar S phase delay (Figure 2.3). Because *cdc9-2* mutants have higher DNA ligase I levels and are not as temperature sensitive as *cdc9-1* alleles, they arrested later in S phase (Figures 2.1 and 2.3). These results support the notion that the completion of S phase is dependent on the activity of DNA ligase I.

The delay in S phase in *cdc9* mutants relied on the activation of the S phase checkpoint kinase, Rad53, in an Mrc1- and Rad9-dependent manner, indicating the presence of both stalled replication forks and DSBs, respectively (Figures 2.4a and b). Complete inactivation of Rad53 in *cdc9-1* cells by inhibiting both Mrc1 and Rad9 or by overexpressing a dominant-negative Rad53 kinase-dead mutant (Rad53-K221A/D339A) [Pellicioli *et al.*, 1999; Szyjka *et al.*, 2008] failed to arrest in S phase (Figures 2.4b and 4.3b). Altogether, we conclude that cells can sense lesions that arise "behind" the

replication fork in the absence of Cdc9 and activate the S phase checkpoint kinase Rad53 in an Mrc1- and Rad9-dependent manner.

Defects in DNA ligase I activity induce PCNA ubiquitination at K107.

Besides the S phase checkpoint pathway, we established that nicked replication intermediates caused by defects in DNA ligase I trigger PCNA mono- and poly-ubiquitination at K107 and independently of K164 (Figures 2.6, 2.14, and 4.1). In support of this notion, expression of either *S. cerevisiae* wild-type Cdc9 or *Chlorella* virus DNA ligase was able to complement *cdc9-1* cell viability and revert PCNA ubiquitination (Figures 4.1 and 4.2). A previous study in *cdc9* mutants demonstrated that Okazaki fragments accumulate as ligatable nicks that can be readily joined upon induction of DNA ligase I expression [Bielinsky and Gerbi, 1999]. Moreover, despite the failure to ligate Okazaki fragments, replicated DNA is still assembled into nucleosomes [Smith and Whitehouse, 2012], and DNA ligase I is active on nucleosomal substrates *in vitro* [Chafin *et al.*, 2000]. This would suggest that cells can recognize both "clean" (3'-OH and 5'-PO_4) and "dirty" (3'-OH and 5'-AMP) nicks in the context of chromatin. It is likely that limited amounts of DNA ligase I cause abortive ligation reactions leaving behind adenylated nicks. This idea is also consistent with previous reports demonstrating the presence of both types of nicks in human 46BR.1G1 DNA ligase I-deficient cells [Prigent *et al.*, 1994], raising a possibility that PCNA ubiquitination pathway may be present in high eukaryotes. Indeed, we demonstrated that depleting hLIG1 results in PCNA ubiquitination in human cells (Figure 2.16). The data presented in this study leads

us to propose that both types of nicks trigger PCNA ubiquitination, at least in budding yeast. Since this lower eukaryote lacks a homolog of the mammalian nick sensor poly (ADP-ribose) polymerase-1 (PARP-1) [Collinge and Althaus, 1994], we further postulate that PCNA ubiquitination serves as an integral part of a nick-sensory pathway during DNA replication.

Although PCNA K107 is not present in humans, our results indicate that at least the PCNA ubiquitination pathway in response to accumulation of nicks is conserved from yeasts to humans. Thus, future experiments aimed to identify the lysine residue will be necessary to understand the significance of PCNA ubiquitination in human DNA ligase I-deficient cells. Nevertheless, we conclude that *S. cerevisiae* can recognize the accumulation of unligated Okazaki fragments and trigger PCNA ubiquitination at K107, not K164.

PCNA ubiquitination at K107 is dependent on the *UBC4/MMS2/RAD5* E2-E3 ubiquitin complex.

Because PCNA is ubiquitinated at K107 in *cdc9-1* mutants, we speculated that the genetic requirements for PCNA ubiquitination would be different than PCNA ubiquitination at K164. Indeed, we identified a different E2-E3 ubiquitin complex, *UBC4/MMS2/RAD5,* that mono- and poly-ubiquitinates PCNA at K107 in *cdc9-1* cells, where the poly-ubiquitin chain is linked through K29, instead of K63 (Figure 2.7 and 2.8) [Hoege *et al.*, 2002]. The human homolog of Ubc4, UBCH5A, synthesizes K29 linked poly-ubiquitin chain *in vitro* [Mastrandrea *et al.*, 1999], which agrees with our *in vivo*

data. To date, the *in vivo* function of K29 poly-ubiquitin chain linkage is not well characterized. In humans, K29-linked poly-ubiquitin chains have been indicated to promote protein degradation via the lysosome [Chastagner *et al.*, 2006]. Alternatively, in budding yeast, a K29-linked poly-ubiquitin chain was shown to interact with Doa1 (degradation of alpha 1) [Russell and Wilkinson, 2004]. Doa1 is involved in replenishing free ubiquitin pools [Lis and Romesberg, 2006]. Thus, it is unclear whether PCNA K29-linked poly-ubiquitin signals for protein recruitment or protein degradation.

Our genetic evidence showing that *MMS2* interacts with *UBC4* for PCNA ubiquitination in *cdc9-1* mutants is intriguing because Mms2 is thought to interact only with Ubc13, an E2 conjugating enzyme [Hofmann and Pickart, 1999; 2001] (Figure 2.7 and 2.8). Since we did not detect any interactions between Ubc4 and Mms2 by co-immunoprecitipation experiments (data not shown), this raised a question as to whether other proteins can interact with Mms2 to promote Ubc4-dependent PCNA ubiquitination in *cdc9* mutants. Thus, it is possible that we are still missing components in the complex to facilitate PCNA ubiquitination. Identifying additional components involved in PCNA ubiquitination would provide more insight into this novel ubiquitination mechanism.

Lastly, our laboratory has previously reported that Ubc4 is also important for the degradation of Cdc17, the catalytic subunit of pol-α [Haworth *et al.*, 2010], indicating a role of Ubc4 during DNA replication that is currently underappreciated. Dissecting the molecular function of Ubc4 during DNA replication and/or replication stress may provide an additional layer of regulation linking ubiquitination pathways with other replication processes.

PCNA ubiquitination at K107 is required for robust Rad53 hyper-phosphorylation.

From our study, we further established that PCNA mono-ubiquitination at K107 is necessary for robust Rad53 hyper-phosphorylation (Figure 2.9b and 2.14e), illustrating a novel link between PCNA ubiquitination and the S phase checkpoint. Because PCNA ubiquitination only occurs at stalled forks and not at sites of DSBs [Davies *et al.*, 2008], ubiquitination of PCNA at K107 is likely to function in the same pathway as the Mrc1-mediated Rad53 activation. In agreement with this notion, we demonstrate that *cdc9-1* pol30-K107R* double mutants fail to arrest in S phase under semi-permissive conditions, a similar phenotype as observed in *cdc9-1 mrc1*^{AQ} mutants (Figure 2.4c and 4.3a).

How PCNA$^{K107\text{-}Ub}$ promotes S phase checkpoint activation is unclear. Initiating the S phase checkpoint cascade requires replication protein A (RPA) coated ssDNA (ssDNA-RPA) to recruit Mec1/Ddc2 [Zou and Elledge, 2003]. Thus, some processing of the nicks is necessary to generate such ssDNA-RPA structures. It is possible that PCNA ubiquitination facilitates this particular step. The redundant roles of Exo1 and Xrs2, a component of the MRX complex (Mre11/Rad50/Xrs2 (X-ray sensitive 2, a yeast homolog of human NBS1), have been implicated in the degradation of nascent DNA in response to stalled replication forks [Nakada *et al.*, 2004]. Thus, we speculate that they may also facilitate processing in the context of PCNA ubiquitination. Unlike the deletion of MRX components, which is lethal in *cdc9* mutants (Table 4.2) [Davierwala *et al.*, 2005], deletion of *EXO1* in *cdc9-1* cells yielded viable double mutants that displayed a 5-10 fold increase in temperature sensitivity (Figure 5.1a). This result suggested that Exo1 plays some role in *cdc9-1* cell viability. However, Exo1 did not appear to be crucial for

PCNA ubiquitination nor for Rad53 activation in *cdc9-1* mutants (Figures 5.1b and c). At this point we consider it highly likely that multiple different exonuclease activities contribute to the conversion of nicks into ssDNA-RPA structures, and that Exo1 might be one of them (Figure 5.2a). In an alternative model, which is not mutually exclusive to the events proposed above, unligated Okazaki fragments could form flaps, which are subsequently bound by RPA, which in turn, recruits Mec1 (Figure 5.2b). Importantly, there is recent precedence for such a scenario in *dna2* mutants, which accumulate long flaps at the 5'-termini of unprocessed Okazaki fragments [Budd *et al.*, 2011]. We speculate that $PCNA^{K107\text{-}Ub}$ may actively facilitate 5'-flap formation, thereby enabling Mec1 phosphorylation (Figure 5.2b). Consistent with this model, mono-ubiquitinated PCNA still supports DNA synthesis by pol-δ, but prevents Fen1 from accessing 5'-flaps *in vitro* [Zhang *et al.*, 2012]. This would explain why 5'-flaps may have an extended half-life and could serve as a "docking" platform for Mec1 in *cdc9* mutants (Figure 5.2b). Moreover, it would provide a rationale for alternative pathways to deal with 5'-flaps in the absence of Fen1. We propose here that one such pathway depends on Rad59 for the re-annealing of these flaps, which promotes replication fork progression by deactivating Mec1 (Figures 5.2b and c).

How do cells cope with accumulated nicks in *cdc9* mutants?

Since Cdc9 is the only ligase that can seal nicks during lagging strand synthesis, it is unclear what mechanisms are in place to "resolve" persistent nicks in the presence of limited DNA ligase I activity. In *cdc9-1* cells, the observation that Mrc1 and Rad9 are

crucial for S phase delay suggests the presence of both ssDNA at stalled replication forks and DSBs, respectively (Figure 2.4) [Das-Bradoo *et al.*, 2010b]. It is conceivable that DSBs are either ligated by Dnl4 or must await Mec1 inactivation to allow repair by HR as Mec1 activity inhibits HR-mediated DSBs during S phase [Alabert *et al.*, 2009; Barlow and Rothstein, 2009; Irmisch *et al.*, 2009; Lisby *et al.*, 2004]. Rad59 appears to have an important role in aiding Mec1 deactivation by suppressing Mrc1 phosphorylation (Figure 4.9c). Mrc1 is phosphorylated at stalled replication forks and the proximity of Mec1 to phosphorylated Mrc1 is required for enhancing Mec1 activation [Naylor *et al.*, 2009]. Therefore, suppression of Mrc1 phosphorylation will counteract this process and provides one explanation for how Rad59 promotes *cdc9-1* survival (Figures 5.2a and b). How Rad59 suppresses Mrc1 phosphorylation on the molecular level and promotes replication fork progression is not clear. One clue comes from *in vitro* studies demonstrating that Rad59 is required for the annealing of short oligonucleotides [Davis and Symington, 2001; Wu *et al.*, 2006]. If we consider this function in the context of impaired Okazaki fragment ligation, it is possible that Rad52-Rad59 dimers simply promote the re-annealing of detached flaps that might form at the ends of the nicked replication intermediates. For example, DNA polymerase-δ might "invade" a downstream Okazaki fragment during the process of displacement synthesis [Jin *et al.*, 2005; Stith *et al.*, 2008]. As mentioned above, the formation of flaps might be actively induced to generate ssDNA and elicit S phase checkpoint activation [Budd *et al.*, 2011]. The formation of these flaps would likely interfere with proper nucleosome formation behind the fork, which in turn, slows down fork progression [Groth *et al.*, 2007]. This

might in fact be beneficial for *cdc9* mutants, as it provides the cells with extra time to recycle the little DNA ligase I activity that they have at their disposal. We like this idea because the deletion of each of three individual genes, *RLF2*, *MSI1* and *ITC1* which comprise subunits of the chromosome assembly factor 1 [Kaufman *et al.*, 1997], improve *cdc9-1* viability (Table 4.2). However, since replication fork slow down generally increases the risk of replication fork collapse, the cell likely has means to counterbalance this effect. We envision that Rad59 could provide such counterbalance by preserving the structure of the ligatable nicks, thereby promoting nucleosome assembly and ultimately replication fork progression (Figure 5.2c). Whereas the Rad1-Rad10 complex plays a less prominent role in *cdc9-1* survival (Figures 4.7b and c), it could be involved in the removal of 3'-flaps, analogous to its role in SSA between direct repeats (Figure 5.2c). Our original observation that deletion of *RAD14* is beneficial for the survival of *cdc9-1* cells (Figure 4.7d) is consistent with this proposed function for the endonuclease, because loss of Rad14 would prevent the use of Rad1-Rad10 in NER, making it available for other sites of repair. It is noteworthy that the observed separation of function for *RAD1* and *RAD14* that we have described here is rather unique to *cdc9-1* mutants. Among a collection of approximately 1800 SGA queries, we found only one other strain, *scl1* that showed a similar pattern (Figure 4.7a). Scl1 is a subunit of the 20S proteasome and whether this finding has any functional significance is currently unclear.

To date, Rad59's best-documented role is in SSA. Rad59-dependent, but Rad51-independent recombination has been implicated in DSB repair between direct repeats [Ira and Haber, 2002; Ivanov *et al.*, 1996; Sugawara *et al.*, 2000]. However, we demonstrated

that SSA does not play a role in *cdc9-1* cell survival because deletion of *SLX4*, a crucial component of SSA-mediated DSB repair [Flott *et al.*, 2007], exhibited no effect on *cdc9-1* (Figure 4.7f). Based on the finding that deletion of *RAD59* exhibited an increase in Mrc1 phosphorylation in *cdc9* mutants, we favor the view that Rad59 is primarily required for suppression of replication fork stalling. Whether Rad59 functions in the same pathway as $PCNA^{K107\text{-}Ub}$ is unclear. We exclude the possibility that Rad59 promotes Rad53 activation downstream of PCNA ubiquitination. However, it is possible that Rad59 is recruited by ubiquitinated PCNA, either directly or indirectly, to help alleviate fork arrest.

It is also worthwhile to note that there is a limited set of only 23 mutants for which synthetic sickness/lethality with *rad59Δ* has been described [Chanet and Heude, 2003; Costanzo *et al.*, 2010; Pan *et al.*, 2006] (Supplementary Table S1). Intriguingly, among those are four other lagging strand specific mutants, *pol3-13*, defective in the catalytic subunit of the replicative pol-δ, *pol32Δ*, defective in a subunit of pol-δ, *rad27Δ*, defective in flap endonuclease, and *dna2-1* defective in the endonuclease/helicase implicated in flap processing [Budd *et al.*, 2005; Chanet and Heude, 2003; Costanzo *et al.*, 2010; Debrauwere *et al.*, 2001; Loeillet *et al.*, 2005; Pan *et al.*, 2006; Symington, 1998]. This suggests that *RAD59* may play a general role at stalled forks in response to defects in Okazaki fragment maturation. Thus, the molecular function of *RAD59* in *pol3-13*, *pol32Δ, rad27Δ* and *dna2-1* cells needs to be further investigated in the future. We speculate that Rad59 might have a similar role in *dna2-1* mutants as described here for DNA ligase I-deficient cells in suppressing Mrc1 activation.

DNA repair of dirty nicks in *cdc9-1* mutants.

Another aspect that has not been addressed in this thesis is how cells repair dirty nicks. In *cdc9-1* cells, dirty nicks arise from abortive ligation such as in catalytically inactive Cdc9-K598A mutants (Figures 4.1b and c). Because DNA ligase I requires an ATP in the first step to catalyze the ligation reaction, the 5'adenylated end of the dirty nick needs to be removed prior to ligation. Hnt3 (histidine triad nucleotide binding 3), a yeast homolog of human aprataxin (APTX), was shown to remove the 5'adenylate of nicked substrates *in vitro* [Ahel *et al.*, 2006]. To determine whether Hnt3 may be important for *cdc9-1* cell survival, we deleted *HNT3* in both wild-type and *cdc9-1* mutants. Indeed, we observed an increase in temperature sensitivity in *cdc9-1 hnt3Δ* compared to *cdc9-1* mutants (Figure 5.3). However, it is difficult to determine whether cells can repair dirty nicks via a different mechanism such as Rad59-mediated recombination as shown above, since HR is a predominant repair mechanism in budding yeast. Because mutations in the *APTX* gene have been linked to axtaxia oculomotor apraxia-1 (AOA1) syndrome [Date *et al.*, 2001; Moreira *et al.*, 2001], human cells may be more sensitive to the lack of APTX when DNA ligase I is limiting than budding yeast. Future experiments addressing the function of APTX in human DNA ligase I-deficient cells will have to be performed to determine this question.

PCNA ubiquitination in human DNA ligase I-deficient cells.

We demonstrated that PCNA ubiquitination in DNA ligase I-deficient cells is conserved in humans (Figure 2.16). Interestingly, human cells have PARP1, a nick

sensor protein, that is specialized in repairing SSBs [Caldecott, 2008]. Consistent with this notion, BR46.1G1 cells, which have low hLIG1 activity, are hypersensitive to 3-amino benzamide, an inhibitor of PARP1 [Lonn *et al.*, 1989]. Notably, because no known homologs of PARP1 have yet been identified in *S. cerevisiae*, we hypothesize that the PCNA ubiquitination pathway in DNA ligase I-deficient human cells functions either in parallel or as a "back-up" pathway to PARP1. In support of this hypothesis, cells depleted of PARP1 did not exhibit a defect in their ability to repair SSBs during S phase. However, the cells displayed a significant defect when they were treated with a PARP1 inhibitor, which locked PARP1 dimers onto DNA, thereby inactivating them and masking the nicks [Godon *et al.*, 2008]. These results indicate the presence of an additional repair pathway in S phase that is independent of PARP1. Future studies using the same strategy, PARP1 inhibitors versus PARP1-knockdown, which monitor the PCNA ubiquitination status in DNA ligase I-deficient should allow us to determine whether PCNA ubiquitination functions in the same or parallel pathway to PARP1.

PCNA damage code.

In this thesis, we established a novel PCNA ubiquitination pathway at K107 in *cdc9* mutants that is essential for the activation of the S phase checkpoint in response to accumulation of nicks during lagging strand synthesis. This contrasts to the well-known PCNA ubiquitination at K164, which functions independently of the S phase checkpoint, although both are triggered in response to DNA damage-induced stalled replication forks such as UV-irradiation and MMS [Davies *et al.*, 2008; Hoege *et al.*, 2002]. Moreover, a

lagging strand mutant, *pol32*Δ, which is a subunit of pol-δ, caused PCNA ubiquitination at K164, suggesting that cells may monitor different steps during Okazaki fragment processing [Karras and Jentsch, 2010]. Consistent with this result, we also observed PCNA ubiquitination in other lagging strand mutants (Nguyen, Becker, and Bielinsky, unpublished observations). Experiments are currently underway to determine whether K107, K164 or additional lysine residues on PCNA are important to induce appropriate responses. Besides lagging strand mutants, PCNA is also ubiquitinated independently of the *RAD6* pathway in an *asf1* (anti-silencing function 1) mutant, a nucleosome assembly factor [Kats *et al.*, 2009]. Interestingly, in human cells treated with colchicine, a microtubule inhibitor, PCNA ubiquitination at K254 was significantly increased [Xu *et al.*, 2010], further illustrating that PCNA can be ubiquitinated at different lysine residues. Therefore, because PCNA ubiquitination at K107 and K164 are activated in response to different types of DNA structures, nicks versus ssDNA, respectively, we propose a "PCNA damage code" by which cells can distinguish and categorize different types of DNA damage to activate appropriate signaling responses by distinct ubiquitin attachment sites and/or different poly-ubiquitin chains on PCNA.

Future studies

The focus of this thesis was to determine how cells sense and suppress DNA damage that arises "behind" the replication fork in order to maintain genomic stability. This thesis has noted several interesting and novel observations that open up new avenues for future studies. The main observations from this thesis are as followed: (1) PCNA ubiquitination occurs at a novel lysine, K107, when DNA ligase I activity is inhibited; (2) the ubiquitination pathway is mediated by the *UBC4/MMS2/RAD5* E2-E3 ubiquitin ligase complex; (3) PCNA ubiquitination at K107 is required for the activation of the S phase checkpoint kinase Rad53, which is dependent on both Mrc1 and Rad9; (4) Rad59-dependent recombination at stalled forks, either downstream or in a parallel pathway to PCNA ubiquitination, is necessary to suppress Mrc1 phosphorylation and promote S phase progression when DNA ligase I activity is limited; and lastly, (5) this novel PCNA ubiquitination pathway is conserved from yeasts to humans.

Future studies will aim to characterize the *in vivo* function of PCNA ubiquitination with respect to the S phase checkpoint activation and the Rad59-mediated recombination in *cdc9* mutants as described in our proposed model (see discussion). Secondly, different approaches to investigate the "PCNA damage code" will be outlined. Lastly, different strategies will focus on characterizing the PCNA ubiquitination pathway in human DNA ligase I-deficient cells.

Investigating the role of ubiquitinated PCNA at K107 in *S. cerevisiae*.

How does ubiquitinated PCNA at K107 activate the S phase checkpoint?

We have shown that PCNA ubiquitination at K107 is necessary for the activation of the S checkpoint in order to delay cells in S phase (Figure 2.14 and 4.4b). However, the underlying molecular mechanism is unclear. Additionally, in response to DNA damaging agents that induce stalled replication forks such as UV-irradiation or HU, Rad53 activation is dependent on the redundant functions of Exo1 and Xrs2 [Nakada *et al.*, 2004], generating a ssDNA region on the template DNA by degrading nascent DNA in the 5'-3' direction. We speculate that Exo1 and Xrs2 may carry out a similar function in *cdc9* mutants. Whether PCNA ubiquitination cooperates with Exo1 and Xrs2 to activate Rad53 is unclear. Because inhibition of PCNA ubiquitination was sufficient to block Rad53 activation, we propose that Exo1 and/or Xrs2 would function downstream of PCNA ubiquitination. Alternatively, Exo1 and Xrs2 could activate Rad53 in a parallel pathway. We favor the former model because inhibition of PCNA ubiquitination at K107 was sufficient to suppress the S phase delay phenotype in *cdc9* mutants. Because *cdc9 exo1Δ* double mutants do not exhibit any defect in either PCNA ubiquitination or Rad53 phosphorylation, we will delete *XRS2* in *cdc9-1 exo1Δ* strain. Because deletion of the *XRS2* in *cdc9* mutants is likely to be lethal due to its function in DSB repair, we propose to generate the triple mutant in the *cdc9-td* background and monitor both Mrc1 and Rad53 phosphorylation. If Exo1 and Xrs2 function in parallel to PCNA ubiquitination, it would be interesting to test whether overexpression of Exo1 or Xrs2 in *cdc9-td pol30-*

K107R double mutants can induce Rad53 phosphorylation to compensate for the loss of ubiquitinated PCNA.

Alternatively, we propose to adapt the newly developed "split PCNA" protein expression, to express specifically the ubiquitinated PCNA at K107 in *exo1Δ xrs2Δ* mutants and examine whether PCNA ubiquitination can rescue checkpoint activation in these cells. In this approach, PCNA is split and expressed from two different constructs so that the ubiquitin is attached immediately upstream of the lysine residue [Freudenthal *et al.*, 2010]. Split PCNA at K164 has been shown to be functional *in vivo* to interact with TLS polymerase to promote lesion bypass [Freudenthal *et al.*, 2010]. In our case, we will construct an N-terminal half of $PCNA_{1\text{-}106}$ (amino acid 1-106) and C-terminal half of PCNA with an ubiquitin attached immediately upstream of K108, linked with two Gly residues (Ub-GG-$PCNA_{108\text{-}258}$). The Gly-Gly linker is thought to mimic the length of the lysine side chain. Because ubiquitinated PCNA at K107 induces S phase arrest, we will control the split PCNA expression under the control of a galactose promoter. Regardless of the outcome, it would be informative to see whether PCNA ubiquitination at K107 alone is sufficient to activate the S phase checkpoint.

Identifying proteins that interact with PCNA ubiquitinated at K107.

To further characterize the molecular mechanism that leads to checkpoint activation, we will first utilize a candidate approach by determining whether any of the genes identified in our SGA screen interact with ubiquitinated PCNA by co-immunoprecipitation. Interestingly, we identified a putative PIP-box in Rad59 that

potentially mediates interaction with PCNA. Alternatively, we have access to a chemically synthesized form of PCNA that is ubiquitinated at K107 [Chen *et al.*, 2010]. Briefly, a biotin-tagged version of the ubiquitinated PCNA will be synthesized and used in co-immunoprecipitation experiments. We will first bind ubiquitinated PCNA to a streptavidin column prior to incubation with either wild-type or *cdc9-1* yeast extracts. For a candidate approach, interacting proteins such as Rad59 can be examined by western blotting. Alternatively, the method can be coupled with mass-spectroscopy to identify novel candidate interacting proteins.

Determining the binding affinity of ubiquitinated PCNA with replication proteins in vitro.

Taking advantage of the fact that ubiquitinated PCNA can be chemically synthesized, we will also examine how ubiquitinated PCNA affects the binding with other known PCNA-interacting proteins, such as pol-δ and Cdc9. In *cdc9* mutants, two scenarios, not mutually exclusive, could occur with respect to ubiquitinated PCNA. First, ubiquitinated PCNA may reduce the binding affinity of pol-δ, thereby inhibiting DNA synthesis and strand displacement along the lagging strand. Alternatively, ubiquitinated PCNA may "mark" sites of nicks and may have a higher binding affinity to DNA ligase I, thereby aiding in the recruiting of DNA ligase I to unligated Okazaki fragments. Regardless of the outcome, these *in vitro* binding studies may provide insight into the *in vivo* function of PCNA that is ubiquitinated at K107.

Does accumulation of nicks directly trigger PCNA ubiquitination at K107?

To experimentally address this question, we have developed a "nicking" assay in budding yeast by expressing an I-AniI homing endonuclease mutant (I-AniI-K227M) that nicks at a specific 19 bp target sequence (Figure 5.5) [McConnell Smith *et al.*, 2009]. Briefly, we expressed the I-AniI nickase in yeast under the control of a galactose promoter. We also cloned its target sequence into a high copy number plasmid to generate more than a single nick in the cell. As shown in Figure 5.5, we demonstrated that I-AniI nickase, but not the nickase-dead mutant (I-AniI-Q171K/K227M), can produce a SSB at its designated target site in yeast. This approach will allow us to induce nicks in different phases of the cell cycle and monitor the PCNA ubiquitination status independently of the *cdc9* mutation.

"PCNA Damage Code" model.

Does PCNA ubiquitination occur in other lagging strand mutants?

We and the Jentsch laboratory showed that PCNA ubiquitination occurred in lagging strand specific mutants, *cdc9-1* and *pol32Δ*, respectively [Das-Bradoo *et al.*, 2010b; Karras and Jentsch, 2010]. Additionally, these two mutants ubiquitinated PCNA at different lysine residues, K107 in *cdc9-1* and K164 in *pol32Δ* mutants. These results led us to hypothesize that cells can monitor every step during Okazaki fragment maturation and ubiquitinate PCNA at different lysine residues. We have recently tested and observed PCNA ubiquitination a *rad27Δ* mutant in a different background (Becker and Bielinsky unpublished observation). Therefore, we propose to test other lagging

strand mutants (e.g., *DNA2* mutations) to determine whether PCNA ubiquitination does occur in those strains. If we observe PCNA ubiquitination, we will determine the site of ubiquitination (K107, K164 or a different site) and its *in vivo* function.

SGA screen with different PCNA mutants.

Taking advantage of the powerful tool of SGA, we have generated a series of PCNA point mutants (lysine to arginine mutations) to mate with the nonessential gene deletion array to identify genetic interactions with any of the PCNA mutants [Baryshnikova *et al.*, 2010a; Davierwala *et al.*, 2005]. Additionally, these mutants will be tested with a collection of temperature sensitive essential mutants, which includes genes involved in DNA replication. Any genetic interaction between the query strains and the array mutants will be confirmed by tetrad analysis. Once verified, specific array mutants will be tested to determine whether they display any PCNA modification (ubiquitination and/or sumoylation). The extensive SGA data base will then be used to further understand the genetic profile of specific mutants. Theoretically, this approach could also be utilized to analyze query strains that carry more than one mutation in a surface lysine.

PCNA ubiquitination in human DNA ligase I-deficient cells.

We observed PCNA ubiquitination in human DNA ligase I-deficient cells, suggesting that the pathway is conserved. Genetic requirements in yeast for PCNA ubiquitination will be examined in human cells by transient knock-downs using small

interfering (si) RNAs against different ubiquitination pathway components. Additionally, because PCNA K107 is not conserved in humans, the site of PCNA ubiquitination in human DNA ligase I-deficient cells will also need to be determined.

An intriguing difference between yeast and humans is that humans have a nick sensor protein, PARP1, that binds to nicks and triggers SSB repair [Caldecott, 2008]. Thus, it will be interesting to determine whether PCNA ubiquitination observed in human DNA ligase I-deficient cells functions in the same or parallel pathway to PARP1. If PARP1 and PCNA ubiquitination are in the same pathway, inhibition of PARP1 activity either by the use of PARP1 inhibitors or siRNAs in DNA ligase I-deficient cells should not induce PCNA ubiquitination. Alternatively, if they are in separate pathways, blocking PARP1 activity by inhibitors will also hinder PCNA ubiquitination due to the inaccessibility of the nicks, whereas depletion of PARP1 by siRNA will expose nicks that can activate the PCNA ubiquitination pathway. Because PARP1 inhibitors are currently in clinical trials, these studies will be valuable for cancer research regardless of their outcome. Better understanding of PCNA ubiquitination pathway would be advantageous to design more specific target inhibitors against components of the PCNA ubiquitination pathway, since this could potentially inhibit S phase checkpoint activation, similar to yeast. Thus, a combination of inhibitors that target PARP1 to block DNA repair, and components of the PCNA ubiquitination pathway to block the S phase checkpoint, would increase cancer cell sensitivity to cancer therapy drugs.

Figure 5.1

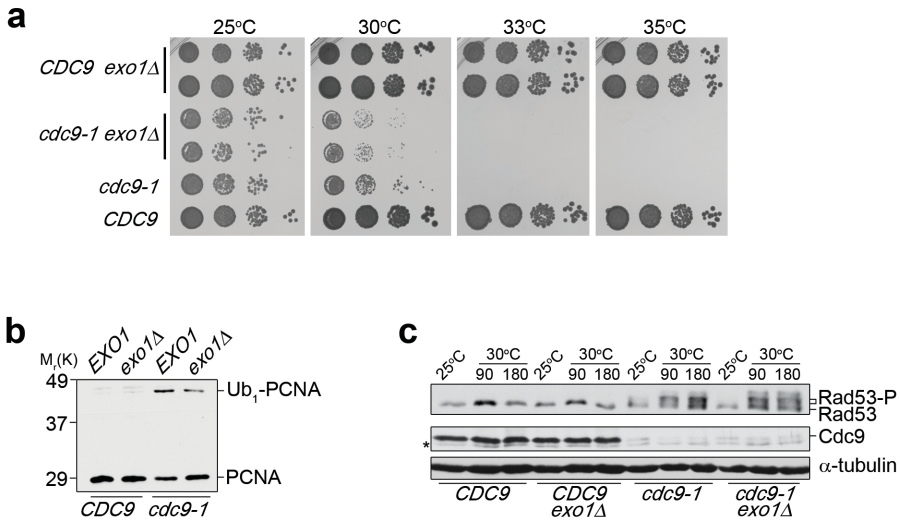

Figure 5.1 Deletion of *EXO1* does not alter PCNA mono-ubiquitination and Rad53 phosphorylation in *cdc9-1* mutants.

(a) Successive 10-fold dilutions of indicated strains were spotted on YPD plates and incubated for 3 days at the indicated temperatures. **(b, c)** Strains in **(a)** were grown asynchronously to mid-log phase at 25°C and subsequently shifted to 30°C for 1.5 and 3h. In **b**, only 3h time point samples were used for PCNA blot. PCNA and its ubiquitinated forms, Cdc9, and Rad53 were detected with anti-PCNA (S871), anti-Cdc9, and anti-Rad53 antibodies, respectively. α-tubulin served as a loading control. An asterisk indicates a non-specific band.

Figure 5.2

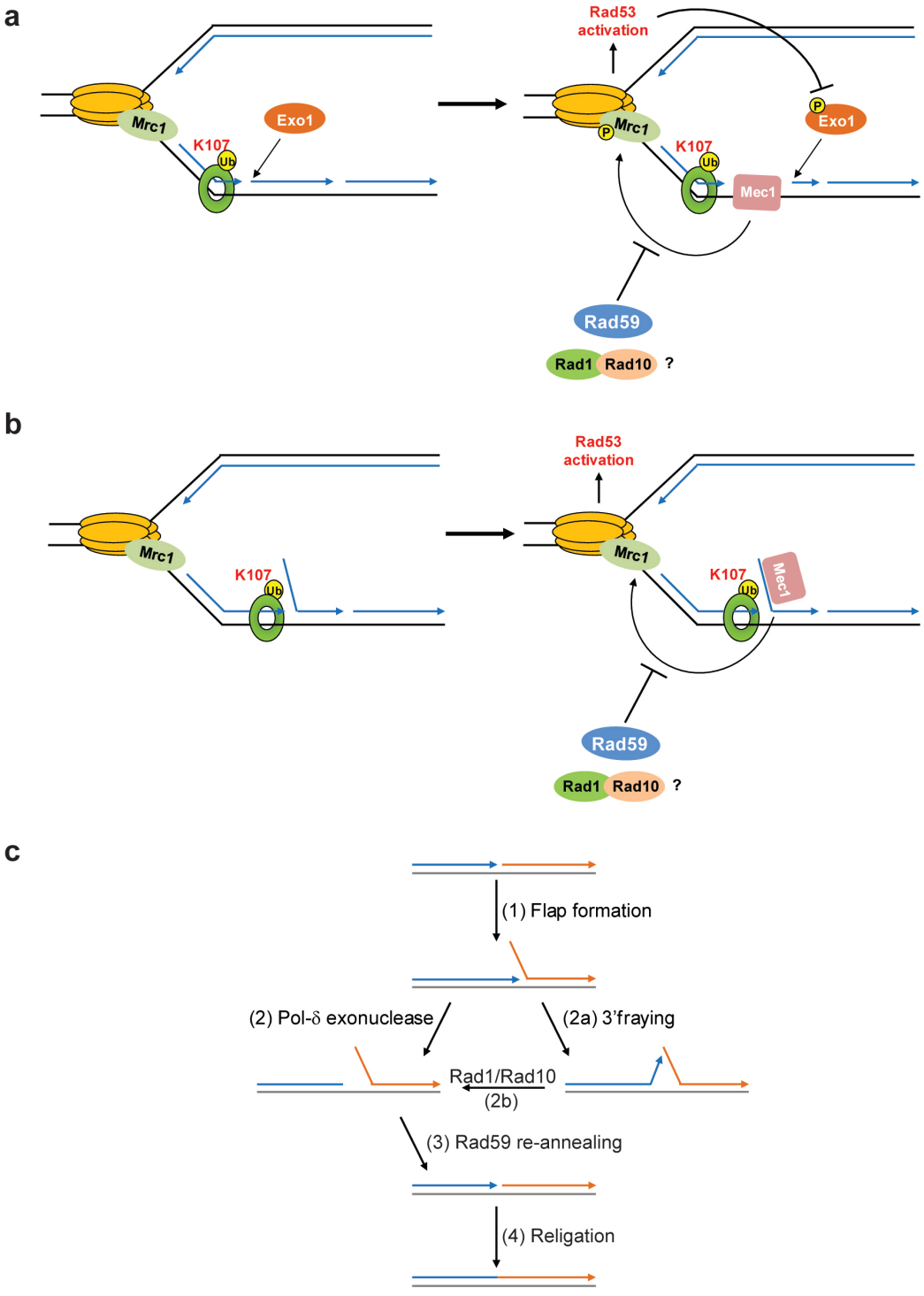
a
Rad53
activation
Mrc1
Exo1
K107
Ub
P
Mec1
Rad59
Rad1
Rad10
?
b
Rad53
activation
Mrc1
K107
Ub
Mec1
Rad59
Rad1
Rad10
?
c
(1) Flap formation
(2) Pol-δ exonuclease
(2a) 3'fraying
Rad1/Rad10
(2b)
(3) Rad59 re-annealing
(4) Religation

Figure 5.2 Alternative models to explain how Rad53 might be regulated at PCNA$^{K107\text{-}Ub}$-flagged replication forks. (a) In the absence of Cdc9 activity, we suggest that PCNA ubiquitination, in conjunction with Exo1, promotes the generation of ssDNA on the lagging strand template (left). ssDNA regions recruit Mec1 to the stalled replication fork. Mec1 then phosphorylates Mrc1 at the fork, which leads to Rad53 hyper-phosphorylation to delay cells in S phase (right). Rad53, in turn, has been shown to phosphorylate Exo1 to inhibit further nascent strand degradation [Morin *et al.*, 2008]. **(b)** Alternatively, pol-δ may displace the downstream Okazaki fragment, generating a 5'-flap (left). The 5'-flap may bind to RPA (not shown), thereby recruiting Mec1 to chromatin in order to phosphorylate Mrc1 at the fork. In both **a** and **b**, Rad59 acts to suppress Mrc1 phosphorylation at the fork. Because we observed synthetic sickness between *cdc9-1* with the *rad1Δ* and *rad10Δ*, we speculate that Rad1 and Rad10 may also function to suppress Mrc1 phosphorylation. **(c)** A model for Rad59 in the suppression of flap formation at unligated Okazaki fragments. In the absence of Cdc9 activity, pol-δ might promote strand displacement synthesis to generate a 5'-flap **(1)**. Pol-δ 3'-5' exonuclease activity can degrade the 3'-end of the Okazaki fragment **(2)**, leaving a short ssDNA region for Rad59 to re-anneal the 5'-flap to the template DNA to maintain the ligatable nick **(3)**. Alternatively, if the 3'-end of the Okazaki fragment is separated from the template DNA **(2a)**, Rad1/10 endonuclease can cleave the 3'-tail **(2b)**, which creates a ssDNA gap for Rad59-mediated strand re-annealing **(3)**. Finally, cells seal the nick with DNA ligase I **(4)**.

Figure 5.3

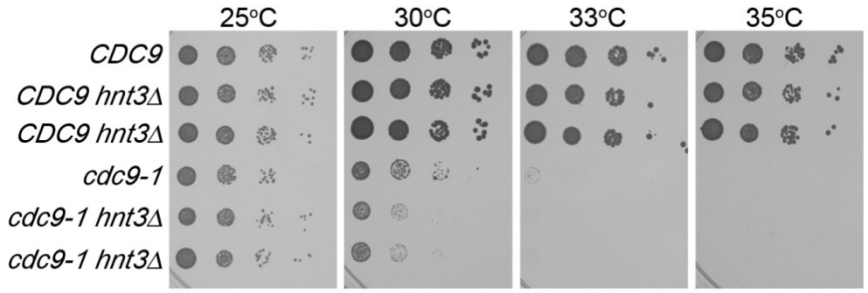

Figure 5.3 Deletion of *HNT3* results in increase temperature sensitivity in *cdc9-1* mutants.

Successive 10-fold dilutions of *CDC9*, *CDC9 hnt3Δ*, *cdc9-1*, and *cdc9-1 hnt3Δ* cells were grown on rich medium plates and incubated at the indicated temperatures.

Figure 5.4

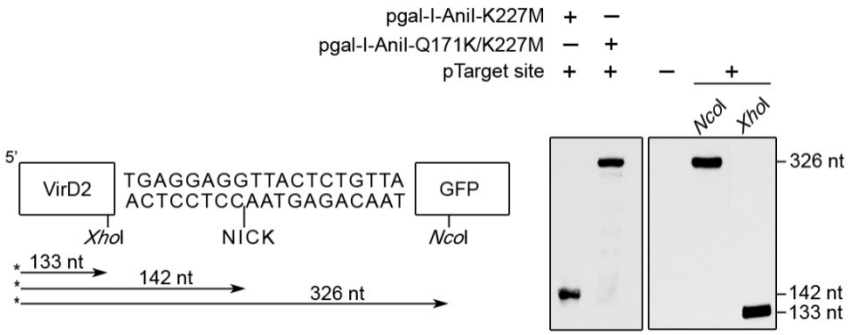

Figure 5.4 Generation of nicked DNA by expressing I-AniI-K227M nickase enzyme in budding yeast.

Left, the I-AniI-K227M nickase target site was cloned into pRS424 vector, flanked by two non-encoding genes, VirD2 from *Agrobacterium tumefaciens* and GFP to yield "pTarget site" plasmid. Asterisks indicate a 5'-biotinylated primer that anneals to the bottom strand of VirD2 region. Right, cells were grown asynchronously at 25°C to mid-log phase and subsequently switched to medium containing 2% galactose to overexpress either the I-AniI-K227M nickase or the enzyme dead mutant I-AniI-Q171K/K227M, which are under the control of a galactose promoter. The 5'-biotinylated primer was used for primer extension. Purified pTarget site plasmid from *E. coli* linearized with either *Nco*I or *Xho*I were used as a positive control for primer extension reactions to produce the indicated ssDNA fragments. A primer extension reaction with no DNA added was used as a negative control.

References

Acharya, N., A. Brahma, et al. (2007). "Mutations in the ubiquitin binding UBZ motif of DNA polymerase eta do not impair its function in translesion synthesis during replication." Mol Cell Biol **27**(20): 7266-7272.

Acharya, N., J. H. Yoon, et al. (2008). "Roles of PCNA-binding and ubiquitin-binding domains in human DNA polymerase eta in translesion DNA synthesis." Proc Natl Acad Sci U S A **105**(46): 17724-17729.

Aguilera, A. and B. Gomez-Gonzalez (2008). "Genome instability: a mechanistic view of its causes and consequences." Nat Rev Genet **9**(3): 204-217.

Ahel, I., U. Rass, et al. (2006). "The neurodegenerative disease protein aprataxin resolves abortive DNA ligation intermediates." Nature **443**(7112): 713-716.

Alabert, C., J. N. Bianco, et al. (2009). "Differential regulation of homologous recombination at DNA breaks and replication forks by the Mrc1 branch of the S-phase checkpoint." EMBO J **28**(8): 1131-1141.

Alcasabas, A. A., A. J. Osborn, et al. (2001). "Mrc1 transduces signals of DNA replication stress to activate Rad53." Nat Cell Biol **3**(11): 958-965.

Alexandru, G., W. Zachariae, et al. (1999). "Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage." EMBO J **18**(10): 2707-2721.

Aparicio, O. M., A. M. Stout, et al. (1999). "Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication." Proc Natl Acad Sci U S A **96**(16): 9130-9135.

Aparicio, O. M., D. M. Weinstein, et al. (1997). "Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase." Cell **91**(1): 59-69.

Araki, Y., Y. Kawasaki, et al. (2003). "Budding yeast mcm10/dna43 mutant requires a novel repair pathway for viability." Genes Cells **8**(5): 465-480.

Armstrong, A. A., F. Mohideen, et al. (2012). "Recognition of SUMO-modified PCNA requires tandem receptor motifs in Srs2." Nature **483**(7387): 59-63.

Ayyagari, R., X. V. Gomes, et al. (2003). "Okazaki fragment maturation in yeast. I. Distribution of functions between FEN1 AND DNA2." J Biol Chem **278**(3): 1618-1625.

Bae, S. H., K. H. Bae, et al. (2001). "RPA governs endonuclease switching during processing of Okazaki fragments in eukaryotes." Nature **412**(6845): 456-461.

Bae, S. H. and Y. S. Seo (2000). "Characterization of the enzymatic properties of the yeast dna2 Helicase/endonuclease suggests a new model for Okazaki fragment processing." J Biol Chem **275**(48): 38022-38031.

Bai, Y., A. P. Davis, et al. (1999). "A novel allele of RAD52 that causes severe DNA repair and recombination deficiencies only in the absence of RAD51 or RAD59." Genetics **153**(3): 1117-1130.

Bai, Y. and L. S. Symington (1996). "A Rad52 homolog is required for RAD51-independent mitotic recombination in Saccharomyces cerevisiae." Genes Dev **10**(16): 2025-2037.

Bailly, V., S. Lauder, et al. (1997a). "Yeast DNA repair proteins Rad6 and Rad18 form a heterodimer that has ubiquitin conjugating, DNA binding, and ATP hydrolytic activities." J Biol Chem **272**(37): 23360-23365.

Bailly, V., S. Prakash, et al. (1997b). "Domains required for dimerization of yeast Rad6 ubiquitin-conjugating enzyme and Rad18 DNA binding protein." Mol Cell Biol **17**(8): 4536-4543.

Bailly, V., C. H. Sommers, et al. (1992). "Specific complex formation between proteins encoded by the yeast DNA repair and recombination genes RAD1 and RAD10." Proc Natl Acad Sci U S A **89**(17): 8273-8277.

Balakrishnan, L. and R. A. Bambara (2011). "Eukaryotic lagging strand DNA replication employs a multi-pathway mechanism that protects genome integrity." J Biol Chem **286**(9): 6865-6870.

Barlow, J. H. and R. Rothstein (2009). "Rad52 recruitment is DNA replication independent and regulated by Cdc28 and the Mec1 kinase." EMBO J **28**(8): 1121-1130.

Barnes, D. E., L. H. Johnston, et al. (1990). "Human DNA ligase I cDNA: cloning and functional expression in Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **87**(17): 6679-6683.

Barnes, D. E., A. E. Tomkinson, et al. (1992). "Mutations in the DNA ligase I gene of an individual with immunodeficiencies and cellular hypersensitivity to DNA-damaging agents." Cell **69**(3): 495-503.

Bartkova, J., Z. Horejsi, et al. (2005). "DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis." Nature **434**(7035): 864-870.

Baryshnikova, A., M. Costanzo, et al. (2010a). "Synthetic genetic array (SGA) analysis in Saccharomyces cerevisiae and Schizosaccharomyces pombe." Methods Enzymol **470**: 145-179.

Baryshnikova, A., M. Costanzo, et al. (2010b). "Quantitative analysis of fitness and genetic interactions in yeast on a genome scale." Nat Methods **7**(12): 1017-1024.

Bell, S. P. and A. Dutta (2002). "DNA replication in eukaryotic cells." Annu Rev Biochem **71**: 333-374.

Bell, S. P. and B. Stillman (1992). "ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex." Nature **357**(6374): 128-134.

Ben-Aroya, S., C. Coombes, et al. (2008). "Toward a comprehensive temperature-sensitive mutant repository of the essential genes of Saccharomyces cerevisiae." Mol Cell **30**(2): 248-258.

Ben-Aroya, S., X. Pan, et al. (2010). "Making temperature-sensitive mutants." Methods Enzymol **470**: 181-204.

Bensimon, A., R. Aebersold, et al. (2011). "Beyond ATM: the protein kinase landscape of the DNA damage response." FEBS Lett **585**(11): 1625-1639.

Benson, F. E., P. Baumann, et al. (1998). "Synergistic actions of Rad51 and Rad52 in recombination and DNA repair." Nature **391**(6665): 401-404.

Bentley, D., J. Selfridge, et al. (1996). "DNA ligase I is required for fetal liver erythropoiesis but is not essential for mammalian cell viability." Nat Genet **13**(4): 489-491.

Bentley, D. J., C. Harrison, et al. (2002). "DNA ligase I null mouse cells show normal DNA repair activity but altered DNA replication and reduced genome stability." J Cell Sci **115**(Pt 7): 1551-1561.

Beranek, D. T. (1990). "Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents." Mutat Res **231**(1): 11-30.

Bergink, S. and S. Jentsch (2009). "Principles of ubiquitin and SUMO modifications in DNA repair." Nature **458**(7237): 461-467.

Bicknell, L. S., E. M. Bongers, et al. (2011a). "Mutations in the pre-replication complex cause Meier-Gorlin syndrome." Nat Genet **43**(4): 356-359.

Bicknell, L. S., S. Walker, et al. (2011b). "Mutations in ORC1, encoding the largest subunit of the origin recognition complex, cause microcephalic primordial dwarfism resembling Meier-Gorlin syndrome." Nat Genet **43**(4): 350-355.

Bielinsky, A. K. and S. A. Gerbi (1999). "Chromosomal ARS1 has a single leading strand start site." Mol Cell **3**(4): 477-486.

Bignell, G. R., C. D. Greenman, et al. (2010). "Signatures of mutation and selection in the cancer genome." Nature **463**(7283): 893-898.

Biton, S., A. Barzilai, et al. (2008). "The neurological phenotype of ataxia-telangiectasia: solving a persistent puzzle." DNA Repair (Amst) **7**(7): 1028-1038.

Blastyak, A., L. Pinter, et al. (2007). "Yeast Rad5 protein required for postreplication repair has a DNA helicase activity specific for replication fork regression." Mol Cell **28**(1): 167-175.

Bloom, J. and F. R. Cross (2007). "Novel role for Cdc14 sequestration: Cdc14 dephosphorylates factors that promote DNA replication." Mol Cell Biol **27**(3): 842-853.

Bochman, M. L., S. P. Bell, et al. (2008). "Subunit organization of Mcm2-7 and the unequal role of active sites in ATP hydrolysis and viability." Mol Cell Biol **28**(19): 5865-5873.

Bowers, J. L., J. C. Randell, et al. (2004). "ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication." Mol Cell **16**(6): 967-978.

Brachmann, C. B., A. Davies, et al. (1998). "Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications." Yeast **14**(2): 115-132.

Branzei, D. and M. Foiani (2006). "The Rad53 signal transduction pathway: Replication fork stabilization, DNA repair, and adaptation." Exp Cell Res **312**(14): 2654-2659.

Branzei, D. and M. Foiani (2007). "Interplay of replication checkpoints and repair proteins at stalled replication forks." DNA Repair (Amst) **6**(7): 994-1003.

Branzei, D. and M. Foiani (2008). "Regulation of DNA repair throughout the cell cycle." Nat Rev Mol Cell Biol **9**(4): 297-308.
Branzei, D. and M. Foiani (2010). "Maintaining genome stability at the replication fork." Nat Rev Mol Cell Biol **11**(3): 208-219.
Branzei, D., J. Sollier, et al. (2006). "Ubc9- and mms21-mediated sumoylation counteracts recombinogenic events at damaged replication forks." Cell **127**(3): 509-522.
Branzei, D., F. Vanoli, et al. (2008). "SUMOylation regulates Rad18-mediated template switch." Nature **456**(7224): 915-920.
Bridges, B. and G. C. Walker (2004). "Presidential honor for Evelyn Witkin." DNA Repair (Amst) **3**(5): 453-454.
Broomfield, S., B. L. Chow, et al. (1998). "MMS2, encoding a ubiquitin-conjugating-enzyme-like protein, is a member of the yeast error-free postreplication repair pathway." Proc Natl Acad Sci U S A **95**(10): 5678-5683.
Brown, E. J. and D. Baltimore (2000). "ATR disruption leads to chromosomal fragmentation and early embryonic lethality." Genes Dev **14**(4): 397-402.
Bruck, I., D. M. Kanter, et al. (2011). "Enabling association of the GINS protein tetramer with the mini chromosome maintenance (Mcm)2-7 protein complex by phosphorylated Sld2 protein and single-stranded origin DNA." J Biol Chem **286**(42): 36414-36426.
Budd, M. E., I. A. Antoshechkin, et al. (2011). "Inviability of a DNA2 deletion mutant is due to the DNA damage checkpoint." Cell Cycle **10**(10): 1690-1698.
Budd, M. E. and J. L. Campbell (1995). "A yeast gene required for DNA replication encodes a protein with homology to DNA helicases." Proc Natl Acad Sci U S A **92**(17): 7642-7646.
Budd, M. E. and J. L. Campbell (1997). "A yeast replicative helicase, Dna2 helicase, interacts with yeast FEN-1 nuclease in carrying out its essential function." Mol Cell Biol **17**(4): 2136-2142.
Budd, M. E., W. Choe, et al. (2000). "The nuclease activity of the yeast DNA2 protein, which is related to the RecB-like nucleases, is essential in vivo." J Biol Chem **275**(22): 16518-16529.
Budd, M. E., C. C. Reis, et al. (2006). "Evidence suggesting that Pif1 helicase functions in DNA replication with the Dna2 helicase/nuclease and DNA polymerase delta." Mol Cell Biol **26**(7): 2490-2500.
Budd, M. E., A. H. Tong, et al. (2005). "A network of multi-tasking proteins at the DNA replication fork preserves genome stability." PLoS Genet **1**(6): e61.
Burgers, P. M. (2009). "Polymerase dynamics at the eukaryotic DNA replication fork." J Biol Chem **284**(7): 4041-4045.
Burgers, P. M. and K. J. Gerik (1998). "Structure and processivity of two forms of Saccharomyces cerevisiae DNA polymerase delta." J Biol Chem **273**(31): 19756-19762.
Cadoret, J. C. and M. N. Prioleau (2010). "Genome-wide approaches to determining origin distribution." Chromosome Res **18**(1): 79-89.

Caldecott, K. W. (2008). "Single-strand break repair and genetic disease." Nat Rev Genet **9**(8): 619-631.
Chabes, A., B. Georgieva, et al. (2003). "Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase." Cell **112**(3): 391-401.
Chafin, D. R., J. M. Vitolo, et al. (2000). "Human DNA ligase I efficiently seals nicks in nucleosomes." EMBO J **19**(20): 5492-5501.
Chanet, R. and M. Heude (2003). "Characterization of mutations that are synthetic lethal with pol3-13, a mutated allele of DNA polymerase delta in Saccharomyces cerevisiae." Curr Genet **43**(5): 337-350.
Chastagner, P., A. Israel, et al. (2006). "Itch/AIP4 mediates Deltex degradation through the formation of K29-linked polyubiquitin chains." EMBO Rep **7**(11): 1147-1153.
Chattopadhyay, S. and A. K. Bielinsky (2007). "Human Mcm10 regulates the catalytic subunit of DNA polymerase-alpha and prevents DNA damage during replication." Mol Biol Cell **18**(10): 4085-4095.
Chen, C. and R. D. Kolodner (1999). "Gross chromosomal rearrangements in Saccharomyces cerevisiae replication and recombination defective mutants." Nat Genet **23**(1): 81-85.
Chen, C., B. J. Merrill, et al. (1999). "Saccharomyces cerevisiae pol30 (proliferating cell nuclear antigen) mutations impair replication fidelity and mismatch repair." Mol Cell Biol **19**(11): 7801-7815.
Chen, J., Y. Ai, et al. (2010). "Chemically ubiquitylated PCNA as a probe for eukaryotic translesion DNA synthesis." Nat Chem Biol **6**(4): 270-272.
Chen, S. and S. P. Bell (2011). "CDK prevents Mcm2-7 helicase loading by inhibiting Cdt1 interaction with Orc6." Genes Dev **25**(4): 363-372.
Chen, S., M. A. de Vries, et al. (2007). "Orc6 is required for dynamic recruitment of Cdt1 during repeated Mcm2-7 loading." Genes Dev **21**(22): 2897-2907.
Cheng, K. C., D. S. Cahill, et al. (1992). "8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G----T and A----C substitutions." J Biol Chem **267**(1): 166-172.
Ciccia, A. and S. J. Elledge (2010). "The DNA damage response: making it safe to play with knives." Mol Cell **40**(2): 179-204.
Cimprich, K. A. and D. Cortez (2008). "ATR: an essential regulator of genome integrity." Nat Rev Mol Cell Biol **9**(8): 616-627.
Coleman, T. R., P. B. Carpenter, et al. (1996). "The Xenopus Cdc6 protein is essential for the initiation of a single round of DNA replication in cell-free extracts." Cell **87**(1): 53-63.
Collinge, M. A. and F. R. Althaus (1994). "Expression of human poly(ADP-ribose) polymerase in Saccharomyces cerevisiae." Mol Gen Genet **245**(6): 686-693.
Cosentino, C., D. Grieco, et al. (2011). "ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair." EMBO J **30**(3): 546-555.
Costa, A., I. Ilves, et al. (2011). "The structural basis for MCM2-7 helicase activation by GINS and Cdc45." Nat Struct Mol Biol **18**(4): 471-477.

Costanzo, M., A. Baryshnikova, et al. (2010). "The genetic landscape of a cell." Science **327**(5964): 425-431.

Cross, F. R. and A. H. Tinkelenberg (1991). "A potential positive feedback loop controlling CLN1 and CLN2 gene expression at the start of the yeast cell cycle." Cell **65**(5): 875-883.

Culotti, J. and L. H. Hartwell (1971). "Genetic control of the cell division cycle in yeast. 3. Seven genes controlling nuclear division." Exp Cell Res **67**(2): 389-401.

Dahmann, C., J. F. Diffley, et al. (1995). "S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state." Curr Biol **5**(11): 1257-1269.

Daigaku, Y., A. A. Davies, et al. (2010). "Ubiquitin-dependent DNA damage bypass is separable from genome replication." Nature **465**(7300): 951-955.

Das-Bradoo, S., H. D. Nguyen, et al. (2010a). "Damage-specific modification of PCNA." Cell Cycle **9**(18): 3674-3679.

Das-Bradoo, S., H. D. Nguyen, et al. (2010b). "Defects in DNA ligase I trigger PCNA ubiquitylation at Lys 107." Nat Cell Biol **12**(1): 74-79.

Das-Bradoo, S., R. M. Ricke, et al. (2006). "Interaction between PCNA and diubiquitinated Mcm10 is essential for cell growth in budding yeast." Mol Cell Biol **26**(13): 4806-4817.

Date, H., O. Onodera, et al. (2001). "Early-onset ataxia with ocular motor apraxia and hypoalbuminemia is caused by mutations in a new HIT superfamily gene." Nat Genet **29**(2): 184-188.

Davierwala, A. P., J. Haynes, et al. (2005). "The synthetic genetic interaction spectrum of essential genes." Nat Genet **37**(10): 1147-1152.

Davies, A. A., D. Huttner, et al. (2008). "Activation of ubiquitin-dependent DNA damage bypass is mediated by replication protein a." Mol Cell **29**(5): 625-636.

Davies, S. L., P. S. North, et al. (2007). "Role for BLM in replication-fork restart and suppression of origin firing after replicative stress." Nat Struct Mol Biol **14**(7): 677-679.

Davis, A. P. and L. S. Symington (2001). "The yeast recombinational repair protein Rad59 interacts with Rad52 and stimulates single-strand annealing." Genetics **159**(2): 515-525.

Davis, A. P. and L. S. Symington (2003). "The Rad52-Rad59 complex interacts with Rad51 and replication protein A." DNA Repair (Amst) **2**(10): 1127-1134.

de Klein, A., M. Muijtjens, et al. (2000). "Targeted disruption of the cell-cycle checkpoint gene ATR leads to early embryonic lethality in mice." Curr Biol **10**(8): 479-482.

Debrauwere, H., S. Loeillet, et al. (2001). "Links between replication and recombination in Saccharomyces cerevisiae: a hypersensitive requirement for homologous recombination in the absence of Rad27 activity." Proc Natl Acad Sci U S A **98**(15): 8263-8269.

Denis, D. and P. A. Bullock (1993). "Primer-DNA formation during simian virus 40 DNA replication in vitro." Mol Cell Biol **13**(5): 2882-2890.

Dereli-Oz, A., G. Versini, et al. (2011). "Studies of genomic copy number changes in human cancers reveal signatures of DNA replication stress." Mol Oncol **5**(4): 308-314.
Detweiler, C. S. and J. J. Li (1998). "Ectopic induction of Clb2 in early G1 phase is sufficient to block prereplicative complex formation in Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **95**(5): 2384-2389.
Diffley, J. F., J. H. Cocker, et al. (1995). "Stepwise assembly of initiation complexes at budding yeast replication origins during the cell cycle." J Cell Sci Suppl **19**: 67-72.
Dionne, I., R. K. Nookala, et al. (2003). "A heterotrimeric PCNA in the hyperthermophilic archaeon Sulfolobus solfataricus." Mol Cell **11**(1): 275-282.
Dirick, L., T. Bohm, et al. (1995). "Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of Saccharomyces cerevisiae." EMBO J **14**(19): 4803-4813.
Dirick, L. and K. Nasmyth (1991). "Positive feedback in the activation of G1 cyclins in yeast." Nature **351**(6329): 754-757.
Ditch, S. and T. T. Paull (2012). "The ATM protein kinase and cellular redox signaling: beyond the DNA damage response." Trends Biochem Sci **37**(1): 15-22.
Dohmen, R. J., P. Wu, et al. (1994). "Heat-inducible degron: a method for constructing temperature-sensitive mutants." Science **263**(5151): 1273-1276.
Dornfeld, K. J. and D. M. Livingston (1991). "Effects of controlled RAD52 expression on repair and recombination in Saccharomyces cerevisiae." Mol Cell Biol **11**(4): 2013-2017.
Drake, J. W., B. Charlesworth, et al. (1998). "Rates of spontaneous mutation." Genetics **148**(4): 1667-1686.
Drury, L. S., G. Perkins, et al. (1997). "The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast." EMBO J **16**(19): 5966-5976.
Drury, L. S., G. Perkins, et al. (2000). "The cyclin-dependent kinase Cdc28p regulates distinct modes of Cdc6p proteolysis during the budding yeast cell cycle." Curr Biol **10**(5): 231-240.
Eddins, M. J., C. M. Carlile, et al. (2006). "Mms2-Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation." Nat Struct Mol Biol **13**(10): 915-920.
Ellenberger, T. and A. E. Tomkinson (2008). "Eukaryotic DNA Ligases: Structural and Functional Insights." Annu Rev Biochem **77**: 313-338.
Ellis, N. A. and J. German (1996). "Molecular genetics of Bloom's syndrome." Hum Mol Genet **5 Spec No**: 1457-1463.
Ellis, N. A., J. Groden, et al. (1995). "The Bloom's syndrome gene product is homologous to RecQ helicases." Cell **83**(4): 655-666.
Ellison, M. J. and M. Hochstrasser (1991). "Epitope-tagged ubiquitin. A new probe for analyzing ubiquitin function." J Biol Chem **266**(31): 21150-21157.
Emili, A. (1998). "MEC1-dependent phosphorylation of Rad9p in response to DNA damage." Mol Cell **2**(2): 183-189.

Epstein, C. B. and F. R. Cross (1992). "CLB5: a novel B cyclin from budding yeast with a role in S phase." Genes Dev **6**(9): 1695-1706.
Evrin, C., P. Clarke, et al. (2009). "A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication." Proc Natl Acad Sci U S A **106**(48): 20240-20245.
Fishman-Lobell, J. and J. E. Haber (1992). "Removal of nonhomologous DNA ends in double-strand break recombination: the role of the yeast ultraviolet repair gene RAD1." Science **258**(5081): 480-484.
Fitch, I., C. Dahmann, et al. (1992). "Characterization of four B-type cyclin genes of the budding yeast Saccharomyces cerevisiae." Mol Biol Cell **3**(7): 805-818.
Flott, S., C. Alabert, et al. (2007). "Phosphorylation of Slx4 by Mec1 and Tel1 regulates the single-strand annealing mode of DNA repair in budding yeast." Mol Cell Biol **27**(18): 6433-6445.
Flynn, R. L. and L. Zou (2011). "ATR: a master conductor of cellular responses to DNA replication stress." Trends Biochem Sci **36**(3): 133-140.
Fortune, J. M., Y. I. Pavlov, et al. (2005). "Saccharomyces cerevisiae DNA polymerase delta: high fidelity for base substitutions but lower fidelity for single- and multi-base deletions." J Biol Chem **280**(33): 29980-29987.
Frampton, J., A. Irmisch, et al. (2006). "Postreplication repair and PCNA modification in Schizosaccharomyces pombe." Mol Biol Cell **17**(7): 2976-2985.
Francis, L. I., J. C. Randell, et al. (2009). "Incorporation into the prereplicative complex activates the Mcm2-7 helicase for Cdc7-Dbf4 phosphorylation." Genes Dev **23**(5): 643-654.
Frank, G., J. Qiu, et al. (1998). "Partial functional deficiency of E160D flap endonuclease-1 mutant in vitro and in vivo is due to defective cleavage of DNA substrates." J Biol Chem **273**(49): 33064-33072.
Freudenthal, B. D., L. Gakhar, et al. (2010). "Structure of monoubiquitinated PCNA and implications for translesion synthesis and DNA polymerase exchange." Nat Struct Mol Biol **17**(4): 479-484.
Fu, Y. V., H. Yardimci, et al. (2011). "Selective bypass of a lagging strand roadblock by the eukaryotic replicative DNA helicase." Cell **146**(6): 931-941.
Gambus, A., R. C. Jones, et al. (2006). "GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks." Nat Cell Biol **8**(4): 358-366.
Gambus, A., G. A. Khoudoli, et al. (2011). "MCM2-7 form double hexamers at licensed origins in Xenopus egg extract." J Biol Chem **286**(13): 11855-11864.
Game, J. C., L. H. Johnston, et al. (1979). "Enhanced mitotic recombination in a ligase-defective mutant of the yeast Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **76**(9): 4589-4592.
Gangavarapu, V., S. Prakash, et al. (2007). "Requirement of RAD52 group genes for postreplication repair of UV-damaged DNA in Saccharomyces cerevisiae." Mol Cell Biol **27**(21): 7758-7764.

Garg, P. and P. M. Burgers (2005a). "DNA polymerases that propagate the eukaryotic DNA replication fork." Crit Rev Biochem Mol Biol **40**(2): 115-128.
Garg, P. and P. M. Burgers (2005b). "Ubiquitinated proliferating cell nuclear antigen activates translesion DNA polymerases eta and REV1." Proc Natl Acad Sci U S A **102**(51): 18361-18366.
Garg, P., C. M. Stith, et al. (2004). "Idling by DNA polymerase delta maintains a ligatable nick during lagging-strand DNA replication." Genes Dev **18**(22): 2764-2773.
Gary, R., M. S. Park, et al. (1999). "A novel role in DNA metabolism for the binding of Fen1/Rad27 to PCNA and implications for genetic risk." Mol Cell Biol **19**(8): 5373-5382.
Gilbert, C. S., C. M. Green, et al. (2001). "Budding yeast Rad9 is an ATP-dependent Rad53 activating machine." Mol Cell **8**(1): 129-136.
Glover, T. W., C. Berger, et al. (1984). "DNA polymerase alpha inhibition by aphidicolin induces gaps and breaks at common fragile sites in human chromosomes." Hum Genet **67**(2): 136-142.
Godon, C., F. P. Cordelieres, et al. (2008). "PARP inhibition versus PARP-1 silencing: different outcomes in terms of single-strand break repair and radiation susceptibility." Nucleic Acids Res **36**(13): 4454-4464.
Green, B. M., K. J. Finn, et al. (2010). "Loss of DNA replication control is a potent inducer of gene amplification." Science **329**(5994): 943-946.
Green, B. M. and J. J. Li (2005). "Loss of rereplication control in Saccharomyces cerevisiae results in extensive DNA damage." Mol Biol Cell **16**(1): 421-432.
Green, B. M., R. J. Morreale, et al. (2006). "Genome-wide mapping of DNA synthesis in Saccharomyces cerevisiae reveals that mechanisms preventing reinitiation of DNA replication are not redundant." Mol Biol Cell **17**(5): 2401-2414.
Groth, A., A. Corpet, et al. (2007). "Regulation of replication fork progression through histone supply and demand." Science **318**(5858): 1928-1931.
Guo, Z., R. Deshpande, et al. (2010a). "ATM activation in the presence of oxidative stress." Cell Cycle **9**(24): 4805-4811.
Guo, Z., S. Kozlov, et al. (2010b). "ATM activation by oxidative stress." Science **330**(6003): 517-521.
Guzder, S. N., C. H. Sommers, et al. (2006). "Complex formation with damage recognition protein Rad14 is essential for Saccharomyces cerevisiae Rad1-Rad10 nuclease to perform its function in nucleotide excision repair in vivo." Mol Cell Biol **26**(3): 1135-1141.
Hand, R. and J. German (1975). "A retarded rate of DNA chain growth in Bloom's syndrome." Proc Natl Acad Sci U S A **72**(2): 758-762.
Hannon, G. J. and D. S. Conklin (2004). "RNA interference by short hairpin RNAs expressed in vertebrate cells." Methods Mol Biol **257**: 255-266.
Haracska, L., C. A. Torres-Ramos, et al. (2004). "Opposing effects of ubiquitin conjugation and SUMO modification of PCNA on replicational bypass of DNA lesions in Saccharomyces cerevisiae." Mol Cell Biol **24**(10): 4267-4274.

Haracska, L., I. Unk, et al. (2001). "Roles of yeast DNA polymerases delta and zeta and of Rev1 in the bypass of abasic sites." Genes Dev **15**(8): 945-954.
Haracska, L., I. Unk, et al. (2006). "Ubiquitylation of yeast proliferating cell nuclear antigen and its implications for translesion DNA synthesis." Proc Natl Acad Sci U S A **103**(17): 6477-6482.
Harrison, C., A. M. Ketchen, et al. (2002). "Replication failure, genome instability, and increased cancer susceptibility in mice with a point mutation in the DNA ligase I gene." Cancer Res **62**(14): 4065-4074.
Hart, E. A., J. A. Bryant, et al. (2002). "Fission yeast Cdc23 interactions with DNA replication initiation proteins." Curr Genet **41**(5): 342-348.
Hartwell, L. H., R. K. Mortimer, et al. (1973). "Genetic Control of the Cell Division Cycle in Yeast: V. Genetic Analysis of cdc Mutants." Genetics **74**(2): 267-286.
Haworth, J., R. C. Alver, et al. (2010). "Ubc4 and Not4 regulate steady-state levels of DNA polymerase-alpha to promote efficient and accurate DNA replication." Mol Biol Cell **21**(18): 3205-3219.
Hecht, F. and T. W. Glover (1984). "Cancer chromosome breakpoints and common fragile sites induced by aphidicolin." Cancer Genet Cytogenet **13**(2): 185-188.
Heller, R. C., S. Kang, et al. (2011). "Eukaryotic origin-dependent DNA replication in vitro reveals sequential action of DDK and S-CDK kinases." Cell **146**(1): 80-91.
Heller, R. C. and K. J. Marians (2006). "Replication fork reactivation downstream of a blocked nascent leading strand." Nature **439**(7076): 557-562.
Henderson, L. M., C. F. Arlett, et al. (1985). "Cells from an immunodeficient patient (46BR) with a defect in DNA ligation are hypomutable but hypersensitive to the induction of sister chromatid exchanges." Proc Natl Acad Sci U S A **82**(7): 2044-2048.
Hershko, A. and A. Ciechanover (1998). "The ubiquitin system." Annu Rev Biochem **67**: 425-479.
Heyer, W. D., K. T. Ehmsen, et al. (2010). "Regulation of homologous recombination in eukaryotes." Annu Rev Genet **44**: 113-139.
Ho, C. K., J. L. Van Etten, et al. (1997). "Characterization of an ATP-dependent DNA ligase encoded by Chlorella virus PBCV-1." J Virol **71**(3): 1931-1937.
Hochstrasser, M. (1996). "Ubiquitin-dependent protein degradation." Annu Rev Genet **30**: 405-439.
Hoege, C., B. Pfander, et al. (2002). "RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO." Nature **419**(6903): 135-141.
Hoeijmakers, J. H. (2001). "Genome maintenance mechanisms for preventing cancer." Nature **411**(6835): 366-374.
Hoeijmakers, J. H. (2009). "DNA damage, aging, and cancer." N Engl J Med **361**(15): 1475-1485.
Hofmann, R. M. and C. M. Pickart (1999). "Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair." Cell **96**(5): 645-653.

Hofmann, R. M. and C. M. Pickart (2001). "In vitro assembly and recognition of Lys-63 polyubiquitin chains." J Biol Chem **276**(30): 27936-27943.
Hubscher, U. and Y. S. Seo (2001). "Replication of the lagging strand: a concert of at least 23 polypeptides." Mol Cells **12**(2): 149-157.
Ira, G. and J. E. Haber (2002). "Characterization of RAD51-independent break-induced replication that acts preferentially with short homologous sequences." Mol Cell Biol **22**(18): 6384-6392.
Ireland, M. J., S. S. Reinke, et al. (2000). "The impact of lagging strand replication mutations on the stability of CAG repeat tracts in yeast." Genetics **155**(4): 1657-1665.
Irmisch, A., E. Ampatzidou, et al. (2009). "Smc5/6 maintains stalled replication forks in a recombination-competent conformation." EMBO J **28**(2): 144-155.
Ivanov, E. L. and J. E. Haber (1995). "RAD1 and RAD10, but not other excision repair genes, are required for double-strand break-induced recombination in Saccharomyces cerevisiae." Mol Cell Biol **15**(4): 2245-2251.
Ivanov, E. L., N. Sugawara, et al. (1996). "Genetic requirements for the single-strand annealing pathway of double-strand break repair in Saccharomyces cerevisiae." Genetics **142**(3): 693-704.
Jablonovich, Z., B. Liefshitz, et al. (1999). "Characterization of the role played by the RAD59 gene of Saccharomyces cerevisiae in ectopic recombination." Curr Genet **36**(1-2): 13-20.
Jackson, L. P., S. I. Reed, et al. (2006). "Distinct mechanisms control the stability of the related S-phase cyclins Clb5 and Clb6." Mol Cell Biol **26**(6): 2456-2466.
Jaspersen, S. L., B. J. Huneycutt, et al. (2004). "Cdc28/Cdk1 regulates spindle pole body duplication through phosphorylation of Spc42 and Mps1." Dev Cell **7**(2): 263-274.
Jentsch, S., J. P. McGrath, et al. (1987). "The yeast DNA repair gene RAD6 encodes a ubiquitin-conjugating enzyme." Nature **329**(6135): 131-134.
Jin, Y. H., R. Ayyagari, et al. (2003). "Okazaki fragment maturation in yeast. II. Cooperation between the polymerase and 3'-5'-exonuclease activities of Pol delta in the creation of a ligatable nick." J Biol Chem **278**(3): 1626-1633.
Jin, Y. H., P. Garg, et al. (2005). "The multiple biological roles of the 3'-->5' exonuclease of Saccharomyces cerevisiae DNA polymerase delta require switching between the polymerase and exonuclease domains." Mol Cell Biol **25**(1): 461-471.
Jiricny, J. (2006). "The multifaceted mismatch-repair system." Nat Rev Mol Cell Biol **7**(5): 335-346.
Johnston, L. H. and K. A. Nasmyth (1978). "Saccharomyces cerevisiae cell cycle mutant cdc9 is defective in DNA ligase." Nature **274**(5674): 891-893.
Kamimura, Y., H. Masumoto, et al. (1998). "Sld2, which interacts with Dpb11 in Saccharomyces cerevisiae, is required for chromosomal DNA replication." Mol Cell Biol **18**(10): 6102-6109.

Kanemaki, M. and K. Labib (2006). "Distinct roles for Sld3 and GINS during establishment and progression of eukaryotic DNA replication forks." EMBO J **25**(8): 1753-1763.

Kanke, M., Y. Kodama, et al. (2012). "Mcm10 plays an essential role in origin DNA unwinding after loading of the CMG components." EMBO J.

Kannouche, P. L., J. Wing, et al. (2004). "Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage." Mol Cell **14**(4): 491-500.

Kanter, D. M. and D. L. Kaplan (2011). "Sld2 binds to origin single-stranded DNA and stimulates DNA annealing." Nucleic Acids Res **39**(7): 2580-2592.

Kao, H. I., J. Veeraraghavan, et al. (2004). "On the roles of Saccharomyces cerevisiae Dna2p and Flap endonuclease 1 in Okazaki fragment processing." J Biol Chem **279**(15): 15014-15024.

Karanja, K. K. and D. M. Livingston (2009). "C-terminal flap endonuclease (rad27) mutations: lethal interactions with a DNA ligase I mutation (cdc9-p) and suppression by proliferating cell nuclear antigen (POL30) in Saccharomyces cerevisiae." Genetics **183**(1): 63-78.

Karras, G. I. and S. Jentsch (2010). "The RAD6 DNA damage tolerance pathway operates uncoupled from the replication fork and is functional beyond S phase." Cell **141**(2): 255-267.

Katou, Y., Y. Kanoh, et al. (2003). "S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex." Nature **424**(6952): 1078-1083.

Kats, E. S., J. M. Enserink, et al. (2009). "The Saccharomyces cerevisiae Rad6 postreplication repair and Siz1/Srs2 homologous recombination-inhibiting pathways process DNA damage that arises in asf1 mutants." Mol Cell Biol **29**(19): 5226-5237.

Kaufman, P. D., R. Kobayashi, et al. (1997). "Ultraviolet radiation sensitivity and reduction of telomeric silencing in Saccharomyces cerevisiae cells lacking chromatin assembly factor-I." Genes Dev **11**(3): 345-357.

Kawabata, T., S. W. Luebben, et al. (2011). "Stalled fork rescue via dormant replication origins in unchallenged S phase promotes proper chromosome segregation and tumor suppression." Mol Cell **41**(5): 543-553.

Kim, S. and T. A. Weinert (1997). "Characterization of the checkpoint gene RAD53/MEC2 in Saccharomyces cerevisiae." Yeast **13**(8): 735-745.

Klemm, R. D., R. J. Austin, et al. (1997). "Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex." Cell **88**(4): 493-502.

Klemm, R. D. and S. P. Bell (2001). "ATP bound to the origin recognition complex is important for preRC formation." Proc Natl Acad Sci U S A **98**(15): 8361-8367.

Koivomagi, M., E. Valk, et al. (2011). "Cascades of multisite phosphorylation control Sic1 destruction at the onset of S phase." Nature **480**(7375): 128-131.

Kolodner, R. D., C. D. Putnam, et al. (2002). "Maintenance of genome stability in Saccharomyces cerevisiae." Science **297**(5581): 552-557.

Krejci, L., S. Van Komen, et al. (2003). "DNA helicase Srs2 disrupts the Rad51 presynaptic filament." Nature **423**(6937): 305-309.
Krogh, B. O. and L. S. Symington (2004). "Recombination proteins in yeast." Annu Rev Genet **38**: 233-271.
Kumagai, A. and W. G. Dunphy (2000). "Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in Xenopus egg extracts." Mol Cell **6**(4): 839-849.
Kuo, A. J., J. Song, et al. (2012). "The BAH domain of ORC1 links H4K20me2 to DNA replication licensing and Meier-Gorlin syndrome." Nature **484**(7392): 115-119.
Labib, K., J. F. Diffley, et al. (1999). "G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus." Nat Cell Biol **1**(7): 415-422.
Labib, K., J. A. Tercero, et al. (2000). "Uninterrupted MCM2-7 function required for DNA replication fork progression." Science **288**(5471): 1643-1647.
Lanker, S., M. H. Valdivieso, et al. (1996). "Rapid degradation of the G1 cyclin Cln2 induced by CDK-dependent phosphorylation." Science **271**(5255): 1597-1601.
Larsen, E., L. Kleppa, et al. (2008). "Early-onset lymphoma and extensive embryonic apoptosis in two domain-specific Fen1 mice mutants." Cancer Res **68**(12): 4571-4579.
Lea, D. and C. Coulson (1949). "The distribution of the numbers of mutants in bacterial populations." J Genet **49**(3): 264-285.
Lee, C., I. Liachko, et al. (2010). "Alternative mechanisms for coordinating polymerase alpha and MCM helicase." Mol Cell Biol **30**(2): 423-435.
Lee, K. H., D. W. Kim, et al. (2000). "The endonuclease activity of the yeast Dna2 enzyme is essential in vivo." Nucleic Acids Res **28**(15): 2873-2881.
Lee, K. Y. and K. Myung (2008). "PCNA modifications for regulation of post-replication repair pathways." Mol Cells **26**(1): 5-11.
Lee, S. H., A. D. Kwong, et al. (1991). "Studies on the activator 1 protein complex, an accessory factor for proliferating cell nuclear antigen-dependent DNA polymerase delta." J Biol Chem **266**(1): 594-602.
Lehmann, A. R. (1972). "Postreplication repair of DNA in ultraviolet-irradiated mammalian cells." J Mol Biol **66**(3): 319-337.
Lehmann, A. R., A. Niimi, et al. (2007). "Translesion synthesis: Y-family polymerases and the polymerase switch." DNA Repair (Amst) **6**(7): 891-899.
Lew, D. J. and S. I. Reed (1993). "Morphogenesis in the yeast cell cycle: regulation by Cdc28 and cyclins." J Cell Biol **120**(6): 1305-1320.
Liang, C., M. Weinreich, et al. (1995). "ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome." Cell **81**(5): 667-676.
Lieber, M. R. (2010). "The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway." Annu Rev Biochem **79**: 181-211.
Lindahl, T. (1993). "Instability and decay of the primary structure of DNA." Nature **362**(6422): 709-715.
Lindahl, T. and B. Nyberg (1972). "Rate of depurination of native deoxyribonucleic acid." Biochemistry **11**(19): 3610-3618.

Lis, E. T. and F. E. Romesberg (2006). "Role of Doa1 in the Saccharomyces cerevisiae DNA damage response." Mol Cell Biol **26**(11): 4122-4133.
Lisby, M., J. H. Barlow, et al. (2004). "Choreography of the DNA damage response: spatiotemporal relationships among checkpoint and repair proteins." Cell **118**(6): 699-713.
Liu, E., X. Li, et al. (2004a). "Cyclin-dependent kinases phosphorylate human Cdt1 and induce its degradation." J Biol Chem **279**(17): 17283-17288.
Liu, Y., H. I. Kao, et al. (2004b). "Flap endonuclease 1: a central component of DNA metabolism." Annu Rev Biochem **73**: 589-615.
Loeillet, S., B. Palancade, et al. (2005). "Genetic network interactions among replication, repair and nuclear pore deficiencies in yeast." DNA Repair (Amst) **4**(4): 459-468.
Lonn, U., S. Lonn, et al. (1989). "Altered formation of DNA replication intermediates in human 46 BR fibroblast cells hypersensitive to 3-aminobenzamide." Carcinogenesis **10**(6): 981-985.
Lonn, U., S. Lonn, et al. (1990). "An abnormal profile of DNA replication intermediates in Bloom's syndrome." Cancer Res **50**(11): 3141-3145.
Lopes, M., C. Cotta-Ramusino, et al. (2001). "The DNA replication checkpoint response stabilizes stalled replication forks." Nature **412**(6846): 557-561.
Lopes, M., M. Foiani, et al. (2006). "Multiple mechanisms control chromosome integrity after replication fork uncoupling and restart at irreparable UV lesions." Mol Cell **21**(1): 15-27.
Lopez-Mosqueda, J., N. L. Maas, et al. (2010). "Damage-induced phosphorylation of Sld3 is important to block late origin firing." Nature **467**(7314): 479-483.
Lorenz, M. C., R. S. Muir, et al. (1995). "Gene disruption with PCR products in Saccharomyces cerevisiae." Gene **158**(1): 113-117.
Lou, H., M. Komata, et al. (2008). "Mrc1 and DNA polymerase epsilon function together in linking DNA replication and the S phase checkpoint." Mol Cell **32**(1): 106-117.
Mackenney, V. J., D. E. Barnes, et al. (1997). "Specific function of DNA ligase I in simian virus 40 DNA replication by human cell-free extracts is mediated by the amino-terminal non-catalytic domain." J Biol Chem **272**(17): 11550-11556.
Maga, G. and U. Hubscher (2003). "Proliferating cell nuclear antigen (PCNA): a dancer with many partners." J Cell Sci **116**(Pt 15): 3051-3060.
Maga, G., G. Villani, et al. (2001). "Okazaki fragment processing: modulation of the strand displacement activity of DNA polymerase delta by the concerted action of replication protein A, proliferating cell nuclear antigen, and flap endonuclease-1." Proc Natl Acad Sci U S A **98**(25): 14298-14303.
Maiorano, D., J. Moreau, et al. (2000). "XCDT1 is required for the assembly of pre-replicative complexes in Xenopus laevis." Nature **404**(6778): 622-625.
Masai, H., C. Taniyama, et al. (2006). "Phosphorylation of MCM4 by Cdc7 kinase facilitates its interaction with Cdc45 on the chromatin." J Biol Chem **281**(51): 39249-39261.
Mastrandrea, L. D., J. You, et al. (1999). "E2/E3-mediated assembly of lysine 29-linked polyubiquitin chains." J Biol Chem **274**(38): 27299-27306.

Matsuoka, S., B. A. Ballif, et al. (2007). "ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage." Science **316**(5828): 1160-1166.
McConnell Smith, A., R. Takeuchi, et al. (2009). "Generation of a nicking enzyme that stimulates site-specific gene conversion from the I-AniI LAGLIDADG homing endonuclease." Proc Natl Acad Sci U S A **106**(13): 5099-5104.
McCulloch, S. D. and T. A. Kunkel (2008). "The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases." Cell Res **18**(1): 148-161.
McGarry, T. J. and M. W. Kirschner (1998). "Geminin, an inhibitor of DNA replication, is degraded during mitosis." Cell **93**(6): 1043-1053.
McGrath, J. P., S. Jentsch, et al. (1991). "UBA 1: an essential yeast gene encoding ubiquitin-activating enzyme." EMBO J **10**(1): 227-236.
Meijers-Heijboer, H., J. Wijnen, et al. (2003). "The CHEK2 1100delC mutation identifies families with a hereditary breast and colorectal cancer phenotype." Am J Hum Genet **72**(5): 1308-1314.
Meira, L. B., N. E. Burgis, et al. (2005). "Base excision repair." Adv Exp Med Biol **570**: 125-173.
Menissier-de Murcia, J., M. Molinete, et al. (1989). "Zinc-binding domain of poly(ADP-ribose)polymerase participates in the recognition of single strand breaks on DNA." J Mol Biol **210**(1): 229-233.
Menoyo, A., H. Alazzouzi, et al. (2001). "Somatic mutations in the DNA damage-response genes ATR and CHK1 in sporadic stomach tumors with microsatellite instability." Cancer Res **61**(21): 7727-7730.
Merchant, A. M., Y. Kawasaki, et al. (1997). "A lesion in the DNA replication initiation factor Mcm10 induces pausing of elongation forks through chromosomal replication origins in Saccharomyces cerevisiae." Mol Cell Biol **17**(6): 3261-3271.
Mimura, S., T. Seki, et al. (2004). "Phosphorylation-dependent binding of mitotic cyclins to Cdc6 contributes to DNA replication control." Nature **431**(7012): 1118-1123.
Minca, E. C. and D. Kowalski (2010). "Multiple Rad5 activities mediate sister chromatid recombination to bypass DNA damage at stalled replication forks." Mol Cell **38**(5): 649-661.
Mitelman, F., B. Johansson, et al. (2007). "The impact of translocations and gene fusions on cancer causation." Nat Rev Cancer **7**(4): 233-245.
Miyata, T., T. Oyama, et al. (2004). "The clamp-loading complex for processive DNA replication." Nat Struct Mol Biol **11**(7): 632-636.
Mizuno, K., S. Lambert, et al. (2009). "Nearby inverted repeats fuse to generate acentric and dicentric palindromic chromosomes by a replication template exchange mechanism." Genes Dev **23**(24): 2876-2886.
Moldovan, G. L., D. Dejsuphong, et al. (2012). "Inhibition of homologous recombination by the PCNA-interacting protein PARI." Mol Cell **45**(1): 75-86.
Moldovan, G. L., B. Pfander, et al. (2007). "PCNA, the maestro of the replication fork." Cell **129**(4): 665-679.

Moll, T., G. Tebb, et al. (1991). "The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the S. cerevisiae transcription factor SWI5." Cell **66**(4): 743-758.

Montecucco, A., R. Rossi, et al. (1998). "DNA ligase I is recruited to sites of DNA replication by an interaction with proliferating cell nuclear antigen: identification of a common targeting mechanism for the assembly of replication factories." EMBO J **17**(13): 3786-3795.

Montelone, B. A., S. Prakash, et al. (1981). "Spontaneous mitotic recombination in mms8-1, an allele of the CDC9 gene of Saccharomyces cerevisiae." J Bacteriol **147**(2): 517-525.

Moreira, M. C., C. Barbot, et al. (2001). "The gene mutated in ataxia-ocular apraxia 1 encodes the new HIT/Zn-finger protein aprataxin." Nat Genet **29**(2): 189-193.

Morin, I., H. P. Ngo, et al. (2008). "Checkpoint-dependent phosphorylation of Exo1 modulates the DNA damage response." EMBO J **27**(18): 2400-2410.

Motegi, A., H. J. Liaw, et al. (2008). "Polyubiquitination of proliferating cell nuclear antigen by HLTF and SHPRH prevents genomic instability from stalled replication forks." Proc Natl Acad Sci U S A **105**(34): 12411-12416.

Motegi, A. and K. Myung (2007). "Measuring the rate of gross chromosomal rearrangements in Saccharomyces cerevisiae: A practical approach to study genomic rearrangements observed in cancer." Methods **41**(2): 168-176.

Mott, C. and L. S. Symington (2011). "RAD51-independent inverted-repeat recombination by a strand-annealing mechanism." DNA Repair (Amst) **10**(4): 408-415.

Moyer, S. E., P. W. Lewis, et al. (2006). "Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase." Proc Natl Acad Sci U S A **103**(27): 10236-10241.

Muramatsu, S., K. Hirai, et al. (2010). "CDK-dependent complex formation between replication proteins Dpb11, Sld2, Pol {epsilon}, and GINS in budding yeast." Genes Dev **24**(6): 602-612.

Myung, K., A. Datta, et al. (2001). "Suppression of spontaneous chromosomal rearrangements by S phase checkpoint functions in Saccharomyces cerevisiae." Cell **104**(3): 397-408.

Myung, K. and R. D. Kolodner (2002). "Suppression of genome instability by redundant S-phase checkpoint pathways in Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **99**(7): 4500-4507.

Naiki, T., T. Wakayama, et al. (2004). "Association of Rad9 with double-strand breaks through a Mec1-dependent mechanism." Mol Cell Biol **24**(8): 3277-3285.

Nair, P. A., J. Nandakumar, et al. (2007). "Structural basis for nick recognition by a minimal pluripotent DNA ligase." Nat Struct Mol Biol **14**(8): 770-778.

Nakada, D., Y. Hirano, et al. (2004). "Requirement of the Mre11 complex and exonuclease 1 for activation of the Mec1 signaling pathway." Mol Cell Biol **24**(22): 10016-10025.

Nash, P., X. Tang, et al. (2001). "Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication." Nature **414**(6863): 514-521.

Naylor, M. L., J. M. Li, et al. (2009). "Mrc1 phosphorylation in response to DNA replication stress is required for Mec1 accumulation at the stalled fork." Proc Natl Acad Sci U S A **106**(31): 12765-12770.

Nguyen, V. Q., C. Co, et al. (2000). "Clb/Cdc28 kinases promote nuclear export of the replication initiator proteins Mcm2-7." Curr Biol **10**(4): 195-205.

Nguyen, V. Q., C. Co, et al. (2001). "Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms." Nature **411**(6841): 1068-1073.

Nick McElhinny, S. A., D. A. Gordenin, et al. (2008). "Division of labor at the eukaryotic replication fork." Mol Cell **30**(2): 137-144.

Nick McElhinny, S. A., G. E. Kissling, et al. (2010a). "Differential correction of lagging-strand replication errors made by DNA polymerases {alpha} and {delta}." Proc Natl Acad Sci U S A **107**(49): 21070-21075.

Nick McElhinny, S. A., D. Kumar, et al. (2010b). "Genome instability due to ribonucleotide incorporation into DNA." Nat Chem Biol **6**(10): 774-781.

Nick McElhinny, S. A., B. E. Watts, et al. (2010c). "Abundant ribonucleotide incorporation into DNA by yeast replicative polymerases." Proc Natl Acad Sci U S A **107**(11): 4949-4954.

Niimi, A., S. Brown, et al. (2008). "Regulation of proliferating cell nuclear antigen ubiquitination in mammalian cells." Proc Natl Acad Sci U S A **105**(42): 16125-16130.

Nishitani, H., Z. Lygerou, et al. (2004). "Proteolysis of DNA replication licensing factor Cdt1 in S-phase is performed independently of geminin through its N-terminal region." J Biol Chem **279**(29): 30807-30816.

Nishitani, H., Z. Lygerou, et al. (2000). "The Cdt1 protein is required to license DNA for replication in fission yeast." Nature **404**(6778): 625-628.

Nishitani, H., N. Sugimoto, et al. (2006). "Two E3 ubiquitin ligases, SCF-Skp2 and DDB1-Cul4, target human Cdt1 for proteolysis." EMBO J **25**(5): 1126-1136.

O'Driscoll, M. (2009). "Mouse models for ATR deficiency." DNA Repair (Amst) **8**(11): 1333-1337.

Ohmori, H., E. C. Friedberg, et al. (2001). "The Y-family of DNA polymerases." Mol Cell **8**(1): 7-8.

Osborn, A. J. and S. J. Elledge (2003). "Mrc1 is a replication fork component whose phosphorylation in response to DNA replication stress activates Rad53." Genes Dev **17**(14): 1755-1767.

Pacek, M., A. V. Tutter, et al. (2006). "Localization of MCM2-7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication." Mol Cell **21**(4): 581-587.

Paek, A. L., S. Kaochar, et al. (2009). "Fusion of nearby inverted repeats by a replication-based mechanism leads to formation of dicentric and acentric chromosomes that cause genome instability in budding yeast." Genes Dev **23**(24): 2861-2875.

Pan, X., P. Ye, et al. (2006). "A DNA integrity network in the yeast Saccharomyces cerevisiae." Cell **124**(5): 1069-1081.
Papouli, E., S. Chen, et al. (2005). "Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p." Mol Cell **19**(1): 123-133.
Paques, F. and J. E. Haber (1999). "Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae." Microbiol Mol Biol Rev **63**(2): 349-404.
Parker, J. L. and H. D. Ulrich (2009). "Mechanistic analysis of PCNA poly-ubiquitylation by the ubiquitin protein ligases Rad18 and Rad5." EMBO J **28**(23): 3657-3666.
Pascal, J. M., P. J. O'Brien, et al. (2004). "Human DNA ligase I completely encircles and partially unwinds nicked DNA." Nature **432**(7016): 473-478.
Pascal, J. M., O. V. Tsodikov, et al. (2006). "A flexible interface between DNA ligase and PCNA supports conformational switching and efficient ligation of DNA." Mol Cell **24**(2): 279-291.
Pellicioli, A., C. Lucca, et al. (1999). "Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase." EMBO J **18**(22): 6561-6572.
Petrini, J. H., Y. Xiao, et al. (1995). "DNA ligase I mediates essential functions in mammalian cells." Mol Cell Biol **15**(8): 4303-4308.
Pettersen, E. F., T. D. Goddard, et al. (2004). "UCSF Chimera--a visualization system for exploratory research and analysis." J Comput Chem **25**(13): 1605-1612.
Pfander, B., G. L. Moldovan, et al. (2005). "SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase." Nature **436**(7049): 428-433.
Piatti, S., T. Bohm, et al. (1996). "Activation of S-phase-promoting CDKs in late G1 defines a "point of no return" after which Cdc6 synthesis cannot promote DNA replication in yeast." Genes Dev **10**(12): 1516-1531.
Piatti, S., C. Lengauer, et al. (1995). "Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast Saccharomyces cerevisiae." EMBO J **14**(15): 3788-3799.
Prakash, L. (1981). "Characterization of postreplication repair in Saccharomyces cerevisiae and effects of rad6, rad18, rev3 and rad52 mutations." Mol Gen Genet **184**(3): 471-478.
Prakash, S., R. E. Johnson, et al. (2005). "Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function." Annu Rev Biochem **74**: 317-353.
Prescott, D. M. and P. L. Kuempel (1972). "Bidirectional replication of the chromosome in Escherichia coli." Proc Natl Acad Sci U S A **69**(10): 2842-2845.
Preston, B. D., T. M. Albertson, et al. (2010). "DNA replication fidelity and cancer." Semin Cancer Biol **20**(5): 281-293.
Prigent, C., M. S. Satoh, et al. (1994). "Aberrant DNA repair and DNA replication due to an inherited enzymatic defect in human DNA ligase I." Mol Cell Biol **14**(1): 310-317.

Pursell, Z. F., I. Isoz, et al. (2007). "Yeast DNA polymerase epsilon participates in leading-strand DNA replication." Science **317**(5834): 127-130.
Rahal, R. and A. Amon (2008). "Mitotic CDKs control the metaphase-anaphase transition and trigger spindle elongation." Genes Dev **22**(11): 1534-1548.
Randell, J. C., J. L. Bowers, et al. (2006). "Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase." Mol Cell **21**(1): 29-39.
Randell, J. C., A. Fan, et al. (2010). "Mec1 is one of multiple kinases that prime the Mcm2-7 helicase for phosphorylation by Cdc7." Mol Cell **40**(3): 353-363.
Reagan, M. S., C. Pittenger, et al. (1995). "Characterization of a mutant strain of Saccharomyces cerevisiae with a deletion of the RAD27 gene, a structural homolog of the RAD2 nucleotide excision repair gene." J Bacteriol **177**(2): 364-371.
Reed, S. I., J. A. Hadwiger, et al. (1985). "Protein kinase activity associated with the product of the yeast cell division cycle gene CDC28." Proc Natl Acad Sci U S A **82**(12): 4055-4059.
Reed, S. I., J. A. Hadwiger, et al. (1989). "Analysis of the Cdc28 protein kinase complex by dosage suppression." J Cell Sci Suppl **12**: 29-37.
Refsland, E. W. and D. M. Livingston (2005). "Interactions among DNA ligase I, the flap endonuclease and proliferating cell nuclear antigen in the expansion and contraction of CAG repeat tracts in yeast." Genetics **171**(3): 923-934.
Remus, D., F. Beuron, et al. (2009). "Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing." Cell **139**(4): 719-730.
Renwick, A., D. Thompson, et al. (2006). "ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles." Nat Genet **38**(8): 873-875.
Riballo, E., S. E. Critchlow, et al. (1999). "Identification of a defect in DNA ligase IV in a radiosensitive leukaemia patient." Curr Biol **9**(13): 699-702.
Richardson, H., D. J. Lew, et al. (1992). "Cyclin-B homologs in Saccharomyces cerevisiae function in S phase and in G2." Genes Dev **6**(11): 2021-2034.
Richardson, H. E., C. Wittenberg, et al. (1989). "An essential G1 function for cyclin-like proteins in yeast." Cell **59**(6): 1127-1133.
Ricke, R. M. and A. K. Bielinsky (2004). "Mcm10 regulates the stability and chromatin association of DNA polymerase-alpha." Mol Cell **16**(2): 173-185.
Ricke, R. M. and A. K. Bielinsky (2006). "A conserved Hsp10-like domain in Mcm10 is required to stabilize the catalytic subunit of DNA polymerase-alpha in budding yeast." J Biol Chem **281**(27): 18414-18425.
Rossi, M. L. and R. A. Bambara (2006). "Reconstituted Okazaki fragment processing indicates two pathways of primer removal." J Biol Chem **281**(36): 26051-26061.
Rossi, M. L., J. E. Pike, et al. (2008). "Pif1 helicase directs eukaryotic Okazaki fragments toward the two-nuclease cleavage pathway for primer removal." J Biol Chem **283**(41): 27483-27493.
Roy, R., J. Chun, et al. (2012). "BRCA1 and BRCA2: different roles in a common pathway of genome protection." Nat Rev Cancer **12**(1): 68-78.

Ruis, B. L., K. R. Fattah, et al. (2008). "The catalytic subunit of DNA-dependent protein kinase regulates proliferation, telomere length, and genomic stability in human somatic cells." Mol Cell Biol **28**(20): 6182-6195.

Rupp, W. D. and P. Howard-Flanders (1968). "Discontinuities in the DNA synthesized in an excision-defective strain of Escherichia coli following ultraviolet irradiation." J Mol Biol **31**(2): 291-304.

Rupp, W. D., C. E. Wilde, 3rd, et al. (1971). "Exchanges between DNA strands in ultraviolet-irradiated Escherichia coli." J Mol Biol **61**(1): 25-44.

Russell, N. S. and K. D. Wilkinson (2004). "Identification of a novel 29-linked polyubiquitin binding protein, Ufd3, using polyubiquitin chain analogues." Biochemistry **43**(16): 4844-4854.

Sakabe, K. and R. Okazaki (1966). "A unique property of the replicating region of chromosomal DNA." Biochim Biophys Acta **129**(3): 651-654.

Sakurai, S., K. Kitano, et al. (2005). "Structural basis for recruitment of human flap endonuclease 1 to PCNA." Embo J **24**(4): 683-693.

Sale, J. E. (2012). "Competition, collaboration and coordination - determining how cells bypass DNA damage." J Cell Sci **125**(Pt 7): 1633-1643.

Sanchez-Diaz, A., M. Kanemaki, et al. (2004). "Rapid depletion of budding yeast proteins by fusion to a heat-inducible degron." Sci STKE **2004**(223): PL8.

Sanchez, M., A. Calzada, et al. (1999). "The Cdc6 protein is ubiquitinated in vivo for proteolysis in Saccharomyces cerevisiae." J Biol Chem **274**(13): 9092-9097.

Sanchez, Y., B. A. Desany, et al. (1996). "Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways." Science **271**(5247): 357-360.

Santocanale, C. and J. F. Diffley (1998). "A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication." Nature **395**(6702): 615-618.

Santocanale, C., M. Foiani, et al. (1993). "The isolated 48,000-dalton subunit of yeast DNA primase is sufficient for RNA primer synthesis." J Biol Chem **268**(2): 1343-1348.

Schar, P., G. Herrmann, et al. (1997). "A newly identified DNA ligase of Saccharomyces cerevisiae involved in RAD52-independent repair of DNA double-strand breaks." Genes Dev **11**(15): 1912-1924.

Schiestl, R. H. and S. Prakash (1988). "RAD1, an excision repair gene of Saccharomyces cerevisiae, is also involved in recombination." Mol Cell Biol **8**(9): 3619-3626.

Schiestl, R. H. and S. Prakash (1990). "RAD10, an excision repair gene of Saccharomyces cerevisiae, is involved in the RAD1 pathway of mitotic recombination." Mol Cell Biol **10**(6): 2485-2491.

Schiestl, R. H., S. Prakash, et al. (1990). "The SRS2 suppressor of rad6 mutations of Saccharomyces cerevisiae acts by channeling DNA lesions into the RAD52 DNA repair pathway." Genetics **124**(4): 817-831.

Schiestl, R. H., P. Reynolds, et al. (1989). "Cloning and sequence analysis of the Saccharomyces cerevisiae RAD9 gene and further evidence that its product is

required for cell cycle arrest induced by DNA damage." Mol Cell Biol **9**(5): 1882-1896.
Schreiber, V., F. Dantzer, et al. (2006). "Poly(ADP-ribose): novel functions for an old molecule." Nat Rev Mol Cell Biol **7**(7): 517-528.
Schwob, E., T. Bohm, et al. (1994). "The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in S. cerevisiae." Cell **79**(2): 233-244.
Schwob, E. and K. Nasmyth (1993). "CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in Saccharomyces cerevisiae." Genes Dev **7**(7A): 1160-1175.
Sclafani, R. A. (2000). "Cdc7p-Dbf4p becomes famous in the cell cycle." J Cell Sci **113 (Pt 12)**: 2111-2117.
Scott, K. L. and S. E. Plon (2003). "Loss of Sin3/Rpd3 histone deacetylase restores the DNA damage response in checkpoint-deficient strains of Saccharomyces cerevisiae." Mol Cell Biol **23**(13): 4522-4531.
Setlow, R. B., P. A. Swenson, et al. (1963). "Thymine Dimers and Inhibition of DNA Synthesis by Ultraviolet Irradiation of Cells." Science **142**: 1464-1466.
Seufert, W., B. Futcher, et al. (1995). "Role of a ubiquitin-conjugating enzyme in degradation of S- and M-phase cyclins." Nature **373**(6509): 78-81.
Seufert, W. and S. Jentsch (1990). "Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins." Embo J **9**(2): 543-550.
Shcherbakova, P. V. and I. J. Fijalkowska (2006). "Translesion synthesis DNA polymerases and control of genome stability." Front Biosci **11**: 2496-2517.
Shcherbakova, P. V., Y. I. Pavlov, et al. (2003). "Unique error signature of the four-subunit yeast DNA polymerase epsilon." J Biol Chem **278**(44): 43770-43780.
Sheu, Y. J. and B. Stillman (2006). "Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression." Mol Cell **24**(1): 101-113.
Shibahara, K. and B. Stillman (1999). "Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin." Cell **96**(4): 575-585.
Shima, N., A. Alcaraz, et al. (2007). "A viable allele of Mcm4 causes chromosome instability and mammary adenocarcinomas in mice." Nat Genet **39**(1): 93-98.
Shiotani, B. and L. Zou (2009a). "ATR signaling at a glance." J Cell Sci **122**(Pt 3): 301-304.
Shiotani, B. and L. Zou (2009b). "Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks." Mol Cell **33**(5): 547-558.
Shirayama, M., A. Toth, et al. (1999). "APC(Cdc20) promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5." Nature **402**(6758): 203-207.
Signon, L., A. Malkova, et al. (2001). "Genetic requirements for RAD51- and RAD54-independent break-induced replication repair of a chromosomal double-strand break." Mol Cell Biol **21**(6): 2048-2056.
Silva, J. M., M. Z. Li, et al. (2005). "Second-generation shRNA libraries covering the mouse and human genomes." Nat Genet **37**(11): 1281-1288.

Simsek, D., A. Furda, et al. (2011). "Crucial role for DNA ligase III in mitochondria but not in Xrcc1-dependent repair." Nature **471**(7337): 245-248.
Skowyra, D., D. M. Koepp, et al. (1999). "Reconstitution of G1 cyclin ubiquitination with complexes containing SCFGrr1 and Rbx1." Science **284**(5414): 662-665.
Smith, D. J. and I. Whitehouse (2012). "Intrinsic coupling of lagging-strand synthesis to chromatin assembly." Nature **483**(7390): 434-438.
Sogo, J. M., M. Lopes, et al. (2002). "Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects." Science **297**(5581): 599-602.
Sommers, C. H., E. J. Miller, et al. (1995). "Conditional lethality of null mutations in RTH1 that encodes the yeast counterpart of a mammalian 5'- to 3'-exonuclease required for lagging strand DNA synthesis in reconstituted systems." J Biol Chem **270**(9): 4193-4196.
Spence, J., S. Sadis, et al. (1995). "A ubiquitin mutant with specific defects in DNA repair and multiubiquitination." Mol Cell Biol **15**(3): 1265-1273.
Sriskanda, V., B. Schwer, et al. (1999). "Mutational analysis of Escherichia coli DNA ligase identifies amino acids required for nick-ligation in vitro and for in vivo complementation of the growth of yeast cells deleted for CDC9 and LIG4." Nucleic Acids Res **27**(20): 3953-3963.
Stelter, P. and H. D. Ulrich (2003). "Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation." Nature **425**(6954): 188-191.
Stewart, G. S., S. Panier, et al. (2009). "The RIDDLE syndrome protein mediates a ubiquitin-dependent signaling cascade at sites of DNA damage." Cell **136**(3): 420-434.
Stinchcomb, D. T., K. Struhl, et al. (1979). "Isolation and characterisation of a yeast chromosomal replicator." Nature **282**(5734): 39-43.
Stith, C. M., J. Sterling, et al. (2008). "Flexibility of eukaryotic Okazaki fragment maturation through regulated strand displacement synthesis." J Biol Chem **283**(49): 34129-34140.
Stokes, M. P. and W. M. Michael (2003). "DNA damage-induced replication arrest in Xenopus egg extracts." J Cell Biol **163**(2): 245-255.
Subramanian, J., S. Vijayakumar, et al. (2005). "Genetic instability induced by overexpression of DNA ligase I in budding yeast." Genetics **171**(2): 427-441.
Sugawara, N., G. Ira, et al. (2000). "DNA length dependence of the single-strand annealing pathway and the role of Saccharomyces cerevisiae RAD59 in double-strand break repair." Mol Cell Biol **20**(14): 5300-5309.
Sugawara, N., E. L. Ivanov, et al. (1995). "DNA structure-dependent requirements for yeast RAD genes in gene conversion." Nature **373**(6509): 84-86.
Sugimoto, N., Y. Tatsumi, et al. (2004). "Cdt1 phosphorylation by cyclin A-dependent kinases negatively regulates its function without affecting geminin binding." J Biol Chem **279**(19): 19691-19697.
Sun, Z., D. S. Fay, et al. (1996). "Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways." Genes Dev **10**(4): 395-406.

Sung, P., P. Reynolds, et al. (1993). "Purification and characterization of the Saccharomyces cerevisiae RAD1/RAD10 endonuclease." J Biol Chem **268**(35): 26391-26399.
Surana, U., H. Robitsch, et al. (1991). "The role of CDC28 and cyclins during mitosis in the budding yeast S. cerevisiae." Cell **65**(1): 145-161.
Sweeney, F. D., F. Yang, et al. (2005). "Saccharomyces cerevisiae Rad9 acts as a Mec1 adaptor to allow Rad53 activation." Curr Biol **15**(15): 1364-1375.
Symington, L. S. (1998). "Homologous recombination is required for the viability of rad27 mutants." Nucleic Acids Res **26**(24): 5589-5595.
Symington, L. S. (2002). "Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair." Microbiol Mol Biol Rev **66**(4): 630-670.
Symington, L. S. and J. Gautier (2011). "Double-strand break end resection and repair pathway choice." Annu Rev Genet **45**: 247-271.
Szyjka, S. J., J. G. Aparicio, et al. (2008). "Rad53 regulates replication fork restart after DNA damage in Saccharomyces cerevisiae." Genes Dev **22**(14): 1906-1920.
Tak, Y. S., Y. Tanaka, et al. (2006). "A CDK-catalysed regulatory phosphorylation for formation of the DNA replication complex Sld2-Dpb11." EMBO J **25**(9): 1987-1996.
Takayama, Y., Y. Kamimura, et al. (2003). "GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast." Genes Dev **17**(9): 1153-1165.
Tanaka, A., S. Weinel, et al. (2012). "Germline Mutation in ATR in Autosomal-Dominant Oropharyngeal Cancer Syndrome." Am J Hum Genet.
Tanaka, K. and P. Russell (2001). "Mrc1 channels the DNA replication arrest signal to checkpoint kinase Cds1." Nat Cell Biol **3**(11): 966-972.
Tanaka, S. and J. F. Diffley (2002). "Interdependent nuclear accumulation of budding yeast Cdt1 and Mcm2-7 during G1 phase." Nat Cell Biol **4**(3): 198-207.
Tanaka, S., T. Umemori, et al. (2007). "CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast." Nature **445**(7125): 328-332.
Tanaka, T. and K. Nasmyth (1998). "Association of RPA with chromosomal replication origins requires an Mcm protein, and is regulated by Rad53, and cyclin- and Dbf4-dependent kinases." EMBO J **17**(17): 5182-5191.
Tanaka, T., T. Umemori, et al. (2011). "Sld7, an Sld3-associated protein required for efficient chromosomal DNA replication in budding yeast." EMBO J **30**(10): 2019-2030.
Tanny, R. E., D. M. MacAlpine, et al. (2006). "Genome-wide analysis of re-replication reveals inhibitory controls that target multiple stages of replication initiation." Mol Biol Cell **17**(5): 2415-2423.
Teo, S. H. and S. P. Jackson (1997). "Identification of Saccharomyces cerevisiae DNA ligase IV: involvement in DNA double-strand break repair." EMBO J **16**(15): 4788-4795.

Terai, K., T. Abbas, et al. (2010). "CRL4(Cdt2) E3 ubiquitin ligase monoubiquitinates PCNA to promote translesion DNA synthesis." Mol Cell **37**(1): 143-149.

Tercero, J. A. and J. F. Diffley (2001). "Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint." Nature **412**(6846): 553-557.

Tercero, J. A., M. P. Longhese, et al. (2003). "A central role for DNA replication forks in checkpoint activation and response." Mol Cell **11**(5): 1323-1336.

Tishkoff, D. X., A. L. Boerger, et al. (1997). "Identification and characterization of Saccharomyces cerevisiae EXO1, a gene encoding an exonuclease that interacts with MSH2." Proc Natl Acad Sci U S A **94**(14): 7487-7492.

Toh, G. W., N. Sugawara, et al. (2010). "Mec1/Tel1-dependent phosphorylation of Slx4 stimulates Rad1-Rad10-dependent cleavage of non-homologous DNA tails." DNA Repair (Amst) **9**(6): 718-726.

Tomkinson, A. E., N. F. Totty, et al. (1991). "Location of the active site for enzyme-adenylate formation in DNA ligases." Proc Natl Acad Sci U S A **88**(2): 400-404.

Tomkinson, A. E., S. Vijayakumar, et al. (2006). "DNA ligases: structure, reaction mechanism, and function." Chem Rev **106**(2): 687-699.

Tong, A. H. and C. Boone (2006). "Synthetic genetic array analysis in Saccharomyces cerevisiae." Methods Mol Biol **313**: 171-192.

Torres-Ramos, C. A., S. Prakash, et al. (2002). "Requirement of RAD5 and MMS2 for postreplication repair of UV-damaged DNA in Saccharomyces cerevisiae." Mol Cell Biol **22**(7): 2419-2426.

Tsurimoto, T. and B. Stillman (1990). "Functions of replication factor C and proliferating-cell nuclear antigen: functional similarity of DNA polymerase accessory proteins from human cells and bacteriophage T4." Proc Natl Acad Sci U S A **87**(3): 1023-1027.

Tsurimoto, T. and B. Stillman (1991). "Replication factors required for SV40 DNA replication in vitro. I. DNA structure-specific recognition of a primer-template junction by eukaryotic DNA polymerases and their accessory proteins." J Biol Chem **266**(3): 1950-1960.

Tyers, M., G. Tokiwa, et al. (1992). "The Cln3-Cdc28 kinase complex of S. cerevisiae is regulated by proteolysis and phosphorylation." EMBO J **11**(5): 1773-1784.

Ulrich, H. D. (2009). "Regulating post-translational modifications of the eukaryotic replication clamp PCNA." DNA Repair (Amst) **8**(4): 461-469.

Ulrich, H. D. and S. Jentsch (2000). "Two RING finger proteins mediate cooperation between ubiquitin-conjugating enzymes in DNA repair." Embo J **19**(13): 3388-3397.

Ulrich, H. D. and H. Walden (2010). "Ubiquitin signalling in DNA replication and repair." Nat Rev Mol Cell Biol **11**(7): 479-489.

van der Kemp, P. A., M. de Padula, et al. (2009). "PCNA monoubiquitylation and DNA polymerase eta ubiquitin-binding domain are required to prevent 8-oxoguanine-induced mutagenesis in Saccharomyces cerevisiae." Nucleic Acids Res **37**(8): 2549-2559.

van Deursen, F., S. Sengupta, et al. (2012). "Mcm10 associates with the loaded DNA helicase at replication origins and defines a novel step in its activation." EMBO J **31**(9): 2195-2206.

VanDemark, A. P., R. M. Hofmann, et al. (2001). "Molecular insights into polyubiquitin chain assembly: crystal structure of the Mms2/Ubc13 heterodimer." Cell **105**(6): 711-720.

Vanoli, F., M. Fumasoni, et al. (2010). "Replication and recombination factors contributing to recombination-dependent bypass of DNA lesions by template switch." PLoS Genet **6**(11): e1001205.

Vaziri, C., S. Saxena, et al. (2003). "A p53-dependent checkpoint pathway prevents rereplication." Mol Cell **11**(4): 997-1008.

Verma, R., R. S. Annan, et al. (1997). "Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase." Science **278**(5337): 455-460.

Vialard, J. E., C. S. Gilbert, et al. (1998). "The budding yeast Rad9 checkpoint protein is subjected to Mec1/Tel1-dependent hyperphosphorylation and interacts with Rad53 after DNA damage." EMBO J **17**(19): 5679-5688.

Vijayakumar, S., B. R. Chapados, et al. (2007). "The C-terminal domain of yeast PCNA is required for physical and functional interactions with Cdc9 DNA ligase." Nucleic Acids Res **35**(5): 1624-1637.

Waga, S., G. Bauer, et al. (1994). "Reconstitution of complete SV40 DNA replication with purified replication factors." J Biol Chem **269**(14): 10923-10934.

Walter, J. and J. Newport (2000). "Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha." Mol Cell **5**(4): 617-627.

Wang, M. and C. M. Pickart (2005). "Different HECT domain ubiquitin ligases employ distinct mechanisms of polyubiquitin chain synthesis." Embo J **24**(24): 4324-4333.

Warren, E. M., H. Huang, et al. (2009). "Physical interactions between Mcm10, DNA, and DNA polymerase alpha." J Biol Chem **284**(36): 24662-24672.

Warren, E. M., S. Vaithiyalingam, et al. (2008). "Structural basis for DNA binding by replication initiator Mcm10." Structure **16**(12): 1892-1901.

Wasch, R. and F. R. Cross (2002). "APC-dependent proteolysis of the mitotic cyclin Clb2 is essential for mitotic exit." Nature **418**(6897): 556-562.

Wasielewski, M., H. Vasen, et al. (2008). "CHEK2 1100delC is a susceptibility allele for HNPCC-related colorectal cancer." Clin Cancer Res **14**(15): 4989-4994.

Watanabe, K., S. Tateishi, et al. (2004). "Rad18 guides poleta to replication stalling sites through physical interaction and PCNA monoubiquitination." EMBO J **23**(19): 3886-3896.

Watase, G., H. Takisawa, et al. (2012). "Mcm10 Plays a Role in Functioning of the Eukaryotic Replicative DNA Helicase, Cdc45-Mcm-GINS." Curr Biol **22**(4): 343-349.

Webster, A. D., D. E. Barnes, et al. (1992). "Growth retardation and immunodeficiency in a patient with mutations in the DNA ligase I gene." Lancet **339**(8808): 1508-1509.
Wei, Y. F., P. Robins, et al. (1995). "Molecular cloning and expression of human cDNAs encoding a novel DNA ligase IV and DNA ligase III, an enzyme active in DNA repair and recombination." Mol Cell Biol **15**(6): 3206-3216.
Weinert, T. and L. Hartwell (1989). "Control of G2 delay by the rad9 gene of Saccharomyces cerevisiae." J Cell Sci Suppl **12**: 145-148.
Weinert, T. A. and L. H. Hartwell (1990). "Characterization of RAD9 of Saccharomyces cerevisiae and evidence that its function acts posttranslationally in cell cycle arrest after DNA damage." Mol Cell Biol **10**(12): 6554-6564.
Weinert, T. A. and L. H. Hartwell (1993). "Cell cycle arrest of cdc mutants and specificity of the RAD9 checkpoint." Genetics **134**(1): 63-80.
Weinert, T. A., G. L. Kiser, et al. (1994). "Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair." Genes Dev **8**(6): 652-665.
Wen, W., J. L. Meinkoth, et al. (1995). "Identification of a signal for rapid export of proteins from the nucleus." Cell **82**(3): 463-473.
Whittaker, A. J., I. Royzman, et al. (2000). "Drosophila double parked: a conserved, essential replication protein that colocalizes with the origin recognition complex and links DNA replication with mitosis and the down-regulation of S phase transcripts." Genes Dev **14**(14): 1765-1776.
Willer, M., M. Rainey, et al. (1999). "The yeast CDC9 gene encodes both a nuclear and a mitochondrial form of DNA ligase I." Curr Biol **9**(19): 1085-1094.
Wilmes, G. M., V. Archambault, et al. (2004). "Interaction of the S-phase cyclin Clb5 with an "RXL" docking sequence in the initiator protein Orc6 provides an origin-localized replication control switch." Genes Dev **18**(9): 981-991.
Wilson, T. E., U. Grawunder, et al. (1997). "Yeast DNA ligase IV mediates non-homologous DNA end joining." Nature **388**(6641): 495-498.
Windecker, H. and H. D. Ulrich (2008). "Architecture and assembly of poly-SUMO chains on PCNA in Saccharomyces cerevisiae." J Mol Biol **376**(1): 221-231.
Winzeler, E. A., D. D. Shoemaker, et al. (1999). "Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis." Science **285**(5429): 901-906.
Wohlschlegel, J. A., B. T. Dwyer, et al. (2000). "Inhibition of eukaryotic DNA replication by geminin binding to Cdt1." Science **290**(5500): 2309-2312.
Wu, X., E. Braithwaite, et al. (1999). "DNA ligation during excision repair in yeast cell-free extracts is specifically catalyzed by the CDC9 gene product." Biochemistry **38**(9): 2628-2635.
Wu, Y., T. Sugiyama, et al. (2006). "DNA annealing mediated by Rad52 and Rad59 proteins." J Biol Chem **281**(22): 15441-15449.

Wyrick, J. J., J. G. Aparicio, et al. (2001). "Genome-wide distribution of ORC and MCM proteins in S. cerevisiae: high-resolution mapping of replication origins." Science **294**(5550): 2357-2360.

Xu, G., J. S. Paige, et al. (2010). "Global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling." Nat Biotechnol **28**(8): 868-873.

Yaglom, J., M. H. Linskens, et al. (1995). "p34Cdc28-mediated control of Cln3 cyclin degradation." Mol Cell Biol **15**(2): 731-741.

Yang, X., J. Gregan, et al. (2005). "Nuclear distribution and chromatin association of DNA polymerase alpha-primase is affected by TEV protease cleavage of Cdc23 (Mcm10) in fission yeast." BMC Mol Biol **6**: 13.

Yang, X. H. and L. Zou (2009). "Dual functions of DNA replication forks in checkpoint signaling and PCNA ubiquitination." Cell Cycle **8**(2): 191-194.

Zegerman, P. and J. F. Diffley (2007). "Phosphorylation of Sld2 and Sld3 by cyclin-dependent kinases promotes DNA replication in budding yeast." Nature **445**(7125): 281-285.

Zegerman, P. and J. F. Diffley (2009). "DNA replication as a target of the DNA damage checkpoint." DNA Repair (Amst) **8**(9): 1077-1088.

Zegerman, P. and J. F. Diffley (2010). "Checkpoint-dependent inhibition of DNA replication initiation by Sld3 and Dbf4 phosphorylation." Nature **467**(7314): 474-478.

Zhang, H. and C. W. Lawrence (2005). "The error-free component of the RAD6/RAD18 DNA damage tolerance pathway of budding yeast employs sister-strand recombination." Proc Natl Acad Sci U S A **102**(44): 15954-15959.

Zhang, Z., K. Shibahara, et al. (2000). "PCNA connects DNA replication to epigenetic inheritance in yeast." Nature **408**(6809): 221-225.

Zhang, Z., S. Zhang, et al. (2012). "Structure of monoubiquitinated PCNA: Implications for DNA polymerase switching and Okazaki fragment maturation." Cell Cycle **11**(11): 2128-2136.

Zhao, X. and R. Rothstein (2002). "The Dun1 checkpoint kinase phosphorylates and regulates the ribonucleotide reductase inhibitor Sml1." Proc Natl Acad Sci U S A **99**(6): 3746-3751.

Zheng, L., H. Dai, et al. (2007). "Fen1 mutations result in autoimmunity, chronic inflammation and cancers." Nat Med **13**(7): 812-819.

Zhu, W., C. Ukomadu, et al. (2007). "Mcm10 and And-1/CTF4 recruit DNA polymerase alpha to chromatin for initiation of DNA replication." Genes Dev **21**(18): 2288-2299.

Zhuang, Z., R. E. Johnson, et al. (2008). "Regulation of polymerase exchange between Poleta and Poldelta by monoubiquitination of PCNA and the movement of DNA polymerase holoenzyme." Proc Natl Acad Sci U S A **105**(14): 5361-5366.

Zou, L. (2007). "Single- and double-stranded DNA: building a trigger of ATR-mediated DNA damage response." Genes Dev **21**(8): 879-885.

Zou, L. and S. J. Elledge (2003). "Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes." Science **300**(5625): 1542-1548.

Zou, L. and B. Stillman (1998). "Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin." Science **280**(5363): 593-596.

Zou, L. and B. Stillman (2000). "Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase." Mol Cell Biol **20**(9): 3086-3096.

Zwerschke, W., H. W. Rottjakob, et al. (1994). "The Saccharomyces cerevisiae CDC6 gene is transcribed at late mitosis and encodes a ATP/GTPase controlling S phase initiation." J Biol Chem **269**(37): 23351-23356.