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The Role of the DNA Helicase Rrm3 under Replication Stress

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by

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Dedication

I would like to dedicate this work to my loving family, my friends, and my wife, who have supported me over the past years. My family who has encouraged me and were always there for me, even all the way from Germany. My friends who helped me enjoy my life outside of the lab. And most of all my loving wife who provided support, love, and motivation.

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Abbreviations	Definitions
APIM	AlkB Homologue 2 PCNA-interacting Motif
ARS	Autonomously Replicating Sequence
ATP	Adensoine 5' Triphosphate
BER	Base Excision Repair
BIR	Break-Induced Repair
CPB	CREB-Binding Protein
CUP1	Copper Chelatin
DDT	DNA Damage Tolerance
D-loop	Displacement Loop
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Triphosphate
DSB	Double Strand Break
dsDNA	Double Stranded DNA
EGFR	Epidermal Growth Factor Receptor
FOB1	FOrk Blocking less
GCRs	Gross Chromosomal Rearrangement
HAT	Histone Acetyltransferase
HIRAN	HIP116 and Rad5 N-terminal
HJs	Holliday Junctions
HLTF	Helicase Like Transcription Factor
HR	Homologous Recombination
HU	Hydroxyurea
IDCL	Inter-Domain Connecting Loop
IDPS	Intrinsically Disordered Proteins
IDR	Intrinsically Disordered Region
MMR	Mismatch Repair
MMS	Methyl Methanesulfonate
MRC1	Mediator of Replication Checkpoint 1
MRX	Mre11-Rad50-Xrs2
Orc5	Origin Recognition Complex 5
PCNA	Proliferating Cell Nuclear Antigen
PIF1	Petite Integration Frequency 1
PIM	PCNA Interacting Motif
PIP-box	PCNA Interacting Protein Binding Motif
RAD5 (REV2)	Reversion-Deficient Mutant
RBs	Replication Barriers
rDNA	ribosomal DNA
RFB	Replication Fork Barrier
RFC	Replication Factor C
RING	Really Interesting New Gene
RLC	Replication Like Complex

RNA	Ribonucleic Acid
RRM3	Ribosomal DNA Recombination Mutation
SCJs	Sister Chromatid Junctions
SDSA	Synthesis-Dependent Strand Annealing
SF1-3	Super Family 1-3
SGS1	Slow Growth Suppressor 1
SHRPH	SNF2 Histone Linker PHD RING Helicase
SILAC	Stable Isotope Labeling with Amino Acids in Cell Culture
SIM	SUMO Interacting Motif
SIOD	Schimke Immuno-Osseous Dysplasia
SLE	Systemic lupus erythematosus
SLIM	Short Linear Motif
SRDs	Substrate Recognition Domain
SRS2	Suppressor of Rad Six
SSA	Single Strand Annealing
SSB	Single Strand Break
ssDNA	Single Stranded DNA
STUbL	Sumo Targeted Ubiquitin Ligase
SUMO	Small Ubiquitin-Related Modifier
SWI/SNF	Mating Type Switching and Sucrose Non-fermenting Family
TLS	Translesion Polymerase
tRNA	Transfer RNA
TS	Template Switching
UB	Ubiquitin
UBD	Ubiquitin Binding Domain
Ubl	Ubiquitin Like
UVR	Ultraviolet Radiation
YPD	Yeast Extract Peptone Dextrose

Abstract

DNA replication needs to be strictly monitored to ensure proper duplication of the genome. During DNA synthesis, the DNA replication machinery encounters multiple obstacles such as incorrect dNTP incorporation, RNA-DNA hybrids, modification of DNA nucleobases, collision with the transcription-replication machinery. These endogenous and exogenous sources of DNA damage may result in single-strand breaks (SSBs) and double-strand DNA breaks (DSBs), thereby impeding fork progression. To prevent stalling of DNA synthesis a multitude of DNA repair pathways are specifically designed to deal with these kinds of blockages. One of the proteins involved in ensuring DNA replication fork progression is the DNA helicase Rrm3 in *Saccharomyces cerevisiae*. Rrm3 belongs to the PIF1 DNA helicase family, which is evolutionary conserved from bacteria to humans. The structure of members of the PIF1 DNA helicase family can be divided into its helicase domain located in the C-terminus and a disordered N-terminus, which promotes protein-protein interaction. Rrm3 interacts with the subunit Orc5 of the origin complex and possesses a PCNA-interacting protein-box (PIP-Box) in its N-terminus. The majority of Rrm3's function can be attributed to its 5'-3' DNA helicase activity as deletion of *RRM3* or disrupting its Walker A motif leads to increased replication fork stalling. Therefore, yeast cells deficient of Rrm3 in *S. cerevisiae* were used as a model organism to study how cells deal with increased stalled replication forks. One of the proteins identified to be upregulated in the presence of increased stalled replication forks is Rad5. Rad5 and its human orthologue, HLTF, belongs into the SWI/SNF family. Rad5 possesses a conserved region encoding an ATPase domain and seven helicase-related sequence motifs in its C-terminus and a HIRAN domain in the N-terminus. Both domains are important for fork reversal of stalled replication forks under replication stress. Furthermore, a really interesting new gene (RING)

ubiquitin-ligase motif is embedded within the helicase domain, allowing Rad5 in a complex with Mms2-Ubc13 to polyubiquitinated PCNA. This in turn initiates the error-free template switching pathways using the newly synthesized sister strand as template to bypass the DNA lesion.

In the second chapter I identify which function of Rad5 is required to deal with increased stalled replication forks in the absence of Rrm3. I determine that the Helicase and HIRAN domain, which are both involved in fork reversal activity, suppress replication stress in the absence of Rrm3, whereas the ubiquitin-ligase activity is not. Furthermore, prolonged fork stalling in the absence of Rrm3 and Rad5 results in recombinogenic DNA lesions, which are being processed by a Rad59-dependent recombination salvage pathway. These recombinogenic DNA lesions are dependent on Rrm3's helicase activity, but not its N-terminus and on Rad5's fork reversal activity. However, the ubiquitin-ligase activity of Rad5 and therefore poly-ubiquitination of PCNA is dispensable. Moreover, I identify the structure-specific endonuclease Mus81 to be required to prevent recombinogenic DNA lesions and gross chromosomal rearrangements (GCRs) in the absence of Rrm3, but not Rad5. Thus, two independent mechanisms, one depending on Rad5's fork reversal activity and the other one on Mus81 dependent cleavage, exist to bypass fork stalling at replication barriers, thereby maintaining genome stability.

The third chapter focuses on further exploring the biological relevance of the interaction between Rrm3 and Pol30. We identified a positive genetic interaction between *RRM3* and *POL30* mutant, which is unable to modify lysine 127, which is located on the inter-domain connecting loop (IDCL). Additionally, deletion of *RRM3* in other *pol30* mutants, when either the ubiquitination site (*pol30-K164R*) or both, the SUMOylating and ubiquitination site (*pol30-K127,164R*) are mutated, did not reveal a genetic interaction. Deletion of *RRM3* suppresses the DNA damage sensitivity and the accumulation of recombinogenic DNA lesions in the *pol30-K127R* mutant. Surprisingly, deletion of Rrm3's N-terminus, specifically, the first 54 amino acids

containing the PIP-box are required for the suppression of the DNA damage sensitivity and for the recombinogenic DNA lesions, but not its helicase activity. Furthermore, the suppression of *pol30-K127R* mutant in the absence of Rrm3 depends on an unknown substrate of the SUMO E3-ligase Siz1 and to a lesser extent Siz2. Thus, it appears that the physical interaction with PCNA, when lysine 127 cannot be SUMOylated, causes DNA damage sensitivity and accumulation of recombinogenic DNA lesions rather than Rrm3's catalytical activity. Finally, studying the cellular functions and biochemical characteristics of Pif1 DNA helicases in yeast, such as Rrm3, can help to gain a better understanding on how the human orthologue PIF1 DNA helicase functions as tumor suppressor.

Chapter One: Introduction

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History of the PIF1 DNA Helicase Family

The PIF1 DNA helicase family is conserved from yeast to mammals [1, 2]. While the yeast *Schizosaccharomyces pombe* and more complex multicellular eukaryotes, including humans, only encode one PIF1 family helicase, *Saccharomyces cerevisiae* expresses two: Rrm3 and ScPif1. The *ScPIF1* gene was originally identified in a screen to determine mutations that change the recombination frequency of tandemly arrayed repeats within mitochondria, and was therefore named after that defect, *p*etite *i*ntegration *f*requency (Sc*PIF1*) [3]. In a quest to determine genes that suppress recombination of tandem repeats of the ribosomal DNA (rDNA) and copper chelatin (*CUP1*) genes in *S. cerevisiae*, the *r*ibosomal DNA *r*ecombination *m*utation 3 (*RRM3*) gene was identified and later classified as a member of the PIF1 DNA helicase family based on sequence similarity [4, 5]. Both Rrm3 and ScPif1 belong to the superfamily 1B and have 5′–3′ translocase activity that is encoded in helicase domains that share 40% identical residues [4, 6-8]. An overview of the domain structure and functional motifs of PIF1 helicase family members in yeasts and humans is provided in Figure 1.1. This leaves the intrinsically disordered N-terminal extensions of Rrm3 and ScPif1 to regulate their enzymatic activity and

their recruitment to specific sites within the yeast genome, where they perform the many distinct cellular functions.

Replication through the rDNA replication fork barrier

After initiation of replication of the highly repetitive ribosomal DNA (rDNA) locus that spans approximately 1.5 Mb on chromosome XII, the leftward-moving replication fork encounters a cis-acting sequence near the 3' end, called the replication fork barrier (RFB) (Figure 1.2.). RFB is located in a non-transcribed spacer and contains two termination sites, Ter1 and Ter2, that are bound by Fob1 to ensure that replication of the rDNA locus occurs in a unidirectional manner [9].

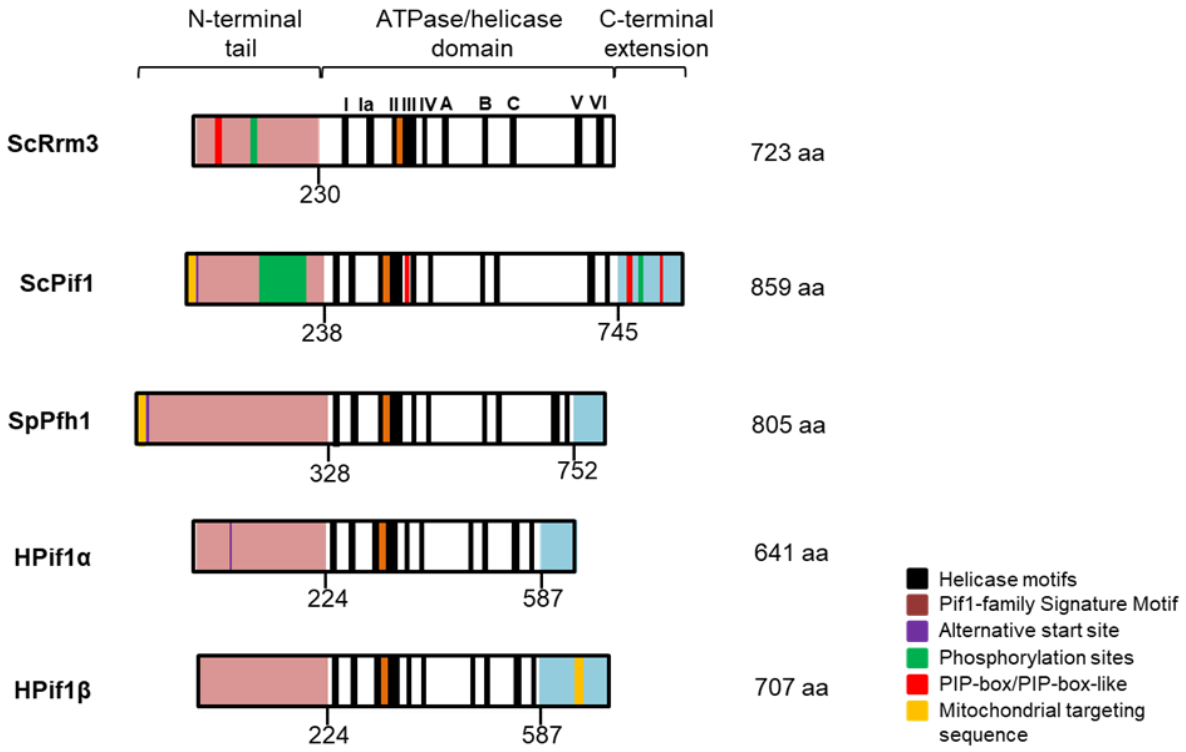

Figure 1.1. Structure and functional motifs of the yeast and human PIF1 family helicases. *Saccharomyces cerevisiae* expresses two members of the PIF1 family, Rrm3, and ScPif1, whereas *Schizosaccharomyces pombe* and higher eukaryotes express one. PIF1 helicases share the conserved ATPase/helicase domain and an intrinsically disordered N-terminal tail of variable sequence. Post-translational modification sites, proliferating cell nuclear antigen (PCNA)-interacting protein (PIP) box and alternative start sites, which give rise to mitochondrial isoforms, are marked.

The PIF1 helicase family members in yeasts whose functions are fairly well-understood (Rrm3, ScPif1, SpPfh1) all accumulate at the RFB and 35S regions of the rDNA, indicating an intimate involvement in the regulation of rDNA replication [10, 11]. However, they appear to have opposite effects; Rrm3 and Pfh1 promote replication through the RFB whereas ScPif1 maintains it, although the molecular mechanisms by which they modulate the RFB remain unclear [10, 11]. These distinct roles in rDNA replication are evidenced by the increased number of chromosomal rDNA repeats, converged forks and fork pausing in *rrm3* and *pfh1* mutants when compared to *pif1* mutants or wildtype cells [10, 12]. It is thought that Rrm3's helicase activity removes DNA-bound proteins, including Fob1, ahead of the replication forks to prevent pausing (Figure 1.2.) [12, 13]. However, deletion of *FOB1* only partially restores replication fork movement through the rDNA locus in *rrm3* mutants, indicating the presence of Fob1-independent barriers, such as the 35S and 5S rRNA genes and the inactive ARS [12, 14]. Additionally, removal of RFBs by deletion of *FOB1* cannot rescue lethal interactions of *rrm3* with deletions of the RecQ helicase gene *SGS1*, or the fork protection complex gene *MRC1*, indicating that forks stalled at RFB and their intermediates are not toxic to *sgs1*Δ and *mrc1*Δ mutants, and that Rrm3 performs crucial functions at genomic loci besides RFB [12, 14]. Two other subunits of the fork protection complex, Tof1 and Csm3, actually inhibit the "sweepase" activity of Rrm3 to remove DNA-bound proteins at termination sites of chromosomal rDNA and RNA polymerase III transcription [14], either directly by inhibiting the helicase activity of Rrm3 or indirectly by causing a conformational change of the replisome leading to restriction of Rrm3 activity (Figure 1.2.) [14]. Although disruption of the fork protection complex abolishes pausing at termination sites, further deletion of *RRM3* leads to a partial re-establishment of the termination site similar to the observation in *rrm3 fob1* mutants [12, 14]. This is consistent with the observation that replication forks in *rrm3* mutants also stall at Fob1-independent sites. Other DNA helicases, such as Sgs1 and Srs2, which are also capable of removing DNA-bound proteins [15, 16], were dispensable for replication through RFB [14]. This raises the possibility

that the factors that promote fork escape in *rrm3 tof1* and *rrm3 csm3* mutants may not be another DNA helicase but could involve DNA motor proteins that remodel DNA or chromatin.

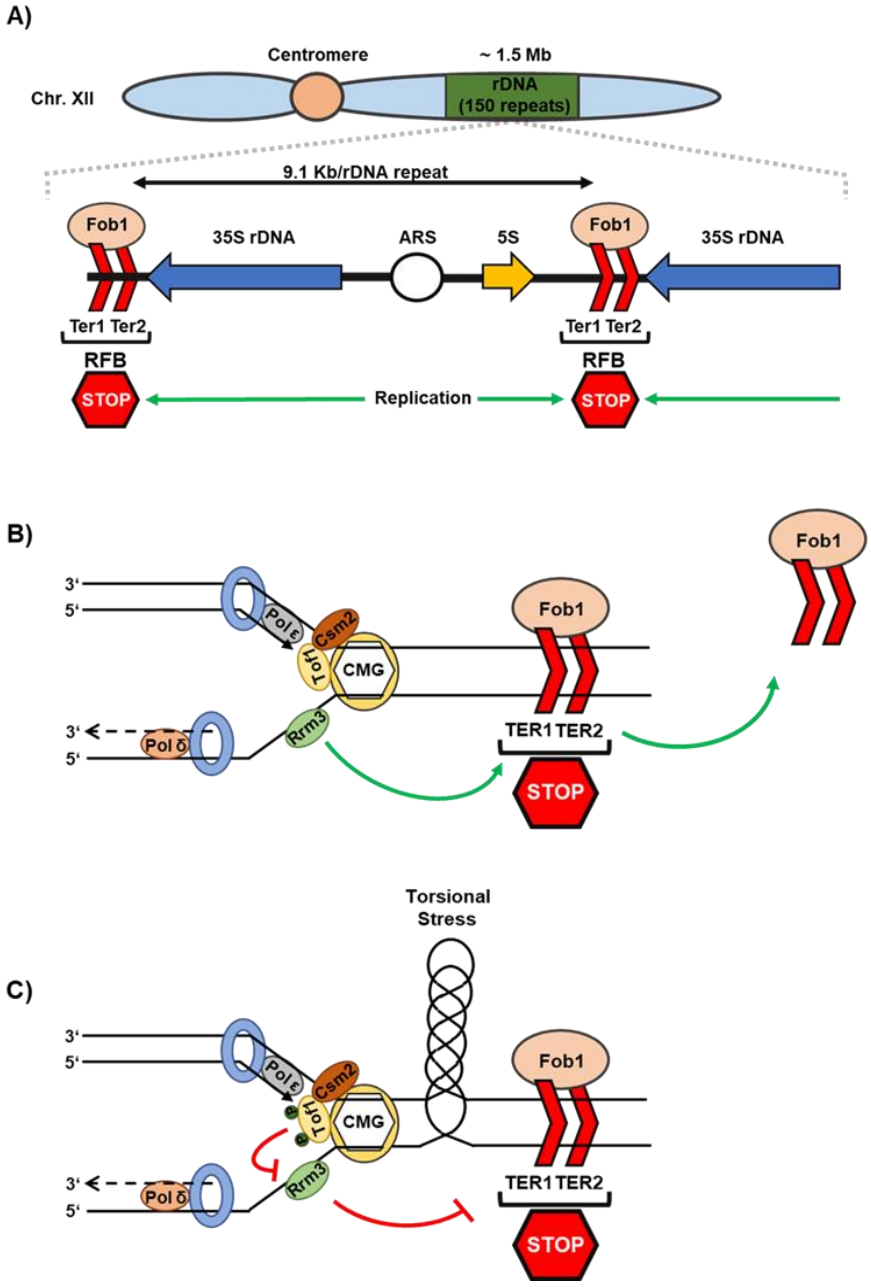

Figure 1.2. Replication through rDNA in *S. cerevisiae*. ***(A)*** Graphical abstract of chromosome XII containing the rDNA region. Close up of the composition of one of the 150 rDNA repeats, which is 9.1 Kb long and is enclosed by the replication fork barrier (RFB) to stop replication. ***(B)*** The DNA helicase Rrm3 travels with the DNA replication complex and removes DNA bound proteins such as Fob1 to promote replication fork progression and minimize torsional stress. ***(C)*** Under replication stress, phosphorylation of Tof1 in a complex with Csm2 inhibits Rrm3's helicase activity. The DNA bound protein such as Fob1 is unable to be removed leading to replication fork stalling. This leads to increased torsional stress. Figures were adapted from [17, 18].

Notably, unlike hydroxyurea (HU)-induced fork pausing, pausing at RFB is independent of the DNA-damage checkpoint kinases Mec1 and Rad53 and DNA synthesis resumes without breakage or recombination at RFBs [19], suggesting that naturally occurring replication pause sites are processed differently than those formed during DNA replication stress. This likely explains why deletion of *MRC1* does not affect replication fork pausing at Fob1-RFBs and why the *rrm3Δ mrc1-AQ* mutant, which is defective in the checkpoint function but not the replication function of Mrc1, is viable [19-22]. A better understanding of the chromatin environment in which natural barriers of DNA replication reside, compared to the environment established at genotoxin-induced paused forks will help to elucidate the mechanisms by which the mechanistically poorly understood Rrm3 and other PIF1 helicases contribute to genome maintenance and stability.

A helicase-independent function of Rrm3 during replication stress

During replication stress, cells lacking Rrm3 continue to progress into S-phase. The ability of Rrm3 to restrict DNA synthesis depends on the integrity of the 230-amino-acid long disordered N-terminal tail of Rrm3, but not its ATPase/helicase activity [13]. Increased nucleotide levels are not sufficient for S-phase progression as helicase-dead *rrm3* mutants also have increased dNTP levels, but do not progress into S-phase in hydroxyurea [13]. Notably, Rrm3 interacts with the origin recognition complex (ORC) subunit Orc5 and the region of the Rrm3 N-terminus required for inhibiting DNA synthesis during replication stress is required for the Rrm3-Orc5 interaction [13, 23]. Moreover, the N-terminal tail of Rrm3 is required for its association with origins of replication during replication stress, but not during the unperturbed cell cycle, raising the possibility that Rrm3 acts at replication origins to restrict DNA synthesis during replication stress.

Cellular response to replication fork stalling in the absence of Rrm3

Populations of *rrm3Δ* cells exhibit a cell cycle defect with a DNA content intermediate between 1N and 2N, indicative of problems with timely progression through S-phase [24]. Deletion of *SRS2* or *SGS1* enhances this S-phase progression defect and causes a severe fitness defect that can be rescued by disrupting HR genes *RAD51* or *RAD55*, suggesting a role for Rrm3 either in preventing the formation of replication-dependent HR substrates or contributing to their repair [24-26]. In contrast, the synthetic lethality between deletions of *RRM3* and *MRE11* or *RAD50,* which code for subunits of the Mre11-Rad50-Xrs2 (MRX) DSB repair complex, is not due to illegitimate HR since it could not be rescued by *RAD54* or *RAD55* deletions [24]. This suggests that, in addition to HR, Rrm3 functions in another MRX-mediated pathway, such as non-homologous end joining, telomere maintenance or S-phase checkpoint activation.

How cells deal with HR substrates and other replication problems that arise from replication forks paused at thousands of protein-bound sites in Rrm3-deficient cells is poorly understood. Syed et al. [13] used Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC), coupled to mass spectrometry, to determine that topoisomerase Top2 and the SWI/SNF ATPases Rad5 and Rdh54 were significantly upregulated in cells lacking Rrm3, implicating their role in replication stress tolerance. Increased Top2 most likely compensates for the loss of Rrm3's contribution to resolving converging replication forks [27]. Rdh54 is important for HR in diploid cells, but roles in haploid cells are also emerging. Notably, the recently identified role for Rdh54 in regulating D-loop formation [28], and thereby HR levels, could become increasingly important in the absence of Rrm3 when greater numbers of HR substrates are likely to form at replication pause sites. That Sgs1-Top3-Rmi1 and Srs2 define two independent pathways of D-loop reversal could contribute to the synthetic lethality of the *rrm3*Δ mutation with *sgs1*Δ and *srs2*Δ mutations as well as its suppression by *RAD51* deletion [24-26,

28]. Upregulation of Mph1, which also functions in D-loop disassembly [29], was also observed in the *rrm3*Δ mutant, albeit to a lesser extent than Top2, Rdh54 and Rad5, and further supports the increased requirement for tight regulation of D-loop formation in the absence of Rrm3 [13, 29].

Rad5 has replication fork reversal activity [30, 31] and its upregulation in the *rrm3*Δ mutant may indicate that fork reversal is a major mechanism to restart forks that are stalled at protein barriers [13]. Considering the association of Rrm3 with replisome components Polε and PCNA [32, 33], one could also speculate that Rrm3 itself can facilitate fork reversal to allow the forks to pass through protein-bound sites in a pathway that functions in parallel to replication stress-induced Rad5-mediated fork reversal.

In the absence of Rrm3, the Rad53-dependent DNA-damage checkpoint is activated in a Rad9-dependent manner and remains active even after preventing the formation of HR intermediates by deleting *RAD51* [24, 25, 34]. During replication stress, Rrm3 itself is phosphorylated in a Rad53-dependent manner; however, phosphorylation is not required for replication across natural pause sites [35]. The biological function of Rrm3 phosphorylation remains unknown but has been suggested to inhibit Rrm3 activity to prevent genome instability during replication stress [35].

SWI/SNF Family of Translocases

Members of the SWI/SNF family play a pivotal role in genome integrity as they are involved in multiple cellular processes. Some proteins function in transcriptional regulation, others in the maintenance of chromosome stability, chromatin remodeling or in nucleotide excision repair, recombinational pathways and post-replication daughter strand gap repair [36-38]. While characterizing mating type switching (SWI) and sucrose nonfermenting (SNF) mutants, hence the name (SWI/SNF family), in *S. cerevisiae*, the first member Snf2, was

discovered [39]. Upon further structural characterization, a conserved region encoding an ATPase domain and seven helicase-related sequence motifs, which have high similarity to other ATP-binding helicases of the DEAD/H family were discovered [40-42]. Proteins containing such helicase motifs can be categorized into superfamilies SF1, SF2 and SF3, depending on the space between motifs and their sequence composition [43-45]. Members of the SWI/SNF family are placed into the SF2 superfamily and can be further subdivided into six sub-families including the Snf2-like, SWI/SNF-related protein-like (Swr1-like), Rad54-like, Rad5/16-like, SSO1653-like, and Distant family, depending on their helicase motif homology [40, 43, 45]. In general, the structure of members of the SWI/SNF family can be divided into an ATP binding and ATP hydrolysis side within the N-terminal containing a RecA-like DExx (I, Ia, II, and III) motif and a DNA translocation function within the C-terminal HELICc (IV, V, and VI) motif (Figure 1.3) [44-46]. A better understanding of the conserved helicase-like motif was gained from the spatial structure of Rad54 from Zebrafish [47]. The outer borders are defined by a Q-motif within the N-terminus and a brace in the C-terminus [40, 44]. Within, the core helicase consistent of two RecA core domains, which are separated by a linker and two helical protrusions, one on each side of the linker [40, 44]. The two core RecA-like domains are farther apart compared to other members of the SF2 family and possess multiple conserved sequence blocks (Figure 1.3.) [40, 44]. This difference could explain why despite of the high similarity among the helicase-related motif to the DEAD/H family, members of the SWI/SNF2 family are unable to unwind DNA [44, 45]. However, likeother helicases, encountering DNA or DNA-protein complexes promotes ATP-hydrolysis activity, which in turn allows members of the SWI/SNF family to translocate along dsDNA instead of unwinding [36, 38, 48-50]. So far recent advances in genome sequencing and homology search have identified 17 genes belonging to the SWI/SNF family in *S. cerevisiae* and 32 in humans [40].

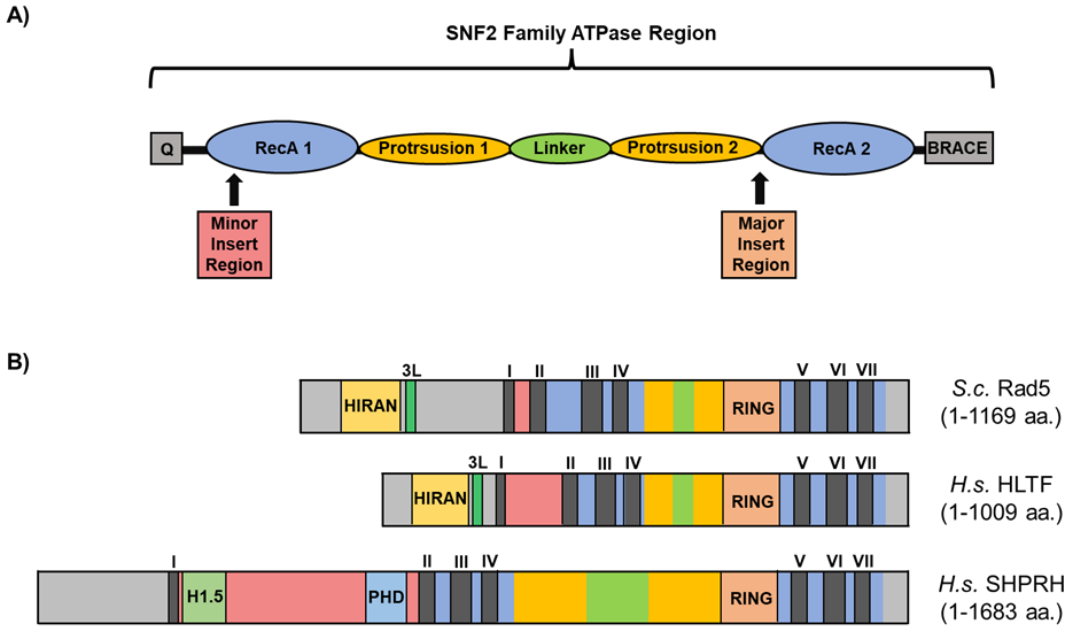

Figure 1.3. Structure and functional motifs of the yeast and human SNF2 family helicases. ***(A)*** SNF2 helicases share the conserved ATPase/helicase domain, which is characterized by two RecA domains connected by two protrusions and a linker. The ATPase domain is embraced by a Q-motif and Brace motif. The minor and major insert region are indicated***. (B)*** *S. cerevisiae's* Rad5 has two human homologues HLTF and SHRPH. The structural features of each protein are color coded to reflect the conserved SNF2 ATPase domain. All three proteins possess the RING domain. In addition, *S.c.* Rad5 and *H.s.* HLTF possess the RING domain, which is in the N-terminus, while *H. s.* SHPRH does not. Instead, *H. s.* SHPRH has a PHD domain and a H1.5 domain inserted in the minor region. Figures were adapted from [51].

Rad5's structure and human homologues

One of the 17 genes belonging to the SWI/SNF family in *S. cerevisiae* encodes for the protein Rad5. Rad5 and its two homologues, helicase like transcription factor (HLTF) and SNF2 histone linker PHD RING helicase (SHPRH), in mammalian cells play critical roles during replication stress [52]. While the overall domains and structure of HLFT resemble more of Rad5's architecture domain, SHRPH has additional motifs and domains (Figure 1.3.) [53]. However, all three possess the seven conserved helicase-like motifs, which are characteristics of members of this family [40, 44]. In addition, all three have a really interesting new gene (RING) ubiquitin ligase domain embedded within the second core of the recA domain [52, 53]. While HLFT and Rad5 have a HIP116 and Rad5 N-terminal (HIRAN) domain at its N-terminus, a putative HIRAN domain has been suggested for SHRPH within the N-terminus [53, 54]. As

mentioned above, SHRPH architectural domain contains additional motifs such as a linker histone H1.5 (linker histone H1 and H5) and PHD (plant homeodomain) domains, which separates the first recA-like domain [53]. Additionally, a putative AlkB homologue 2 PCNA-interacting motif (APIM motif) has been identified in the human homologue HLFT and SHRPH [53, 55, 56].

Mechanistic Insight into Rad5's Cellular Functions: DNA Damage Tolerance: Error-free and Error-prone Pathway

During DNA replication, the replication machinery encounters multiple obstacle such as DNA damage or DNA-bound proteins, which need to be dealt with in a timely manner to ensure replication progression [57]. The DNA damage tolerance pathway is utilized to prevent fork collapsing and thereby ensures continues DNA synthesis (Figure 1.4.) [57, 58]. Upon encountering of a DNA lesion on the leading strand, RPA is recruited to bind to ssDNA, which in turn leads to the recruitment of the Rad6/Rad18 complex [59, 60]. This highly conserved Rad6–Rad18 ubiquitin conjugating enzyme complex targets lysine 164 on the proliferating cell nuclear antigen (PCNA) for mono-ubiquitination [60, 61]. PCNA is a ring-shaped homotrimer and a processivity factor that is loaded onto DNA and binds to replicative DNA polymerases Polδ or Polε [62-66]. After mono-ubiquitination, the replicative DNA polymerase is being replaced by a translesion (TLS) polymerase, which can replicate across the lesion, but at the risk of introducing mutations [67-72]. The TLS pathway is a two-step mechanism, utilizing one type of TLS polymerases to insert nucleotides and a second type of TLS polymerases to extend. The first insertion of nucleotides opposite from DNA damage is performed by members within the Y-family of DNA polymerases, such as REV1, which is only able to incorporate cystine [71-75]. The extension of mismatched nucleotides is catalyzed by the B-family of DNA polymerases such as polymerase Polζ, compromised of the catalytic subunit Rev3 and the accessory subunit Rev7 [69, 75-78]. Thus, this DDT pathway is considered error-prone. However, some sub-

pathways of the TLS pathway is considered error free depending on which TLS polymerase is being used, as the TLS polymerase Rad30 and Rev1 efficiently and accurately repair pyrimidine dimers arising from ultraviolet radiation (UVR) light lesions and 8-oxoguanine lesions or across exocyclic guanine lesions, adducted guanines, and abasic sites, respectively [73, 79-83].

In the early 1990's, the discovery of reversion-deficient mutants *rev1*, *rad5* (previously named rev2), *rev3*, which elevate mutation rates following UVR, provided evidence for a role of Rad5 in the TLS pathway [84]. Rad5's function during the TLS pathway, is independently of its ubiquitin-ligase activity and ATPase activity, but dependent on its physical interaction with Rev1 via Rad5's first 30 amino acids of the N-terminus [85-89]. Recent crystal structure has revealed that Rad5's N-terminus binds within a highly conserved region of Rev1's C-terminus, which correlates with the binding site for polη in mammalian cells [53, 86, 87]. Thus, it is possible that Rad5 competes with polη (Rad30) for binding, especially following UVR. .

Alternatively, PCNA can be further modified leading to poly-ubiquitination on lysine 164, which in turn initiates the error-free pathway via template switching (TS) (Figure 1.4.) [60, 90-92]. Poly-ubiquitination is initiated by a protein complex composed of the E2 heterodimer Ubc13/Mms2 and the E3 ligase Rad5 [93-97]. The ubiquitin-ligase activity of Rad5's RING domain synthesizes K63-linked polyubiquitin chains to the already existing mono-ubiquitination on lysine 164 [93, 98, 99]. One possibility is that stalled forks are converted from the three-way junction into a four-way junction also termed "chicken-foot" and then template switching is initiated using the newly synthesized strands to bypass the damage [57, 77, 85, 100]. This would suggest that template switching occurs in proximity of the stalled fork, on the other hand it was also suggested that the error-free pathway mimics a recombination-like invasion mechanism thereby promoting gap-filling behind the forks [61, 92, 101, 102].

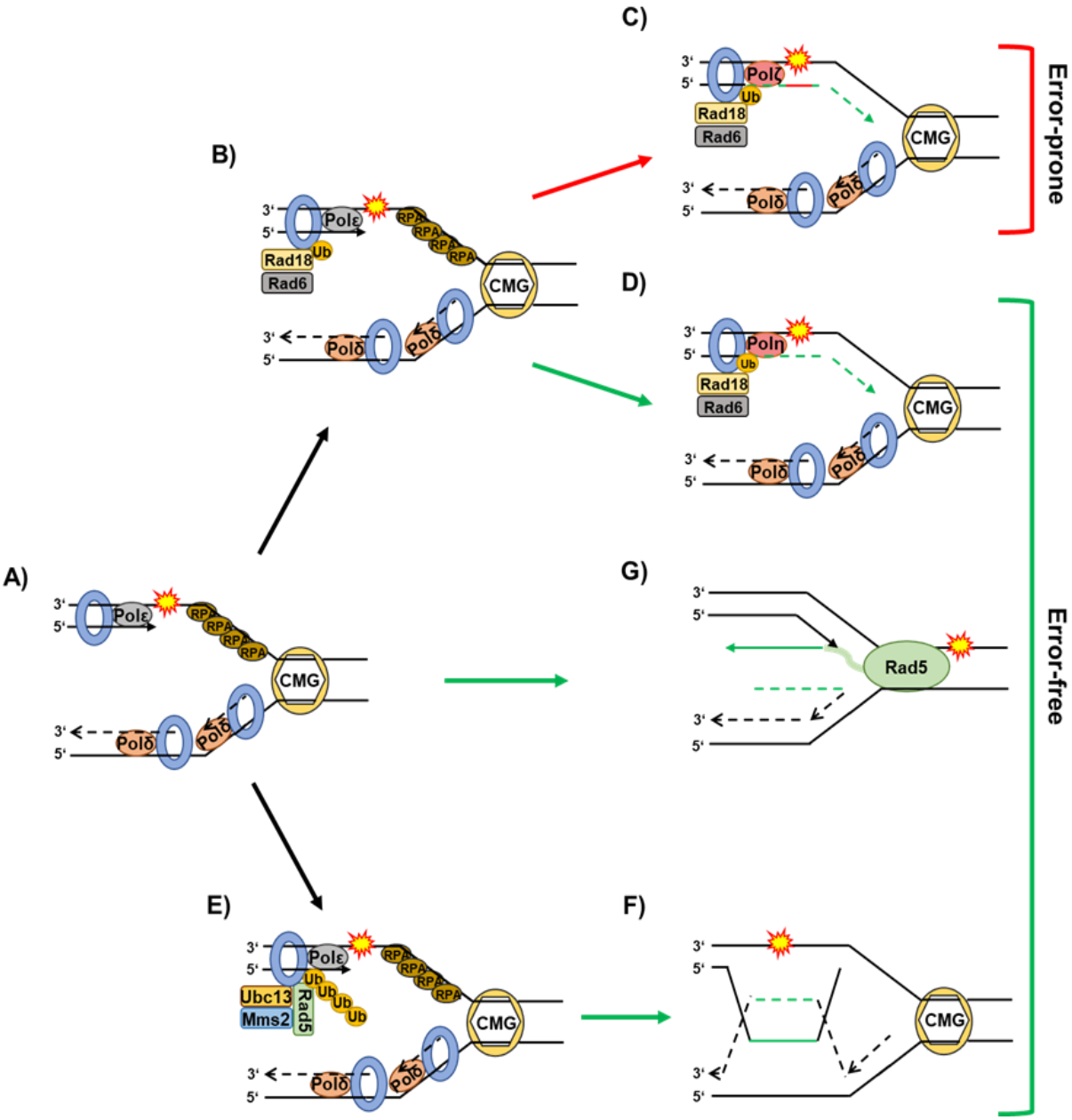

Figure 1.4. Error-Free and Error-prone DNA damage repair pathway. ***(A)*** Upon encountering DNA lesion on the leading strand, RPA accumulates at stalled replication forks leading to ***(B)*** the recruitment of Rad6-Rad18, which monoubiquitinates PCNA. ***(C)*** The replicative DNA polymerase is replaced with a translesion (TLS) polymerase by incorporating non-specific nucleotides to bypass the DNA lesion; error-prone pathway. ***(D)*** Depending on the DNA damage certain TLS polymerases can bypass the DNA lesion in an error free manner. ***(E)*** Alternatively, PCNA can become further poly-ubiquitinated by the Ubc13-Mms2-Rad5 complex, which initiates the error-free pathway. ***(F)*** Poly-ubiquitination of PCNA promotes template switching by utilizing the newly synthesized sister chromatid as a template to bypass the DNA lesion. ***(G)*** An alternative pathway utilizes Rad5's fork reversal activity to generate a four- way junction to bypass the DNA lesion.

Indeed, template switching requires similar proteins that are also used during homologues recombination [100, 103-105]. Furthermore, analyzing of 2-D gels has highlighted a group of DNA replication intermediates resembling X-shaped structures, indicative of sister chromatid junctions (SCJs) and Holliday junctions (HJs) that are dependent on proteins involved in template switching [103, 106, 107]. These recombination intermediates are being resolved by the RecQ helicase Sgs1 in a complex with Top3 and Rmi1 to resume DNA synthesis [108, 109].

It is not quite understood, when or how the cell decides to use error-free vs. error-prone pathway, it has been suggested to be dependent on the cell cycle, but no other protein so far has been shown to interact with poly-ubiquitinated PCNA to be the driving force to signal for the error-free template switching pathway [102, 110-112].

Rad5's/HLTF's Fork Reversal Activity

Fork reversal or fork regression is utilized as another template switch mechanism to deal with DNA replication stress to maintain genome stability (Figure 1.4.) [60, 92]. During fork reversal, the newly synthesized DNA strands dissociate from their parental strains and re-anneal with each other, forming a four-way structure [97, 113]. Using fork reversal, protects the cells from accumulation of ssDNA and bypasses DNA lesions in the template strand by providing a recombination like pathway such as template switching [97, 113, 114]. The proteins with fork reversal activity that have been identified so far are Rad5 in *S. cerevisiae* and its human ortholog, HLTF as well as two other members of the SWI2 /SNF2 family in mammalians, SMARCAL1 and ZRANB3 [115-118]. All of them possess a substrate recognition domain (SRDs), which allows them to interact with specific DNA substrates [119, 120]. In Rad5 and HLTF this recognition site is the HIRAN (HIP116, Rad5p, N-terminal) domain, which recognizes ssDNA, specifically the free 3′-end of the newly synthesized leading strand [121, 122]. The HIRAN domain is in the N-terminus of Rad5 (170aa–310aa) and HLTF (57aa–167aa) and is separated by a flexible linker from the ATPase motor domain [54]. The HIRAN domain is highly

structured, possessing helical and beta-strand propensities (b1–b2–b3–b4–a1–b5–b6–a2), which is thought to form a binding pocket and an oligonucleotide/ oligosaccharide-fold for the 3' end's hydroxyl group and nucleobase of the nascent ssDNA, respectively [54, 118, 121, 122]. Besides the HIRAN domain, fork reversal also depends on Rad5's/HLTF's dsDNA translocase, activated by ATP hydrolysis, leading to branch migration of the four-way structure [115, 118]. Therefore, upon encountering a DNA lesion in the leading strand, fork reversal is initiated by binding of the HIRAN domain to the free hydroxyl group of the 3' end of the newly synthesized leading strand [52, 118, 121, 123]. Based on the crystal structure of the HIRAN domain bound to DNA, it is possible that the HIRAN domain is able to unzip the nascent strand from the template or the DNA translocase activity causes topological constraints, thereby disassociating the 3' end of the nascent strand from the template to be captured by the HIRAN domain [53, 118, 122]. It is also possible that the DNA translocase activity moves along dsDNA ahead of the fork, thereby removing any DNA bound proteins. Indeed, it was shown that Rad5/HLTF displaces DNA bound proteins from dsDNA and ssDNA, independently of the placement in regards to the replication fork, in an ATP-dependent manner [124].

Implications of Impaired Fork Remodeling in Cancer

Mutations found in members of the SWI/SNF family have been associated with human diseases, such as the autosomal recessive disorder Schimke immuno-osseous dysplasia (SIOD) or African-specific type-2 diabetes and various cancers such as endometrial cancer, kidney cancer and many more [125-128]. While SIOD, which is associated with multiple symptoms such as T-cell immunodeficiency, growth retardation, renal failure and spondyloepiphyseal dysplasia is caused by a biallelic mutation of SMARCAL1, African-specific type-2 diabetes results from mutations in *ZRAMN3* [126-129]. While these genetic disorders might arise outside of SMARCAL1 and ZRAMN3 fork remodeling activity,

The expression levels of fork remodeler HLTF are associated with multiple types of cancer in humans. While HLTF's downregulation, caused by methylation of its promoter, is associated with human colorectal and gastric cancers, overexpression of HLTF facilitates tumor metastasis and negatively impacts survival rate in non-small cell lung cancer patients [130-133]. Additionally, different types of cancer possess variations of *HLTF* mRNA resulting in overexpressed dominant negative HLTF mutants, deficient in their DNA repair domains [134]. The poor prognosis could be associated with fork instability, as overexpression of HLTF's yeast ortholog, Rad5, causes hyper-recombination at stalled forks [135]. In contrast, loss or downregulation of HLTF, but not of the remodelers SMARCAL1 and ZRAMN3, reduces DNA damage checkpoint signaling, double-strand break formation, sensitivity to replication stress induced by HU or mitomycin C and thereby, promotes proliferation in osteosarcoma U2OS cell lines [136-140]. Just like *HLTF*, *SHPRH* is also believed to be a tumor-suppressor gene as the expression of non-functional SHRPH protein or loss of heterozygosity on chromosome 6 is detected in various cancers such as in melanoma, cervical and ovarian. The loss of heterozygosity on the chromosome 6 is mapped to the region 6q24–q2, correlating with the location of the *SHPRH* gene [141-144].

Thus, the expression levels and functions of replication fork remodelers need to be tightly controlled to maintain genome stability, since being able to perform replication fork remodeling or not could be the difference between life or death.

Structure and Function of Proliferating Cell Nuclear Antigen (PCNA)

In *S. cerevisiae*, PCNA is encoded by the gene *POL30* and is irreplaceable for efficient and timely DNA synthesis [77, 145, 146]. Before a direct involvement of PCNA during DNA replication was proposed, it was originally identified as a target for autoantibodies extracted from the sera of patients with systemic lupus erythematosus (SLE) in the late 1970's [147]. The sliding clamp PCNA is a ring-shaped homotrimer that is composed of three identical monomers.

During DNA replication PCNA functions as a processivity factor for the replicative DNA polymerases (Pol) δ, Polα and β, and Polε and also functions in lagging strand maturation and DNA damage checkpoint pathway activation [63, 64, 66, 148-153]. Because of the interaction with varies polymerases, soon a direct role for PCNA in nucleotide excision repair was discovered by facilitating the repair of UV-induced DNA damage together with DNA polymerase δ [154-157]. More and more the involvement of PCNA, outside of its role as a processivity factor, in other DNA repair pathways such as base excision repair (BER), double-strand break repair, DNA mismatch repair, resolution of collision between replication and transcription machinery and in the error-prone (translesion synthesis) and error-free (template switching) DNA damage tolerance (DDT) pathway, has emerged [92, 97, 125, 158-170]. Besides its function in DNA damage response, PCNA is viewed as a regulatory platform involved in multiple other biological processes including sister chromatid cohesion formation, chromatin assembly, gene expression, epigenetic conversions, and inheritance [171-181].

In general, PCNA belongs into the family of β-clamps, which are highly conserved among all three domains, Bacteria, Archaea and Eukaryote [182-186]. While all β-clamps adopt the characteristic pseudo-six-fold symmetry, their monomer composition varies with the bacterial sliding clamp forming homodimers compared to homotrimers and heterotrimers of the archaeal and eukaryotic sliding clamps [182, 184, 186, 187]. Despite of only 10% sequence homology among the different domains, superimposing their tertiary structures reveal a highly structural similarity, indicating a common ancestor and more importantly conserved biological processes [77, 186]. Each PCNA monomer has two similar globular domains linked by the flexible inter-domain connecting loop (IDCL) [182, 188]. The homotrimer adopts the characteristic pseudo-six-fold symmetry, which allows for the negatively outer surface, composed of six β-sheets, to face away from the DNA binding site, while the positively inner surface, composed of twelve α-helices faces towards it [182, 187, 188]. This inner surface

provides a channel (~ 35 Å) that is nearly twice the diameter of B-form DNA (~ 20 Å) allowing for the binding of DNA, which is facilitated by five basic lysine residues interacting with the phosphate backbones of DNA [182, 187, 188].

Two modes of sliding of PCNA along DNA have been proposed. One is termed “cogwheeling”, which requires the PCNA homotrimer to adjust to the helical propensity of DNA, thereby being tilted by 30° and tracks along the helix alternating between forward and backward tilts every one-half turn of DNA [185, 187]. The orientation of the tilt at the 3′ end of the DNA molecule functions as an additional regulatory mechanism in selection PCNA interacting binding partners [187]. The other mechanism is termed “tilt switch”, which is a non-helical tracking mechanism. This allows PCNA to track along DNA independently of the helical pitch [185, 187]. This mode of action by PCNA could grant greater freedom of rotation leading to a switch of the DNA binding site from the bound five α-helices to any of the other twelve α-helices, thereby altering the tilt relative to a 3′ terminus [185, 187]. This conformational change would allow additional binding of PCNA interacting partners or allow for the proof-reading activity of polymerases by redirecting the primer terminus from the polymerase site to the exonuclease site [185, 187].

Before PCNA can perform its biological processes, it needs to be loaded onto duplex DNA, by proteins known as clamp loaders, which belong to the family of AAA+ ATPase [189, 190]. The replication factor C (RFC) complex interacts with PCNA and generates force in an ATP-dependent manner to open the ring-shape structures of PCNA to load at primer terminus of the RNA-DNA hybrid or at lesions in the DNA back bones [189, 191-195]. The other clamp loaders, which are RFC-like complexes (RLCs) are composed of the same smaller subunits Rfc 2, 3, 4 and 5 but differ in their large subunit being Ctf18, Elg1 and Rad24 in S. cerevisiae or CTF18, ATAD5 and RAD17 in humans, respectively [190, 196, 197]. This indicates that different clamp loaders perform individual functions. Indeed, the RFC complex primarily loads PCNA

onto the lagging strand, while the Ctf18 RLC loads PCNA onto the leading strand [175, 198]. In contrast unloading of PCNA from DNA is performed by ATAD5-RLC or Elg1-RLC in mammalian cells and yeast, respectively [199-202]. The dissociation from DNA can also occur independently of clamp loaders or is regulated by post-translational modification of PCNA such as ubiquitination or acetylation [203-206]. Moreover, topological changes in DNA structure caused by replisome and nucleosome recruitment allows for the dissociation [198, 204].

PCNA is loaded onto DNA in an orientation-dependent manner, allowing the front face to point towards the direction of DNA synthesis [207-209]. This is important because the front face is composed of each monomer's C-terminus and the flexible interdomain-connecting loop (IDCL) [77, 207, 210]. The inter-domain connecting loop creates a hydrophobic pocket, which allows for the interaction with proteins containing the PCNA-interacting protein (PIP) motifs [210, 211]. This motif is found in the replicative polymerase and thus the proper orientation of PCNA allows for DNA synthesis to progress in the correct direction [210, 212]. On the other hand, the back face contains extended loops and is the site for post-translational modifications such as SUMOylation or ubiquitination on lysine 164, allowing for the recruitment of other proteins and holding them in proximity until they are needed [60, 213, 214].

The PCNA-Interacting-Protein Binding Motif (PIP-Box)

PCNA-**I**nteracting **P**roteins have a common motif that can be identified as a PIP-box (Table 1.1) [215]. The consensus motif of the PIP-box was discovered by analyzing the crystal structure of human PCNA with cyclin-dependent protein inhibitor $p21^{CIP1/WAF1}$ and revealed the following characteristic sequence: Q_1-x_2-x_3-h_4-x_5-x_6-a_7-a_8 [210, 215]. While position "x" represents random amino acids, position "h" is always occupied by a hydrophobic (I/L/M/V) amino acids and positions "a" by aromatic (F/Y) amino acids [210, 215]. This PIP-box motif can be termed "canonical" or "non-canonical", depending on the presence or absence of Q and aromatic amino acids F or Y at the first or the 7^{th} and 8^{th} residues of the motif, respectively [77,

215-217]. The consensus motif folds into a 3_{10} helix, allowing the conserved hydrophobic and aromatic amino acids to bind to the hydrophobic PCNA cleft located within the IDCL [210, 218]. Furthermore, orientation of the PIP-box sequence is irrelevant since both forward and reverse can interact with the hydrophobic pocket. To accommodate all PCNA-protein interactors, it is believed that depending on the composition of the PIP-box sequence proteins have different binding affinities for the hydrophobic pocket located in the IDCL [208]. Indeed, biochemical studies have shown that the PIP-box sequence Q_1-x_2-x_3-h_4-T-D-a_7-a_8 exhibit higher PCNA-binding affinity than proteins who do not possess the TD motif [218, 219]. If in addition to the PIP-box a positively charged residue (K or R) is found downstream at position 4, this protein will be marked for degradation, hence termed the PIP-degron [219]. Another PCNA-interacting motif that has been discovered is the AlkB homologue 2 PCNA interacting motif (APIM), which is composed of the sequence (K/R)-(F/Y/W)-(L/I/V/A)-(L/I/V/A)-(K/R) [211, 220, 221]. The APIM motif is mainly utilized when cells encounter replication stress and mediates the interaction with proteins involved in DNA repair [208]. An additional sequence, characteristic of the first two residues being lysine (K) and alanine (A), K-A-(A/L/I)-(A/L/Q)-x-x-(L/V), hence, termed the KA box, was discovered in proteins interacting with PCNA [222, 223]. Additionally, post-translation modifications such as SUMO and ubiquitin have shown to impact PCNA-interacting protein binding, while phosphorylation and acetylation regulate protein stability [60, 90]. Indeed, some binding partners of PCNA interact with the back face of PCNA by possessing ubiquitin-binding domain (UBD) and SUMO-interacting motif (SIM), which increases their binding affinity for modified PCNA [217, 224, 225].

Since each monomer possesses a hydrophobic pocket, it is possible that the homotrimer can interact with up to three different proteins via the PIP-box motif. In contrast, it is also possible for a protein to have multiple binding sites with PCNA via its different types of PIP-boxes or independently of them, thereby switching from an inactive state to an active state and

vice versa depending on the cellular response [226-230]. Additionally, many proteins interacting with PCNA have a short linear motif (SLiM) and are either intrinsically disordered proteins (IDPs) or hold intrinsically disordered regions (IDRs), which upon connecting with their binding partner exhibit highly stable structure [231-233]. Thus, a complex and multilayer mechanism exists for the binding of PCNA-interacting proteins and thereby regulating PCNA's multiple biological processes to ensure genome integrity.

Table 1.1. Known various PCNA interacting binding motifs (PIP-box)

Short Linear Motifs (SLiMs)	Sequence	Example	Protein	Species
Canonical PIP-Box	Q-x-x-H-x-x-a-a	YRQQTLSSFFMGSGKK SRQSTLYSFFPKSPAL	Rrm3 MSH6	*S. cerevisiae* *H. sapiens*
Non-Canonical PIP-Box	x-x-x-H-x-x-x-x	IKNKSLDSFFQKVNGE SGMKSIDTFFGVKNKK	Apn2 RNase H2B	*S. cerevisiae* *H. sapiens*
Canonical PIP-Degron	Q-x-x-H-T-D-a-a-x-x-x-(K/R)	FYQIGLTDFANFGKIN MEQRRVTDFFARRRPG	Mlh1 CDT1	*S. cerevisiae* *H. sapiens*
AlkB homologue 2 (APIM)	(K/R)-(F/Y/W)-(L/I/V/A)-(L/I/V/A)-(K/R)	IEITKRFILRRTNAI ASKQLRFALKTYFPY	Rdh54 FACC	*S. cerevisiae* *H. sapiens*
KA-Box	K-A-(A/L/I)-(A/L/Q)-x-x-(L/V)	DHIKKAILLMLMGGV KEKATQCQLEADV	Mcm3 POL δ	*S. cerevisiae* *H. sapiens*

Listing of the different binding motifs found in PCNA interacting proteins. The conserved amino acids are highlighted and examples are given for each unique PCNA binding motif in *S. cerevisiae* and *H.sapiens.* An extensive summary of PIP-box proteins in yeast and humans has been provided elsewhere [234, 235].

Ubiquitin and SUMO pathway

Small ubiquitin-related modifier (SUMO) and ubiquitin (UB) belong to the superfamily of ubiquitin-like (Ubl) modifiers [236]. *S. cerevisiae* only possesses one SUMO gene, encoding the protein Smt3, which is a 10-kDa highly conserved protein modifier [237] . While in higher eukaryotes the SUMO gene encodes for five different SUMO variants, SUMO1-SUMO5, the UB gene, which encodes for a 76-kDa protein modifier, is highly conserved from yeast to mammalian cells, differing only in three amino acids [237, 238]. In general, SUMOylation or ubiquitination occurs on specific lysine residues, which alter protein-protein interaction, protein localization and protein stability [237].

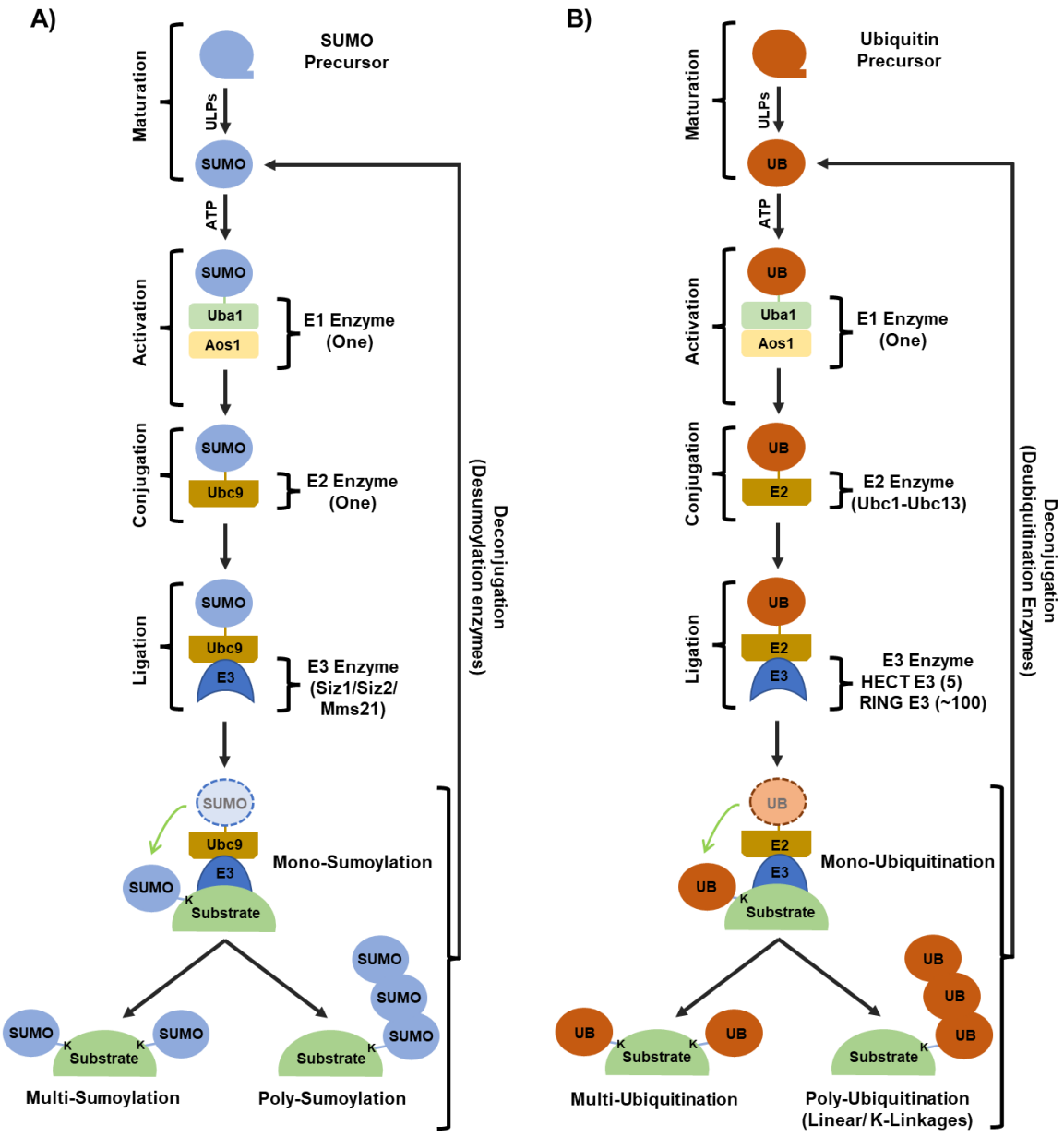

Figure 1.5. Enzymatic cascade of ubiquitin-like modifiers. Summary of the pathway to process and transport the precursor of the ***(A)*** small ubiquitin-related modifier (SUMO) and ***(B)*** ubiquitin (UB) to the target protein. After maturation of the precursor, both pathways follow the same major steps of activation, conjugation and ligation. The E1, E2 and E3 enzymes are depicted in the pictures and the number of enzymes that are known to perform each step. The E3 ligase directs the SUMO/ubiquitin-conjugated enzyme E2 to the target site and promotes either mono-, multi- or poly-SUMOylation/ubiquitination, respectively. Figures were adapted from [239].

These small motifs are being processed and transported by a well-conserved enzymatic cascade leading to SUMOylation or ubiquitination of certain targeted proteins (Figure 1.5.) [236, 237]. Firstly, the immature precursors of SUMO/Ub are processed by a cysteine-specific

protease, thereby exposing a di-glycine motif, which allows for covalent binding to the activating enzyme E1 in an ATP-dependent manner [237, 240]. Next, SUMO/Ub is transferred from the E1 to a conjugated enzyme E2 forming thioester linage [240, 241]. In the final step, an E3 ligase directs the SUMO/ubiquitin-conjugated enzyme E2 to the target site and promotes SUMOylation or ubiquitination, respectively [242, 243]. So far it was shown that organism usually contains one or two E1s, a few E2s and multiple E3-ligases for substrate specificity [237, 244].

Besides modifying one lysine on the target protein, additional lysines of the same protein can be modified, but also oligomeric chain structures can form [237, 245]. While SUMO chains are formed through K15 linkage, which in turn is marked for degradation by the SUMO targeted ubiquitin ligases (STUbLs), ubiquitin-chains are either formed through lys-48- or lys-63-linkage, acting as a degradation signal, as well, or in the DNA damage response pathway, respectively [240, 246, 247]. Additionally, other ubiquitin-chains are being discovered forming linkages at positions M1, K6, K11, K27, K29 and K33. Indicating that multiple cellular processes are being regulated depending on the type of linkages that result in ubiquitin-like modifier chains [245, 248, 249]. The removal of SUMOylation or ubiquitination is performed by de-SUMOylation and deubiquitinating enzymes maintaining the balance between free, and substrate bound Ubl modifiers [237, 250].

PCNA's Post-translational Modifications and its Implications

Post-translational modifications of proteins lead to conformational changes and can tailor the mode of action in response to cellular stress [209, 216]. One main regulator of cellular activity, PCNA, can be modified at 13 of 16 lysine residues [216]. The identified post-translation modifications of these lysine residues include ubiquitination, SUMOylation, acetylation, NEDDylation, methylation and ISGylation (Figure 1.6.) [216]. This modification is not limited to one per lysine, but the same lysine can be targeted by different modification allowing for multiple different pathways.

SUMOylation and ubiquitination of PCNA were originally identified in *S. cerevisiae*, but since the structure of PCNA is highly conserved across the different domains of life so are most of the post-translation modifications [60]. Crystal structure of modified PCNA has revealed closed and discrete positions for ubiquitin, while SUMOylation appears to be in a more open and flexible state [251]. This allows for Rad18 to be recruited via its SIM motif and placed in proximity of lysine 164 of the adjacent PCNA monomer, resulting in mono-ubiquitination of lysine 164 by the Rad6-Rad18 complex [59, 60, 252]. Mono-ubiquitination of lysine 164 leads to the replacement of the replicative helicase with a TLS polymerase to bypass the DNA damage [57, 97]. Depending on the lesion and choice of TLS polymerase this pathway can be error-free or error-prone [67]. Furthermore, a ubiquitin-chain linkage can form by the E3 RING ligase Rad5 in a complex with Mms2-Ubc13 leading to polyubiquitination, which leads to the error-free template switching pathway using the newly synthesized sister chromatid as a template to bypass the DNA lesion [60, 90, 92-94]. Another protein shown to bind to polyubiquitinated PCNA is Mgs1 (WRNIP in humans), which protects the stalled fork from unloading of PCNA and Srs2, thereby inhibiting illegitimate recombination [253]. Besides ubiquitination of lysine 164, other ubiquitination sites have been identified. If maturation of Okazaki fragments or the DNA ligase 1 activity are impaired, the E3 Ring ligase Rad5 in a complex with Mms2-Ubc4, not Ubc13, ubiquitinates lysine 107 (K110 in humans) [254, 255]. This modification acts as a sensor to activate S-phase checkpoints and cell cycle delay [254, 255]. Furthermore, in fission yeast ubiquitination of lysine 107 contributes to gross-chromosomal rearrangements (GCRs) in an Rad52-dependent manner [256]. Lysine 107 is located between PCNA monomers and ubiquitination at this site could alter the structure of PCNA thereby inhibiting its function [256]. Another site, lysine 242, is also ubiquitinated in response to impaired maturation of Okazaki fragments resulting in higher mutation rate by promoting TLS [257]. Finally, one modified site of unknown function is the ubiquitination of lysine 117 in response to UV-exposure [258].

Alternatively, PCNA is SUMOylated at the same lysine residue 164 and at lysine 127 by the E3 ligase Siz1 and Siz2, respectively [60, 68]. While lysine 127 correlates with the canonical sumo consensus motif ΨKx(D/E), in which the lysine residue is embedded by a hydrophobic residue (Ψ) and any amino acid (x) followed by aspartic or glutamic acid, located at the IDCL, SUMOlyation of lysine 164 occurs at a non-canonical motif [259-262]. PCNA SUMOylation of lysine 127 and lysine 164 leads to the recruitment of the DNA helicase Srs2, which antagonizes homologous recombination by physically interacting with Rad51 and thereby removing Rad51 nucleofilaments [263, 264]. Srs2 has a SIM and a non-canonical PIP-motif (PIP-degron), which is required for efficient binding to PCNA [214, 224]. While Srs2 can interact with unmodified PCNA, it possesses a higher binding affinity for SUMOylated PCNA [265]. Besides acting as an anti-recombinase, directly binding to SUMOylated PCNA allows for the displacement of Pol32, thereby limiting the quantity of spontaneous recombination intermediates [266]. Furthermore, Srs2 is downregulated at the replication fork by Esc2 in two ways [267]. Firstly, SUMOylated PCNA interacting with Srs2 is targeted by the clamp unloader Elg1, which is recruited to PCNA by SUMOylation on lysine 164 [267]. Secondly, Esc2 forms a complex with the Slx5/Slx8 SUMO-targeted ubiquitin ligase (STUbL) marking Srs2 for degradation via its PIP-degron [267]. This allows for Rad51 filament formation to promote the error-free Rad5 template switching pathway [268]. Alternatively, SUMOylation of lysine 127 inhibits the recruitment of Eco1, thereby impairing cohesion establishment [174, 216]. Thus, it is possible that SUMOylation at lysine 127 could act as a reset button for new protein-protein interaction [77]. While lysine at position 127 of PCNA is not conserved among species, its surrounding residues leucine 126 and isoleucine 128 are [268]. Therefore, SUMOylation of lysine 127 and its function might be specific for *S. cerevisiae*.

Besides ubiquitination and SUMOylation of PCNA, acetylation of lysine 20 and lysine 77 in response to DNA damage also protects the cells from genome instability [269, 270]. While

acetylation of lysine 20 is performed by the Eco1 acetyltransferase, which promotes repair in an HR dependent pathway using the sister chromatids, modification of lysine 77 and its function remain to be determined [269, 271]. Besides promoting repair by HR, acetylation of K20 could also lead to conformational changes of the trimer and thereby impairing DNA synthesis by disrupting the interaction with the replicative polymerases or PCNA sliding on DNA [187, 269, 271]. In mammalian cells PCNA acetylation is performed by the histone acetyltransferase (HAT), p300, and CREB-binding protein (CPB) at lysine 13, 14, 77, and 80, which leads to the removal and degradation of DNA bound PCNA following UV-exposure [203, 270, 272].

Additionally, mass spectrometry reveals 13 phosphorylation sites on PCNA in mammalian cells [216], with the strongest signal captured from tyrosine 211 and serine 261 [273, 274]. Phosphorylation of tyrosine 211 is performed by the epidermal growth factor receptor (EGFR) [273]. This modification inhibits the mismatch repair (MMR) pathway by disrupting the interaction with MMR proteins allowing for error-prone DNA replication to occur [274]. Furthermore, phosphorylation of tyrosine 211 functions as degradation protection mechanism [273, 275]. Moreover, phosphorylation of tyrosine 60, 133 and 250 stimulates PCNA ubiquitination in response to DNA damage [276].

Certain post-translation modifications have only been identified in mammalian cells (Figure 1.6.). One of these, are methylation of lysine 110, lysine 248 and di-methylation of lysine 10, which contributes to the stability of the PCNA trimer and to the interaction with Polδ, respectively [277, 278]. Another one modifies aspartate and glutamate residues by attaching ADP-ribose monomer, which also can form polymers, known as ADP-ribosylation [279]. This modification is thought to occur in the IDCL domain and therefore, might inhibit the binding site for PIP-box containing proteins [280]. Lastly, the resumption of normal replication is initiated by ISGylation in mammalian cells, which leads to the recruitment of deubiquitinating enzymes and

therefore leads to the restoration of unmodified PCNA allowing for reloading of the replicative DNA polymerases [281, 282].

In conclusion, PCNA is a main regulator of cellular activity, and its post-translational modifications determine the mode of action. In the future, identifying additionally modified residues and pathways of known modified sites will help us gain a better insight into the mechanisms which maintain genome stability.

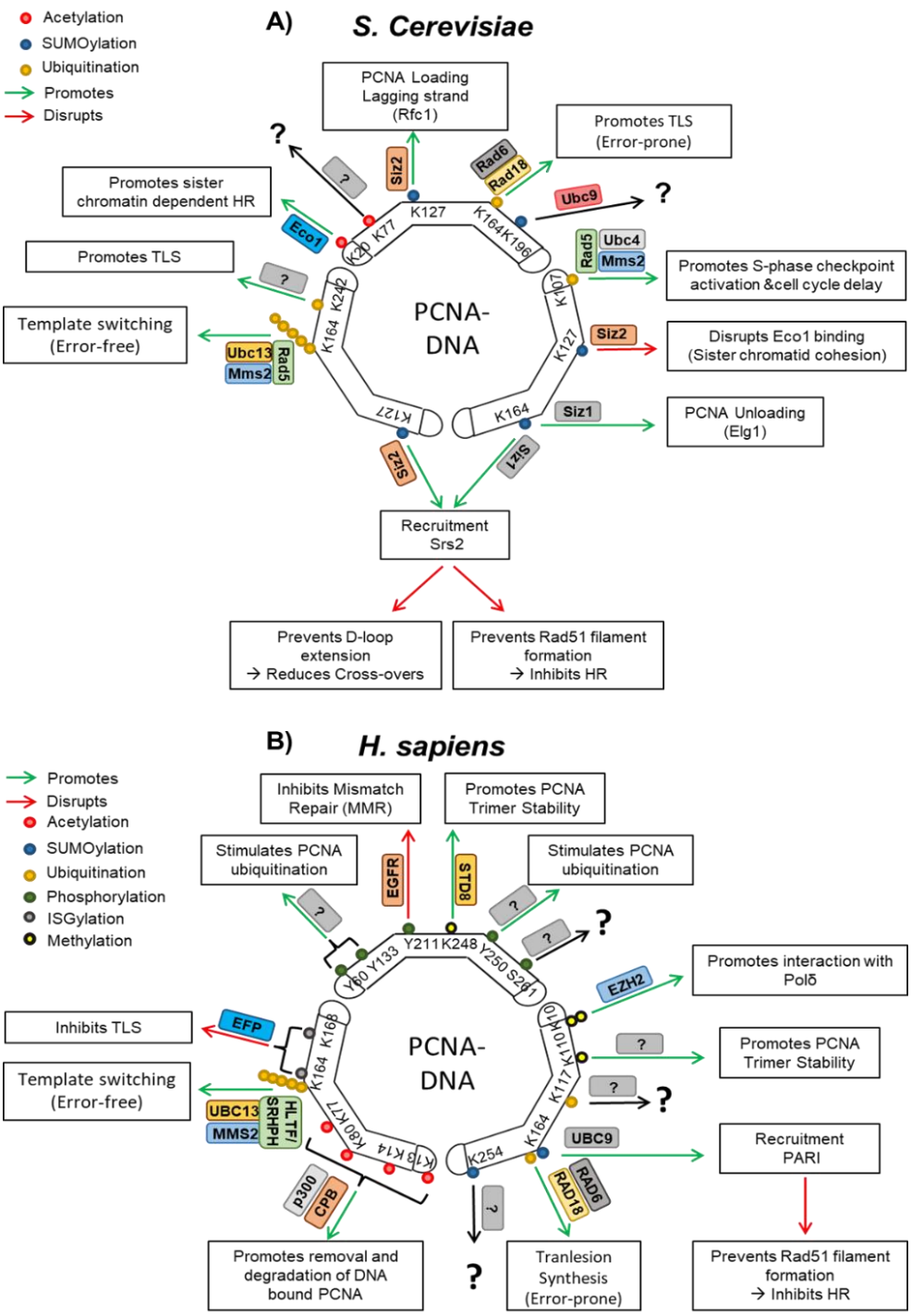

Figure 1.6. Post-translational Modifications of PCNA and their functions. Summarizing the various post-translational modifications of PCNA in ***(A)*** *S. cerevisiae* and in ***(B)*** *H. sapiens.* The known enzymes responsible for the modification and the downstream applications are shown. Certain post-translational modifications are unique to PCNA in *H. sapiens.* Unknown enzymes or downstream targets are marked with a question mark. Arrows indicating if the modification promotes (green) or inhibits (red) downstream targets.

References

1. Boule, J.B. and V.A. Zakian, *Roles of Pif1-like helicases in the maintenance of genomic stability.* Nucleic Acids Res, 2006. **34**(15): p. 4147-53.
2. Bochman, M.L., C.P. Judge, and V.A. Zakian, *The Pif1 family in prokaryotes: what are our helicases doing in your bacteria?* Mol Biol Cell, 2011. **22**(12): p. 1955-9.
3. Foury, F. and J. Kolodynski, *pif mutation blocks recombination between mitochondrial rho+ and rho- genomes having tandemly arrayed repeat units in Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 1983. **80**(17): p. 5345-9.
4. Bessler, J.B., J.Z. Torres, and V.A. Zakian, *The Pif1p subfamily of helicases: region-specific DNA helicases?* Trends Cell Biol, 2001. **11**(2): p. 60-5.
5. Keil, R.L. and A.D. McWilliams, *A gene with specific and global effects on recombination of sequences from tandemly repeated genes in Saccharomyces cerevisiae.* Genetics, 1993. **135**(3): p. 711-8.
6. Enemark, E.J. and L. Joshua-Tor, *On helicases and other motor proteins.* Curr Opin Struct Biol, 2008. **18**(2): p. 243-57.
7. Lohman, T.M., E.J. Tomko, and C.G. Wu, *Non-hexameric DNA helicases and translocases: mechanisms and regulation.* Nat Rev Mol Cell Biol, 2008. **9**(5): p. 391-401.
8. Singleton, M.R., M.S. Dillingham, and D.B. Wigley, *Structure and mechanism of helicases and nucleic acid translocases.* Annu Rev Biochem, 2007. **76**: p. 23-50.
9. Kobayashi, T. and T. Horiuchi, *A yeast gene product, Fob1 protein, required for both replication fork blocking and recombinational hotspot activities.* Genes Cells, 1996. **1**(5): p. 465-74.
10. Ivessa, A.S., J.Q. Zhou, and V.A. Zakian, *The Saccharomyces Pif1p DNA helicase and the highly related Rrm3p have opposite effects on replication fork progression in ribosomal DNA.* Cell, 2000. **100**(4): p. 479-89.
11. Sabouri, N., et al., *DNA replication through hard-to-replicate sites, including both highly transcribed RNA Pol II and Pol III genes, requires the S. pombe Pfh1 helicase.* Genes Dev, 2012. **26**(6): p. 581-93.
12. Torres, J.Z., J.B. Bessler, and V.A. Zakian, *Local chromatin structure at the ribosomal DNA causes replication fork pausing and genome instability in the absence of the S. cerevisiae DNA helicase Rrm3p.* Genes Dev, 2004. **18**(5): p. 498-503.
13. Syed, S., et al., *A Novel Rrm3 Function in Restricting DNA Replication via an Orc5-Binding Domain Is Genetically Separable from Rrm3 Function as an ATPase/Helicase in Facilitating Fork Progression.* PLoS Genet, 2016. **12**(12): p. e1006451.
14. Mohanty, B.K., N.K. Bairwa, and D. Bastia, *The Tof1p-Csm3p protein complex counteracts the Rrm3p helicase to control replication termination of Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 2006. **103**(4): p. 897-902.
15. Crickard, J.B., et al., *The RecQ helicase Sgs1 drives ATP-dependent disruption of Rad51 filaments.* Nucleic Acids Res, 2019. **47**(9): p. 4694-4706.
16. Krejci, L., et al., *DNA helicase Srs2 disrupts the Rad51 presynaptic filament.* Nature, 2003. **423**(6937): p. 305-9.
17. Leman, A.R. and E. Noguchi, *The replication fork: understanding the eukaryotic replication machinery and the challenges to genome duplication.* Genes (Basel), 2013. **4**(1): p. 1-32.
18. Larcher, M.V. and P. Pasero, *Top1 and Top2 promote replication fork arrest at a programmed pause site.* Genes Dev, 2020. **34**(1-2): p. 1-3.
19. Calzada, A., et al., *Molecular anatomy and regulation of a stable replisome at a paused eukaryotic DNA replication fork.* Genes Dev, 2005. **19**(16): p. 1905-19.

20. Osborn, A.J. and S.J. Elledge, *Mrc1 is a replication fork component whose phosphorylation in response to DNA replication stress activates Rad53.* Genes Dev, 2003. **17**(14): p. 1755-67.
21. Schmidt, K.H. and R.D. Kolodner, *Suppression of spontaneous genome rearrangements in yeast DNA helicase mutants.* Proc Natl Acad Sci U S A, 2006. **103**(48): p. 18196-201.
22. Szyjka, S.J., C.J. Viggiani, and O.M. Aparicio, *Mrc1 is required for normal progression of replication forks throughout chromatin in S. cerevisiae.* Mol Cell, 2005. **19**(5): p. 691-7.
23. Matsuda, K., et al., *Yeast two-hybrid analysis of the origin recognition complex of Saccharomyces cerevisiae: interaction between subunits and identification of binding proteins.* FEMS Yeast Res, 2007. **7**(8): p. 1263-9.
24. Schmidt, K.H. and R.D. Kolodner, *Requirement of Rrm3 helicase for repair of spontaneous DNA lesions in cells lacking Srs2 or Sgs1 helicase.* Mol Cell Biol, 2004. **24**(8): p. 3213-26.
25. Torres, J.Z., S.L. Schnakenberg, and V.A. Zakian, *Saccharomyces cerevisiae Rrm3p DNA helicase promotes genome integrity by preventing replication fork stalling: viability of rrm3 cells requires the intra-S-phase checkpoint and fork restart activities.* Mol Cell Biol, 2004. **24**(8): p. 3198-212.
26. Ooi, S.L., D.D. Shoemaker, and J.D. Boeke, *DNA helicase gene interaction network defined using synthetic lethality analyzed by microarray.* Nat Genet, 2003. **35**(3): p. 277-86.
27. Deegan, T.D., et al., *Pif1-Family Helicases Support Fork Convergence during DNA Replication Termination in Eukaryotes.* Mol Cell, 2019. **74**(2): p. 231-244 e9.
28. Piazza, A., et al., *Dynamic Processing of Displacement Loops during Recombinational DNA Repair.* Mol Cell, 2019. **73**(6): p. 1255-1266 e4.
29. Prakash, R., et al., *Yeast Mph1 helicase dissociates Rad51-made D-loops: implications for crossover control in mitotic recombination.* Genes Dev, 2009. **23**(1): p. 67-79.
30. Quinet, A., D. Lemacon, and A. Vindigni, *Replication Fork Reversal: Players and Guardians.* Mol Cell, 2017. **68**(5): p. 830-833.
31. Shin, S., et al., *ATP Binding to Rad5 Initiates Replication Fork Reversal by Inducing the Unwinding of the Leading Arm and the Formation of the Holliday Junction.* Cell Rep, 2018. **23**(6): p. 1831-1839.
32. Azvolinsky, A., et al., *The S. cerevisiae Rrm3p DNA helicase moves with the replication fork and affects replication of all yeast chromosomes.* Genes Dev, 2006. **20**(22): p. 3104-16.
33. Schmidt, K.H., K.L. Derry, and R.D. Kolodner, *Saccharomyces cerevisiae RRM3, a 5' to 3' DNA helicase, physically interacts with proliferating cell nuclear antigen.* J Biol Chem, 2002. **277**(47): p. 45331-7.
34. Hashash, N., A.L. Johnson, and R.S. Cha, *Regulation of fragile sites expression in budding yeast by MEC1, RRM3 and hydroxyurea.* J Cell Sci, 2011. **124**(Pt 2): p. 181-5.
35. Rossi, S.E., et al., *Rad53-Mediated Regulation of Rrm3 and Pif1 DNA Helicases Contributes to Prevention of Aberrant Fork Transitions under Replication Stress.* Cell Rep, 2015. **13**(1): p. 80-92.
36. Auble, D.T., et al., *Molecular analysis of the SNF2/SWI2 protein family member MOT1, an ATP-driven enzyme that dissociates TATA-binding protein from DNA.* Mol Cell Biol, 1997. **17**(8): p. 4842-51.
37. Davis, J.L., R. Kunisawa, and J. Thorner, *A presumptive helicase (MOT1 gene product) affects gene expression and is required for viability in the yeast Saccharomyces cerevisiae.* Mol Cell Biol, 1992. **12**(4): p. 1879-92.
38. Swagemakers, S.M., et al., *The human RAD54 recombinational DNA repair protein is a double-stranded DNA-dependent ATPase.* J Biol Chem, 1998. **273**(43): p. 28292-7.

39. Becker, P.B. and W. Horz, *ATP-dependent nucleosome remodeling.* Annu Rev Biochem, 2002. **71**: p. 247-73.
40. Flaus, A., et al., *Identification of multiple distinct Snf2 subfamilies with conserved structural motifs.* Nucleic Acids Res, 2006. **34**(10): p. 2887-905.
41. Subramanya, H.S., et al., *Crystal structure of a DExx box DNA helicase.* Nature, 1996. **384**(6607): p. 379-83.
42. Laurent, B.C., X. Yang, and M. Carlson, *An essential Saccharomyces cerevisiae gene homologous to SNF2 encodes a helicase-related protein in a new family.* Mol Cell Biol, 1992. **12**(4): p. 1893-902.
43. Eisen, J.A., K.S. Sweder, and P.C. Hanawalt, *Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions.* Nucleic Acids Res, 1995. **23**(14): p. 2715-23.
44. Caruthers, J.M. and D.B. McKay, *Helicase structure and mechanism.* Curr Opin Struct Biol, 2002. **12**(1): p. 123-33.
45. Durr, H., et al., *Snf2 family ATPases and DExx box helicases: differences and unifying concepts from high-resolution crystal structures.* Nucleic Acids Res, 2006. **34**(15): p. 4160-7.
46. Clapier, C.R. and B.R. Cairns, *The biology of chromatin remodeling complexes.* Annu Rev Biochem, 2009. **78**: p. 273-304.
47. Thoma, N.H., et al., *Structure of the SWI2/SNF2 chromatin-remodeling domain of eukaryotic Rad54.* Nat Struct Mol Biol, 2005. **12**(4): p. 350-6.
48. Laurent, B.C., I. Treich, and M. Carlson, *The yeast SNF2/SWI2 protein has DNA-stimulated ATPase activity required for transcriptional activation.* Genes Dev, 1993. **7**(4): p. 583-91.
49. Phelan, M.L., et al., *Reconstitution of a core chromatin remodeling complex from SWI/SNF subunits.* Mol Cell, 1999. **3**(2): p. 247-53.
50. Richmond, E. and C.L. Peterson, *Functional analysis of the DNA-stimulated ATPase domain of yeast SWI2/SNF2.* Nucleic Acids Res, 1996. **24**(19): p. 3685-92.
51. Machado, L.E., et al., *PHD domain from human SHPRH.* J Biomol NMR, 2013. **56**(4): p. 393-9.
52. Unk, I., et al., *Role of yeast Rad5 and its human orthologs, HLTF and SHPRH in DNA damage tolerance.* DNA Repair (Amst), 2010. **9**(3): p. 257-67.
53. Shen, M., et al., *Mechanistic Insights into the Multiple Activities of the Rad5 Family of Enzymes.* J Mol Biol, 2022. **434**(10): p. 167581.
54. Korzhnev, D.M., et al., *Solution NMR structure of the HLTF HIRAN domain: a conserved module in SWI2/SNF2 DNA damage tolerance proteins.* J Biomol NMR, 2016. **66**(3): p. 209-219.
55. Seelinger, M. and M. Otterlei, *Helicase-Like Transcription Factor HLTF and E3 Ubiquitin Ligase SHPRH Confer DNA Damage Tolerance through Direct Interactions with Proliferating Cell Nuclear Antigen (PCNA).* Int J Mol Sci, 2020. **21**(3).
56. Masuda, Y., et al., *Regulation of HLTF-mediated PCNA polyubiquitination by RFC and PCNA monoubiquitination levels determines choice of damage tolerance pathway.* Nucleic Acids Res, 2018. **46**(21): p. 11340-11356.
57. Branzei, D. and M. Foiani, *Maintaining genome stability at the replication fork.* Nat Rev Mol Cell Biol, 2010. **11**(3): p. 208-19.
58. Prakash, L., *Characterization of postreplication repair in Saccharomyces cerevisiae and effects of rad6, rad18, rev3 and rad52 mutations.* Mol Gen Genet, 1981. **184**(3): p. 471-8.
59. Davies, A.A., et al., *Activation of ubiquitin-dependent DNA damage bypass is mediated by replication protein a.* Mol Cell, 2008. **29**(5): p. 625-36.

60. Hoege, C., et al., *RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.* Nature, 2002. **419**(6903): p. 135-41.
61. Torres-Ramos, C.A., S. Prakash, and L. Prakash, *Requirement of RAD5 and MMS2 for postreplication repair of UV-damaged DNA in Saccharomyces cerevisiae.* Mol Cell Biol, 2002. **22**(7): p. 2419-26.
62. Podust, V.N. and U. Hubscher, *Lagging strand DNA synthesis by calf thymus DNA polymerases alpha, beta, delta and epsilon in the presence of auxiliary proteins.* Nucleic Acids Res, 1993. **21**(4): p. 841-6.
63. Lee, S.H., et al., *Synthesis of DNA by DNA polymerase epsilon in vitro.* J Biol Chem, 1991. **266**(33): p. 22707-17.
64. Prelich, G., et al., *Functional identity of proliferating cell nuclear antigen and a DNA polymerase-delta auxiliary protein.* Nature, 1987. **326**(6112): p. 517-20.
65. Burgers, P.M., *Saccharomyces cerevisiae replication factor C. II. Formation and activity of complexes with the proliferating cell nuclear antigen and with DNA polymerases delta and epsilon.* J Biol Chem, 1991. **266**(33): p. 22698-706.
66. Bravo, R., et al., *Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta.* Nature, 1987. **326**(6112): p. 515-7.
67. Prakash, S., R.E. Johnson, and L. Prakash, *Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function.* Annu Rev Biochem, 2005. **74**: p. 317-53.
68. Stelter, P. and H.D. Ulrich, *Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation.* Nature, 2003. **425**(6954): p. 188-91.
69. Johnson, R.E., et al., *Eukaryotic polymerases iota and zeta act sequentially to bypass DNA lesions.* Nature, 2000. **406**(6799): p. 1015-9.
70. Kannouche, P.L., J. Wing, and A.R. Lehmann, *Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage.* Mol Cell, 2004. **14**(4): p. 491-500.
71. Lawrence, C.W., *Cellular functions of DNA polymerase zeta and Rev1 protein.* Adv Protein Chem, 2004. **69**: p. 167-203.
72. Sharma, S., C.M. Helchowski, and C.E. Canman, *The roles of DNA polymerase zeta and the Y family DNA polymerases in promoting or preventing genome instability.* Mutat Res, 2013. **743-744**: p. 97-110.
73. Haracska, L., et al., *Roles of yeast DNA polymerases delta and zeta and of Rev1 in the bypass of abasic sites.* Genes Dev, 2001. **15**(8): p. 945-54.
74. Sale, J.E., A.R. Lehmann, and R. Woodgate, *Y-family DNA polymerases and their role in tolerance of cellular DNA damage.* Nat Rev Mol Cell Biol, 2012. **13**(3): p. 141-52.
75. Friedberg, E.C., A.R. Lehmann, and R.P. Fuchs, *Trading places: how do DNA polymerases switch during translesion DNA synthesis?* Mol Cell, 2005. **18**(5): p. 499-505.
76. Shachar, S., et al., *Two-polymerase mechanisms dictate error-free and error-prone translesion DNA synthesis in mammals.* EMBO J, 2009. **28**(4): p. 383-93.
77. Moldovan, G.L., B. Pfander, and S. Jentsch, *PCNA, the maestro of the replication fork.* Cell, 2007. **129**(4): p. 665-79.
78. Ohmori, H., et al., *The Y-family of DNA polymerases.* Mol Cell, 2001. **8**(1): p. 7-8.
79. Johnson, R.E., S. Prakash, and L. Prakash, *Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Poleta.* Science, 1999. **283**(5404): p. 1001-4.
80. Haracska, L., et al., *Efficient and accurate replication in the presence of 7,8-dihydro-8-oxoguanine by DNA polymerase eta.* Nat Genet, 2000. **25**(4): p. 458-61.
81. Washington, M.T., et al., *Efficient and error-free replication past a minor-groove N2-guanine adduct by the sequential action of yeast Rev1 and DNA polymerase zeta.* Mol Cell Biol, 2004. **24**(16): p. 6900-6.

82. Pryor, J.M. and M.T. Washington, *Pre-steady state kinetic studies show that an abasic site is a cognate lesion for the yeast Rev1 protein.* DNA Repair (Amst), 2011. **10**(11): p. 1138-44.
83. McDonald, J.P., A.S. Levine, and R. Woodgate, *The Saccharomyces cerevisiae RAD30 gene, a homologue of Escherichia coli dinB and umuC, is DNA damage inducible and functions in a novel error-free postreplication repair mechanism.* Genetics, 1997. **147**(4): p. 1557-68.
84. Lemontt, J.F., *Mutants of yeast defective in mutation induced by ultraviolet light.* Genetics, 1971. **68**(1): p. 21-33.
85. Gangavarapu, V., et al., *Mms2-Ubc13-dependent and -independent roles of Rad5 ubiquitin ligase in postreplication repair and translesion DNA synthesis in Saccharomyces cerevisiae.* Mol Cell Biol, 2006. **26**(20): p. 7783-90.
86. Xu, X., et al., *Involvement of budding yeast Rad5 in translesion DNA synthesis through physical interaction with Rev1.* Nucleic Acids Res, 2016. **44**(11): p. 5231-45.
87. Kuang, L., et al., *A non-catalytic function of Rev1 in translesion DNA synthesis and mutagenesis is mediated by its stable interaction with Rad5.* DNA Repair (Amst), 2013. **12**(1): p. 27-37.
88. Fan, Q., et al., *Rad5 coordinates translesion DNA synthesis pathway by recognizing specific DNA structures in saccharomyces cerevisiae.* Curr Genet, 2018. **64**(4): p. 889-899.
89. Pages, V., et al., *Requirement of Rad5 for DNA polymerase zeta-dependent translesion synthesis in Saccharomyces cerevisiae.* Genetics, 2008. **180**(1): p. 73-82.
90. Ulrich, H.D., *Regulating post-translational modifications of the eukaryotic replication clamp PCNA.* DNA Repair (Amst), 2009. **8**(4): p. 461-9.
91. Haracska, L., et al., *Opposing effects of ubiquitin conjugation and SUMO modification of PCNA on replicational bypass of DNA lesions in Saccharomyces cerevisiae.* Mol Cell Biol, 2004. **24**(10): p. 4267-74.
92. Branzei, D., M. Seki, and T. Enomoto, *Rad18/Rad5/Mms2-mediated polyubiquitination of PCNA is implicated in replication completion during replication stress.* Genes Cells, 2004. **9**(11): p. 1031-42.
93. Ulrich, H.D. and S. Jentsch, *Two RING finger proteins mediate cooperation between ubiquitin-conjugating enzymes in DNA repair.* EMBO J, 2000. **19**(13): p. 3388-97.
94. Zhang, H. and C.W. Lawrence, *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, 2005. **102**(44): p. 15954-9.
95. Broomfield, S., B.L. Chow, and W. Xiao, *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, 1998. **95**(10): p. 5678-83.
96. Brusky, J., Y. Zhu, and W. Xiao, *UBC13, a DNA-damage-inducible gene, is a member of the error-free postreplication repair pathway in Saccharomyces cerevisiae.* Curr Genet, 2000. **37**(3): p. 168-74.
97. Branzei, D. and I. Psakhye, *DNA damage tolerance.* Curr Opin Cell Biol, 2016. **40**: p. 137-144.
98. Carlile, C.M., et al., *Synthesis of free and proliferating cell nuclear antigen-bound polyubiquitin chains by the RING E3 ubiquitin ligase Rad5.* J Biol Chem, 2009. **284**(43): p. 29326-34.
99. Zheng, N. and N. Shabek, *Ubiquitin Ligases: Structure, Function, and Regulation.* Annu Rev Biochem, 2017. **86**: p. 129-157.
100. Minca, E.C. and D. Kowalski, *Multiple Rad5 activities mediate sister chromatid recombination to bypass DNA damage at stalled replication forks.* Mol Cell, 2010. **38**(5): p. 649-61.

101. Gangavarapu, V., S. Prakash, and L. Prakash, *Requirement of RAD52 group genes for postreplication repair of UV-damaged DNA in Saccharomyces cerevisiae.* Mol Cell Biol, 2007. **27**(21): p. 7758-64.
102. Karras, G.I. and S. Jentsch, *The RAD6 DNA damage tolerance pathway operates uncoupled from the replication fork and is functional beyond S phase.* Cell, 2010. **141**(2): p. 255-67.
103. Branzei, D., F. Vanoli, and M. Foiani, *SUMOylation regulates Rad18-mediated template switch.* Nature, 2008. **456**(7224): p. 915-20.
104. Ball, L.G., et al., *The yeast Shu complex couples error-free post-replication repair to homologous recombination.* Mol Microbiol, 2009. **73**(1): p. 89-102.
105. Mott, C. and L.S. Symington, *RAD51-independent inverted-repeat recombination by a strand-annealing mechanism.* DNA Repair (Amst), 2011. **10**(4): p. 408-15.
106. Giannattasio, M., et al., *Visualization of recombination-mediated damage bypass by template switching.* Nat Struct Mol Biol, 2014. **21**(10): p. 884-92.
107. Liberi, G., et al., *Rad51-dependent DNA structures accumulate at damaged replication forks in sgs1 mutants defective in the yeast ortholog of BLM RecQ helicase.* Genes Dev, 2005. **19**(3): p. 339-50.
108. Mankouri, H.W. and I.D. Hickson, *Top3 processes recombination intermediates and modulates checkpoint activity after DNA damage.* Mol Biol Cell, 2006. **17**(10): p. 4473-83.
109. Wu, L. and I.D. Hickson, *The Bloom's syndrome helicase suppresses crossing over during homologous recombination.* Nature, 2003. **426**(6968): p. 870-4.
110. Ortiz-Bazan, M.A., et al., *Rad5 plays a major role in the cellular response to DNA damage during chromosome replication.* Cell Rep, 2014. **9**(2): p. 460-8.
111. Karras, G.I., et al., *Noncanonical role of the 9-1-1 clamp in the error-free DNA damage tolerance pathway.* Mol Cell, 2013. **49**(3): p. 536-46.
112. Daigaku, Y., A.A. Davies, and H.D. Ulrich, *Ubiquitin-dependent DNA damage bypass is separable from genome replication.* Nature, 2010. **465**(7300): p. 951-5.
113. Friedberg, E.C., *Suffering in silence: the tolerance of DNA damage.* Nat Rev Mol Cell Biol, 2005. **6**(12): p. 943-53.
114. Neelsen, K.J. and M. Lopes, *Replication fork reversal in eukaryotes: from dead end to dynamic response.* Nat Rev Mol Cell Biol, 2015. **16**(4): p. 207-20.
115. Blastyak, A., et al., *Yeast Rad5 protein required for postreplication repair has a DNA helicase activity specific for replication fork regression.* Mol Cell, 2007. **28**(1): p. 167-75.
116. Bansbach, C.E., et al., *The annealing helicase SMARCAL1 maintains genome integrity at stalled replication forks.* Genes Dev, 2009. **23**(20): p. 2405-14.
117. Poole, L.A. and D. Cortez, *Functions of SMARCAL1, ZRANB3, and HLTF in maintaining genome stability.* Crit Rev Biochem Mol Biol, 2017. **52**(6): p. 696-714.
118. Chavez, D.A., B.H. Greer, and B.F. Eichman, *The HIRAN domain of helicase-like transcription factor positions the DNA translocase motor to drive efficient DNA fork regression.* J Biol Chem, 2018. **293**(22): p. 8484-8494.
119. Badu-Nkansah, A., et al., *Identification of a Substrate Recognition Domain in the Replication Stress Response Protein Zinc Finger Ran-binding Domain-containing Protein 3 (ZRANB3).* J Biol Chem, 2016. **291**(15): p. 8251-7.
120. Mason, A.C., et al., *A structure-specific nucleic acid-binding domain conserved among DNA repair proteins.* Proc Natl Acad Sci U S A, 2014. **111**(21): p. 7618-23.
121. Achar, Y.J., et al., *Human HLTF mediates postreplication repair by its HIRAN domain-dependent replication fork remodelling.* Nucleic Acids Res, 2015. **43**(21): p. 10277-91.
122. Hishiki, A., et al., *Structure of a Novel DNA-binding Domain of Helicase-like Transcription Factor (HLTF) and Its Functional Implication in DNA Damage Tolerance.* J Biol Chem, 2015. **290**(21): p. 13215-23.

123. Kile, A.C., et al., *HLTF's Ancient HIRAN Domain Binds 3' DNA Ends to Drive Replication Fork Reversal.* Mol Cell, 2015. **58**(6): p. 1090-100.
124. Longerich, S. and P. Sung, *Clearance of roadblocks in replication fork restart.* Proc Natl Acad Sci U S A, 2011. **108**(34): p. 13881-2.
125. Adeyemo, A.A., et al., *ZRANB3 is an African-specific type 2 diabetes locus associated with beta-cell mass and insulin response.* Nat Commun, 2019. **10**(1): p. 3195.
126. Carroll, C., et al., *Schimke Immunoosseous Dysplasia associated with undifferentiated carcinoma and a novel SMARCAL1 mutation in a child.* Pediatr Blood Cancer, 2013. **60**(9): p. E88-90.
127. Carroll, C., et al., *A novel splice site mutation in SMARCAL1 results in aberrant exon definition in a child with Schimke immunoosseous dysplasia.* Am J Med Genet A, 2015. **167A**(10): p. 2260-4.
128. Spranger, J., et al., *Schimke immuno-osseous dysplasia: a newly recognized multisystem disease.* J Pediatr, 1991. **119**(1 Pt 1): p. 64-72.
129. Hunter, K.B., et al., *Schimke immunoosseous dysplasia: defining skeletal features.* Eur J Pediatr, 2010. **169**(7): p. 801-11.
130. Moinova, H.R., et al., *HLTF gene silencing in human colon cancer.* Proc Natl Acad Sci U S A, 2002. **99**(7): p. 4562-7.
131. Leung, W.K., et al., *Inactivation of helicase-like transcription factor by promoter hypermethylation in human gastric cancer.* Mol Carcinog, 2003. **37**(2): p. 91-7.
132. Capouillez, A., et al., *Helicase-like transcription factor exhibits increased expression and altered intracellular distribution during tumor progression in hypopharyngeal and laryngeal squamous cell carcinomas.* Virchows Arch, 2008. **453**(5): p. 491-9.
133. Debauve, G., et al., *Early expression of the Helicase-Like Transcription Factor (HLTF/SMARCA3) in an experimental model of estrogen-induced renal carcinogenesis.* Mol Cancer, 2006. **5**: p. 23.
134. Piao, S., et al., *ALDH1A1 and HLTF modulate the activity of lysosomal autophagy inhibitors in cancer cells.* Autophagy, 2017. **13**(12): p. 2056-2071.
135. Bryant, E.E., et al., *Rad5 dysregulation drives hyperactive recombination at replication forks resulting in cisplatin sensitivity and genome instability.* Nucleic Acids Res, 2019. **47**(17): p. 9144-9159.
136. Bai, G., et al., *HLTF Promotes Fork Reversal, Limiting Replication Stress Resistance and Preventing Multiple Mechanisms of Unrestrained DNA Synthesis.* Mol Cell, 2020. **78**(6): p. 1237-1251 e7.
137. Li, J., et al., *DCUN1D1 facilitates tumor metastasis by activating FAK signaling and up-regulates PD-L1 in non-small-cell lung cancer.* Exp Cell Res, 2019. **374**(2): p. 304-314.
138. Betous, R., et al., *SMARCAL1 catalyzes fork regression and Holliday junction migration to maintain genome stability during DNA replication.* Genes Dev, 2012. **26**(2): p. 151-62.
139. Ciccia, A., et al., *The SIOD disorder protein SMARCAL1 is an RPA-interacting protein involved in replication fork restart.* Genes Dev, 2009. **23**(20): p. 2415-25.
140. Yuan, J., G. Ghosal, and J. Chen, *The HARP-like domain-containing protein AH2/ZRANB3 binds to PCNA and participates in cellular response to replication stress.* Mol Cell, 2012. **47**(3): p. 410-21.
141. Lee, J.H., et al., *Frequent loss of heterozygosity on chromosomes 6q, 11, and 17 in human ovarian carcinomas.* Cancer Res, 1990. **50**(9): p. 2724-8.
142. Mazurenko, N., et al., *High resolution mapping of chromosome 6 deletions in cervical cancer.* Oncol Rep, 1999. **6**(4): p. 859-63.
143. Millikin, D., et al., *Loss of heterozygosity for loci on the long arm of chromosome 6 in human malignant melanoma.* Cancer Res, 1991. **51**(20): p. 5449-53.

144. Sood, R., et al., *Cloning and characterization of a novel gene, SHPRH, encoding a conserved putative protein with SNF2/helicase and PHD-finger domains from the 6q24 region.* Genomics, 2003. **82**(2): p. 153-61.
145. Bauer, G.A. and P.M. Burgers, *Molecular cloning, structure and expression of the yeast proliferating cell nuclear antigen gene.* Nucleic Acids Res, 1990. **18**(2): p. 261-5.
146. Waga, S. and B. Stillman, *The DNA replication fork in eukaryotic cells.* Annu Rev Biochem, 1998. **67**: p. 721-51.
147. Miyachi, K., M.J. Fritzler, and E.M. Tan, *Autoantibody to a nuclear antigen in proliferating cells.* J Immunol, 1978. **121**(6): p. 2228-34.
148. Tan, C.K., et al., *An auxiliary protein for DNA polymerase-delta from fetal calf thymus.* J Biol Chem, 1986. **261**(26): p. 12310-6.
149. Fairman, M., et al., *Identification of cellular components required for SV40 DNA replication in vitro.* Biochim Biophys Acta, 1988. **951**(2-3): p. 382-7.
150. Yoder, B.L. and P.M. Burgers, *Saccharomyces cerevisiae replication factor C. I. Purification and characterization of its ATPase activity.* J Biol Chem, 1991. **266**(33): p. 22689-97.
151. Maga, G., et al., *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, 2001. **98**(25): p. 14298-303.
152. Sporbert, A., et al., *PCNA acts as a stationary loading platform for transiently interacting Okazaki fragment maturation proteins.* Nucleic Acids Res, 2005. **33**(11): p. 3521-8.
153. Tournier, S., et al., *Heterologous expression of the human cyclin-dependent kinase inhibitor p21Cip1 in the fission yeast, Schizosaccharomyces pombe reveals a role for PCNA in the chk1+ cell cycle checkpoint pathway.* Mol Biol Cell, 1996. **7**(4): p. 651-62.
154. Celis, J.E. and P. Madsen, *Increased nuclear cyclin/PCNA antigen staining of non S-phase transformed human amnion cells engaged in nucleotide excision DNA repair.* FEBS Lett, 1986. **209**(2): p. 277-83.
155. Toschi, L. and R. Bravo, *Changes in cyclin/proliferating cell nuclear antigen distribution during DNA repair synthesis.* J Cell Biol, 1988. **107**(5): p. 1623-8.
156. Nishida, C., P. Reinhard, and S. Linn, *DNA repair synthesis in human fibroblasts requires DNA polymerase delta.* J Biol Chem, 1988. **263**(1): p. 501-10.
157. Dresler, S.L. and K.S. Kimbro, *2',3'-Dideoxythymidine 5'-triphosphate inhibition of DNA replication and ultraviolet-induced DNA repair synthesis in human cells: evidence for involvement of DNA polymerase delta.* Biochemistry, 1987. **26**(10): p. 2664-8.
158. Nichols, A.F. and A. Sancar, *Purification of PCNA as a nucleotide excision repair protein.* Nucleic Acids Res, 1992. **20**(13): p. 2441-6.
159. Shivji, K.K., M.K. Kenny, and R.D. Wood, *Proliferating cell nuclear antigen is required for DNA excision repair.* Cell, 1992. **69**(2): p. 367-74.
160. Johnson, R.E., et al., *Evidence for involvement of yeast proliferating cell nuclear antigen in DNA mismatch repair.* J Biol Chem, 1996. **271**(45): p. 27987-90.
161. Umar, A., et al., *Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis.* Cell, 1996. **87**(1): p. 65-73.
162. Lau, P.J. and R.D. Kolodner, *Transfer of the MSH2.MSH6 complex from proliferating cell nuclear antigen to mispaired bases in DNA.* J Biol Chem, 2003. **278**(1): p. 14-7.
163. Klungland, A. and T. Lindahl, *Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1).* EMBO J, 1997. **16**(11): p. 3341-8.
164. Gary, R., et al., *Proliferating cell nuclear antigen facilitates excision in long-patch base excision repair.* J Biol Chem, 1999. **274**(7): p. 4354-63.

165. Levin, D.S., et al., *Interaction between PCNA and DNA ligase I is critical for joining of Okazaki fragments and long-patch base-excision repair.* Curr Biol, 2000. **10**(15): p. 919-22.
166. Liberti, S.E., et al., *Bi-directional routing of DNA mismatch repair protein human exonuclease 1 to replication foci and DNA double strand breaks.* DNA Repair (Amst), 2011. **10**(1): p. 73-86.
167. Haracska, L., et al., *Targeting of human DNA polymerase iota to the replication machinery via interaction with PCNA.* Proc Natl Acad Sci U S A, 2001. **98**(25): p. 14256-61.
168. Haracska, L., et al., *Stimulation of DNA synthesis activity of human DNA polymerase kappa by PCNA.* Mol Cell Biol, 2002. **22**(3): p. 784-91.
169. Kannouche, P., et al., *Localization of DNA polymerases eta and iota to the replication machinery is tightly co-ordinated in human cells.* EMBO J, 2003. **22**(5): p. 1223-33.
170. Li, M., et al., *TRIM28 functions as the SUMO E3 ligase for PCNA in prevention of transcription induced DNA breaks.* Proc Natl Acad Sci U S A, 2020. **117**(38): p. 23588-23596.
171. Henderson, D.S., et al., *Mutagen sensitivity and suppression of position-effect variegation result from mutations in mus209, the Drosophila gene encoding PCNA.* EMBO J, 1994. **13**(6): p. 1450-9.
172. Nomura, A., *Nuclear distribution of proliferating cell nuclear antigen (PCNA) in fertilized eggs of the starfish Asterina pectinifera.* J Cell Sci, 1994. **107 (Pt 12)**: p. 3291-300.
173. Poot, R.A., et al., *The Williams syndrome transcription factor interacts with PCNA to target chromatin remodelling by ISWI to replication foci.* Nat Cell Biol, 2004. **6**(12): p. 1236-44.
174. Moldovan, G.L., B. Pfander, and S. Jentsch, *PCNA controls establishment of sister chromatid cohesion during S phase.* Mol Cell, 2006. **23**(5): p. 723-32.
175. Liu, H.W., et al., *Division of Labor between PCNA Loaders in DNA Replication and Sister Chromatid Cohesion Establishment.* Mol Cell, 2020. **78**(4): p. 725-738 e4.
176. Zuilkoski, C.M. and R.V. Skibbens, *PCNA promotes context-specific sister chromatid cohesion establishment separate from that of chromatin condensation.* Cell Cycle, 2020. **19**(19): p. 2436-2450.
177. Wyse, B., et al., *RRM3 regulates epigenetic conversions in Saccharomyces cerevisiae in conjunction with Chromatin Assembly Factor I.* Nucleus, 2016. **7**(4): p. 405-14.
178. Hervouet, E., et al., *Specific or not specific recruitment of DNMTs for DNA methylation, an epigenetic dilemma.* Clin Epigenetics, 2018. **10**: p. 17.
179. Zhang, Z., K. Shibahara, and B. Stillman, *PCNA connects DNA replication to epigenetic inheritance in yeast.* Nature, 2000. **408**(6809): p. 221-5.
180. Ehrenhofer-Murray, A.E., R.T. Kamakaka, and J. Rine, *A role for the replication proteins PCNA, RF-C, polymerase epsilon and Cdc45 in transcriptional silencing in Saccharomyces cerevisiae.* Genetics, 1999. **153**(3): p. 1171-82.
181. Iida, T., et al., *PCNA clamp facilitates action of DNA cytosine methyltransferase 1 on hemimethylated DNA.* Genes Cells, 2002. **7**(10): p. 997-1007.
182. Krishna, T.S., et al., *Crystal structure of the eukaryotic DNA polymerase processivity factor PCNA.* Cell, 1994. **79**(7): p. 1233-43.
183. Kelman, Z. and M. O'Donnell, *Structural and functional similarities of prokaryotic and eukaryotic DNA polymerase sliding clamps.* Nucleic Acids Res, 1995. **23**(18): p. 3613-20.
184. Fukuda, K., et al., *Structure-function relationship of the eukaryotic DNA replication factor, proliferating cell nuclear antigen.* J Biol Chem, 1995. **270**(38): p. 22527-34.
185. Kochaniak, A.B., et al., *Proliferating cell nuclear antigen uses two distinct modes to move along DNA.* J Biol Chem, 2009. **284**(26): p. 17700-10.

186. Indiani, C. and M. O'Donnell, *The replication clamp-loading machine at work in the three domains of life.* Nat Rev Mol Cell Biol, 2006. **7**(10): p. 751-61.
187. De March, M., et al., *Structural basis of human PCNA sliding on DNA.* Nat Commun, 2017. **8**: p. 13935.
188. Kondratick, C.M., et al., *Crystal structures of PCNA mutant proteins defective in gene silencing suggest a novel interaction site on the front face of the PCNA ring.* PLoS One, 2018. **13**(3): p. e0193333.
189. Erzberger, J.P. and J.M. Berger, *Evolutionary relationships and structural mechanisms of AAA+ proteins.* Annu Rev Biophys Biomol Struct, 2006. **35**: p. 93-114.
190. Ogura, T. and A.J. Wilkinson, *AAA+ superfamily ATPases: common structure--diverse function.* Genes Cells, 2001. **6**(7): p. 575-97.
191. Majka, J. and P.M. Burgers, *The PCNA-RFC families of DNA clamps and clamp loaders.* Prog Nucleic Acid Res Mol Biol, 2004. **78**: p. 227-60.
192. Tsurimoto, T. and B. Stillman, *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, 1991. **266**(3): p. 1950-60.
193. Podust, L.M., et al., *Mammalian DNA polymerase auxiliary proteins: analysis of replication factor C-catalyzed proliferating cell nuclear antigen loading onto circular double-stranded DNA.* Mol Cell Biol, 1995. **15**(6): p. 3072-81.
194. Zhuang, Z., et al., *The structure of a ring-opened proliferating cell nuclear antigen-replication factor C complex revealed by fluorescence energy transfer.* Proc Natl Acad Sci U S A, 2006. **103**(8): p. 2546-51.
195. Sakato, M., Y. Zhou, and M.M. Hingorani, *ATP binding and hydrolysis-driven rate-determining events in the RFC-catalyzed PCNA clamp loading reaction.* J Mol Biol, 2012. **416**(2): p. 176-91.
196. Davey, M.J., et al., *Motors and switches: AAA+ machines within the replisome.* Nat Rev Mol Cell Biol, 2002. **3**(11): p. 826-35.
197. Miyata, T., et al., *Open clamp structure in the clamp-loading complex visualized by electron microscopic image analysis.* Proc Natl Acad Sci U S A, 2005. **102**(39): p. 13795-800.
198. Kang, M.S., et al., *Regulation of PCNA cycling on replicating DNA by RFC and RFC-like complexes.* Nat Commun, 2019. **10**(1): p. 2420.
199. Johnson, C., et al., *PCNA Retention on DNA into G2/M Phase Causes Genome Instability in Cells Lacking Elg1.* Cell Rep, 2016. **16**(3): p. 684-95.
200. Lee, K.Y., et al., *ATAD5 regulates the lifespan of DNA replication factories by modulating PCNA level on the chromatin.* J Cell Biol, 2013. **200**(1): p. 31-44.
201. Kubota, T., et al., *Replication-Coupled PCNA Unloading by the Elg1 Complex Occurs Genome-wide and Requires Okazaki Fragment Ligation.* Cell Rep, 2015. **12**(5): p. 774-87.
202. Shemesh, K., et al., *A structure-function analysis of the yeast Elg1 protein reveals the importance of PCNA unloading in genome stability maintenance.* Nucleic Acids Res, 2017. **45**(6): p. 3189-3203.
203. Cazzalini, O., et al., *CBP and p300 acetylate PCNA to link its degradation with nucleotide excision repair synthesis.* Nucleic Acids Res, 2014. **42**(13): p. 8433-48.
204. Shibahara, K. and B. Stillman, *Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin.* Cell, 1999. **96**(4): p. 575-85.
205. Thakar, T., et al., *Ubiquitinated-PCNA protects replication forks from DNA2-mediated degradation by regulating Okazaki fragment maturation and chromatin assembly.* Nat Commun, 2020. **11**(1): p. 2147.
206. Yao, N.Y., et al., *Mechanism of proliferating cell nuclear antigen clamp opening by replication factor C.* J Biol Chem, 2006. **281**(25): p. 17528-17539.

207. Boehm, E.M., M.S. Gildenberg, and M.T. Washington, *The Many Roles of PCNA in Eukaryotic DNA Replication.* Enzymes, 2016. **39**: p. 231-54.
208. Mailand, N., I. Gibbs-Seymour, and S. Bekker-Jensen, *Regulation of PCNA-protein interactions for genome stability.* Nat Rev Mol Cell Biol, 2013. **14**(5): p. 269-82.
209. Choe, K.N. and G.L. Moldovan, *Forging Ahead through Darkness: PCNA, Still the Principal Conductor at the Replication Fork.* Mol Cell, 2017. **65**(3): p. 380-392.
210. Gulbis, J.M., et al., *Structure of the C-terminal region of p21(WAF1/CIP1) complexed with human PCNA.* Cell, 1996. **87**(2): p. 297-306.
211. Hishiki, A., et al., *Structural basis for novel interactions between human translesion synthesis polymerases and proliferating cell nuclear antigen.* J Biol Chem, 2009. **284**(16): p. 10552-60.
212. Acharya, N., et al., *'PIPs' in DNA polymerase: PCNA interaction affairs.* Biochem Soc Trans, 2020. **48**(6): p. 2811-2822.
213. Dieckman, L.M., B.D. Freudenthal, and M.T. Washington, *PCNA structure and function: insights from structures of PCNA complexes and post-translationally modified PCNA.* Subcell Biochem, 2012. **62**: p. 281-99.
214. Freudenthal, B.D., et al., *Crystal structure of SUMO-modified proliferating cell nuclear antigen.* J Mol Biol, 2011. **406**(1): p. 9-17.
215. Warbrick, E., *PCNA binding through a conserved motif.* Bioessays, 1998. **20**(3): p. 195-9.
216. Slade, D., *Maneuvers on PCNA Rings during DNA Replication and Repair.* Genes (Basel), 2018. **9**(8).
217. Kim, S.O., et al., *Srs2 possesses a non-canonical PIP box in front of its SBM for precise recognition of SUMOylated PCNA.* J Mol Cell Biol, 2012. **4**(4): p. 258-61.
218. Bruning, J.B. and Y. Shamoo, *Structural and thermodynamic analysis of human PCNA with peptides derived from DNA polymerase-delta p66 subunit and flap endonuclease-1.* Structure, 2004. **12**(12): p. 2209-19.
219. Havens, C.G. and J.C. Walter, *Docking of a specialized PIP Box onto chromatin-bound PCNA creates a degron for the ubiquitin ligase CRL4Cdt2.* Mol Cell, 2009. **35**(1): p. 93-104.
220. Gilljam, K.M., et al., *Identification of a novel, widespread, and functionally important PCNA-binding motif.* J Cell Biol, 2009. **186**(5): p. 645-54.
221. Lancey, C., et al., *Structure of the processive human Pol delta holoenzyme.* Nat Commun, 2020. **11**(1): p. 1109.
222. Xu, H., et al., *A novel PCNA-binding motif identified by the panning of a random peptide display library.* Biochemistry, 2001. **40**(14): p. 4512-20.
223. Liang, Z., et al., *Proliferating cell nuclear antigen is required for loading of the SMCX/KMD5C histone demethylase onto chromatin.* Epigenetics Chromatin, 2011. **4**(1): p. 18.
224. Armstrong, A.A., F. Mohideen, and C.D. Lima, *Recognition of SUMO-modified PCNA requires tandem receptor motifs in Srs2.* Nature, 2012. **483**(7387): p. 59-63.
225. Bienko, M., et al., *Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis.* Science, 2005. **310**(5755): p. 1821-4.
226. Mayanagi, K., et al., *Architecture of the DNA polymerase B-proliferating cell nuclear antigen (PCNA)-DNA ternary complex.* Proc Natl Acad Sci U S A, 2011. **108**(5): p. 1845-9.
227. Mayanagi, K., et al., *Mechanism of replication machinery assembly as revealed by the DNA ligase-PCNA-DNA complex architecture.* Proc Natl Acad Sci U S A, 2009. **106**(12): p. 4647-52.
228. Sakurai, S., et al., *Structural basis for recruitment of human flap endonuclease 1 to PCNA.* EMBO J, 2005. **24**(4): p. 683-93.

229. Xing, G., et al., *Structural insight into recruitment of translesion DNA polymerase Dpo4 to sliding clamp PCNA.* Mol Microbiol, 2009. **71**(3): p. 678-91.
230. Bubeck, D., et al., *PCNA directs type 2 RNase H activity on DNA replication and repair substrates.* Nucleic Acids Res, 2011. **39**(9): p. 3652-66.
231. Yoon, M.K., et al., *Residual structure within the disordered C-terminal segment of p21(Waf1/Cip1/Sdi1) and its implications for molecular recognition.* Protein Sci, 2009. **18**(2): p. 337-47.
232. Click, T.H., D. Ganguly, and J. Chen, *Intrinsically disordered proteins in a physics-based world.* Int J Mol Sci, 2010. **11**(12): p. 5292-309.
233. Cordeiro, T.N., et al., *Disentangling polydispersity in the PCNA-p15PAF complex, a disordered, transient and multivalent macromolecular assembly.* Nucleic Acids Res, 2017. **45**(3): p. 1501-1515.
234. Prestel, A., et al., *The PCNA interaction motifs revisited: thinking outside the PIP-box.* Cell Mol Life Sci, 2019. **76**(24): p. 4923-4943.
235. Olaisen, C., et al., *The role of PCNA as a scaffold protein in cellular signaling is functionally conserved between yeast and humans.* FEBS Open Bio, 2018. **8**(7): p. 1135-1145.
236. Melchior, F., *SUMO--nonclassical ubiquitin.* Annu Rev Cell Dev Biol, 2000. **16**: p. 591-626.
237. Pichler, A., et al., *SUMO conjugation - a mechanistic view.* Biomol Concepts, 2017. **8**(1): p. 13-36.
238. Nakasone, M.A. and D.T. Huang, *Ubiquitination Accomplished: E1 and E2 Enzymes Were Not Necessary.* Mol Cell, 2016. **62**(6): p. 807-809.
239. Wang, L., et al., *Novel insights into the impact of the SUMOylation pathway in hematological malignancies (Review).* Int J Oncol, 2021. **59**(3).
240. Schulman, B.A. and J.W. Harper, *Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways.* Nat Rev Mol Cell Biol, 2009. **10**(5): p. 319-31.
241. Wang, J., et al., *Crystal structure of UBA2(ufd)-Ubc9: insights into E1-E2 interactions in Sumo pathways.* PLoS One, 2010. **5**(12): p. e15805.
242. Johnson, E.S. and G. Blobel, *Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p.* J Biol Chem, 1997. **272**(43): p. 26799-802.
243. Mahajan, R., L. Gerace, and F. Melchior, *Molecular characterization of the SUMO-1 modification of RanGAP1 and its role in nuclear envelope association.* J Cell Biol, 1998. **140**(2): p. 259-70.
244. Pickart, C.M., *Back to the future with ubiquitin.* Cell, 2004. **116**(2): p. 181-90.
245. Tsui, C., A. Raguraj, and C.M. Pickart, *Ubiquitin binding site of the ubiquitin E2 variant (UEV) protein Mms2 is required for DNA damage tolerance in the yeast RAD6 pathway.* J Biol Chem, 2005. **280**(20): p. 19829-35.
246. Tatham, M.H., et al., *Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9.* J Biol Chem, 2001. **276**(38): p. 35368-74.
247. Chiu, R.K., et al., *Lysine 63-polyubiquitination guards against translesion synthesis-induced mutations.* PLoS Genet, 2006. **2**(7): p. e116.
248. Srikumar, T., et al., *Global analysis of SUMO chain function reveals multiple roles in chromatin regulation.* J Cell Biol, 2013. **201**(1): p. 145-63.
249. Glickman, M.H. and A. Ciechanover, *The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction.* Physiol Rev, 2002. **82**(2): p. 373-428.
250. Komander, D., M.J. Clague, and S. Urbe, *Breaking the chains: structure and function of the deubiquitinases.* Nat Rev Mol Cell Biol, 2009. **10**(8): p. 550-63.
251. Tsutakawa, S.E., et al., *Structurally distinct ubiquitin- and sumo-modified PCNA: implications for their distinct roles in the DNA damage response.* Structure, 2015. **23**(4): p. 724-733.

252. Parker, J.L. and H.D. Ulrich, *A SUMO-interacting motif activates budding yeast ubiquitin ligase Rad18 towards SUMO-modified PCNA.* Nucleic Acids Res, 2012. **40**(22): p. 11380-8.
253. Jimenez-Martin, A., et al., *The Mgs1/WRNIP1 ATPase is required to prevent a recombination salvage pathway at damaged replication forks.* Sci Adv, 2020. **6**(15): p. eaaz3327.
254. Das-Bradoo, S., et al., *Defects in DNA ligase I trigger PCNA ubiquitylation at Lys 107.* Nat Cell Biol, 2010. **12**(1): p. 74-9; sup pp 1-20.
255. Nguyen, H.D., et al., *Unligated Okazaki Fragments Induce PCNA Ubiquitination and a Requirement for Rad59-Dependent Replication Fork Progression.* PLoS One, 2013. **8**(6): p. e66379.
256. Su, J., et al., *Fission yeast Rad8/HLTF facilitates Rad52-dependent chromosomal rearrangements through PCNA lysine 107 ubiquitination.* PLoS Genet, 2021. **17**(7): p. e1009671.
257. Becker, J.R., et al., *Flap endonuclease overexpression drives genome instability and DNA damage hypersensitivity in a PCNA-dependent manner.* Nucleic Acids Res, 2018. **46**(11): p. 5634-5650.
258. Elia, A.E., et al., *Quantitative Proteomic Atlas of Ubiquitination and Acetylation in the DNA Damage Response.* Mol Cell, 2015. **59**(5): p. 867-81.
259. Rodriguez, M.S., C. Dargemont, and R.T. Hay, *SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting.* J Biol Chem, 2001. **276**(16): p. 12654-9.
260. Hendriks, I.A. and A.C. Vertegaal, *A comprehensive compilation of SUMO proteomics.* Nat Rev Mol Cell Biol, 2016. **17**(9): p. 581-95.
261. Blomster, H.A., et al., *In vivo identification of sumoylation sites by a signature tag and cysteine-targeted affinity purification.* J Biol Chem, 2010. **285**(25): p. 19324-9.
262. Zhu, J., et al., *Small ubiquitin-related modifier (SUMO) binding determines substrate recognition and paralog-selective SUMO modification.* J Biol Chem, 2008. **283**(43): p. 29405-15.
263. Pfander, B., et al., *SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase.* Nature, 2005. **436**(7049): p. 428-33.
264. Niu, H. and H.L. Klein, *Multifunctional roles of Saccharomyces cerevisiae Srs2 protein in replication, recombination and repair.* FEMS Yeast Res, 2017. **17**(2).
265. Papouli, E., et al., *Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p.* Mol Cell, 2005. **19**(1): p. 123-33.
266. Burkovics, P., et al., *Srs2 mediates PCNA-SUMO-dependent inhibition of DNA repair synthesis.* EMBO J, 2013. **32**(5): p. 742-55.
267. Urulangodi, M., et al., *Local regulation of the Srs2 helicase by the SUMO-like domain protein Esc2 promotes recombination at sites of stalled replication.* Genes Dev, 2015. **29**(19): p. 2067-80.
268. Belli, G., et al., *Post-Translational Modifications of PCNA: Guiding for the Best DNA Damage Tolerance Choice.* J Fungi (Basel), 2022. **8**(6).
269. Billon, P., et al., *Acetylation of PCNA Sliding Surface by Eco1 Promotes Genome Stability through Homologous Recombination.* Mol Cell, 2017. **65**(1): p. 78-90.
270. Naryzhny, S.N. and H. Lee, *The post-translational modifications of proliferating cell nuclear antigen: acetylation, not phosphorylation, plays an important role in the regulation of its function.* J Biol Chem, 2004. **279**(19): p. 20194-9.
271. Billon, P. and J. Cote, *Novel mechanism of PCNA control through acetylation of its sliding surface.* Mol Cell Oncol, 2017. **4**(2): p. e1279724.
272. Yu, Y., et al., *Proliferating cell nuclear antigen is protected from degradation by forming a complex with MutT Homolog2.* J Biol Chem, 2009. **284**(29): p. 19310-20.

273. Wang, S.C., et al., *Tyrosine phosphorylation controls PCNA function through protein stability.* Nat Cell Biol, 2006. **8**(12): p. 1359-68.
274. Ortega, J., et al., *Phosphorylation of PCNA by EGFR inhibits mismatch repair and promotes misincorporation during DNA synthesis.* Proc Natl Acad Sci U S A, 2015. **112**(18): p. 5667-72.
275. Lo, Y.H., P.C. Ho, and S.C. Wang, *Epidermal growth factor receptor protects proliferating cell nuclear antigen from cullin 4A protein-mediated proteolysis.* J Biol Chem, 2012. **287**(32): p. 27148-57.
276. Waraky, A., et al., *Nuclear insulin-like growth factor 1 receptor phosphorylates proliferating cell nuclear antigen and rescues stalled replication forks after DNA damage.* J Biol Chem, 2017. **292**(44): p. 18227-18239.
277. A, P., et al., *EZH2 promotes DNA replication by stabilizing interaction of POLdelta and PCNA via methylation-mediated PCNA trimerization.* Epigenetics Chromatin, 2018. **11**(1): p. 44.
278. Takawa, M., et al., *Histone lysine methyltransferase SETD8 promotes carcinogenesis by deregulating PCNA expression.* Cancer Res, 2012. **72**(13): p. 3217-27.
279. Zhou, H., et al., *Toward a comprehensive characterization of a human cancer cell phosphoproteome.* J Proteome Res, 2013. **12**(1): p. 260-71.
280. Leung, A.K., *Poly(ADP-ribose): an organizer of cellular architecture.* J Cell Biol, 2014. **205**(5): p. 613-9.
281. Leung, W., et al., *Mechanisms of DNA Damage Tolerance: Post-Translational Regulation of PCNA.* Genes (Basel), 2018. **10**(1).
282. Park, J.M., et al., *Modification of PCNA by ISG15 plays a crucial role in termination of error-prone translesion DNA synthesis.* Mol Cell, 2014. **54**(4): p. 626-38.

Chapter Two: Helicase activities of Rad5 and Rrm3 genetically interact in the prevention of recombinogenic DNA lesions in *Saccharomyces cerevisiae*

Abstract

The genome must be monitored to ensure its duplication is completed accurately to prevent genome instability. In *Saccharomyces cerevisiae,* the 5' to 3' DNA helicase Rrm3, a member of the conserved PIF1 family, facilitates replication fork progression through an unknown mechanism. Disruption of Rrm3's helicase activity leads to increased replication fork pausing throughout the yeast genome. Here, we show that Rrm3 contributes to replication stress tolerance in the absence of the fork reversal activity of Rad5, defined by its HIRAN domain and DNA helicase activity, but not in the absence of Rad5's ubiquitin ligase activity. The Rrm3 and Rad5 helicase activities also interact in the prevention of recombinogenic DNA lesions, and stalled forks that accumulate in their absence need to be salvaged by a Rad59-dependent recombination pathway. Disruption of the structure-specific endonuclease Mus81 leads to accumulation of recombinogenic DNA lesions and chromosomal rearrangements in the absence of Rrm3, but not Rad5. Thus, at least two mechanisms exist to overcome fork stalling at replication barriers, defined by Rad5-mediated fork reversal and Mus81-mediated cleavage, and contribute to the maintenance of chromosome stability in the absence of Rrm3.

Introduction

DNA bound proteins and secondary DNA structures such as G-quadruplexes or hairpin-loops, need to be removed to facilitate the progression of the replication fork and maintain genome stability. Thus, cells have developed mechanisms to deal with different kinds of barriers encountered during replication that restart blocked or collapsed replication forks and to repair or

bypass damaged DNA. In *Saccharomyces cerevisiae,* the 5' to 3' DNA helicase Rrm3 ensures replication fork progression through nonhistone DNA-bound proteins. In the absence of Rrm3, paused replication forks accumulate at many sites throughout the genome including the rDNA locus, tRNA genes, telomeres, the mating type loci, centromeres, inactive origins of replication, and RNA polymerase II-transcribed genes [1-3]. *RRM3,* the *r*ibosomal DNA *r*ecombination *m*utation *3* gene, was initially identified as a suppressor of tandem repeat instability at the rDNA and the copper chelatin (*CUP1*) genes [1]. The *RRM3* gene codes for 723 amino acids, including the seven helicase motifs of the PIF1 helicase family [4, 5] (Figure 2.1A). In general, it is thought that Rrm3 helicase activity removes DNA-bound proteins, such as replication-fork blocking Fob1 in the rDNA gene array, before the replication fork encounters the obstacle. The ATPase activity of Rrm3 for the removal of DNA-bound proteins is regulated by subunits of the fork protection complex, Tof1 and Csm3 [6]. However, there are Fob1-independent pause sites and deleting Fob1 does not rescue synthetic lethality between *rrm3*Δ and *sgs1*Δ or *mrc1*Δ, indicating a role for Rrm3 outside of simply "sweeping" Fob1 in front of the DNA replication machinery [6, 7].

In response to *RRM3* deletion, Rad5 levels increase, suggesting that Rad5 may allow cells to deal with increased replication fork stalling [8]. Rad5 is a member of the SWI/SNF2 family and a multi-functional protein defined by its three domains: an N-terminal HIRAN domain (residues 170-300), a C-terminal domain with the seven conserved helicase motifs (residues 430-1169), and a RING domain between helicase motifs III and IV (residues 910-990) (Figure 2.1A) [9-12]. The best understood role of Rad5 is in the DNA damage tolerance pathway, also known as the post-replicative repair pathway. If the DNA replication machinery stalls, accumulating ssDNA is coated by RPA, which in turn recruits RAD6/Rad18 to mono-ubiquitinate PCNA [13]. Rad5 then catalyzes poly-ubiquitination of PCNA in a complex with Mms2 and Ubc13, which acts as a signal to utilize the newly synthesized sister chromatid for template

switching to bypass fork-blocking lesions, known as the error-free pathway [12, 14-16]. Mono-ubiquitination of PCNA replaces the replicative DNA polymerase with a translesion (TLS) polymerase, such as Rev1, to bypass the DNA lesion in an error-prone manner [14-16]. The first 30 residues of Rad5 contain a binding site for Rev1, possibly indicating a connection of Rad5 to this error-prone pathway [17, 18]. Rad5 has been identified to initiate fork reversal in *S. cerevisiae*, which is dependent on its HIRAN domain and the integrity of the helicase motifs [19-22]. During fork reversal the newly synthesized strands are paired, generating a fourth regressed arm, thus transforming the three-armed replication fork into a Holliday-junction-like four-way structure, also referred to as a “chicken foot” [23, 24].

Despite growing evidence for the importance of fork reversal to maintain genomic integrity, the mechanism by which it occurs and when it is activated remains largely unknown. Here, I use Rrm3-deficient yeast as a model system to further elucidate the cellular response to replication fork pausing, with a focus on the genetic interactions between Rrm3 and the multiple DNA-damage response functions of Rad5 in replication stress tolerance and the prevention of recombinogenic DNA lesions.

Materials and Methods

Yeast strains and media

Yeast strains were derived from S288C-derived strain KHSY802 (MATa, ura3-52, trp1Δ63, his3Δ200, leu2Δ1, lys2-Bgl, hom3-10, ade2Δ1, ade8, hxt13::URA3). RAD5 point mutations that disrupt the HIRAN domain (rad5-K194E) [20], ubiquitin-ligase activity (rad5-C914/917A) [12], or helicase activity (rad5-Q1106D) [22] were introduced into plasmid pR5-28 [25] (gift from L. Prakash) by site-directed mutagenesis and confirmed by sequencing. These rad5 point mutations have been described and characterized elsewhere [12, 20, 22]. A myc-HIS3MX6 tag was amplified from plasmid pFA6a-13Myc-His3MX6 [26] and inserted into the

plasmid at the 3' end of the rad5 mutant alleles. The plasmid-borne rad5 alleles were amplified by PCR and inserted at the chromosomal RAD5 locus by LiAc-mediated yeast transformation as described [27]; integration of the desired mutant alleles (rad5-K194E, rad5-C914/917A, rad5-Q1106D) at the chromosomal RAD5 ORF was verified by PCR, followed by sequencing of the alleles. *RRM3* truncation alleles were first created in CEN/ARS plasmid pRS315, containing a genomic fragment encompassing the *RRM3* ORF and promoter linked to a *TRP1* selection cassette (pRS315-*RRM3.TRP1)* using HR-mediated integration in NHEJ-deficient yeast strain KHSY2331 (*lig4*Δ) and verified by sequencing. The truncated *rrm3* allele was then amplified by PCR and integrated at the chromosomal *RRM3* locus, followed by verification of the truncation by sequencing. Similarly, the C-terminus of Rad52 was tagged with GFP using plasmid pFA6a-GFP(S65T)-HIS3MX6 [26] as a template. Haploid strains with more than one modified genomic locus were obtained by sporulating diploids heterozygous for the desired mutations and genotyping spores on selective media or by PCR. Yeast were grown at 30 °C in yeast extract (10g/L) peptone (20g/L) and dextrose (20g/L) (YPD) or synthetic complete (SC) media, as previously described [28]. Solid media was supplemented with agar (20 g/L) agar. Yeast strains used in this study are listed in Table 2.1.

Spot assay

The sensitivity of yeast cells to DNA damaging agents or replication stress was tested via spot assay as previously described [29]. Briefly, cell cultures were grown exponentially to OD_{600} = 0.5 in YPD and either 5-fold or 10-fold serial dilutions were spotted on YPD supplemented with hydroxyurea (HU) (US Biological) at the indicated concentrations. Colony growth was recorded every 12h for 2-4d of incubation at 30 °C with a Gel-Doc IT Imaging system (UVP).

Gross-chromosomal rearrangement (GCR) assay

Gross-chromosomal rearrangement (GCR) rates were determined by fluctuation analysis as previously described [30-32]. Briefly, fifteen cultures from three independent isolates were grown from single colonies in 10 ml YPD to saturation (2 days) at 30 °C. Dilutions of cells were plated on YPD agar plates to determine the viable cell count. The remaining culture was plated on synthetic media lacking arginine and uracil and supplemented with canavanine (60 mg/L, Sigma) and 5-fluoro-orotic acid (1 g/L, US Biological). Clones with GCRs were identified by their resistance to canavanine and 5-fluoro-orotic acid (Can^r 5-FOA^r), which is indicative of simultaneous inactivation of the *CAN1* and *URA3* genes on chromosome V. The rate of accumulating GCRs was determined as previously described and is reported as the median rate with 95% confidence intervals [30].

Fluorescence microscopy

To determine the fraction of cells with nuclear Rad52 foci, cells were grown in YPD to OD_{600} = 0.5 and arrested in G1 phase by adding alpha-factor (2 µg/µL) and continuing incubation at 30 °C for 90 minutes, with a second addition of alpha-factor at 45 min. Cells were washed in water and released into prewarmed YPD with 200 mM HU for 90 min. For fluorescence microscopy, cells were spotted on agarose pads and images acquired on a BZ-X800 (Keyence) fitted with a 60x objective. The presence of Rad52-GFP foci was evaluated in at least 250 cells from three independent isolates per yeast strain. The mean and standard deviation is reported. Unpaired *t* tests were performed to determine statistical significance of differences between the fraction of cells with Rad52-GFP foci in different mutant strains.

Doubling time measurement

A single colony was incubated overnight in YPD at 30°C. To measure doubling time, a 15ml starting culture of to OD_{600} = 0.2 was set up and placed in a shaking incubator at 30 °C. Cell

density was measured for every hour for a total of 8 hours at to OD_{600}. Individual measurements for each strain were graphed on a logarithmic scale to determine the exponential growth phase. Measurements were performed in triplicates and the mean and standard deviation is reported. Unpaired t tests were performed to determine statistical significance of differences between cell proliferation.

Results

Integrity of the HIRAN and helicase domains of Rad5 is required for tolerance of replication stress in the absence of Rrm3

Deletion of *RAD5* (*rad5*Δ) causes hypersensitivity to DNA damage and, to a lesser extent, to replication stress, whereas deletion of *RRM3* (*rrm3*Δ) does not cause hypersensitivity to these agents [8, 12, 19]. Deleting *RRM3* in the *rad5*Δ mutant (*rad5*Δ *rrm3*Δ) leads to a further increase in sensitivity to replication stress induced by hydroxyurea (HU) (Figure 2.1B). To determine which of the multiple Rad5 domains are required for replication stress tolerance in the *rrm3*Δ mutant we replaced the chromosomal *RAD5* ORF with *rad5* mutant alleles and assessed sensitivity of these mutants to HU using the spot assay. Specifically, we disrupted the HIRAN domain (*rad5-K194E* [20]), which captures the 3'OH of the nascent leading strand end during fork reversal [19, 20, 22], helicase motif VI (*rad5-Q1106D* [22]) whose integrity is required for ATPase and helicase activity and for fork reversal, and the RING domain (*rad5-C914/917A* [12]), which mediates poly-ubiquitination of PCNA [12, 14, 15] (Figure 2.1A). Whereas the *rad5-Q1106D* and *rrm3*Δ single mutants were no more sensitive to HU-induced replication stress than wildtype, even at the highest HU concentration, combining the *rrm3*Δ and *rad5-Q1106D* mutations led to HU hypersensitivity (Figure 2.1B), revealing a genetic interaction between the two helicases in tolerating replication stress. In contrast to the *rad5-Q1106D* mutant, the *rad5-K194E* mutant was hypersensitive to HU, suggesting a greater importance of the HIRAN domain for dealing with replication stress, which increased further in the *rrm3*Δ mutant (Figure 2.1B).

Combining HIRAN domain and helicase motif VI mutations (*rad5-K194E/Q1106D)* increased HU sensitivity compared to either single mutant, suggesting at least some redundant roles for the two activities in HU tolerance, which was further exacerbated by deletion of *RRM3* (Figure 2.1B). The ubiquitin-ligase activity of Rad5, defined by its RING domain, was also required for replication stress tolerance, as indicated by HU hypersensitivity of the *rad5-C914/917A* (Figure 2.1B). However, in contrast to the *rad5-Q1106D* and *rad5-K194E* mutations, we observed no genetic interaction between the *rad5-C914/917A* and *rrm3*Δ mutations on HU (Figure 2.1B). Together, these observations indicated that the HIRAN domain of Rad5 is important for dealing with replication stress whereas the helicase activity of Rad5 is dispensable, but that both activities of Rad5 are required for dealing with replication stress in cells lacking Rrm3. Notably, the helicase activity of Rad5 only becomes necessary in the absence of Rrm3 or in the absence of HIRAN domain activity. In contrast, Rad5 ubiquitin-ligase activity, implicated in poly-ubiquitination of PCNA [12, 14, 15], is equally required for dealing with replication stress in Rrm3-deficient cells and in Rrm3-proficient cells.

Recombinogenic DNA lesions accumulate in the absence of Rrm3 and Rad5 activities

Since deletion of *RRM3* increases replication fork pausing genome wide [1-3], we hypothesized that prolonged fork stalling in the absence of Rad5 fork reversal activities could lead to recombinogenic DNA lesions. To test this, we arrested cells in G1 with α-factor, released them into fresh media supplemented with HU, and evaluated Rad52-GFP foci accumulation as a marker of recombinogenic DNA lesions [33] (Figure 2.2A). Using fluorescence microscopy, we observed that cells lacking Rad5 had about twice as many Rad52-GFP foci as wildtype cells ($p < 0.01$), whereas we observed no significant difference in Rad52-GFP foci accumulation between wildtype cells and the *rrm3*Δ mutant either in the presence or absence of HU (Figure 2.2B). However, deletion of both *RRM3* and *RAD5* led to significantly more Rad52-GFP foci

during HU exposure than in either single mutant ($p < 0.05$), suggesting that *RRM3* and *RAD5* genetically interact to suppress recombinogenic DNA lesion formation in response to replication stress.

Rrm3 can be divided into a structured C-terminal helicase domain (residues 213-723) and an unstructured N-terminal tail (residues 1-212), which contains a PIP-box for interaction with PCNA [34] and two motifs that are important for the response to DNA replication stress [8, 35] (Figure 2.1A). As expected from the wildtype level of Rad52-GFP foci in the *rrm3*Δ mutant, neither deleting the N-terminal tail of Rrm3 (*rrm3-*Δ*N212*), which leaves the helicase activity of Rrm3 intact [8, 34], nor disrupting its helicase activity (*rrm3-K260D*) led to Rad52-GFP foci accumulation above wildtype levels in the presence or absence of HU (Figure 2.3A). Deleting *RAD5* in these two *rrm3* mutants had no effect on Rad52-GFP foci accumulation in the absence of HU (Figure 2.3A, left panel), but in the presence of HU deleting *RAD5* in the *rrm3-K260D* mutant increased Rad52-GFP foci formation (Figure 2.3A, right panel, $p < 0.01$) to the same level as in the *rrm3*Δ mutant whereas deletion of *RAD5* in the *rrm3-*Δ*212* mutant had no effect. This indicates that cells that lack the helicase activity of Rrm3, which normally prevents replication fork pausing throughout the yeast genome [1-3], need Rad5 to prevent the accumulation of recombinogenic DNA lesions under replication stress whereas cells that lack the N-terminal, regulatory tail of Rrm3 do not.

Next, we determined which functional domains of Rad5 were required for the suppression of recombinogenic DNA lesions. Disrupting either the RING domain (*rad5-C914/917A*) or the HIRAN domain (*rad5-K194E*) led to accumulation of Rad52-GFP foci in the presence ($p < 0.01$) or absence ($p<0.5$) of replication stress similar to deletion of *RAD5 (rad5*Δ*)*, whereas Rad52-GFP foci stayed at wildtype levels after disrupting the helicase domain of Rad5 (*rad5-Q1106D*) (Figure 2.3B). Rad5 helicase activity (*rad5-Q1106D*), however, was required for suppression of Rad52-GFP foci when *RRM3* was deleted (Figure 2.3B, left panel, $p < 0.01$).

Deletion of *RRM3* also further increased Rad52-GFP foci accumulation in the HIRAN-domain mutant (Figure 2.3B, right panel, *rad5-K194E*, $p < 0.01$), reaching the same level as in the *rad5*Δ mutant, but did not increase Rad52-GFP foci formation in the RING-domain mutant (*rad5-C914/917A*) (Figure 2.3B). Combining the HIRAN domain mutation and the helicase domain mutations (*rad5-K194E/Q1106D*) did not further increase Rad52-GFP foci formation (Figure 2.3B, right panel). Finally, disrupting Rad5 helicase activity (*rad5-Q1106D*) in a *rrm3* mutant specifically defective in helicase activity (*rrm3-K260D*) significantly increased Rad52-GFP foci accumulation both in the absence (Figure 2.3C, left panel, $p < 0.01$) and presence (Figure 2.3C, right panel, $p < 0.01$) of replication stress. Together, these findings demonstrate a genetic interaction between the helicase activity of Rrm3 and Rad5 activities involved in fork reversal (3'OH end capture, helicase activity), but not error-free DNA-damage tolerance pathways triggered by PCNA poly-ubiquitination (RING domain), in the prevention of recombinogenic DNA lesions during replication stress. Notably, the helicase activity of Rad5 (*rad5-Q1106D*) only became necessary for the suppression of recombinogenic DNA lesions in the absence of the helicase activity of Rrm3 (Figure 2.3C).

Requirement of Mus81 endonuclease for genome stability and fitness under replication stress in the absence of Rrm3

Stalled replication forks can be substrates for endonucleases to initiate rescue processes [36, 37]. One of these endonucleases, Mus81, preferably recognizes four-way DNA structures, such as Holliday junctions, but also stalled replication forks and 3' flaps, and plays a major role in the restart of stalled replication forks [38-41]. Deletion of *MUS81* in wildtype cells (*mus81*Δ) did not lead to an increase in Rad52-GFP foci in the absence (Figure 2.4A, left panel) or presence of HU (Figure 2.4A, right panel), but did so in Rrm3-helicase-defective cells (Figure 2.4A, *rrm3*Δ *mus81*Δ, $p < 0.001$; *rrm3-K260D mus81*Δ, $p < 0.001$), indicating that Mus81 prevents recombinogenic DNA lesions in cells with replication fork stalling caused by the

absence of Rrm3 helicase activity. In contrast to the *rrm3* mutants, deletion of *MUS81* in the *rad5*Δ mutant did not increase Rad52-GFP foci accumulation in the absence of replication stress (Figure 2.4A, left panel) and only had a minor effect in its presence (Figure 2.4A, right panel, $p < 0.01$). The further increase in Rad52-GFP foci in the *rrm3*Δ *mus81*Δ mutant upon deletion of *RAD5* (Figure 2.4A, *rrm3*Δ *mus81*Δ *rad5*Δ, $p < 0.01$) indicates that Rad5 and Mus81 independently contribute to the prevention of recombinogenic DNA lesions in cells lacking Rrm3.

Increased formation of recombinogenic DNA lesions due to the deletion of *MUS81* in the *rrm3*Δ mutant (Figure 2.4A) was associated with impaired tolerance of induced replication stress (Figure 2.4B), suggesting that absence of Mus81 could lead to inappropriate or untimely processing of paused forks that arise in the absence of Rrm3. To test this, we used the gross-chromosomal rearrangement (GCR) assay, which measures the rate of simultaneous loss of two counter-selectable markers (*URA3, CAN1*) on the non-essential end of chromosome V, typically due to a chromosome break that is healed by a chromosome rearrangement (e.g., translocation, interstitial deletion, inversion) or *de novo* telomere addition [30, 42]. Using this GCR assay, we observed that combining *RRM3* and *MUS81* deletions (*rrm3*Δ *mus81*Δ) caused a synergistic increase (3.8-fold) in genome instability compared to the single mutants (Table 2.1), demonstrating the importance of Mus81 not only for the suppression of recombinogenic DNA lesions (Figure 2.4A) and tolerance of replication stress in the *rrm3*Δ mutant (Figure 2.4B), but also for the maintenance of genome stability (Table 2.1).

Combining *MUS81* and *RAD5* deletions (*rad5*Δ *mus81*Δ) also led to increased sensitivity to replication stress (Figure 2.4C), notably at a lower concentration of HU (25 mM versus 125 mM) than for the *rrm3*Δ *mus81*Δ mutant (Figure 2.4B), but no significant increase in the GCR rate over that of the *rad5*Δ single mutant (Table 2.1). This finding indicates that, in contrast to the *rrm3*Δ mutant, DNA lesions that form in the *rad5*Δ mutant do not appear to require Mus81

for error-free repair. Compared to the double mutants, genome instability and sensitivity to replication stress did not change in the *rrm3*Δ *rad5*Δ *mus81*Δ triple mutant (Figure 2.4C, Table 2.1). Considering that Rad52-GFP foci increased in this triple mutant and we did not see a loss in viable cell count in the GCR assay, the lack of a GCR rate increase in the *rrm3*Δ *rad5*Δ *mus81*Δ triple mutant suggests that some of the recombinogenic DNA lesions in this strain do not go on to form mutagenic genome rearrangements.

To gain insight into how replication-stress-induced recombinogenic DNA lesions in the *rrm3*Δ *rad5*Δ mutant are repaired, we considered the Rad51- and Rad59-dependent branches of homology-directed DSB repair. Rad51 forms the recombinogenic filament for strand invasion in synthesis-dependent strand annealing (SDSA) whereas Rad59 can act on shorter 3' overhangs and is required for single-strand annealing (SSA). While Rad59 is also involved in break-induced replication (BIR), which initiates from one-ended DSBs that arise from collapsed replication forks, BIR can also take place in the absence of Rad51 [43-47]. We observed that combining *RAD5* and *RRM3* deletions (*rrm3*Δ *rad5*Δ) or a *RAD59* deletion with an *RRM3* deletion (*rrm3*Δ *rad59*Δ) or a *RAD5* deletion (*rad5*Δ *rad59*Δ) had no effect on cell proliferation as measured by the doubling time (Figure 2.4D), but the doubling time increased significantly (~30%, $p < 0.01$) upon deletion of all three genes (*rrm3*Δ *rad5*Δ *rad59*Δ). In contrast, combining a *RAD51* deletion with an *RRM3* deletion (*rrm3*Δ *rad5*Δ) had no effect on the doubling time ($p = 0.29$). Although combination of *RAD51* and *RAD5* deletions (*rad5*Δ *rad51*Δ) significantly increased the doubling time (~18%, $p < 0.05$), there was no further increase upon *RRM3* deletion (*rad5*Δ *rrm3*Δ *rad51*Δ, $p = 0.13$). Overall, these findings show that Rad51 is important for normal growth in the absence of Rad5 whereas Rad59 only becomes important in the absence of both Rad5 and Rrm3.

Discussion

The ATPase/helicase and HIRAN domains of Rad5 collaborate to reverse replication forks *in vitro,* with the ATPase/helicase thought to power leading strand unwinding to initiate four-way junction formation and the HIRAN domain capturing the 3' OH at the nascent leading strand end to allow ATP-hydrolysis-driven branch migration of the four-way-junction, including reversal of the regressed fork back to a three-way junction [19, 20]. Although mapping to opposite ends of Rad5, the HIRAN and ATPase/helicase domains appear to engage in intra-molecular interactions, consistent with their concerted actions in fork reversal [48]. In this study, we have investigated the importance of this fork reversal activity of Rad5 in cells experiencing replication stress either due to deletion of *RRM3* or due to exposure to HU, or both. We show that the HIRAN domain is required for tolerating HU and for preventing recombinogenic DNA lesions in wildtype cells, and this requirement increases if Rrm3 is absent. Intriguingly, Rad5 helicase activity is required for HU tolerance and recombinogenic DNA lesion prevention only if Rrm3 is absent. The ubiquitin-ligase activity of Rad5 was required for HU tolerance and recombinogenic lesion prevention, but unlike the fork reversal activities of Rad5, it was no more important in the absence of Rrm3 than in its presence.

Considering that the HIRAN and ATPase/helicase domains cooperate in fork reversal *in vitro*, it was surprising that the HIRAN domain is required for recombinogenic DNA lesion suppression and HU tolerance of wildtype cells whereas the ATPase/helicase activity was only required for these functions in the absence of Rrm3. This difference could be explained by the recent discovery that besides fork reversal the HIRAN domain of Rad5 contributes to binding and ubiquitination of PCNA [49]. Moreover, the spot assay results of the *rad5-K194E/Q1106D* mutant indicate at least some redundancy between the function of the HIRAN domain and the ATPase/helicase activity of Rad5 in tolerating HU-induced replication stress. However, analysis

of additional genetic interactions will be required to identify and better understand overlapping and nonoverlapping roles of these two Rad5 activities *in vivo*.

Previous studies established functions of the ubiquitin-ligase activity of Rad5 in PCNA poly-ubiquitination and recombination-based, error-free DNA lesion bypass, and the role of the Rev1 binding site of Rad5 in recruitment of TLS polymerases for error-prone DNA lesion bypass and replication across single-strand DNA gaps [50-52]. However, while *in vitro* experiments demonstrated that Rad5 has helicase activity and that this activity can mediate fork regression *in vitro* [19], the role of Rad5 helicase activity *in vivo* is poorly understood. The difficulty stems in part from the initially unrecognized overlap of conserved helicase motifs with the RING domain, which led to mutations being used for the *in vivo* characterization of Rad5 helicase activity that also affected PCNA poly-ubiquitination [22, 53-56]. Recently, mutation of the conserved Q1106 residue in helicase motif VI (*rad5-Q1106D*), which is also used in this study, was established as a true separation-of-function mutation that disrupts Rad5 helicase activity without affecting PCNA poly-ubiquitination [22]. Using this mutation, previous reports showed that the helicase activity of Rad5 was not required for survival of replication stress induced by HU [50], consistent with my observation, and dispensable for error-free or error-prone DNA lesion bypass [22, 51, 55]. This study is one of the first to identify a specific biological requirement for the helicase activity of Rad5 *in vivo*, namely the prevention of recombinogenic DNA lesions in cells with impaired replication fork progression.

That the helicase activity of Rad5 was required under conditions of replication fork stalling due to *RRM3* deletion but not due to nucleotide pool depletion by HU suggests that the Rad5 helicase reverses specific types of forks. For example, nucleotide pool depletion due to HU treatment impairs DNA synthesis by leading and lagging strand DNA polymerases, generating long single-stranded regions behind the fork [57, 58] whereas DNA-bound proteins and G-quadruplexes that impair fork progression in the *rrm3*Δ mutant [2, 3, 59] would act in front

of the fork, thus impairing CMG helicase progression but not causing large ssDNA regions behind the fork. Indeed, a recent *in vitro* study suggests that Rad5 helicase-mediated leading strand unwinding may not be required for fork reversal if there is a sufficiently large ssDNA gap on the leading strand [20]. Additionally, HU-arrested replication forks may be stabilized by multiple pathways whereas survival of forks in the *rrm3*Δ mutant may depend more strongly on Rad5 helicase activity. In support of the latter, Rad5 was one of the top three proteins (in addition to Rdh54, Top2) upregulated in cells lacking Rrm3 [8].

The recombinogenic DNA lesions that arise in *rrm3*Δ cells when Rad5 is disrupted appear to be preferentially repaired by Rad59-dependent rather than Rad51-dependent recombination (Figure 2.4D). This is consistent with previous reports that Rad59 is preferred for homology-dependent repair of DNA lesions associated with DNA replication [60-62], and there is evidence that Rad51-dependent recombination events may even be downregulated during DNA replication [60]. The role of Rad59 in lesion repair in the *rrm3*Δ *rad5*Δ mutant could also be regulatory, such as inhibiting detrimental Rad51-dependent events at stalled forks. I observed a similar interplay between Rad51 and Rad59 when DSBs could not be properly processed due to the absence of Sgs1 and Exo1 [63]. The DNA structures that arise at replication forks in *rrm3*Δ cells when fork regression is prevented could also allow Rad59 to be directly involved in fork recovery by sister-chromatid recombination.

The genetic interaction between *rrm3* and *mus81* mutations provides some additional insight into events at blocked replication forks in the absence of Rrm3. As a subunit of a structure-specific endonuclease, Mus81 prevents the accumulation of arrested or broken forks [38, 39, 64]. Our findings indicate that some forks that stall due to the absence of Rrm3 could form recombination structures that become substrates for Mus81, which faithfully restarts these forks. In the absence of Mus81, stalled forks that arise due to lack of Rrm3 could become unstable, mostly likely due to attack by other nucleases, causing the accumulation of

recombinogenic DNA lesions and giving rise to genome instability. A similar negative genetic interaction between *RRM3* and *MUS81* was recently identified at yeast telomeres [41]. I find that inactivation of Rad5 increased recombinogenic lesions in the *rrm3*Δ *mus81*Δ mutant and that inactivation of Rad5 in the *mus81*Δ mutant increased hypersensitivity to replication stress, suggesting that the Rad5- and Mus81-mediated fork rescue mechanisms can act independently on stalled forks, probably because they have different fork substrates, and that a fraction of the recombinogenic lesions, especially those generated by the lack of Rad5 in the *rrm3*Δ *mus81*Δ mutant, may be lethal.

In summary, I propose the following pathways for the rescue of blocked replication forks and prevention of recombinogenic DNA lesions and chromosome instability (Figure 2.5): To ensure replication fork progression, Rrm3 helicase activity allows replisomes to move through replication barriers (RBs), such as DNA-protein complexes, most prominently the replication-fork-blocking protein Fob1 in the rDNA genes but also countless other RBs throughout the genome, and may also unfold hard-to-replicate DNA secondary structures, such as G-quadruplexes [65, 66]. If these blocks to replisome progression cannot be overcome due to the absence of Rrm3, our findings indicate two pathways for fork rescue: First, collaboration between the helicase activity and the 3' OH end capture activity of Rad5 regresses the stalled fork into a chicken-foot-like structure to set it up for restart [67]. We find that only this fork reversal activity of Rad5 is required in the absence of Rrm3 to prevent recombinogenic lesions, most likely a sign of fork stalling or collapse, whereas the Rad5 ubiquitin-ligase activity, which leads to PCNA poly-ubiquitination thought to remodel replisome components to allow resumption of DNA synthesis by template switching [68, 69], is not. Additionally, it is conceivable that movement of Rad5 on the DNA in the vicinity of a stalled replisome could remove RBs similar to Rrm3 and contribute to resumption of some forks. In the absence of Rad5 or Rad5 fork reversal activity, forks that stalled due to lack of Rrm3 helicase persist,

leading to accumulation of recombinogenic DNA lesions and increased sensitivity to additional replication stress. We observed that a recombination mechanism dependent on Rad59 is specifically required for normal growth when both Rrm3 and Rad5 are absent. As Rad59 does not appear to be required for template switching [70], a Rad59-dependent recombination-driven replication salvage pathway that also requires Rad52 and Pol δ [71] may act on forks in cells that lack the Rrm3- and Rad5-mediated pathways for fork progression and fork rescue, respectively. Finally, a subset of stalled forks are processed by Mus81, most likely in a mechanism that suppresses chromosome rearrangements that result from BIR, for example by converting mutagenic BIR-induced D-loops [72, 73] to normal replication forks [74].

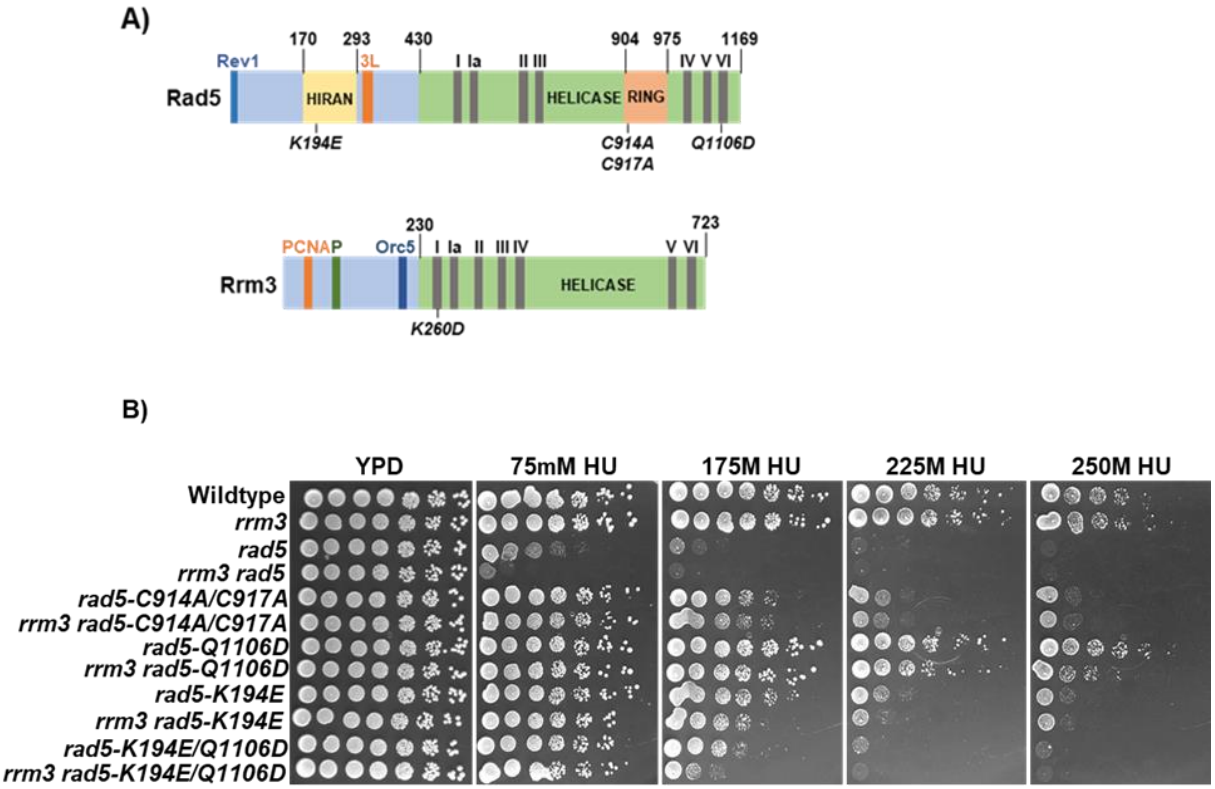

Figure 2.1. Genetic interaction between functional domains of *RAD5* and *RRM3* in the suppression of replication stress. ***(A)*** Domain structures of Rad5 and Rrm3. Both enzymes have structurally ordered C-terminal domains (green), which contain the seven conserved helicase motifs I-VI; a RING domain is located between helicase motifs III and IV of Rad5. The disordered N-terminal domains (blue) contain protein-protein interaction sites (Rev1, Orc5, PCNA), a leucine zipper (3L), and a phosphorylation cluster (P) [8, 34, 35]. A structured HIRAN domain is located within the N-terminus of Rad5 [18]. Amino acid positions of approximate domain boundaries are indicated above the drawing. Point mutations used in this study are shown below the drawing. ***(B)*** Spot assay to test for genetic interactions between *rad5* and *rrm3* mutations in the suppression of DNA replication stress induced by chronic exposure to hydroxyurea (HU). Serial dilutions of exponentially growing yeast strains with deletions of *RAD5* and/or *RRM3* or with chromosomally integrated mutations in functional domains of *RAD5* (RING-domain: *rad5-CC914/917AA,* helicase-domain: *rad5-Q1106D,* HIRAN-domain*: rad5-K194E,* or both HIRAN- and helicase-domain: *rad5-K194E /Q1106D*) were spotted onto YPD agar plates containing various concentrations of HU. Images were acquired on a GelDoc-It system (UVP) after 2-3 days of incubation at 30 ˚C.

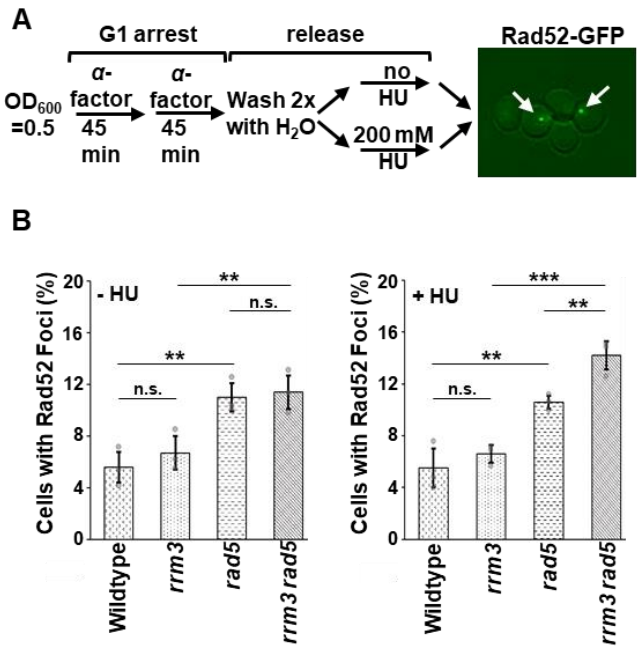

Figure 2.2 Genetic interactions between *rad5*Δ and *rrm3*Δ in the prevention of recombinogenic DNA lesions. ***(A)*** Yeast strains bearing deletions of *RRM3* and/or *RAD5* and expressing GFP-tagged Rad52 (Rad52-GFP) were grown to O.D.=0.5, arrested in G1 phase by incubation with α-factor, and released into fresh YPD with or without 200 mM hydroxyurea (HU). Images of at least 250 cells per yeast strain were acquired on a BZ-X800 microscope (Keyence) and the fraction of cells with Rad52-GFP foci (white arrows) scored. ***(B)*** Quantification of percentage of cells with Rad52-GFP foci in *rrm3*Δ, *rad5*Δ and *rrm3*Δ *rad5*Δ mutants in the presence or absence of replication stress induced by HU. Experiments were repeated three times and the mean ± SD is reported.

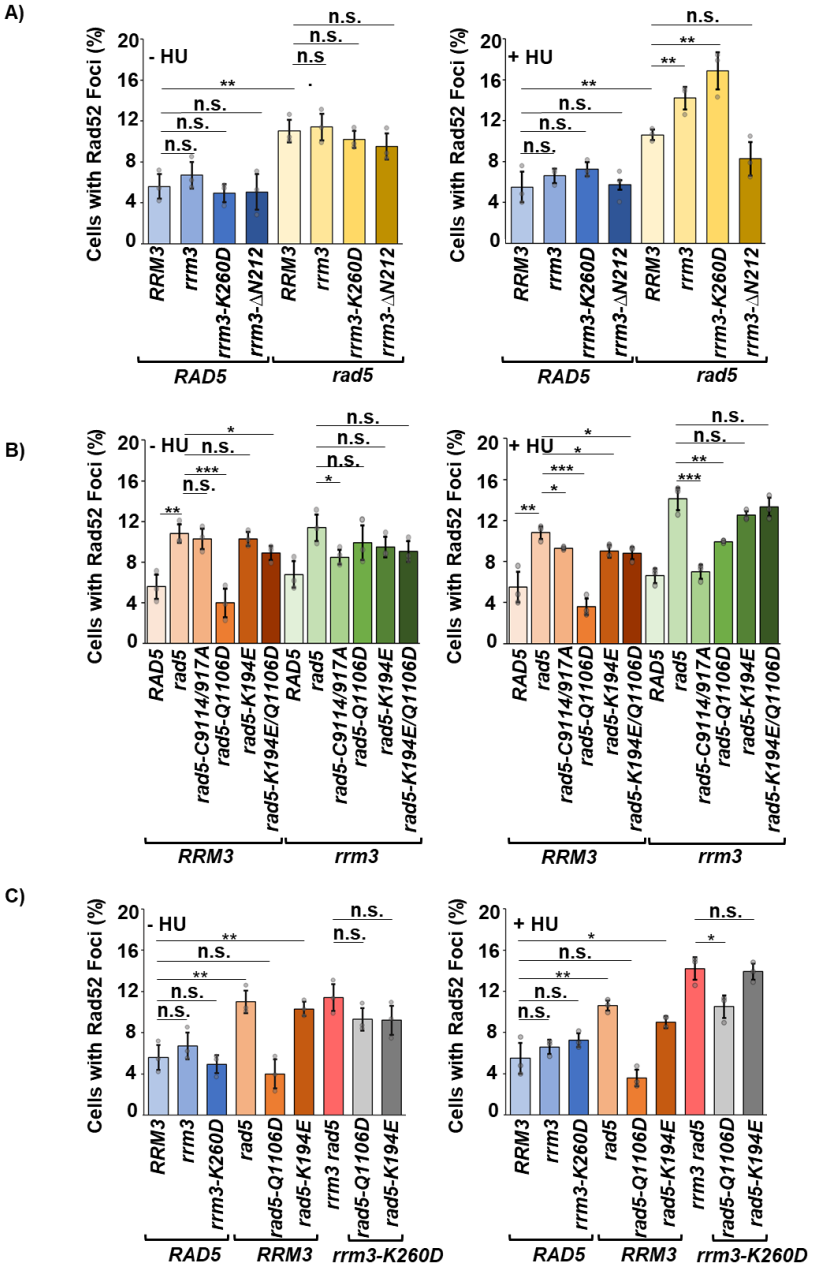

Figure 2.3. Interaction between *RRM3* and *RAD5* functions in the suppression of recombinogenic DNA lesions. ***(A)*** Quantification of Rad52-GFP foci in cells harboring an *RRM3* deletion (*rrm3*Δ), a disruption of Rrm3 helicase activity (*rrm3-K260D*), or a truncation of the Rrm3 N-terminal tail (*rrm3-*Δ*N212*). Effect of *rrm3* mutations was assessed in the presence (*RAD5*) or absence (*rad5*Δ) of Rad5 and in the presence or absence of replication stress induced by hydroxyurea (HU) following the scheme in Figure 2A. ***(B)*** Quantification of Rad52-GFP foci in cells harboring a *RAD5* deletion (*rad5*Δ), a disruption of the Rad5 RING domain (*rad5-CC914/917AA*), Rad5 HIRAN domain (*rad5-K194E*), Rad5 helicase motif VI (*rad5-Q1106D*), or both HIRAN- and helicase-domain (*rad5-K194E/Q1106D*). The effect of *rad5* mutations on Rad52-GFP foci formation was assessed in the presence (*RRM3*) or absence (*rrm3*Δ) of Rrm3 and in the presence or absence of replication stress induced by hydroxyurea (HU) following the scheme in Figure 2A. ***(C)*** Quantification of Rad52-GFP foci in cells harboring point mutations disrupting fork reversal activity of Rad5 (*rad5-K194E, rad5-Q1106D*) and helicase activity of Rrm3 (*rrm3-K260D*).

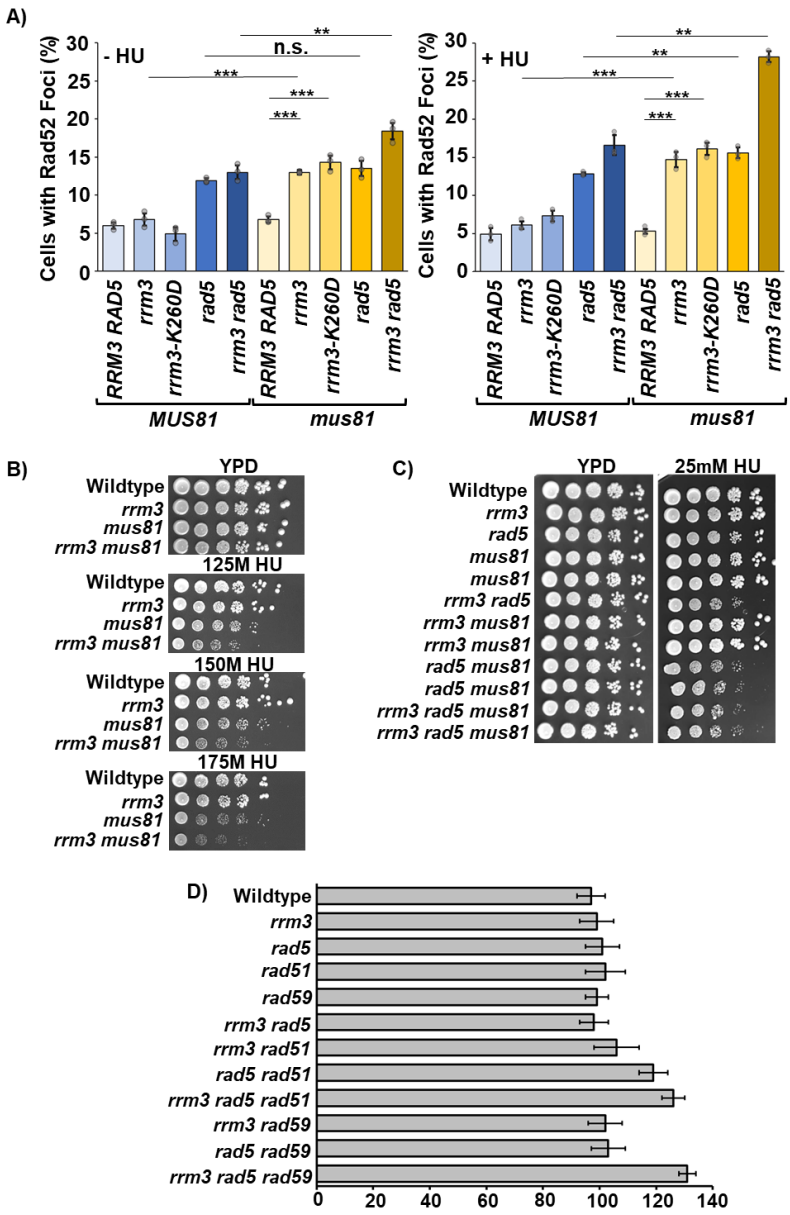

Figure 2.4. Effect of *MUS81* deletion on recombinogenic DNA lesion formation and replication stress tolerance in *rrm3*Δ and *rad5*Δ mutants. ***(A)*** Quantification of Rad52-GFP foci in cells with deletions of *RRM3* (*rrm3*Δ), *RAD5* (*rad5*Δ) or both (*rrm3*Δ *rad5*Δ*)* in the presence (*MUS81*) or absence (*mus81*Δ) of Mus81. All experiments were performed in triplicate in the presence or absence of replication stress induced by hydroxyurea (HU) following the scheme in Figure 2A. A minimum of 250 cells were scored for absence or presence of Rad52-GFP foci by fluorescence microscopy. The mean percentage of cells with Rad52-GFP foci in all three experiments is reported with standard deviation. The mean percentage of cells with Rad52-GFP foci for each of the three experiments is indicated in the graphs by gray dots. Statistical significance of differences between mutants was determined by *t* test and reported as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. ***(B)*** Spots of serial dilutions of exponentially growing *rrm3*Δ mutants harboring wildtype *MUS81* or a *mus81*Δ deletion on YPD agar plates supplemented with hydroxyurea (HU). ***(C)*** Spots of serial dilutions of exponentially growing *rrm3*Δ, *rad5*Δ and *rrm3*Δ *rad5*Δ mutants harboring wildtype *MUS81* or a *mus81*Δ deletion on YPD agar plates supplemented with hydroxyurea (HU). ***(D)*** Effect of *RAD51* and *RAD59* deletions on the doubling time of *rrm3*Δ*, rad5*Δ, and *rrm3*Δ *rad5*Δ mutants. Measurements were performed in triplicate and the mean ± SD is shown.

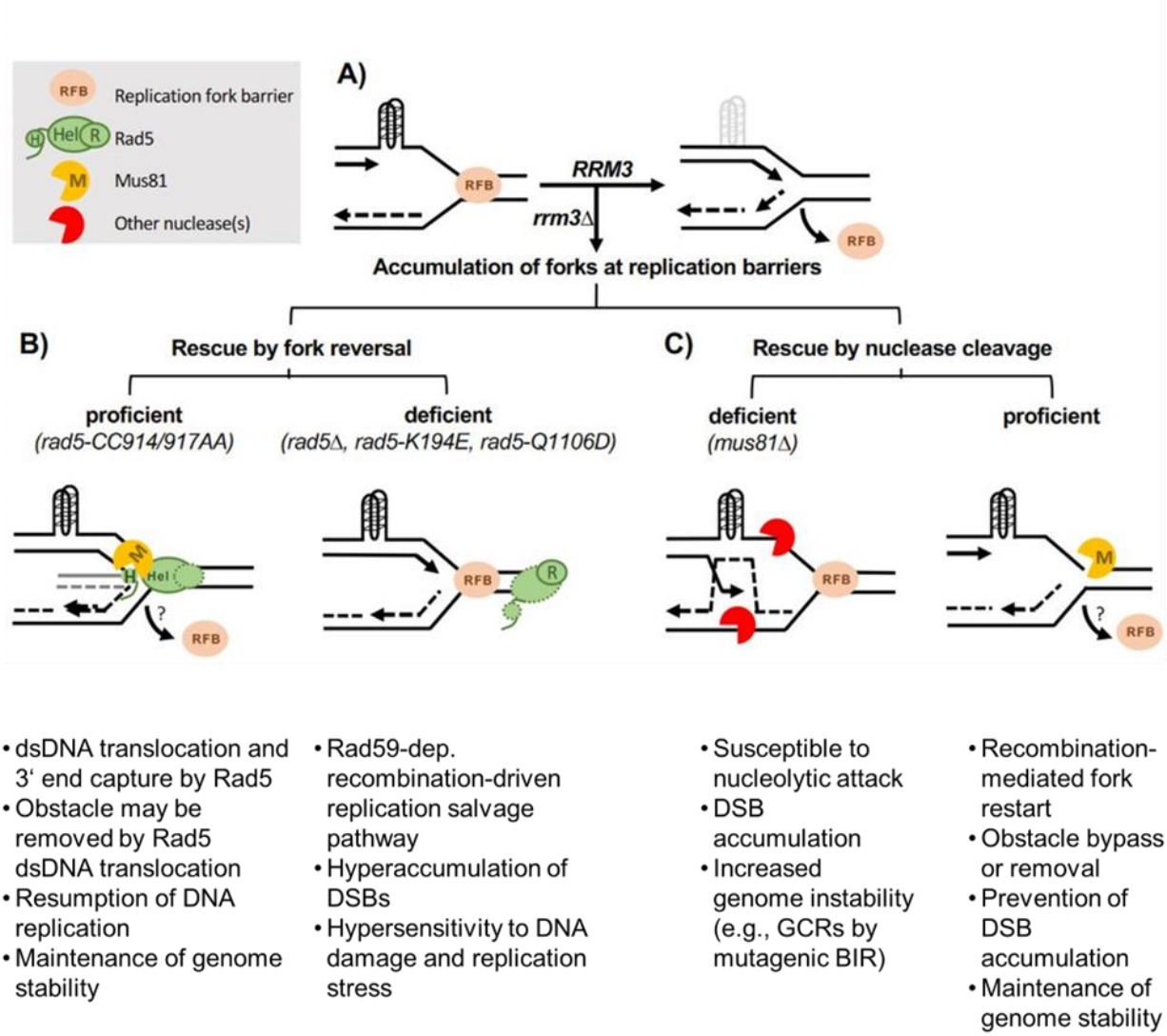

Figure 2.5. Model for overcoming replication fork barriers that accumulate in the absence of Rrm3. ***(A)*** Rrm3 facilitates fork progression by removing replication blockages (RBs), such as those formed by DNA-associated proteins. The PIF1 helicase family to which Rrm3 belongs has also been shown to unwind polymerase-blocking DNA secondary structures, such as G-quadruplexes. ***(B)*** In the absence of Rrm3, when RBs cannot be readily removed, blocked forks can be rescued by Rad5 fork reversal activity, which requires intact HIRAN (H) and helicase (Hel) motifs, preventing accumulation of recombinogenic DNA lesions. Mus81 endonuclease may act on 3-way or 4-way junctions formed during fork reversal. Rad5 translocation on DNA may also lead to removal of DNA-associated proteins. Ubiquitin-ligase-deficiency of Rad5 (*rad5-CC914/917AA*) does not impair this pathway whereas HIRAN and helicase motif mutations in Rad5 (*rad5-K194E, rad5-Q1106D*) cause hyperaccumulation of recombinogenic DNA lesions and hypersensitivity to replication stress and DNA damage. ***(C)*** Structure-specific endonuclease Mus81 can act independently of Rad5 to prevent recombinogenic DNA lesions, possibly on a subset of stalled forks that is not amenable to reversal by Rad5. In the absence of Mus81 those blocked forks persist and become susceptible to nucleolytic attack, causing recombinogenic DNA lesions and chromosome rearrangements.

Table 2.1. Effect of deletions of *RRM3*, *RAD5*, *MUS81* on accumulation of gross-chromosomal rearrangements

Relevant Genotype	GCR rate (95% Cl) Can^r $5\text{-}FOA^r$ x 10^{-10}
Wildtype	1.1 (<1-6)
rrm3	13.7 (12-16)
mus81	23.2 (18-33)
rrm3 mus81	86.6 (71-124)
rad5	103 (67-122)
rrm3 rad5	89 (81-105)
rad5 mus81	138 (61-196)
rrm3 rad5 mus81	128 (98-153)

Table 2.2. Yeast strains used in this study

Strain	Genotype
KHSY802	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3*
KHSY211	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, mus81::HIS3*
KHSY1064	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1*
KHSY2746	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad51::HIS3*
KHSY5145	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad5::HIS3*
KHSY5146	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad5::HIS3*
KHSY5194	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-ΔN212.Myc.HIS3*
KHSY5196	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-K260D.Myc.HIS3*
KHSY5520	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, Rad52.GFP.HIS3*
KHSY6428	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, Rad52.GFP.HIS3*
KHSY6429	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad5::HIS3, Rad52.GFP.HIS3*
KHSY6438	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad5::HIS3, Rad52.GFP.HIS3*
KHSY7038	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad5-CC914/917AA.Myc.HIS3*
KHSY7039	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad5-Q1106D.Myc.HIS3*
KHSY7040	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad5-K194E.Myc.HIS3*

Table 2.2 (continued)

KHSY7044	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad5-CC914/917AA.Myc.HIS3*
KHSY7045	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad5-Q1106D.Myc.HIS3*
KHSY7046	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad5-K194E.Myc.HIS3*
KHSY7049	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-K260D.Myc.HIS3, Rad52.GFP.HIS3*
KHSY7050	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-ΔN212.Myc.HIS3, Rad52.GFP.HIS3*
KHSY7051	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-K260D.Myc.HIS3, rad5::HIS3, Rad52.GFP.HIS3*
KHSY7052	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-ΔN212.Myc.HIS3, rad5::HIS3, Rad52.GFP.HIS3*
KHSY7056	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad5-CC914/917AA.Myc.HIS3, Rad52.GFP.HIS3*
KHSY7057	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad5-Q1106D.Myc.HIS3, Rad5.GFP.HIS3*
KHSY7058	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad5-K194E.Myc.HIS3, Rad52.GFP.HIS3*
KHSY7059	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad5-CC914/917AA.Myc.HIS3, Rad52.GFP.HIS3*

Table 2.2 (continued)

KHSY7060	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad5-Q1106D.Myc.HIS3, Rad52.GFP.HIS3*
KHSY7061	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad5-K194E.Myc.HIS3, Rad52.GFP.HIS3*
KHSY7064	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-K260D.Myc.HIS3, rad5-Q1106D.Myc.HIS3, Rad52.GFP.HIS3*
KHSY7065	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-K260D.Myc.HIS3, rad5-K194E.Myc.HIS3, Rad52.GFP.HIS3*
KHSY7067	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, mus81::HIS3*
KHSY7068	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad5::HIS3, mus81::HIS3*
KHSY7069	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad5::HIS3, mus81::HIS3*
KHSY7070	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, mus81::HIS3, Rad52.GFP.HIS3*
KHSY7071	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, mus81::HIS3, Rad52.GFP.HIS3*
KHSY7072	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad5::HIS3, mus81::HIS3, Rad52.GFP.HIS3*
KHSY7073	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad5::HIS3, mus81::HIS3, Rad52.GFP.HIS3*

Table 2.2 (continued)

KHSY7075	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-K260D.Myc.HIS3, mus81::HIS3, Rad52.GFP.HIS3*
KHSY7076	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad59::HIS3*
KHSY7078	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad59::HIS3*
KHSY7079	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad5::HIS3, rad59::HIS3*
KHSY7080	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad5::HIS3, rad59::HIS3*
KHSY7082	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad51::HIS3*
KHSY7083	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad5::HIS3, rad51::HIS3*
KHSY7084	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad5::HIS3, rad51::HIS3*
KHSY7125	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3,rad5-K194E/Q1106D.Myc.HIS3*
KHSY7126	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad5-K194E/Q1106D.Myc.HIS3*
KHSY7127	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad5-K194E/Q1106D.Myc.HIS3, Rad52.GFP.HIS3*
KHSY7128	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad5-K194E/Q1106D.Myc.HIS3, Rad52.GFP.HIS3*

References

1. Keil, R.L. and A.D. McWilliams, *A gene with specific and global effects on recombination of sequences from tandemly repeated genes in Saccharomyces cerevisiae.* Genetics, 1993. **135**(3): p. 711-8.
2. Ivessa, A.S., et al., *The Saccharomyces cerevisiae helicase Rrm3p facilitates replication past nonhistone protein-DNA complexes.* Mol Cell, 2003. **12**(6): p. 1525-36.
3. Ivessa, A.S., et al., *Saccharomyces Rrm3p, a 5' to 3' DNA helicase that promotes replication fork progression through telomeric and subtelomeric DNA.* Genes Dev, 2002. **16**(11): p. 1383-96.
4. Lohman, T.M., E.J. Tomko, and C.G. Wu, *Non-hexameric DNA helicases and translocases: mechanisms and regulation.* Nat Rev Mol Cell Biol, 2008. **9**(5): p. 391-401.
5. Singleton, M.R., M.S. Dillingham, and D.B. Wigley, *Structure and mechanism of helicases and nucleic acid translocases.* Annu Rev Biochem, 2007. **76**: p. 23-50.
6. Mohanty, B.K., N.K. Bairwa, and D. Bastia, *The Tof1p-Csm3p protein complex counteracts the Rrm3p helicase to control replication termination of Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 2006. **103**(4): p. 897-902.
7. Torres, J.Z., J.B. Bessler, and V.A. Zakian, *Local chromatin structure at the ribosomal DNA causes replication fork pausing and genome instability in the absence of the S. cerevisiae DNA helicase Rrm3p.* Genes Dev, 2004. **18**(5): p. 498-503.
8. Syed, S., et al., *A Novel Rrm3 Function in Restricting DNA Replication via an Orc5-Binding Domain Is Genetically Separable from Rrm3 Function as an ATPase/Helicase in Facilitating Fork Progression.* PLoS Genet, 2016. **12**(12): p. e1006451.
9. Iyer, L.M., M.M. Babu, and L. Aravind, *The HIRAN domain and recruitment of chromatin remodeling and repair activities to damaged DNA.* Cell Cycle, 2006. **5**(7): p. 775-82.
10. Johnson, R.E., et al., *Saccharomyces cerevisiae RAD5-encoded DNA repair protein contains DNA helicase and zinc-binding sequence motifs and affects the stability of simple repetitive sequences in the genome.* Mol Cell Biol, 1992. **12**(9): p. 3807-18.
11. Lorick, K.L., et al., *RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination.* Proc Natl Acad Sci U S A, 1999. **96**(20): p. 11364-9.
12. Gangavarapu, V., et al., *Mms2-Ubc13-dependent and -independent roles of Rad5 ubiquitin ligase in postreplication repair and translesion DNA synthesis in Saccharomyces cerevisiae.* Mol Cell Biol, 2006. **26**(20): p. 7783-90.
13. Davies, A.A., et al., *Activation of ubiquitin-dependent DNA damage bypass is mediated by replication protein a.* Mol Cell, 2008. **29**(5): p. 625-36.
14. Lee, K.Y. and K. Myung, *PCNA modifications for regulation of post-replication repair pathways.* Mol Cells, 2008. **26**(1): p. 5-11.
15. Hoege, C., et al., *RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.* Nature, 2002. **419**(6903): p. 135-41.
16. Ulrich, H.D., *Regulating post-translational modifications of the eukaryotic replication clamp PCNA.* DNA Repair (Amst), 2009. **8**(4): p. 461-9.
17. Kuang, L., et al., *A non-catalytic function of Rev1 in translesion DNA synthesis and mutagenesis is mediated by its stable interaction with Rad5.* DNA Repair (Amst), 2013. **12**(1): p. 27-37.
18. Xu, X., et al., *Involvement of budding yeast Rad5 in translesion DNA synthesis through physical interaction with Rev1.* Nucleic Acids Res, 2016. **44**(11): p. 5231-45.
19. Blastyak, A., et al., *Yeast Rad5 protein required for postreplication repair has a DNA helicase activity specific for replication fork regression.* Mol Cell, 2007. **28**(1): p. 167-75.

20. Shin, S., et al., *ATP Binding to Rad5 Initiates Replication Fork Reversal by Inducing the Unwinding of the Leading Arm and the Formation of the Holliday Junction.* Cell Rep, 2018. **23**(6): p. 1831-1839.
21. Kile, A.C., et al., *HLTF's Ancient HIRAN Domain Binds 3' DNA Ends to Drive Replication Fork Reversal.* Mol Cell, 2015. **58**(6): p. 1090-100.
22. Choi, K., et al., *Concerted and differential actions of two enzymatic domains underlie Rad5 contributions to DNA damage tolerance.* Nucleic Acids Res, 2015. **43**(5): p. 2666-77.
23. Fujiwara, Y. and M. Tatsumi, *Replicative bypass repair of ultraviolet damage to DNA of mammalian cells: caffeine sensitive and caffeine resistant mechanisms.* Mutat Res, 1976. **37**(1): p. 91-110.
24. Postow, L., et al., *Positive torsional strain causes the formation of a four-way junction at replication forks.* J Biol Chem, 2001. **276**(4): p. 2790-6.
25. Pages, V., et al., *Requirement of Rad5 for DNA polymerase zeta-dependent translesion synthesis in Saccharomyces cerevisiae.* Genetics, 2008. **180**(1): p. 73-82.
26. Longtine, M.S., et al., *Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae.* Yeast, 1998. **14**(10): p. 953-61.
27. Gietz, R.D. and R.A. Woods, *Yeast transformation by the LiAc/SS Carrier DNA/PEG method.* Methods Mol Biol, 2006. **313**: p. 107-20.
28. Mirzaei, H., et al., *Sgs1 truncations induce genome rearrangements but suppress detrimental effects of BLM overexpression in Saccharomyces cerevisiae.* J Mol Biol, 2011. **405**(4): p. 877-91.
29. Mirzaei, H. and K.H. Schmidt, *Non-Bloom syndrome-associated partial and total loss-of-function variants of BLM helicase.* Proc Natl Acad Sci U S A, 2012. **109**(47): p. 19357-62.
30. Schmidt, K.H., et al., *Analysis of gross-chromosomal rearrangements in Saccharomyces cerevisiae.* Methods Enzymol, 2006. **409**: p. 462-76.
31. Schmidt, K.H., et al., *Defects in DNA lesion bypass lead to spontaneous chromosomal rearrangements and increased cell death.* Eukaryot Cell, 2010. **9**(2): p. 315-24.
32. Lea, D.E. and C.A. Coulson, *The distribution of the numbers of mutants in bacterial populations.* J Genet, 1949. **49**(3): p. 264-85.
33. Lisby, M., R. Rothstein, and U.H. Mortensen, *Rad52 forms DNA repair and recombination centers during S phase.* Proc Natl Acad Sci U S A, 2001. **98**(15): p. 8276-82.
34. Schmidt, K.H., K.L. Derry, and R.D. Kolodner, *Saccharomyces cerevisiae RRM3, a 5' to 3' DNA helicase, physically interacts with proliferating cell nuclear antigen.* J Biol Chem, 2002. **277**(47): p. 45331-7.
35. Rossi, S.E., et al., *Rad53-Mediated Regulation of Rrm3 and Pif1 DNA Helicases Contributes to Prevention of Aberrant Fork Transitions under Replication Stress.* Cell Rep, 2015. **13**(1): p. 80-92.
36. Cotta-Ramusino, C., et al., *Exo1 processes stalled replication forks and counteracts fork reversal in checkpoint-defective cells.* Mol Cell, 2005. **17**(1): p. 153-9.
37. Froget, B., et al., *Cleavage of stalled forks by fission yeast Mus81/Eme1 in absence of DNA replication checkpoint.* Mol Biol Cell, 2008. **19**(2): p. 445-56.
38. Ehmsen, K.T. and W.D. Heyer, *A junction branch point adjacent to a DNA backbone nick directs substrate cleavage by Saccharomyces cerevisiae Mus81-Mms4.* Nucleic Acids Res, 2009. **37**(6): p. 2026-36.
39. Fricke, W.M., S.A. Bastin-Shanower, and S.J. Brill, *Substrate specificity of the Saccharomyces cerevisiae Mus81-Mms4 endonuclease.* DNA Repair (Amst), 2005. **4**(2): p. 243-51.

40. Wyatt, H.D. and S.C. West, *Holliday junction resolvases.* Cold Spring Harb Perspect Biol, 2014. **6**(9): p. a023192.
41. Schwartz, E.K., et al., *Saccharomyces cerevisiae Mus81-Mms4 prevents accelerated senescence in telomerase-deficient cells.* PLoS Genet, 2020. **16**(5): p. e1008816.
42. Chen, C. and R.D. Kolodner, *Gross chromosomal rearrangements in Saccharomyces cerevisiae replication and recombination defective mutants.* Nat Genet, 1999. **23**(1): p. 81-5.
43. Lee, S.E., et al., *Yeast Rad52 and Rad51 recombination proteins define a second pathway of DNA damage assessment in response to a single double-strand break.* Mol Cell Biol, 2003. **23**(23): p. 8913-23.
44. Symington, L.S., *Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair.* Microbiol Mol Biol Rev, 2002. **66**(4): p. 630-70, table of contents.
45. Bai, Y. and L.S. Symington, *A Rad52 homolog is required for RAD51-independent mitotic recombination in Saccharomyces cerevisiae.* Genes Dev, 1996. **10**(16): p. 2025-37.
46. Ira, G. and J.E. Haber, *Characterization of RAD51-independent break-induced replication that acts preferentially with short homologous sequences.* Mol Cell Biol, 2002. **22**(18): p. 6384-92.
47. Signon, L., et al., *Genetic requirements for RAD51- and RAD54-independent break-induced replication repair of a chromosomal double-strand break.* Mol Cell Biol, 2001. **21**(6): p. 2048-56.
48. Gildenberg, M.S. and M.T. Washington, *Conformational flexibility of fork-remodeling helicase Rad5 shown by full-ensemble hybrid methods.* PLoS One, 2019. **14**(10): p. e0223875.
49. Shen, M., et al., *Structural basis for the multi-activity factor Rad5 in replication stress tolerance.* Nat Commun, 2021. **12**(1): p. 321.
50. Gallo, D., et al., *Rad5 Recruits Error-Prone DNA Polymerases for Mutagenic Repair of ssDNA Gaps on Undamaged Templates.* Mol Cell, 2019. **73**(5): p. 900-914 e9.
51. Masłowska, K. and V. Pagès, *Rad5 participates in lesion bypass through its Rev1-binding and ubiquitin ligase domains, but not through its helicase function.* Front. Mol. Biosci., 2022. **9**(1062027): p. doi: 10.3389/fmolb.2022.1062027.
52. Gallo, D. and G.W. Brown, *Post-replication repair: Rad5/HLTF regulation, activity on undamaged templates, and relationship to cancer.* Crit Rev Biochem Mol Biol, 2019. **54**(3): p. 301-332.
53. Chen, S., et al., *The RING finger ATPase Rad5p of Saccharomyces cerevisiae contributes to DNA double-strand break repair in a ubiquitin-independent manner.* Nucleic Acids Res, 2005. **33**(18): p. 5878-86.
54. Minca, E.C. and D. Kowalski, *Multiple Rad5 activities mediate sister chromatid recombination to bypass DNA damage at stalled replication forks.* Mol Cell, 2010. **38**(5): p. 649-61.
55. Ball, L.G., et al., *The Rad5 helicase activity is dispensable for error-free DNA post-replication repair.* DNA Repair (Amst), 2014. **16**: p. 74-83.
56. Ortiz-Bazan, M.A., et al., *Rad5 plays a major role in the cellular response to DNA damage during chromosome replication.* Cell Rep, 2014. **9**(2): p. 460-8.
57. Sogo, J.M., M. Lopes, and M. Foiani, *Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects.* Science, 2002. **297**(5581): p. 599-602.
58. Feng, W., et al., *Genomic mapping of single-stranded DNA in hydroxyurea-challenged yeasts identifies origins of replication.* Nat Cell Biol, 2006. **8**(2): p. 148-55.

59. Ivessa, A.S., J.Q. Zhou, and V.A. Zakian, *The Saccharomyces Pif1p DNA helicase and the highly related Rrm3p have opposite effects on replication fork progression in ribosomal DNA.* Cell, 2000. **100**(4): p. 479-89.
60. Lee, M., et al., *Rad52/Rad59-dependent recombination as a means to rectify faulty Okazaki fragment processing.* J Biol Chem, 2014. **289**(21): p. 15064-79.
61. Zou, H. and R. Rothstein, *Holliday junctions accumulate in replication mutants via a RecA homolog-independent mechanism.* Cell, 1997. **90**(1): p. 87-96.
62. Irmisch, A., et al., *Smc5/6 maintains stalled replication forks in a recombination-competent conformation.* EMBO J, 2009. **28**(2): p. 144-55.
63. Campos-Doerfler, L., S. Syed, and K.H. Schmidt, *Sgs1 Binding to Rad51 Stimulates Homology-Directed DNA Repair in Saccharomyces cerevisiae.* Genetics, 2018. **208**(1): p. 125-138.
64. McGlynn, P. and R.G. Lloyd, *Recombinational repair and restart of damaged replication forks.* Nat Rev Mol Cell Biol, 2002. **3**(11): p. 859-70.
65. Paeschke, K., et al., *Pif1 family helicases suppress genome instability at G-quadruplex motifs.* Nature, 2013. **497**(7450): p. 458-62.
66. Muellner, J. and K.H. Schmidt, *Yeast Genome Maintenance by the Multifunctional PIF1 DNA Helicase Family.* Genes (Basel), 2020. **11**(2).
67. Pasero, P. and A. Vindigni, *Nucleases Acting at Stalled Forks: How to Reboot the Replication Program with a Few Shortcuts.* Annu Rev Genet, 2017. **51**: p. 477-499.
68. Branzei, D., M. Seki, and T. Enomoto, *Rad18/Rad5/Mms2-mediated polyubiquitination of PCNA is implicated in replication completion during replication stress.* Genes Cells, 2004. **9**(11): p. 1031-42.
69. Branzei, D. and B. Szakal, *DNA damage tolerance by recombination: Molecular pathways and DNA structures.* DNA Repair (Amst), 2016. **44**: p. 68-75.
70. Vanoli, F., et al., *Replication and recombination factors contributing to recombination-dependent bypass of DNA lesions by template switch.* PLoS Genet, 2010. **6**(11): p. e1001205.
71. Jimenez-Martin, A., et al., *The Mgs1/WRNIP1 ATPase is required to prevent a recombination salvage pathway at damaged replication forks.* Sci Adv, 2020. **6**(15): p. eaaz3327.
72. Mayle, R., et al., *DNA REPAIR. Mus81 and converging forks limit the mutagenicity of replication fork breakage.* Science, 2015. **349**(6249): p. 742-7.
73. Deem, A., et al., *Break-induced replication is highly inaccurate.* PLoS Biol, 2011. **9**(2): p. e1000594.
74. Schwartz, E.K. and W.D. Heyer, *Processing of joint molecule intermediates by structure-selective endonucleases during homologous recombination in eukaryotes.* Chromosoma, 2011. **120**(2): p. 109-27.

Chapter Three: Physical interaction of Rrm3 and PCNA results in aberrant recombination when PCNA's inter-domain connecting loop cannot be modified at lysine 127

Abstract

DNA synthesis needs to be executed in a timely and accurate manner. To ensure DNA replication fork progression, the cell utilizes multiple pathways, which are specifically designed to deal with the endogenous and exogenous sources of DNA damage. In *Saccharomyces cerevisiae*, Rrm3, a member of the Pif1 DNA helicase family, is required to ensure DNA replication fork progression as deletion of *RRM3* or disruption of its 5'-3' helicase activity leads to the accumulation of stalled replication forks genome wide. While these helicase-mediated functions of Rrm3 are becoming better understood, a biological role for the physical interaction of Rrm3 with PCNA has remained elusive. Here, we define a novel genetic interaction between PCNA and Rrm3 under replication stress that sheds light on the physical interaction between the two proteins. We find that Rrm3 is toxic to cells if PCNA cannot be modified at lysine 127 but not when PCNA cannot be modified at lysine 164. Notably, this toxicity of Rrm3 is caused by a disordered segment of the first 54 residues which contains a PCNA-interaction-protein (PIP)-box motif, whereas the DNA helicase activity of Rrm3 is nontoxic. The deleterious effect of the PIP-box domain is associated with an accumulation of DNA lesions that bind the homologous recombination factor Rad52. Restoration of replication stress tolerance by interrupting binding between Rrm3 and PCNA non-modifiable at lysine 127 is dependent on the SUMO E3-ligase Siz1, and to a lesser extent Siz2; together with the observation that the Rrm3 toxicity is independent of its DNA helicase activity this suggests that Rrm3 may prevent another protein from binding to PCNA. In conclusion, our findings suggest that the physical interaction between Rrm3 and PCNA needs to be tightly regulated under replication stress by modification of lysine

127 near the interdomain-connector loop of PCNA where Rrm3 binds to achieve proper repair of replication-stress-induced DNA lesions.

Introduction

DNA replication needs to be performed in an efficient and precise manner for accurately duplication of the genome. During DNA synthesis, the DNA replication machinery encounters multiple obstacles, which could impair replication fork progression [1, 2]. Therefore, the cell possesses multiple pathways to deal with specific sources of DNA damage [1, 2]. One of the proteins promoting replication fork progression is Rrm3 [3-5]. Rrm3 belongs into the PIF1 DNA helicase family, which are conserved from bacteria to humans [6, 7]. Members of this family possess a 5'-3' translocase activity which is embedded in the highly conserved helicase motif of the C-terminus [8-10]. In the absence of Rrm3 or disrupting its catalytical activity results in the accumulation of stalled replication forks at multiple sites such as rDNA locus, tRNA genes, centromeres, and many more [4, 5, 11, 12]. In contrast, the N-terminus is highly disordered and varies among members of the PIF1 DNA helicase family, promoting different protein-protein interaction or are site of post-translational modifications and thereby executing unique functions [13-15]. Indeed, the N-terminus becomes phosphorylated and harbors the binding site for Orc5, a subunit of the origin complex [13, 14]. Moreover, the binding site of the proliferating cell nuclear antigen (PCNA) resides within Rrm3's N-terminus [16]. Rrm3, just like other PCNA interacting proteins, possesses a canonical PCNA-interacting protein motif (PIP-box) with the consensus motif Q-x-x-(I/L/M/V)-x-x-(F/Y)-(F/Y) within the first 54 amino acids of the N-terminus [16-18]. In *S. cerevisiae*, PCNA is encoded by the gene *POL30* and is an essential gene in DNA synthesis [19]. PCNA is a sliding clamp, which is composed of three monomers, forming a functional homotrimer. During DNA synthesis PCNA functions as a processivity factor for the replicative DNA polymerases to promote DNA replication [20-23]. Upon DNA damage, PCNA can undergo post-translational modifications resulting in SUMOylation or ubiquitination of lysine

residues to deal with replication stress [1, 2, 24-26]. Mono-ubiquitination of lysine 164 by the Rad6-Rad18 complex results in the replacement of the replicative DNA polymerase with a translesion (TLS) DNA polymerase [26-30]. Depending on the DNA lesion and choice of TLS polymerase, this pathway can be error-prone or error-free [31-34]. Furthermore, lysine 164 can be poly-ubiquitinated by the formation of ubiquitin-chains, which is promoted by the E3 RING ligase Rad5 in a complex with Mms2 and Ubc13 [24, 26, 35-38]. This modification initiated the error-free pathway, using the synthesized sister chromatid for template switching to bypass DNA lesions [2, 24, 38-40]. On the other hand, lysine 164 and lysine 127 can be SUMOylated by the E3 ligases Siz1 and Siz2, respectively [26, 29, 41]. While lysine 127, which is in the inter-domain connecting loop (IDCL), possesses a canonical SUMO consensus motif ΨKx(D/E), lysine 164 does not [42-44]. Sumoylation of lysine 127 and lysine 164 promotes the recruitment of the anti-recombinase Srs2, which inhibits homologous recombination by dismantling Rad51 nucleofilaments [45, 46]. Additionally, binding of Srs2 with SUMOylated PCNA results in the displacement of Pol32 and thereby, limiting D-loop extension [47]. Sumoylation of PCNA at lysine 127 suppresses homologous recombination but also leads to the displacement of Eco1, which is important in cohesion establishment [48-50]. Thus, it is possible that modification at lysine 127 functions as a reset button to promote new protein interactions in response to DNA replication stress [19].

Despite of the known binding site of PCNA for the past 20 years, the biological relevance of the interaction between the DNA helicase Rrm3 and PCNA has remained unknown. Here, we are investigating the genetic interaction between *POL30* mutants and *RRM3* mutants.

Materials and Methods

Yeast strains and media

Yeast strains were derived from S288C-derived strain KHSY802 (*MATa, ura3-52, trp1Δ63, his3Δ200, leu2Δ1, lys2-Bgl, hom3-10, ade2Δ1, ade8, hxt13::URA3*). *POL30* point mutations were purchased from Addgene and incorporated at the endogenous *POL30 locus* by LiAc-mediated transformation and replaced by 5-FOA selection as described [14, 51]. The presence of the desired mutations and successful replacement of the endogenous locus was verified by sequencing the *POL30* open reading frame. The C-terminus of Rad52 was tagged with GFP using plasmid pFA6a-GFP(S65T)-HIS3MX6 as a template [52]. Desired Srs2 point mutations or deletions were generated by site-directed mutagenesis and PCR respectively. The replacement of the native SRS2 locus was confirmed by PCR. Haploid strains with more than one modified genomic locus were obtained by sporulating diploids heterozygous for the desired mutations and genotyping spores on selective media or by PCR. Yeast were grown at 30°C in yeast extract (10g/L) peptone (20g/L) and dextrose (20g/L) (YPD) or synthetic complete (SC) media, as previously described. Solid media was supplemented with agar (20 g/l) agar. Yeast strains used in this study are listed in Table 3.1.

Spot assay

The sensitivity of yeast cells to DNA damaging agents or replication stress was tested via spot assay as previously described [14]. Briefly, cell cultures were grown in exponential phase to an OD_{600} = 0.5 in yeast extract (10g/L) peptone (20g/L) and dextrose (20g/L) (YPD) and either 5-fold or 10-fold serial dilutions were spotted on YPD supplemented with hydroxyurea (HU) (US Biological) or methyl methane sulfate (MMS) at the indicated concentrations. Colony growth was recorded every 12h for 3-5 days of incubation at 30 °C with a Gel-Doc IT Imaging system (UVP).

Fluorescence microscopy

To determine the fraction of cells with nuclear Rad52 foci, cell cultures were grown in exponential phase to an OD_{600} = 0.5 in YPD and arrested in G1 phase by adding alpha-factor (2 µg/µL) and continuing incubation at 30° C for 90 min, with a second addition of alpha-factor at 45 min. Cells were washed in water and released into prewarmed YPD with 200 mM HU for 90 min at 30° C. For fluorescence microscopy, cells were spotted on agarose pads and images acquired on a BZ-X800 (Keyence) fitted with a 60x objective. Presence of Rad52-GFP foci was evaluated in at least 250 cells from three independent isolates per yeast strain. The mean with standard deviation is reported. Student t-tests were performed to determine statistical significance of differences between the fraction of cells with Rad52-GFP foci in different yeast strains.

Results

DNA helicase Rrm3 becomes toxic under replication stress when the interdomain-connecting loop of PCNA cannot be modified at lysine 127

The physical interaction between Rrm3 and PCNA via an N-terminal PIP-box in Rrm3 was identified over 20 years ago yet a biological role of this interaction has remained elusive [16]. Our recent identification of a novel functional interaction during replication stress between the helicase-activities of Rrm3 and Rad5, the latter of which also ubiquitinates PCNA, prompted us to investigate the possibility of genetic interactions between *RRM3* and *POL30*. To this end, we replaced the endogenous *POL30* ORF with previously described *pol30* mutant alleles, specifically *pol30-K127R*, coding for PCNA that cannot be SUMOylated at lysine 127, *pol30-K164R*, coding for PCNA that cannot be SUMOylated or ubiquitinated on lysine 164, and *pol30-K127/164*, coding for PCNA defective in modification at both lysine 127 and 164 [26, 48, 51]. Inability to modify lysine 164 (*pol30-K164R*) caused hypersensitivity to HU-induced replication

stress and to MMS-induced DNA damage (Figure 3.1A), consistent with previous reports [48, 51]. This hypersensitivity was suppressed by disrupting PCNA modification on lysine 127, which maps to the IDCL of PCNA and is embedded in the SUMOylation consensus motif ΨKXE, (*pol30-K127/K164R*) [48, 51] (Figure 3.1A). In contrast, only mutating lysine 127 (*pol30-K127R*) increases sensitivity to both replication stress and DNA damage, albeit to a lesser extent than mutating lysine 164 (*pol30-K164R*) or both (*pol30-K127/164R*) [42, 48, 51] (Figure 3.1A). When we combined these *pol30* mutant alleles with an *RRM3* deletion (*rrm3*Δ) we observed suppression of the hypersensitivity of the *pol30-K127R* mutant to HU but not MMS (Figure 3.1B), but no suppression of either HU or MMS hypersensitivity of the *pol30-K164R* mutant (Figure 3.1C.) or the *pol30-K127R/K164R* double mutant (Figure 3.1D). This genetic analysis indicated that Rrm3 is toxic in cells under replication stress if they cannot SUMOylate PCNA at lysine 127. In contrast, Rrm3 does not contribute to HU/MMS hypersensitivity of cells that cannot modify PCNA at lysine 164.

The N-terminal disordered region of Rrm3, not its ATPase/helicase activity, suppresses the sensitivity of HU-induced replication stress and accumulation of Rad52 foci in the *pol30-K127R* mutant

Rrm3's protein structure can be divided into a conserved C-terminus (residues 213-723), harboring its 5'-3' DNA helicase activity, and into its disordered N-terminus (residues 1-212), which includes the PIP-Box motif, a phosphorylation cluster and binding site for protein-protein interaction [13, 14, 16]. To distinguish which function of Rrm3 leads to sensitivity when lysine 127 cannot be modified under replication stress, we deleted the disordered N-terminus of Rrm3 (*rrm3-*Δ*N212*), which leaves the ATPase/helicase activity of Rrm3 intact, or disrupted the helicase activity of Rrm3 (*rrm3-K260D*). Unexpectedly, the *rrm3-*Δ*N212* mutation but not the *rrm3-K260D* mutation suppressed the sensitivity of HU-induced replication stress in the *pol30-K127R* mutant similar to the effect of deleting *RRM3* (*rrm3*Δ) (Figure 3.2A), suggesting that a

function mapping to the first 212 residues of Rrm3, encompassing the disordered N-terminal tail, is toxic when cells under replication stress cannot SUMOylate PCNA at K127. We found that removing the first 54 residues of Rrm3 (*rrm3-Δ54)* was sufficient to suppress the sensitivity of the *pol30-K127R* mutant to HU (Figure 3.2B). This segment of 54 residues of Rrm3 contains a PCNA-interaction-protein (PIP)-box motif which was previously verified to interact with PCNA in vitro and in a yeast-two hybrid assay [16]. Thus, it appears that the HU hypersensitivity of the *pol30-K127R* mutant is caused by a toxic physical interaction between PCNA-K127R and Rrm3, whereas the enzymatic activity of Rrm3 is harmless.

Since mutation of lysine 127 of PCNA reduces the hypersensitivity to replication stress (HU) and DNA damage (MMS) when lysine 164 of PCNA cannot be modified, it was proposed that SUMOylation on lysine 127 inhibits DNA repair [48]. Indeed, SUMOylation of lysine 127 and lysine 164 lead to the recruitment of anti-recombinase Srs2 [45], which prevents Rad51 filament formation and therefore, inhibits HR-dependent DNA repair [45, 46]. Based on these previous findings, I hypothesized that preventing SUMOylation at lysine 127 of PCNA leads to diminished Srs2 recruitment which in turn results in hyperrecombination and, thus, increased sensitivity to HU-induced replication stress in the *pol30-K127R* mutant. To test this hypothesis, we used fluorescence microscopy to measure the appearance of nuclear Rad52-GFP foci as an indicator of accumulating recombinogenic DNA lesions. First, we observed a significant increase in cells with Rad52-GFP foci in cultures of the *pol30-K127R* mutant compared to cultures of wildtype cells in the presence or absence of HU, whereas Rad52-GFP foci appearance did not change in any of the *rrm3* mutants we tested (*rrm3Δ, rrm3-K260D, rrm3-ΔN212, rrm3-ΔN54, or rrm3-K260D/ΔN212).* (Figure 3.3A-B). However, when we deleted *RRM3* (*rrm3Δ*) in the *pol30*-K127R mutant accumulation of Rad52-GFP foci was suppressed to wildtype levels, both in the presence or absence of replication stress (Figure 3.3A-B). This suppression of Rad52-foci accumulation in the *pol30-K127R* mutant in the presence or absence of replication stress could

be achieved by removing the N-terminal tail of Rrm3 (*rrm3-ΔN212, rrm3-K260D/ΔN212*), specifically the first 54 residues, but not by disrupting its helicase activity (*rrm3-K260D*) (Figure 3.3A-B). Thus, a deleterious physical interaction between Rrm3 and PCNA-K127R not only causes hypersensitivity to HU-induced replication stress but also accumulation of recombinogenic DNA lesions.

SUMO E3-ligases Siz1 and Siz2 are required, whereas Rad18 is not, for the suppression of *pol30-K127R* in the absence of Rrm3

In *S. cerevisiae* SUMOylation of PCNA is performed by the E3-ligases Siz1 and Siz2, which bind to lysine 164 and lysine 127, respectively [26, 29, 41]. If the absence of Rrm3 suppresses the sensitivity in the *pol30-K127R* mutant under replication stress because PCNA cannot be SUMOylated at lysine 127, then further deletion of *SIZ2* (*siz2*Δ) should not alter the DNA damage sensitivity. Deletion of *SIZ2* (*siz2*Δ) does not result in sensitivity in the presence or absence of Rrm3 under replication stress. As expected, I also did not observe changes in sensitivity when deleting the E3 ligase *SIZ2* (*siz2*Δ) in the *pol30-K127R* mutant (Figure 3.4B) under DNA damage and replication stress. Interestingly, I observed an increased DNA damage sensitivity when further deleting *SIZ2* (*siz2*Δ) in the *rrm3*Δ *pol30-K127R* mutant indicating that the suppression of the DNA damage sensitivity in the absence of Rrm3 depends on Siz2's function outside of SUMOylating PCNA.

Additionally, we determined that deletion of *RRM3* suppresses the DNA damage sensitivity to the same extent as mutating lysine 164 (*pol30-K127/164R*). Since lysine 164 can undergo SUMOylation or ubiquitination, we deleted the genes *SIZ1* (*siz1*Δ) and *RAD18* (*rad18*Δ), respectively, to distinguish between them. Deletion of *SIZ*1 (*siz1*Δ) does not cause DNA damage sensitivity in the presence or absence of Rrm3 or in the *pol30-K127R* mutant. However, further deletion of *SIZ*1 (*siz1*Δ) in the *rrm3*Δ *pol30-K127R* mutant causes increased

DNA damage sensitivity (Figure 3.4A). In contrast, inhibiting ubiquitination of lysine 164 by deleting *RAD18* (*rad18*Δ) increased DNA damage sensitivity in the presence or absence of Rrm3. We also observe an increased DNA damage sensitivity when further deleting *RAD18* in the *pol30-K127R* mutant (Figure 3.4C). Unlike deletion of *SIZ1* (*siz1*Δ), further deletion of *RAD18* (*rad18*Δ) in *rrm3*Δ *pol30-K127R* mutants, does not lead to increased DNA damage sensitivity compared to either *rrm3*Δ *rad18*Δ or *rad18*Δ *pol30-K*127R mutants.

Even though, deletion of *RRM3* suppresses the DNA damage sensitivity to the same extent as mutating lysine 164 (*pol30-K127/164R*), this occurs independently of SUMOylation or ubiquitination of lysine 164. Nonetheless, a downstream target of Siz1 and to a lesser extent of Siz2 is required for the suppression of the DNA damage sensitivity of the *pol30-K127R* mutant in the absence of Rrm3.

Srs2's functions, not Eco1's, are responsible for sensitivity in the *pol30-K127R* mutant under DNA damage, but not under replication stress

To better understand how deletion of *RRM3* suppresses the sensitivity of HU-induced replication stress in the *pol30-K127R* mutant, we considered the role of Srs2. Deletion of *SRS2* (*srs2*Δ) in the *rrm3*Δ mutant leads to a severe fitness defect [53] and SUMOylation of PCNA at K127 leads to the recruitment of Srs2. To determine which roles of Srs2 are important for normal growth of the *rrm3*Δ mutant we generated *srs2* mutations that disrupt the helicase activity (*srs2-K41A*), Rad51 binding (*srs2*Δ*875-902*), D-loop extension (*srs2-SIM*) and PCNA binding (*srs2-A1159E*) [46, 47, 54-56]. When combining these *srs2* mutations with the *rrm3*Δ mutation we found that only the *rrm3*Δ *srs2-K41A* mutant had a severe fitness defect, indicated by slow growth on YPD, and exhibited hypersensitivity to HU and MMS. This indicates that cells lacking the Rrm3 helicase require the helicase activity of Srs2 for viability and DNA damage tolerance whereas the other functions of Srs2 are dispensable (Figure 3.5A-C). The *rrm3*Δ *srs2-*

*K41A pol30-K127*R triple mutant had the same growth defect and HU/MMS sensitivity as the *rrm3*Δ *srs2-K41A* mutant indicating that Rrm3 and Srs2 helicase activity are required for normal growth and HU/MMS tolerance irrespective of PCNA status (Figure 3.5D).

While none of the other srs2 mutations (*srs2*Δ*875-902*, *srs2-SIM*, *srs2-A1159E*) were synthetically sick with *rrm3*Δ or affected the sensitivity of the *rrm3*Δ mutant to HU-induced replication stress (Figure 3.5A-D) they did increase sensitivity after MMS-induced DNA damage when combined with an *RRM3* deletion (*rrm3*Δ) (Figure 3.5A-C). Notably, the *srs2* mutations *srs2*Δ*875-902*, *srs2-SIM* and *srs2-A1159E* suppressed the MMS hypersensitivity in the *pol30-K127R* mutant while having no effect on HU hypersensitivity in the *pol30-K127R* mutant (Figure 3.5A-C). This contrasts with the *rrm3* mutations, which suppress the HU hypersensitivity of the *pol30-K127R* mutant, but not its MMS hypersensitivity. The ability of the *srs2* mutations to suppress the MMS hypersensitivity of the *pol30-K127R* mutant was at least partially dependent on Rrm3, indicating that Rrm3-mediated events deal with DNA damage in these *srs2 pol30-K127R* mutants (Figure 3.5A-D).

Unmodified PCNA residue 127 also contributes to the binding site for the acetyltransferase Eco1 as interaction between PCNA and Eco1 is disrupted upon SUMOylation of lysine 127 [50]. It was therefore conceivable that the *pol30-K127R* mutation stabilizes Eco1 binding to PCNA and impairs the regulation of the binding event in response to replication stress and DNA damage. Eco1 binding leads to acetylation of lysine 20 on PCNA, which promotes DNA damage tolerance by an HR-dependent pathway using the sister chromatid (template switching) [57].Thus, in cells exposed to genotoxic agents like HU and MMS, SUMOylation of PCNA K127 would be expected to disrupt Eco1 binding, K20 acetylation and, thus, HR at the DNA replication fork. In contrast, in the *pol30-K127R* mutant, Eco1 binding would not be disrupted upon genotoxin exposure, maintaining (undesirable) K20 acetylation and HR at the replication fork. Thus, like the pol30-K127R mutation, a *pol30-K20Q* mutation, which mimics

K20 acetylation, would be expected to cause hypersensitivity to genotoxins. If so, this hypersensitivity might be suppressed by an *RRM3* deletion similar to suppression of genotoxin hypersensitivity caused by the *pol30-K127R* mutation. To test this, we constructed strains expressing PCNA-K20Q (*pol30-K20Q)* or PCNA-K20A (*pol30*-K20A), which mimic or inhibit lysine 20 acetylation, respectively, and combined them with an *RRM3* deletion. While both *pol30-K20Q* and *pol30-K20A* caused genotoxin hypersensitivity, *RRM3* deletion could not suppress it (Figure 3.6). This suggests that the toxicity of Rrm3 in the HU-treated *pol30-K127R* mutant is not related to HR dysregulation by PCNA K20 (hyper)acetylation.

Physical interaction between Rrm3 and PCNA is toxic in the *pol32*∆ mutant

As shown above, the interaction between PCNA and the PIP-box containing N-terminal 54 residues of Rrm3 is toxic when PCNA cannot be modified at lysine 127 during replication stress. To test if Rrm3 was also toxic in cells carrying other replisome defects, we tested a *POL32* deletion. *POL32* encodes the nonessential subunit of DNA polymerase δ, linking the polymerase to PCNA through its PIP-box to ensure high processivity and fidelity of DNA replication [58-61]. While the other two subunits of DNA polymerase δ, Pol3 and Pol31, are essential, Pol32 is not, but its absence results in DNA replication defects and renders cells sensitive to replication stress [60, 61]. Remarkably, deletion of *RRM3* suppressed the hypersensitivity of the *pol32*∆ mutant to HU-induced replication stress. To determine which function of Rrm3 was toxic in the *pol32*∆ mutant, we introduced the *rrm3-K260D* or *rrm3-*∆*N54* mutations into the *pol32*∆ mutant. Like the *pol30-K127R* mutant, the first 54 residues of Rrm3, containing the PIP-box, were toxic in the *pol32*∆ mutant whereas the ATPase/helicase activity of Rrm3 was nontoxic (Figure 3.7.). This suggests that the PIP-box mediated interaction of Rrm3 with PCNA contributes to the replication-stress hypersensitivity of cells in which Pol δ has been uncoupled from PCNA.

Discussion

During the DNA damage response PCNA undergoes post-translational modifications and acts as a platform to recruit proteins to bypass the DNA lesions [19, 62]. One of the proteins shown to interact with PCNA via its PIP-box is Rrm3 [16]. In this study I identified a positive genetic interaction between *RRM3* and *POL30*, when lysine 127 cannot be modified, under replication stress. We further showed that disrupting the interaction with PCNA (*rrm3-ΔN54*), rather than Rrm3's helicase activity (*rrm3-K260D*) suppresses the sensitivity of HU-induced replication stress and accumulation of Rad52-foci in the *pol30-K127R* mutant.

We investigated the genetic interaction between Rrm3 and *pol30* mutants that cannot be modified at lysine 127 (*pol30-K127R*), lysine 164 (*pol30-K164R*), or both (*pol30-K127/164R*). In our previous publication, we did not observe a requirement for Rad5's ubiquitin-ligase activity, which is needed for poly-ubiquitination of lysine 164, in the absence of Rrm3 to deal with replication stress or DNA damage [26, 63]. In accordance with this observation, we also do not identify a genetic interaction between *RRM3* and *POL30* when lysine 164 cannot be modified. Further confirming that the error-free pathway is no more required for dealing with replication stress or DNA damage in the *rrm3Δ* mutant than in wildtype cells. In contrast, deleting *RRM3* (*rrm3Δ*) suppresses the sensitivity of cells that are unable to modify PCNA on lysine 127 under replication stress. Lysine 127 resides within the canonical SUMO consensus motif ΨKx(D/E) and is located on the IDCL, which is a flexible linker between PCNA monomers [42]. The function of SUMOylation of lysine 127 is poorly understood, in comparison to lysine 164. However, mutating lysine 127 suppresses the DNA damage sensitivity of the *pol30-K164R* mutant, which is unable to utilize the error-free or error-prone DNA repair pathway [26, 27, 29, 40]. Thus, it was proposed that SUMOylation of lysine 127 is inhibiting DNA repair [48]. Indeed, we provide further evidence that SUMOylation of lysine 127 of PCNA inhibits DNA repair using Rad52-foci as an indicator of recombinogenic DNA lesions. We identified a significant increase

in recombinogenic DNA lesions when PCNA cannot be modified at lysine 127 in the presence or absence of replication stress. One possible explanation for the increase in recombinogenic DNA lesions in the *pol30-K127R* mutant could be the diminished recruitment of the anti-recombinase Srs2, which is facilitated by SUMOylation of PCNA [25, 26]. In turn, the increased recombinogenic DNA lesions lead to sensitivity of the *pol30-K127R* mutant under replication stress.

When further investigating the positive genetic interaction between *RRM3* and *POL30-K127R,* we discovered that deleting the first 54 amino acids of Rrm3's N-terminus suppresses the sensitivity of HU-induced replication stress and the accumulation of Rad52-foci of PCNA when lysine 127 cannot be SUMOylated, while Rrm3's helicase activity is dispensable. The first 54 amino acids of Rrm3's N-terminus contain the PCNA interacting binding motif (PIP-box) [16]. This short linear motif in Rrm3's N-terminus is a canonical PIP-box motif following the characteristic sequence Q-x-x-(I/L/M/V)-x-x-(F/Y)-(F/Y) [18]. Proteins with a PIP-box were shown to bind specifically to the hydrophobic pocket formed in the IDCL domain of PCNA, where lysine 127 is located [19]. Thus, the inability to deal with replication stress and increased recombinogenic DNA lesions I observe in the *pol30-K127R* mutant is caused by the physical interaction between PCNA and Rrm3 rather than Rrm3's helicase activity generating toxic DNA substrates.

Previously, it has been proposed that SUMOylation of PCNA at lysine 127 could act as a "reset button" for new protein interaction [19]. Therefore, a possible model could be that SUMOylation of lysine 127 disrupts its interaction with Rrm3 to encourage interaction of an unknown protein under replication stress. A possible candidate could be the chromatin assembly factor 1, which has been shown to compete with Rrm3 for PCNA binding [64]. Alternatively, the acetyltransferase Eco1 binds to unmodified PCNA and the interaction is disrupted upon SUMOylation of lysine 127 [50]. Besides, acetylating lysine 20 of PCNA, Eco1 is

required for cohesion establishment [50, 57, 65, 66]. Thus, prolonged interaction with PCNA could lead to robust cohesion establishment, which leads to sensitivity and recombinogenic DNA lesion in *pol30-K127R* mutant under HU-induced replication stress. Furthermore, Rrm3 was also shown to promote cohesion, thus deletion of *RRM3* (*rrm3*Δ) or disrupting its interaction with PCNA (*rrm3-*Δ*N54*) might suppress the cohesion establishment induced by Eco1 [67].

Additionally, we discovered that the E3 ligase Siz1 is required for the suppression of *pol30-K127R* mutant in the absence of Rrm3. Siz1, Siz2 and Mms21 are the only E3 ligases responsible for SUMOylation of the whole yeast genome [68, 69]. While Siz2 substrates are mainly HR proteins, the substrates for Siz1 are unclear, as they also can have overlapping targets and compensate for each other [68, 69]. However, one downstream target of Siz1 could be Sae2, which was shown to function in the error-free and error-prone pathway [70, 71]. Additionally, a negative genetic interaction between Rrm3 and Sae2 has been suggested [72]. Sae2 functions together with MRX in resecting ssDNA at stalled replication forks, which then leads to the recruitment of Rad6-Rad18 [70]. Furthermore, mono- and diubiquitinated PCNA are reduced in a *sae2* mutant [70]. Thus, the simplest explanation for the requirement of Siz1 would be that in its absence Sae2 cannot be SUMOylated, which results in insufficient ssDNA resection, thereby preventing the recruitment of Rad6-Rad18. This in turn inhibits mono- or polyubiquitination of lysine 164 leading to the impairment of the TLS and error-free pathway resulting in DNA damage sensitivity [2, 25, 26, 29]. In addition, deletion of *RAD18* causes increased DNA damage sensitivity when PCNA cannot be modified at lysine 127 as well. However, the *pol30-K127/164R* mutant is less sensitivity compared to either mutating lysine 127 or lysine 164 [48]. Therefore, simple inhibition of ubiquitination at lysine 164 cannot explain the requirement of Siz1 in the *rrm3 pol30-K127R* mutant. Alternatively, a separate pathway depending on the modification of lysine 127 but independent of the error-free and error-prone pathway could be utilized. Indeed, just recently another pathway termed the salvage

recombination (SR) pathway, which is independent of PCNA ubiquitination has been described [73-75].

In conclusion, while most of Rrm3 functions to date have been associated with its helicase activity, this study provides the first evidence for a function of Rrm3's N-terminus under replication stress. Further studies will determine how the interaction between Rrm3 and PCNA is regulated and whether other proteins, such as Caf1, are competing with Rrm3 for binding to PCNA under different physiological conditions.

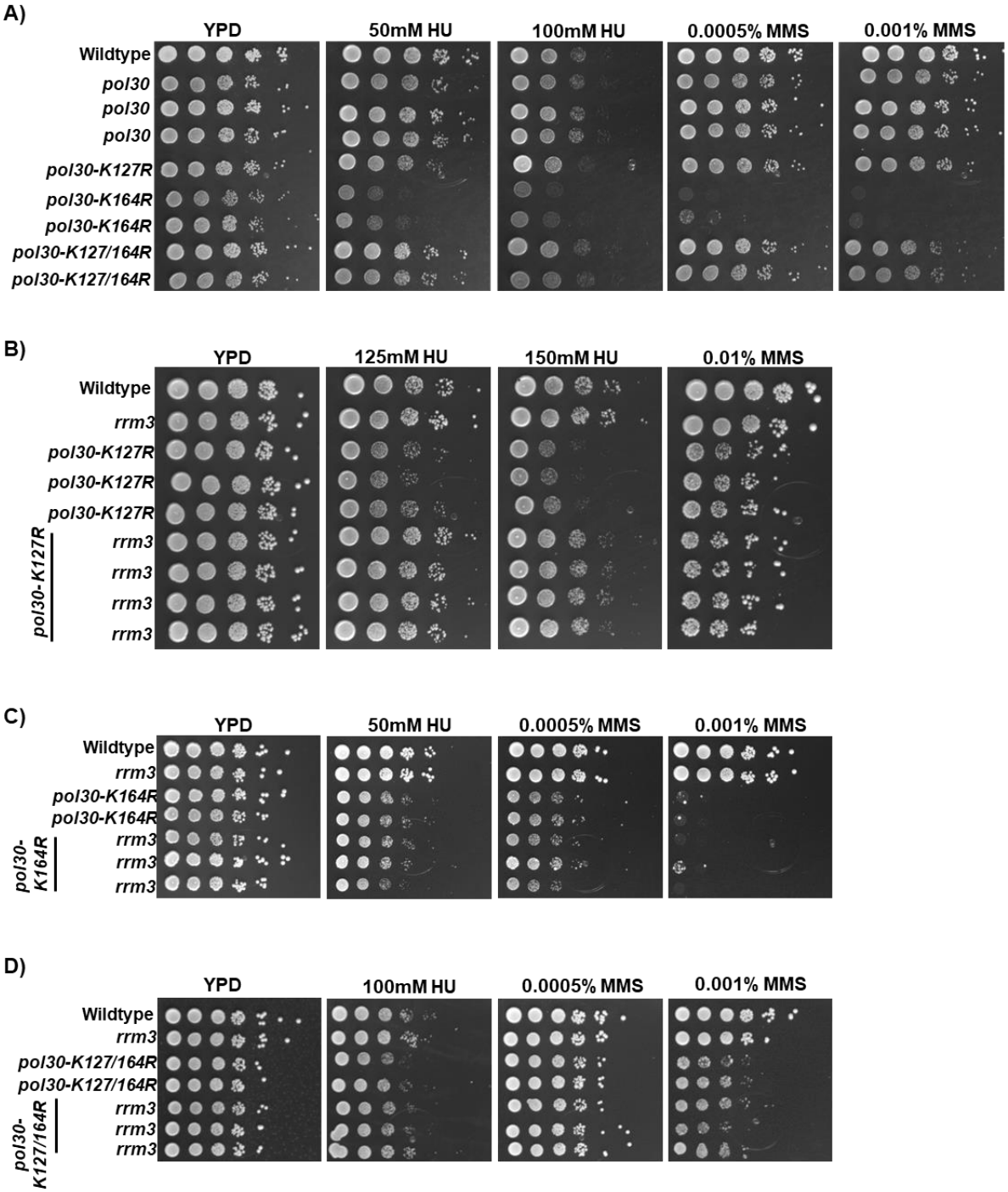

Figure 3.1. Genetic interaction between *POL30* and *RRM3* in the suppression of replication stress. ***(A)*** Different pol30 mutants on various Hydroxyurea (HU) and methylmethane sulfonate (MMS) concentrations. The *pol30* mutants are 6xHIS tagged on the N-terminus and have point mutations at position 127 (*pol30-K127R*), position 164 (*pol30-K164R*), or on both (*pol30-K127/164R*). Spot assay to test for genetic interactions between ***(B)*** *pol30-K127R,* ***(C)*** *pol30-K164R,* ***(D)*** *pol30-K127/164R* and *rrm3* mutations in the suppression of DNA replication stress induced by chronic exposure to hydroxyurea (HU). Images were acquired on a GelDoc-It system (UVP) after 2-3 days of incubation at 30˚C.

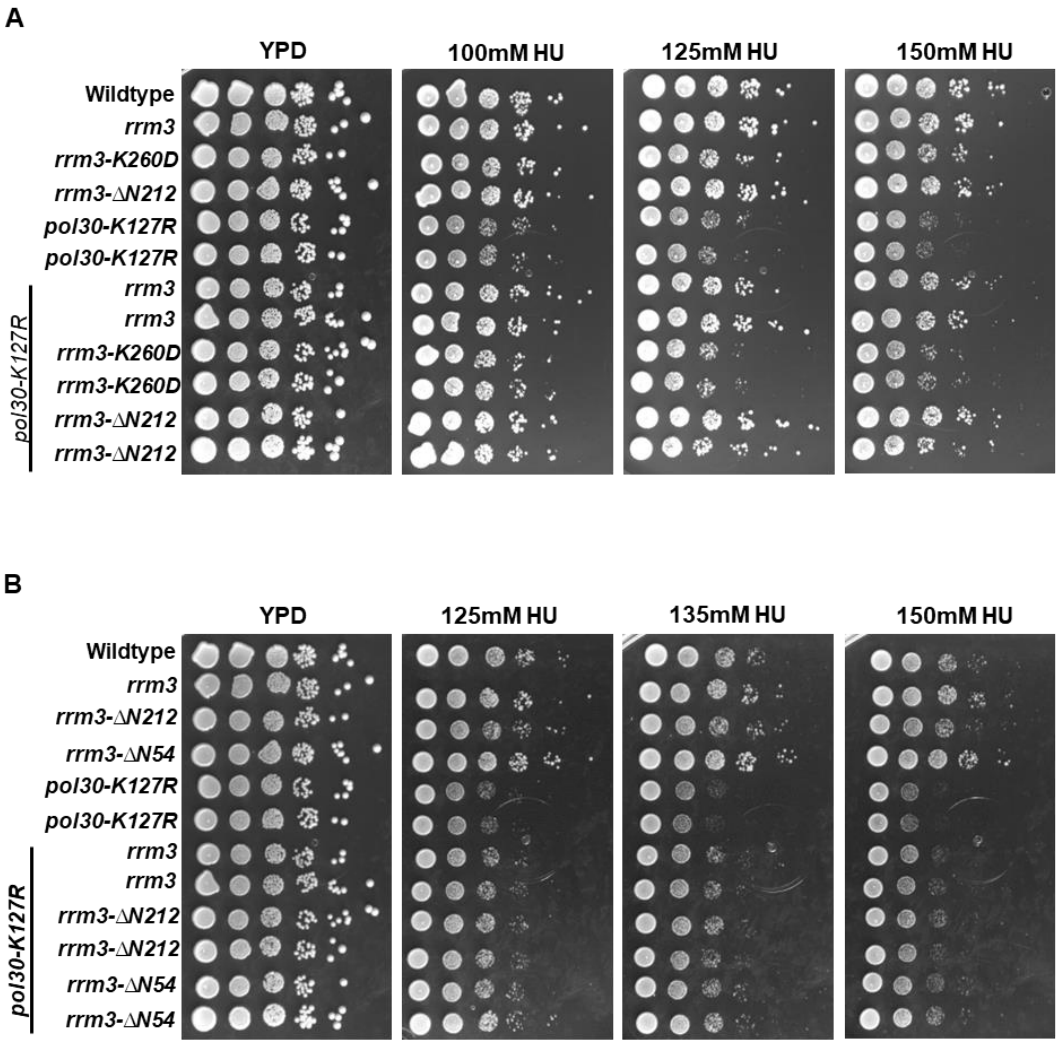

Figure 3.2. Genetic interaction between functional domains of *RRM3* and *POL30,* when lysine 127 cannot be modified, in the suppression of replication stress *(A)* Assessing DNA damage sensitivity in cells harboring an *RRM3* deletion (*rrm3*Δ), a disruption of Rrm3's helicase activity (*rrm3-K260D*), or a truncation of Rrm3's N-terminal tail (*rrm3-*Δ*N212*) following exposure to concentrations of HU and MMS ***(B)*** Effect of deleting first 54 amino acids of Rrm3's N-terminus (*rrm3-*Δ*N54*) on the suppression of *pol30-K127R* mutant on HU and MMS. Images were acquired on a GelDoc-It system (UVP) after 2-3 days of incubation at 30˚C.

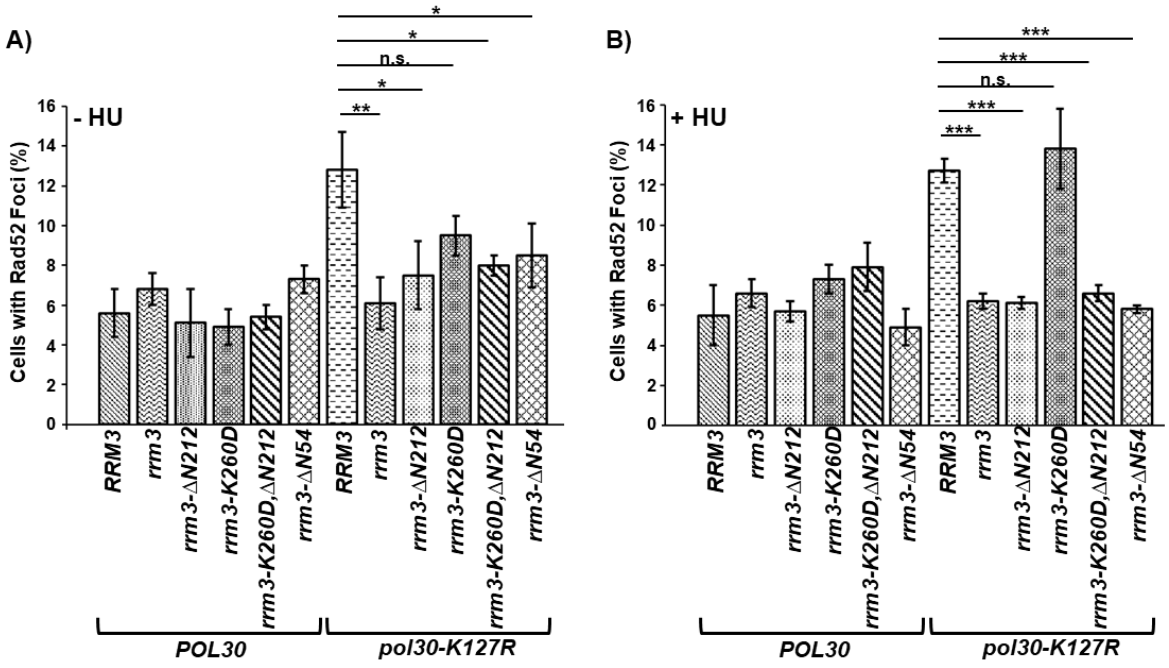

Figure 3.3. Interaction between *POL30* and *RRM3* mutations in the suppression of recombinogenic DNA lesions. ***(A)*** Quantification of Rad52-GFP foci in cells harboring an *RRM3* deletion (*rrm3*Δ), a disruption of Rrm3 helicase activity (*rrm3-K260D*), or deletion of first 54 amino acids (*rrm3-*Δ*N54*), or a truncation of the Rrm3 N-terminal tail (*rrm3-*Δ*N212*), or both (*rrm3-K260D,* Δ*N212*). Effect of *rrm3* mutations was assessed in *pol30* mutant cells, when PCNA cannot be modified at lysine 127 and in the absence or ***(B)*** presence of replication stress induced by hydroxyurea (HU). Images of at least 250 cells per yeast strain were acquired on a BZ-X800 microscope (Keyence). All experiments were repeated three times and the mean percentage of cells with Rad52-gfp foci in all three experiments is reported with standard deviation. The mean percentage of cells with Rad52-GFP foci for each of the three experiments is indicated by a gray dot. Statistical significance of differences between mutants was determined with a Student's t-test and reported as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; n.s., not significant.

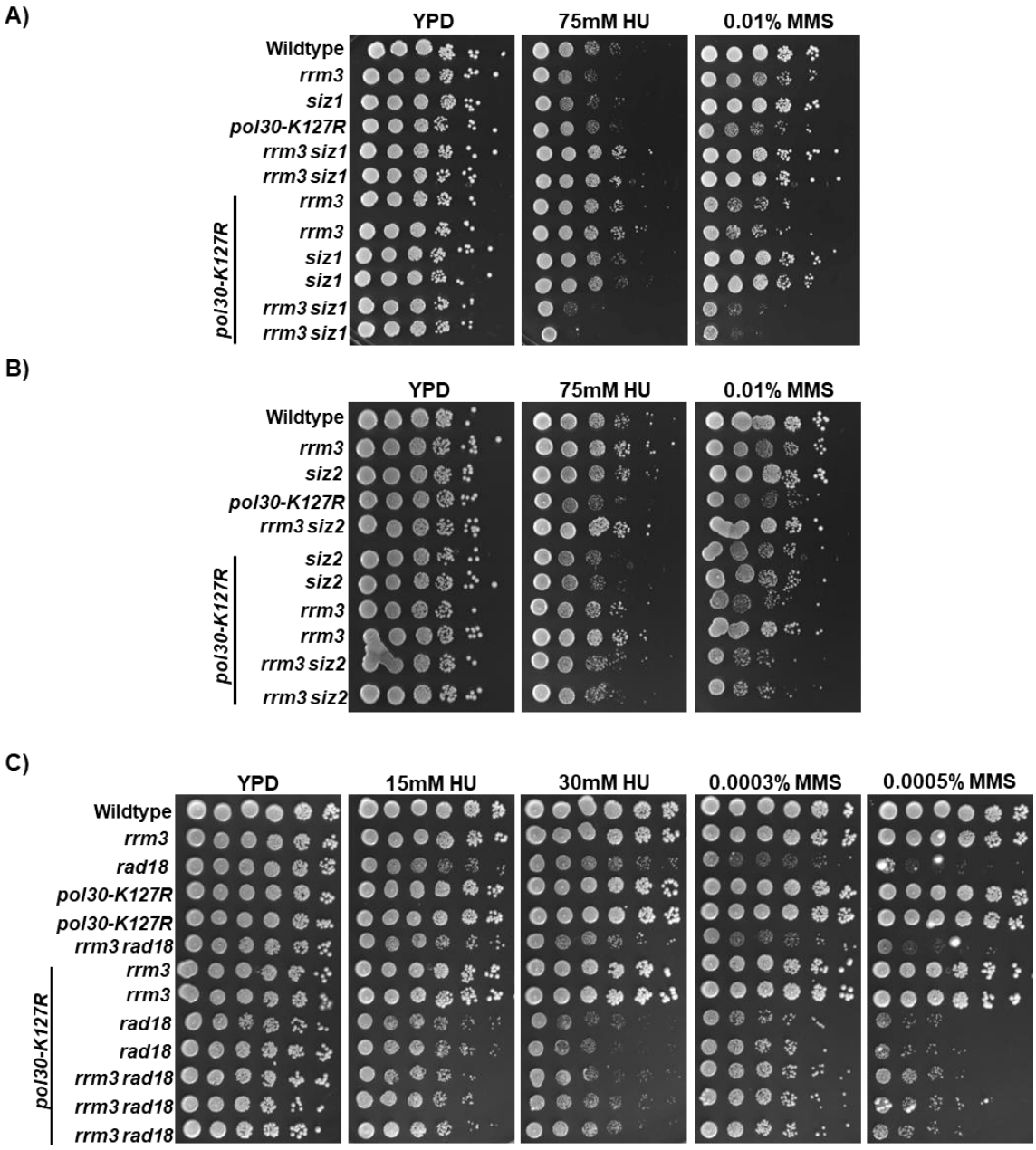

Figure 3.4. Function of Lysine 164 modifications in *rrm3*Δ *pol30-K127R* mutant. ***(A)*** Spot assay of serial dilutions of exponentially growing cells to determine the effect of deleting *SIZ1* or ***(B)*** deleting *RAD18* on the suppression of the DNA damage sensitivity of *pol30-K127R* mutant in the absence of Rrm3 on HU and MMS.

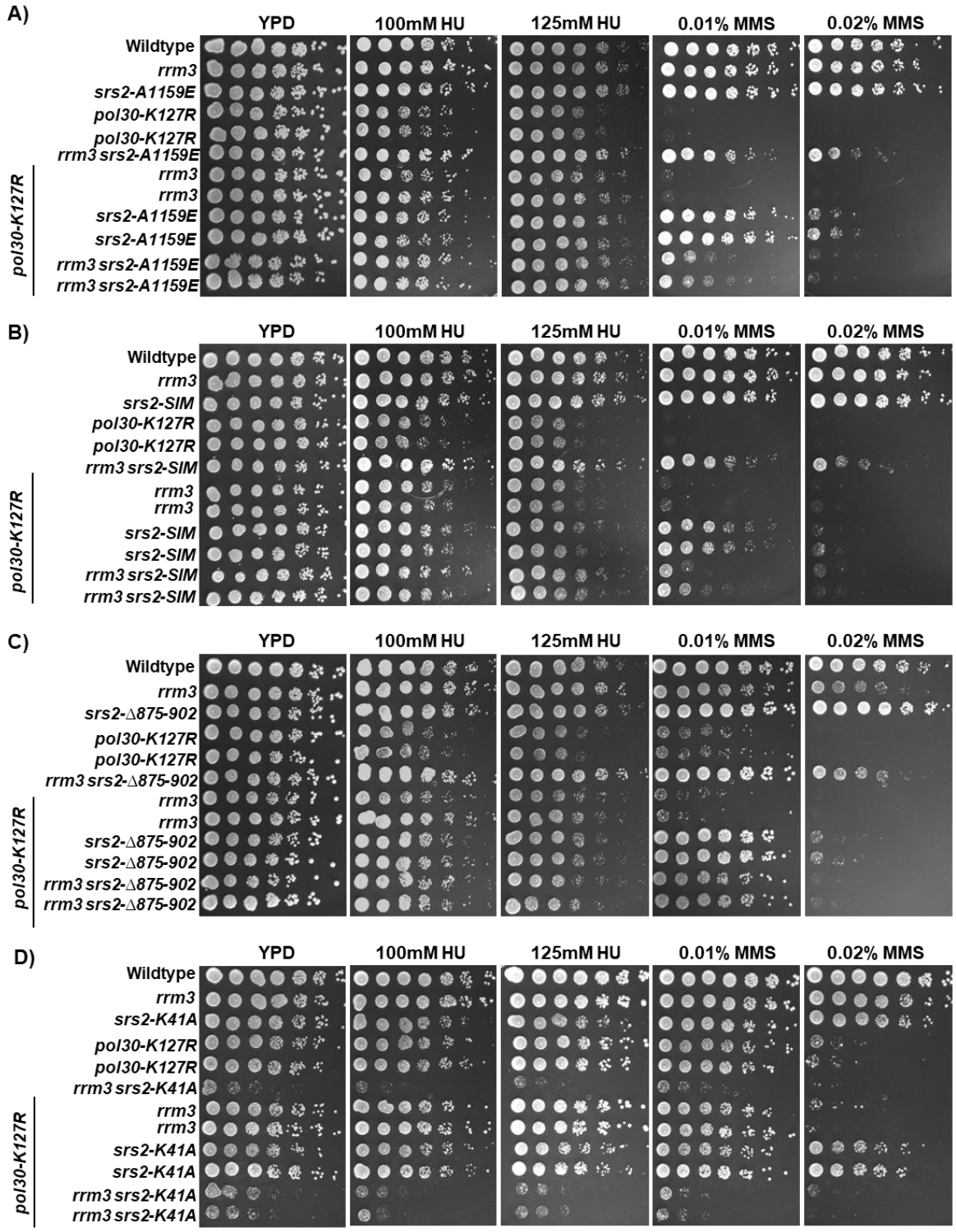

Figure 3.5. Identifying functional domains of Srs2 in the suppression of DNA damage sensitivity in *rrm3 pol30-K127R*. Assessing DNA damage sensitivity of cells harboring mutations in Srs2's ***(A)*** PCNA interacting motif (*srs2-A1159E*), ***(B)*** D-loop activity (*srs2-SIM*), ***(C)*** Rad51-binding site (*srs2-875-902*) and ***(D)*** helicase activity (*srs2-K41A*) in the absence of Rrm3 and *pol30-K127R* following exposure to MMS and HU.

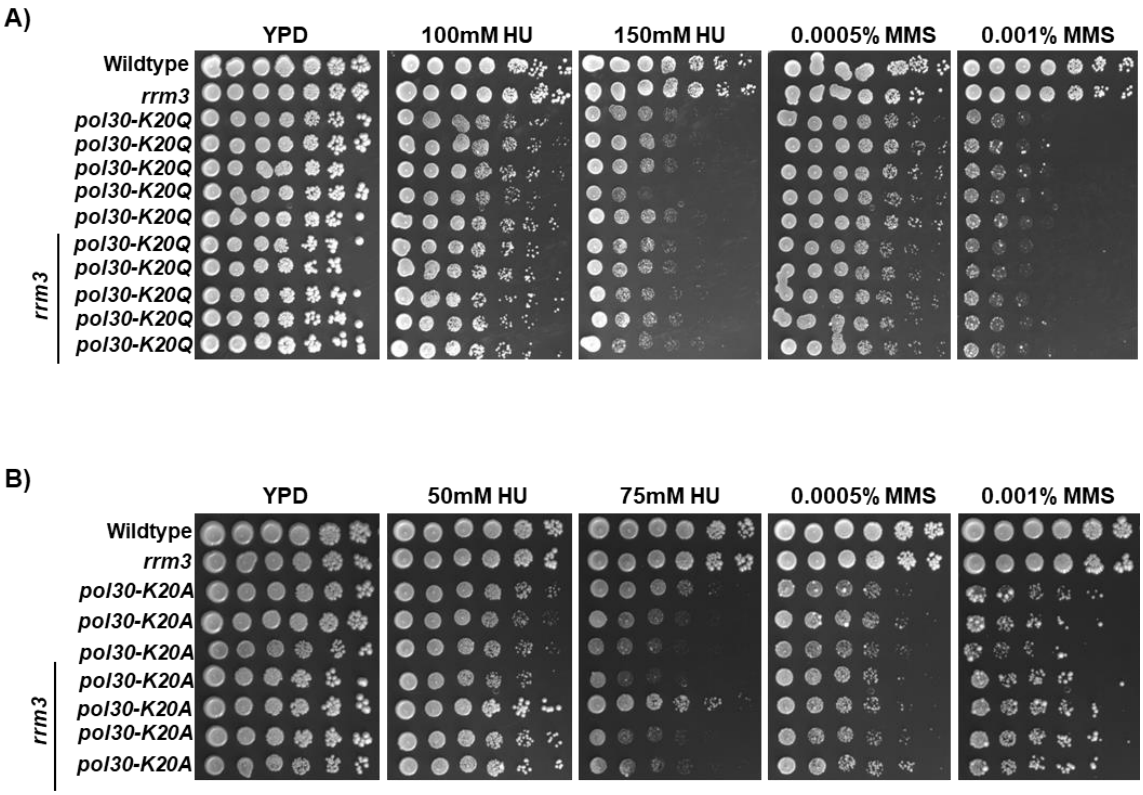

Figure 3.6. Genetic interaction between *RRM3* and *POL30* mutants when lysine 20 cannot be modified under replication stress. Assessing the DNA damage sensitivity of *pol30* mutants that ***(A)*** mimic acetylation (*pol30-K20Q*) or ***(B)*** inhibits acetylation (*pol30-K20A*) in the absence of Rrm3 under replication stress induced by HU and MMS.

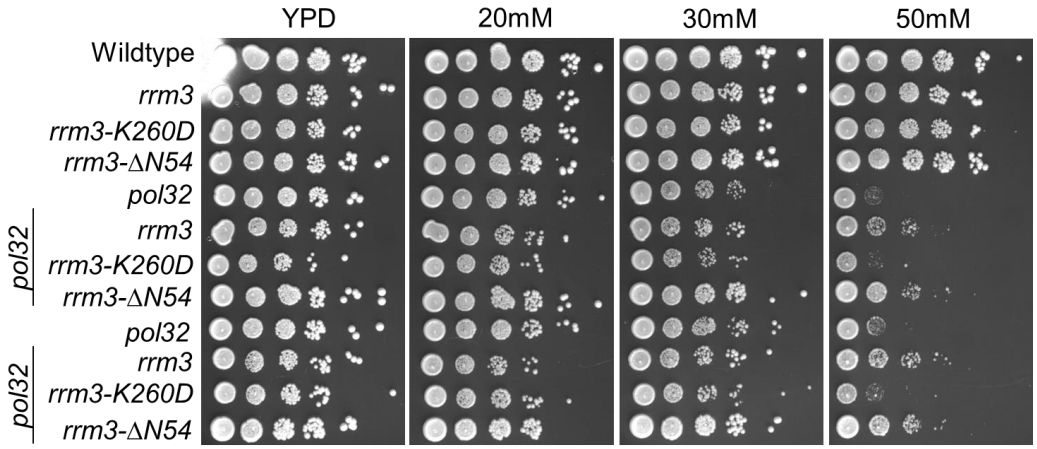

Figure 3.7 Genetic interaction between functional domains of *RRM3* and *POL32*. Assessing DNA damage sensitivity in cells harboring an *RRM3* deletion (*rrm3*Δ), a disruption of Rrm3's helicase activity (*rrm3-K260D*), or a truncation of Rrm3's PIP-box motif (*rrm3-ΔN54*) in the absence of Pol32 following exposure to concentrations of HU and MMS.

Table 3.1. Yeast strains used in this study

Strain	Genotype
KHSY55	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3*, *rrm3-∆54.Myc.HIS3*
KHSY802	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3,*
KHSY885	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad51::HIS3*
KHSY886	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad51::HIS3, rrm3::TRP1*
KHSY1064	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1*
KHSY5194	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-∆N212.Myc.HIS3*
KHSY5196	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-K260D.Myc.HIS3*
KHSY5520	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, Rad52.GFP.HIS3*
KHSY5898	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, 6xHIS pol30-K164R*
KHSY5901	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, 6xHIS pol30-K164*
KHSY5906	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, 6xHIS pol30-K127,164R*
KHSY5910	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, 6xHIS pol30-K127,164R*
KHSY5930	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, 6xHIS pol30*
KHSY5977	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, 6xHIS pol30-K127R*
KHSY5981	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, 6xHIS pol30-K127R*

Table 3.1. (Continued)

KHSY6566	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, 6xHIS pol30-K127R, Rad52.GFP.HIS3*
KHSY6607	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-ΔN212.Myc.HIS3, 6xHIS pol30-K127R*
KHSY6646	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-ΔN212.Myc.HIS3, 6xHIS pol30-K127R, Rad52.GFP.HIS3*
KHSY6671	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, siz1::HIS3*
KHSY6672	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad51::HIS3, 6xHIS pol30-K127R*
KHSY6673	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad51::HIS3, 6xHIS pol30-K127R*
KHSY6674	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, siz1::HIS3*
KHSY6675	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, siz1::HIS3, 6xHIS pol30-K127R*
KHSY6676	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, siz1::HIS3, 6xHIS pol30-K127R*
KHSY6731	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1,siz2::HIS3*
KHSY6735	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, siz2::HIS3, 6xHIS pol30-K127R*

Table 3.1. (continued)

KHSY6740	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, siz2::HIS3, 6xHIS pol30-K127R*
KHSY6774	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-ΔN54.Myc.HIS3, 6xHIS pol30-K127R*
KHSY7130	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-ΔN212/K260D.Myc.HIS3*
KHSY7131	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-K260D.Myc.HIS3, 6xHIS pol30-K127R*
KHSY7133	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, 6xHIS pol30-K127R, Rad52.GFP.HIS3*
KHSY7134	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-K260D.Myc.HIS3, 6xHIS pol30-K127R, Rad52.GFP.HIS3*
KHSY7136	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-ΔN212/K260D.Myc.HIS3, 6xHIS pol30-K127R, Rad52.GFP.HIS3*
KHSY7137	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-ΔN212/K260D.Myc.HIS3, Rad52.GFP.HIS3*
KHSY7138	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-ΔN54.Myc.HIS3, 6xHIS pol30-K127R, Rad52.GFP.HIS3*
KHSY7139	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3-ΔN54.Myc.HIS3, Rad52.GFP.HIS3*
KHSY7140	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, srs2-A1159E.Myc.HIS3*

Table 3.1. (continued)

KHSY7141	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, srs2-SIM.Myc.HIS3*
KHSY7142	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, srs2-SIM.Myc.HIS3*
KHSY7143	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, srs2-SIM.Myc.HIS3, 6xHIS pol30-K127R*
KHSY7144	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, srs2-SIM.Myc.HIS3, 6xHIS pol30-K127R*
KHSY7145	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, srs2-A1159E.Myc.HIS3*
KHSY7146	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, srs2-A1159E.Myc.HIS3, 6xHIS pol30-K127R*
KHSY7147	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, srs2-A1159E.Myc.HIS3, 6xHIS pol30-K127R*
KHSY7148	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, srs2-∆875-902.Myc.HIS3*
KHSY7149	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, srs2-∆875-902.Myc.HIS3*
KHSY7150	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, srs2-∆875-902.Myc.HIS3, 6xHIS pol30-K127R*
KHSY7151	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, srs2-∆875-902.Myc.HIS3, 6xHIS pol30-K127R*

Table 3.1. (continued)

KHSY7152	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, srs2-K41A.Myc.HIS3*
KHSY7153	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad18::HIS3*
KHSY7154	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, srs2-K41A.Myc.HIS3*
KHSY7155	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, srs2-K41A.Myc.HIS3, 6xHIS pol30-K127R*
KHSY7156	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, srs2-K41A.Myc.HIS3, 6xHIS pol30-K127R*
KHSY7157	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad18::HIS3*
KHSY7158	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rad18::HIS3, 6xHIS pol30-K127R*
KHSY7159	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, rad18::HIS3, 6xHIS pol30-K127R*
KHSY7160	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, 6xHIS pol30-K20A*
KHSY7161	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, 6xHIS pol30-K20Q*
KHSY7162	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, 6xHIS pol30-K20A*
KHSY7163	*MATa ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, rrm3::TRP1, 6xHIS pol30-K20Q*

References

1. Branzei, D. and M. Foiani, *Maintaining genome stability at the replication fork.* Nat Rev Mol Cell Biol, 2010. **11**(3): p. 208-19.
2. Branzei, D. and I. Psakhye, *DNA damage tolerance.* Curr Opin Cell Biol, 2016. **40**: p. 137-144.
3. Azvolinsky, A., et al., *The S. cerevisiae Rrm3p DNA helicase moves with the replication fork and affects replication of all yeast chromosomes.* Genes Dev, 2006. **20**(22): p. 3104-16.
4. Torres, J.Z., J.B. Bessler, and V.A. Zakian, *Local chromatin structure at the ribosomal DNA causes replication fork pausing and genome instability in the absence of the S. cerevisiae DNA helicase Rrm3p.* Genes Dev, 2004. **18**(5): p. 498-503.
5. Torres, J.Z., S.L. Schnakenberg, and V.A. Zakian, *Saccharomyces cerevisiae Rrm3p DNA helicase promotes genome integrity by preventing replication fork stalling: viability of rrm3 cells requires the intra-S-phase checkpoint and fork restart activities.* Mol Cell Biol, 2004. **24**(8): p. 3198-212.
6. Boule, J.B. and V.A. Zakian, *Roles of Pif1-like helicases in the maintenance of genomic stability.* Nucleic Acids Res, 2006. **34**(15): p. 4147-53.
7. Bochman, M.L., C.P. Judge, and V.A. Zakian, *The Pif1 family in prokaryotes: what are our helicases doing in your bacteria?* Mol Biol Cell, 2011. **22**(12): p. 1955-9.
8. Bessler, J.B., J.Z. Torres, and V.A. Zakian, *The Pif1p subfamily of helicases: region-specific DNA helicases?* Trends Cell Biol, 2001. **11**(2): p. 60-5.
9. Enemark, E.J. and L. Joshua-Tor, *On helicases and other motor proteins.* Curr Opin Struct Biol, 2008. **18**(2): p. 243-57.
10. Singleton, M.R., M.S. Dillingham, and D.B. Wigley, *Structure and mechanism of helicases and nucleic acid translocases.* Annu Rev Biochem, 2007. **76**: p. 23-50.
11. Ivessa, A.S., et al., *The Saccharomyces cerevisiae helicase Rrm3p facilitates replication past nonhistone protein-DNA complexes.* Mol Cell, 2003. **12**(6): p. 1525-36.
12. Ivessa, A.S., et al., *Saccharomyces Rrm3p, a 5' to 3' DNA helicase that promotes replication fork progression through telomeric and subtelomeric DNA.* Genes Dev, 2002. **16**(11): p. 1383-96.
13. Rossi, S.E., et al., *Rad53-Mediated Regulation of Rrm3 and Pif1 DNA Helicases Contributes to Prevention of Aberrant Fork Transitions under Replication Stress.* Cell Rep, 2015. **13**(1): p. 80-92.
14. Syed, S., et al., *A Novel Rrm3 Function in Restricting DNA Replication via an Orc5-Binding Domain Is Genetically Separable from Rrm3 Function as an ATPase/Helicase in Facilitating Fork Progression.* PLoS Genet, 2016. **12**(12): p. e1006451.
15. Muellner, J. and K.H. Schmidt, *Yeast Genome Maintenance by the Multifunctional PIF1 DNA Helicase Family.* Genes (Basel), 2020. **11**(2).
16. Schmidt, K.H., K.L. Derry, and R.D. Kolodner, *Saccharomyces cerevisiae RRM3, a 5' to 3' DNA helicase, physically interacts with proliferating cell nuclear antigen.* J Biol Chem, 2002. **277**(47): p. 45331-7.
17. Gulbis, J.M., et al., *Structure of the C-terminal region of p21(WAF1/CIP1) complexed with human PCNA.* Cell, 1996. **87**(2): p. 297-306.
18. Warbrick, E., *PCNA binding through a conserved motif.* Bioessays, 1998. **20**(3): p. 195-9.
19. Moldovan, G.L., B. Pfander, and S. Jentsch, *PCNA, the maestro of the replication fork.* Cell, 2007. **129**(4): p. 665-79.
20. Prelich, G., et al., *Functional identity of proliferating cell nuclear antigen and a DNA polymerase-delta auxiliary protein.* Nature, 1987. **326**(6112): p. 517-20.

21. Bravo, R., et al., *Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta.* Nature, 1987. **326**(6112): p. 515-7.
22. Maga, G., et al., *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, 2001. **98**(25): p. 14298-303.
23. Sporbert, A., et al., *PCNA acts as a stationary loading platform for transiently interacting Okazaki fragment maturation proteins.* Nucleic Acids Res, 2005. **33**(11): p. 3521-8.
24. Branzei, D., M. Seki, and T. Enomoto, *Rad18/Rad5/Mms2-mediated polyubiquitination of PCNA is implicated in replication completion during replication stress.* Genes Cells, 2004. **9**(11): p. 1031-42.
25. Branzei, D., F. Vanoli, and M. Foiani, *SUMOylation regulates Rad18-mediated template switch.* Nature, 2008. **456**(7224): p. 915-20.
26. Hoege, C., et al., *RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.* Nature, 2002. **419**(6903): p. 135-41.
27. Davies, A.A., et al., *Activation of ubiquitin-dependent DNA damage bypass is mediated by replication protein a.* Mol Cell, 2008. **29**(5): p. 625-36.
28. Prakash, S., R.E. Johnson, and L. Prakash, *Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function.* Annu Rev Biochem, 2005. **74**: p. 317-53.
29. Stelter, P. and H.D. Ulrich, *Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation.* Nature, 2003. **425**(6954): p. 188-91.
30. Johnson, R.E., et al., *Eukaryotic polymerases iota and zeta act sequentially to bypass DNA lesions.* Nature, 2000. **406**(6799): p. 1015-9.
31. Haracska, L., et al., *Efficient and accurate replication in the presence of 7,8-dihydro-8-oxoguanine by DNA polymerase eta.* Nat Genet, 2000. **25**(4): p. 458-61.
32. Haracska, L., et al., *Roles of yeast DNA polymerases delta and zeta and of Rev1 in the bypass of abasic sites.* Genes Dev, 2001. **15**(8): p. 945-54.
33. Washington, M.T., et al., *Efficient and error-free replication past a minor-groove N2-guanine adduct by the sequential action of yeast Rev1 and DNA polymerase zeta.* Mol Cell Biol, 2004. **24**(16): p. 6900-6.
34. Johnson, R.E., S. Prakash, and L. Prakash, *Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Poleta.* Science, 1999. **283**(5404): p. 1001-4.
35. Ulrich, H.D., *Regulating post-translational modifications of the eukaryotic replication clamp PCNA.* DNA Repair (Amst), 2009. **8**(4): p. 461-9.
36. Ulrich, H.D. and S. Jentsch, *Two RING finger proteins mediate cooperation between ubiquitin-conjugating enzymes in DNA repair.* EMBO J, 2000. **19**(13): p. 3388-97.
37. Broomfield, S., B.L. Chow, and W. Xiao, *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, 1998. **95**(10): p. 5678-83.
38. Brusky, J., Y. Zhu, and W. Xiao, *UBC13, a DNA-damage-inducible gene, is a member of the error-free postreplication repair pathway in Saccharomyces cerevisiae.* Curr Genet, 2000. **37**(3): p. 168-74.
39. Daigaku, Y., A.A. Davies, and H.D. Ulrich, *Ubiquitin-dependent DNA damage bypass is separable from genome replication.* Nature, 2010. **465**(7300): p. 951-5.
40. Zhang, H. and C.W. Lawrence, *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, 2005. **102**(44): p. 15954-9.
41. Parker, J.L., et al., *SUMO modification of PCNA is controlled by DNA.* EMBO J, 2008. **27**(18): p. 2422-31.

42. Rodriguez, M.S., C. Dargemont, and R.T. Hay, *SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting.* J Biol Chem, 2001. **276**(16): p. 12654-9.
43. Hendriks, I.A. and A.C. Vertegaal, *A comprehensive compilation of SUMO proteomics.* Nat Rev Mol Cell Biol, 2016. **17**(9): p. 581-95.
44. Zhu, J., et al., *Small ubiquitin-related modifier (SUMO) binding determines substrate recognition and paralog-selective SUMO modification.* J Biol Chem, 2008. **283**(43): p. 29405-15.
45. Pfander, B., et al., *SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase.* Nature, 2005. **436**(7049): p. 428-33.
46. Niu, H. and H.L. Klein, *Multifunctional roles of Saccharomyces cerevisiae Srs2 protein in replication, recombination and repair.* FEMS Yeast Res, 2017. **17**(2).
47. Burkovics, P., et al., *Srs2 mediates PCNA-SUMO-dependent inhibition of DNA repair synthesis.* EMBO J, 2013. **32**(5): p. 742-55.
48. Papouli, E., et al., *Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p.* Mol Cell, 2005. **19**(1): p. 123-33.
49. Slade, D., *Maneuvers on PCNA Rings during DNA Replication and Repair.* Genes (Basel), 2018. **9**(8).
50. Moldovan, G.L., B. Pfander, and S. Jentsch, *PCNA controls establishment of sister chromatid cohesion during S phase.* Mol Cell, 2006. **23**(5): p. 723-32.
51. Davies, A.A. and H.D. Ulrich, *Detection of PCNA modifications in Saccharomyces cerevisiae.* Methods Mol Biol, 2012. **920**: p. 543-67.
52. Longtine, M.S., et al., *Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae.* Yeast, 1998. **14**(10): p. 953-61.
53. Schmidt, K.H. and R.D. Kolodner, *Requirement of Rrm3 helicase for repair of spontaneous DNA lesions in cells lacking Srs2 or Sgs1 helicase.* Mol Cell Biol, 2004. **24**(8): p. 3213-26.
54. Krejci, L., et al., *DNA helicase Srs2 disrupts the Rad51 presynaptic filament.* Nature, 2003. **423**(6937): p. 305-9.
55. Kim, S.O., et al., *Srs2 possesses a non-canonical PIP box in front of its SBM for precise recognition of SUMOylated PCNA.* J Mol Cell Biol, 2012. **4**(4): p. 258-61.
56. Krejci, L., et al., *Role of ATP hydrolysis in the antirecombinase function of Saccharomyces cerevisiae Srs2 protein.* J Biol Chem, 2004. **279**(22): p. 23193-9.
57. Billon, P., et al., *Acetylation of PCNA Sliding Surface by Eco1 Promotes Genome Stability through Homologous Recombination.* Mol Cell, 2017. **65**(1): p. 78-90.
58. Boulet, A., et al., *Structure and function of the Saccharomyces cerevisiae CDC2 gene encoding the large subunit of DNA polymerase III.* EMBO J, 1989. **8**(6): p. 1849-54.
59. Simon, M., L. Giot, and G. Faye, *The 3' to 5' exonuclease activity located in the DNA polymerase delta subunit of Saccharomyces cerevisiae is required for accurate replication.* EMBO J, 1991. **10**(8): p. 2165-70.
60. Gerik, K.J., et al., *Characterization of the two small subunits of Saccharomyces cerevisiae DNA polymerase delta.* J Biol Chem, 1998. **273**(31): p. 19747-55.
61. Burgers, P.M. and K.J. Gerik, *Structure and processivity of two forms of Saccharomyces cerevisiae DNA polymerase delta.* J Biol Chem, 1998. **273**(31): p. 19756-62.
62. Belli, G., et al., *Post-Translational Modifications of PCNA: Guiding for the Best DNA Damage Tolerance Choice.* J Fungi (Basel), 2022. **8**(6).
63. Lee, K.Y. and K. Myung, *PCNA modifications for regulation of post-replication repair pathways.* Mol Cells, 2008. **26**(1): p. 5-11.
64. Wyse, B., et al., *RRM3 regulates epigenetic conversions in Saccharomyces cerevisiae in conjunction with Chromatin Assembly Factor I.* Nucleus, 2016. **7**(4): p. 405-14.

65. Liu, H.W., et al., *Division of Labor between PCNA Loaders in DNA Replication and Sister Chromatid Cohesion Establishment.* Mol Cell, 2020. **78**(4): p. 725-738 e4.
66. Zuilkoski, C.M. and R.V. Skibbens, *PCNA promotes context-specific sister chromatid cohesion establishment separate from that of chromatin condensation.* Cell Cycle, 2020. **19**(19): p. 2436-2450.
67. Warren, C.D., et al., *S-phase checkpoint genes safeguard high-fidelity sister chromatid cohesion.* Mol Biol Cell, 2004. **15**(4): p. 1724-35.
68. Reindle, A., et al., *Multiple domains in Siz SUMO ligases contribute to substrate selectivity.* J Cell Sci, 2006. **119**(Pt 22): p. 4749-57.
69. Gareau, J.R. and C.D. Lima, *The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition.* Nat Rev Mol Cell Biol, 2010. **11**(12): p. 861-71.
70. Ball, L.G., et al., *The Mre11-Rad50-Xrs2 complex is required for yeast DNA postreplication repair.* PLoS One, 2014. **9**(10): p. e109292.
71. Sarangi, P., et al., *Sumoylation influences DNA break repair partly by increasing the solubility of a conserved end resection protein.* PLoS Genet, 2015. **11**(1): p. e1004899.
72. Costanzo, M., et al., *A global genetic interaction network maps a wiring diagram of cellular function.* Science, 2016. **353**(6306).
73. Jimenez-Martin, A., et al., *The Mgs1/WRNIP1 ATPase is required to prevent a recombination salvage pathway at damaged replication forks.* Sci Adv, 2020. **6**(15): p. eaaz3327.
74. Branzei, D. and B. Szakal, *DNA damage tolerance by recombination: Molecular pathways and DNA structures.* DNA Repair (Amst), 2016. **44**: p. 68-75.
75. Arbel, M., et al., *Access to PCNA by Srs2 and Elg1 Controls the Choice between Alternative Repair Pathways in Saccharomyces cerevisiae.* mBio, 2020. **11**(3).

Chapter Four: Conclusions and Future Directions

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The cell utilizes multiple pathways to ensure progression of the DNA replication machinery through numerous obstacles to transmit genetic information in an accurate and timely manner. Endogenously- and exogenously-induced obstacles impeding the DNA replication machinery, causes replication fork stalling or collapse, which can result in genome instability [1]. While a common understanding that DNA lesions inhibit replication fork progression was established decades ago, the discovery of how frequently replication fork stalling occurs reignited interest in this field [2-4]. Therefore, the importance of identifying proteins that promote replication fork progression has emerged in the last decade.

The PIF1 DNA helicase Rrm3 was shown to promote fork progression genome wide [5-7]. Therefore, in the second chapter we used cells deficient of Rrm3 in *S. cerevisiae*, as a model organism to study how cells deal with increased stalled replication forks. It is thought that Rrm3 removes DNA bound protein ahead of the fork, therefore, it could be possible that Rad5, which was shown to be upregulated to deal with increased stalled replication forks in the absence of Rrm3 performs a similar function [6, 8]. Indeed, Rad5 and its human homolog, HLTF efficiently displace DNA bound proteins in front of the replication fork [9]. Thus, Rad5’s ATPase translocate

activity can act as a backup mechanism to Rrm3's helicase activity in removing non-histone bound proteins ahead of the replication forks. Furthermore, we identified another protein, the endonuclease Mus81, that deals with increased replication fork stalling. While *RRM3* and *RAD5* genetically function together to prevent recombinogenic DNA lesions under replication stress, Mus81 specifically acts on replication forks that stall in the absence of Rrm3, but not under replication stress. This further provides evidence that replication forks stalling at natural pausing sites, such as the replication fork barrier, is structurally distinct from those arising under replication stress.

In mammalian and yeast cells, replication forks stalled at natural pausing sites are processed independently of the DNA damage checkpoint kinases Mec1 and Rad53 in comparison to genotoxin-induced forks [10]. While DNA structures found at these sites resemble reversed forks, fork reversal is regulated by the S-phase checkpoint under replication stress, induced by hydroxyurea (HU) [11, 12]. Specifically, phosphorylation of Rrm3 and Pif1 by Rad53 reduces replication fork reversal and resection, thus, indicating that members of the Pif1 family can induce fork remodeling [13]. Indeed, *in-vitro* analysis showed that human PIF1 can generate DNA structures resembling reversed forks as well as unwind DNA structures representing stalled replication forks [14]. This further supports the proposal that Rad5 is required to deal with increased stalled replication forks, especially its fork reversal activity in the absence of Rrm3.

Originally, the established mutations distinguishing Rad5's helicase and ubiquitin-ligase activity overlapped, hence some of the conclusions drawn from these *rad5* mutants contradict each other [15-17]. Just recently, several point-mutations separating the individual functions of Rad5 have emerged and provided a foundation to properly characterize Rad5 [18, 19]. Furthermore, while fork reversal activity of Rad5 and its human orthologue, HLTF, has been extensively studied on model substrates *in-vitro*, which only contain a fraction of the proteins found at stalled replication forks, their functional contributions *in-vivo* are scarce [9, 20, 21].

Here in this study, I have used the newly established Rad5 separated-by-functions alleles, providing the first evidence for a biological relevance of Rad5's fork reversal activity in dealing with increased stalled replication forks and preventing recombinogenic DNA lesions under replication stress *in-vivo*. These recombinogenic DNA lesions arise due to prolonged fork stalling or inability to perform remodeling by fork reversal, which results in fork breakage and one-ended DSBs in the absence of Rrm3 and Rad5 under replication stress.

Early on, fork reversal was only observed in replication-checkpoint deficient yeast cells, thus were assumed to be DNA structures arising from failed replication fork progression [13, 22]. However, in recent years the importance of fork reversal was highlighted by the discovery that inducing replication stress by activating oncogenes causes atypical replication intermediates resembling reversed replication forks in cancerous cells [23]. Furthermore, HLTF is silenced in nearly 50% of colon cancers, in other types of cancers HLTF is amplified, thus, highlighting the importance of studying the mechanism of fork reversal as a therapeutic target [24-26]. In addition to Rad5's human orthologue, HTLF, other members of the SWI/SNF family, SMARCAL1, ZRANB3 promote fork reversal in mammalian cells, indicating that fork reversal in higher eukaryotes is a common occurrence and plays an important physiological role in maintaining genome stability [9, 20, 27, 28]. This raises multiple questions: Do other proteins in yeast also promote fork reversal? Do these proteins collaborate with each other during fork remodeling? Does a specific DNA-damage induced DNA structure require a certain member of the SWI/SNF family to perform fork reversal?

In the third chapter, we identified that the presence of Rrm3 is toxic when PCNA cannot be modified at lysine 127 under replication stress (HU), but is not toxic following the induction of methyl methanesulfonate (MMS). MMS is a DNA alkylating agent modifying both guanine to 7-methylguanine and adenine to 3-methlyladenine, thereby impeding replication fork progression [29]. Thus, the genetic interaction between *RRM3* and *POL30*, when lysine 127 cannot be

modified, is not specifically required after alkylation damage. Additionally, other proteins were shown to be sensitive to MMS, but not HU, further supporting the notion that HU and MMS result in unique specific modes of action to deal with the different DNA structures [30]. The checkpoint signaling pathways utilized are different depending on the lesion, while replisome stalling (in this case caused by HU) initiates a Mrc1-dependent DNA replication response, polymerase-blocking lesions (such as those induced by MMS) result in activation of Rad9-dependent DNA damage response [31-33]. Additionally, HU-induced replication stress leads to the unloading of PCNA from the lagging strand, thereby inhibiting elongation to reduced ssDNA formation [34, 35]. Therefore, significantly more ssDNA is found when cells are treated with MMS, but not HU, which in contrast significantly reduces the amount of ssDNA compared to wildtype [31]. The increased accumulation of ssDNA after MMS-induced DNA damage corresponds to post-replicative daughter-strand gaps, which are processed by the Rad6/Rad18 pathways [31, 36, 37].

Indeed, increased PCNA ubiquitination is observed after MMS, but not HU treatment, indicating an importance for the error-free or error-prone pathways in dealing with alkylated DNA lesions [36, 38, 39]. Besides ubiquitination, PCNA is also highly sumoylated after MMS treatment, which recruits the anti-recombinase Srs2 to regulate recombination during the post-replicative repair pathways of the daughter-strand gaps [37, 40, 41]. Therefore, we only observed *srs2* mutants suppressing the DNA damage sensitivity of PCNA when lysine 127 cannot be modified on MMS but had no effect on HU. Thus, a different potential recombination pathway outside of template switching and independently of Srs2 is utilized by modification of lysine 127 under replication stress. It was shown that the recombination proteins Rad52 and Rad51 are recruited to the nucleus following prolonged HU exposure to initiate fork restart [42-45]. However, this needs to be tightly controlled since hyperrecombination prevent fork restart [46-49]. Thus, one could hypothesize that the increased recombinogenic DNA lesions in *pol30-*

K127R could impede fork restart. Subsequently, deletion of Rrm3 or its first 54 amino acids, harboring the PCNA binding sites, suppresses recombinogenic DNA lesion, therefore, promoting fork restart.

However, future studies are required to gain a better understanding on how the interaction between Rrm3 and PCNA lead to replication stress when PCNA cannot be modified at lysine 127. These studies may focus on determining at what stage during the cell cycle Rrm3 interacts with PCNA and whether the interaction changes under different physiological conditions. Alternatively, unmodified PCNA at lysine 127 interacts with the acetyltransferase Eco1, which is important in cohesion establishment and Rrm3 is known to have a defect in cohesion [34, 50-52]. Thus, it is possible that defects in cohesion establishments or prolonged cohesions give rise to recombinogenic DNA lesions in *pol30-K127R* mutant and restoration of a dynamic assembly/disassembly of cohesion is restored in the absence of Rrm3 under replication stress.

Finally, I would like to highlight the importance of functional DNA helicase PIF1 in maintaining genome stability. It is thought that Human Pif1 acts as a tumor suppressor [53]. Multiple PIF1 variants that code for single amino changes of uncertain significance have been identified in cancer patients and L319P functionally evaluated in yeast. The completely conserved L319 is located in the helicase domain of hPif1 and mutation of its corresponding residue in Pfh1 was lethal, suggesting that it disrupts both nuclear and mitochondrial functions of Pfh1 and that L319P likely inactivates hPif1 [53]. Other hPif1 mutations from cancer genomes map near conserved helicase motifs (S223T) or affect other relatively conserved residues in the helicase domain (P357L, R592C), suggesting that they could also impair hPif1 function [53]. P109S, although located far upstream of the conserved helicase domain, also affects a completely conserved residue, but of unknown function [53]. The physical interaction between

hPif1 and Brca1 in the resolution of G4 structures also supports a potential role for hPif1 in cancer suppression [54].

Even though we know that PIF1 DNA helicases associate with over a thousand discrete sites in the yeast genome, including 274 tRNA genes and ~900 sites in the rDNA array, as well as replication origins, boundary elements, and sites of replication fork convergence, some of the underlying mechanisms by which helicases of the PIF1 family prevent fork stalling at these sites remain unknown [7, 55-58]. Despite Rrm3's and ScPif1's common binding regions in the yeast genome, such as tRNA genes, rDNA locus, centromeres, and telomeres, their mode of action at those sites appears distinct. Their highly disordered N-terminal tails, which are not conserved at the amino acid level, may be responsible for this difference by recruiting distinct sets of genome maintenance factors and being subject to distinct post-translational modification. Indeed, the recent identification of Rrm3 functions that map to the N-terminal tail rather than the helicase domain, N-terminal phosphorylation sites that regulate the helicase activity, and distinct as well as shared N-terminal binding partners keep adding to the ever-expanding properties of the PIF1 helicase family and their roles in maintaining genome integrity in unperturbed and stressed cells [13, 59, 60].

In addition to further elucidating the regulatory function of the N-terminal tails of PIF1 family helicases, other puzzling observations still await explanations. What is so special about genome maintenance in some yeasts that it requires two PIF1 helicases when eukaryotes with more complex genomes, including humans, cope with one? What extra functions make *S. pombe's* Pfh1 essential for survival? Are these functions performed by other proteins in other eukaryotes or are these functions not required? Besides providing detailed insights into the growing number of cellular functions and biochemical characteristics of the PIF1 helicase family, yeast can also serve as a powerful model system for the functional evaluation of hPif1, and potentially disease-associated hPif1 mutations.

References

1. Zeman, M.K. and K.A. Cimprich, *Causes and consequences of replication stress.* Nat Cell Biol, 2014. **16**(1): p. 2-9.
2. Setlow, R.B., P.A. Swenson, and W.L. Carrier, *Thymine Dimers and Inhibition of DNA Synthesis by Ultraviolet Irradiation of Cells.* Science, 1963. **142**(3598): p. 1464-6.
3. Hanawalt, P.C., *The U.V. sensitivity of bacteria: its relation to the DNA replication cycle.* Photochem Photobiol, 1966. **5**(1): p. 1-12.
4. Cox, M.M., et al., *The importance of repairing stalled replication forks.* Nature, 2000. **404**(6773): p. 37-41.
5. Keil, R.L. and A.D. McWilliams, *A gene with specific and global effects on recombination of sequences from tandemly repeated genes in Saccharomyces cerevisiae.* Genetics, 1993. **135**(3): p. 711-8.
6. Ivessa, A.S., et al., *The Saccharomyces cerevisiae helicase Rrm3p facilitates replication past nonhistone protein-DNA complexes.* Mol Cell, 2003. **12**(6): p. 1525-36.
7. Ivessa, A.S., et al., *Saccharomyces Rrm3p, a 5' to 3' DNA helicase that promotes replication fork progression through telomeric and subtelomeric DNA.* Genes Dev, 2002. **16**(11): p. 1383-96.
8. Ivessa, A.S., J.Q. Zhou, and V.A. Zakian, *The Saccharomyces Pif1p DNA helicase and the highly related Rrm3p have opposite effects on replication fork progression in ribosomal DNA.* Cell, 2000. **100**(4): p. 479-89.
9. Achar, Y.J., D. Balogh, and L. Haracska, *Coordinated protein and DNA remodeling by human HLTF on stalled replication fork.* Proc Natl Acad Sci U S A, 2011. **108**(34): p. 14073-8.
10. Calzada, A., et al., *Molecular anatomy and regulation of a stable replisome at a paused eukaryotic DNA replication fork.* Genes Dev, 2005. **19**(16): p. 1905-19.
11. Zou, H. and R. Rothstein, *Holliday junctions accumulate in replication mutants via a RecA homolog-independent mechanism.* Cell, 1997. **90**(1): p. 87-96.
12. Fumasoni, M., et al., *Error-free DNA damage tolerance and sister chromatid proximity during DNA replication rely on the Polalpha/Primase/Ctf4 Complex.* Mol Cell, 2015. **57**(5): p. 812-823.
13. Rossi, S.E., et al., *Rad53-Mediated Regulation of Rrm3 and Pif1 DNA Helicases Contributes to Prevention of Aberrant Fork Transitions under Replication Stress.* Cell Rep, 2015. **13**(1): p. 80-92.
14. George, T., et al., *Human Pif1 helicase unwinds synthetic DNA structures resembling stalled DNA replication forks.* Nucleic Acids Res, 2009. **37**(19): p. 6491-502.
15. Minca, E.C. and D. Kowalski, *Multiple Rad5 activities mediate sister chromatid recombination to bypass DNA damage at stalled replication forks.* Mol Cell, 2010. **38**(5): p. 649-61.
16. Ortiz-Bazan, M.A., et al., *Rad5 plays a major role in the cellular response to DNA damage during chromosome replication.* Cell Rep, 2014. **9**(2): p. 460-8.
17. Chen, S., et al., *The RING finger ATPase Rad5p of Saccharomyces cerevisiae contributes to DNA double-strand break repair in a ubiquitin-independent manner.* Nucleic Acids Res, 2005. **33**(18): p. 5878-86.
18. Ball, L.G., et al., *The Rad5 helicase activity is dispensable for error-free DNA post-replication repair.* DNA Repair (Amst), 2014. **16**: p. 74-83.
19. Choi, K., et al., *Concerted and differential actions of two enzymatic domains underlie Rad5 contributions to DNA damage tolerance.* Nucleic Acids Res, 2015. **43**(5): p. 2666-77.
20. Blastyak, A., et al., *Yeast Rad5 protein required for postreplication repair has a DNA helicase activity specific for replication fork regression.* Mol Cell, 2007. **28**(1): p. 167-75.

21. Achar, Y.J., et al., *Human HLTF mediates postreplication repair by its HIRAN domain-dependent replication fork remodelling.* Nucleic Acids Res, 2015. **43**(21): p. 10277-91.
22. Neelsen, K.J. and M. Lopes, *Replication fork reversal in eukaryotes: from dead end to dynamic response.* Nat Rev Mol Cell Biol, 2015. **16**(4): p. 207-20.
23. Neelsen, K.J., et al., *Oncogenes induce genotoxic stress by mitotic processing of unusual replication intermediates.* J Cell Biol, 2013. **200**(6): p. 699-708.
24. Unk, I., et al., *Role of yeast Rad5 and its human orthologs, HLTF and SHPRH in DNA damage tolerance.* DNA Repair (Amst), 2010. **9**(3): p. 257-67.
25. Sandhu, S., et al., *Loss of HLTF function promotes intestinal carcinogenesis.* Mol Cancer, 2012. **11**: p. 18.
26. Kile, A.C., et al., *HLTF's Ancient HIRAN Domain Binds 3' DNA Ends to Drive Replication Fork Reversal.* Mol Cell, 2015. **58**(6): p. 1090-100.
27. Poole, L.A. and D. Cortez, *Functions of SMARCAL1, ZRANB3, and HLTF in maintaining genome stability.* Crit Rev Biochem Mol Biol, 2017. **52**(6): p. 696-714.
28. Ciccia, A., et al., *Polyubiquitinated PCNA recruits the ZRANB3 translocase to maintain genomic integrity after replication stress.* Mol Cell, 2012. **47**(3): p. 396-409.
29. Beranek, D.T., *Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents.* Mutat Res, 1990. **231**(1): p. 11-30.
30. Chang, M., et al., *A genome-wide screen for methyl methanesulfonate-sensitive mutants reveals genes required for S phase progression in the presence of DNA damage.* Proc Natl Acad Sci U S A, 2002. **99**(26): p. 16934-9.
31. Garcia-Rodriguez, N., et al., *Spatial separation between replisome- and template-induced replication stress signaling.* EMBO J, 2018. **37**(9).
32. Pardo, B., L. Crabbe, and P. Pasero, *Signaling pathways of replication stress in yeast.* FEMS Yeast Res, 2017. **17**(2).
33. Nielsen, I., et al., *A Rad53 independent function of Rad9 becomes crucial for genome maintenance in the absence of the Recq helicase Sgs1.* PLoS One, 2013. **8**(11): p. e81015.
34. Zuilkoski, C.M. and R.V. Skibbens, *PCNA antagonizes cohesin-dependent roles in genomic stability.* PLoS One, 2020. **15**(10): p. e0235103.
35. Yu, C., et al., *Strand-specific analysis shows protein binding at replication forks and PCNA unloading from lagging strands when forks stall.* Mol Cell, 2014. **56**(4): p. 551-63.
36. Hoege, C., et al., *RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.* Nature, 2002. **419**(6903): p. 135-41.
37. Davies, A.A., et al., *Activation of ubiquitin-dependent DNA damage bypass is mediated by replication protein a.* Mol Cell, 2008. **29**(5): p. 625-36.
38. Davies, A.A. and H.D. Ulrich, *Detection of PCNA modifications in Saccharomyces cerevisiae.* Methods Mol Biol, 2012. **920**: p. 543-67.
39. Ulrich, H.D., *Regulating post-translational modifications of the eukaryotic replication clamp PCNA.* DNA Repair (Amst), 2009. **8**(4): p. 461-9.
40. Papouli, E., et al., *Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p.* Mol Cell, 2005. **19**(1): p. 123-33.
41. Krejci, L., et al., *DNA helicase Srs2 disrupts the Rad51 presynaptic filament.* Nature, 2003. **423**(6937): p. 305-9.
42. Torres, J.Z., S.L. Schnakenberg, and V.A. Zakian, *Saccharomyces cerevisiae Rrm3p DNA helicase promotes genome integrity by preventing replication fork stalling: viability of rrm3 cells requires the intra-S-phase checkpoint and fork restart activities.* Mol Cell Biol, 2004. **24**(8): p. 3198-212.
43. Petermann, E., et al., *Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair.* Mol Cell, 2010. **37**(4): p. 492-502.

44. Irmisch, A., et al., *Smc5/6 maintains stalled replication forks in a recombination-competent conformation.* EMBO J, 2009. **28**(2): p. 144-55.
45. Hanada, K., et al., *The structure-specific endonuclease Mus81 contributes to replication restart by generating double-strand DNA breaks.* Nat Struct Mol Biol, 2007. **14**(11): p. 1096-104.
46. Bernstein, K.A., et al., *Sgs1 function in the repair of DNA replication intermediates is separable from its role in homologous recombinational repair.* EMBO J, 2009. **28**(7): p. 915-25.
47. Willis, N. and N. Rhind, *Mus81, Rhp51(Rad51), and Rqh1 form an epistatic pathway required for the S-phase DNA damage checkpoint.* Mol Biol Cell, 2009. **20**(3): p. 819-33.
48. Yodh, J.G., et al., *BLM helicase measures DNA unwound before switching strands and hRPA promotes unwinding reinitiation.* EMBO J, 2009. **28**(4): p. 405-16.
49. Liberi, G., et al., *Rad51-dependent DNA structures accumulate at damaged replication forks in sgs1 mutants defective in the yeast ortholog of BLM RecQ helicase.* Genes Dev, 2005. **19**(3): p. 339-50.
50. Moldovan, G.L., B. Pfander, and S. Jentsch, *PCNA controls establishment of sister chromatid cohesion during S phase.* Mol Cell, 2006. **23**(5): p. 723-32.
51. Zuilkoski, C.M. and R.V. Skibbens, *PCNA promotes context-specific sister chromatid cohesion establishment separate from that of chromatin condensation.* Cell Cycle, 2020. **19**(19): p. 2436-2450.
52. Warren, C.D., et al., *S-phase checkpoint genes safeguard high-fidelity sister chromatid cohesion.* Mol Biol Cell, 2004. **15**(4): p. 1724-35.
53. Chisholm, K.M., et al., *A genomewide screen for suppressors of Alu-mediated rearrangements reveals a role for PIF1.* PLoS One, 2012. **7**(2): p. e30748.
54. Jimeno, S., et al., *The Helicase PIF1 Facilitates Resection over Sequences Prone to Forming G4 Structures.* Cell Rep, 2018. **24**(12): p. 3262-3273 e4.
55. Chung, W.H., *To peep into Pif1 helicase: multifaceted all the way from genome stability to repair-associated DNA synthesis.* J Microbiol, 2014. **52**(2): p. 89-98.
56. Tran, P.L.T., et al., *PIF1 family DNA helicases suppress R-loop mediated genome instability at tRNA genes.* Nat Commun, 2017. **8**: p. 15025.
57. Osmundson, J.S., et al., *Pif1-family helicases cooperatively suppress widespread replication-fork arrest at tRNA genes.* Nat Struct Mol Biol, 2017. **24**(2): p. 162-170.
58. Azvolinsky, A., et al., *Highly transcribed RNA polymerase II genes are impediments to replication fork progression in Saccharomyces cerevisiae.* Mol Cell, 2009. **34**(6): p. 722-34.
59. Syed, S., et al., *A Novel Rrm3 Function in Restricting DNA Replication via an Orc5-Binding Domain Is Genetically Separable from Rrm3 Function as an ATPase/Helicase in Facilitating Fork Progression.* PLoS Genet, 2016. **12**(12): p. e1006451.
60. Bessler, J.B. and V.A. Zakian, *The amino terminus of the Saccharomyces cerevisiae DNA helicase Rrm3p modulates protein function altering replication and checkpoint activity.* Genetics, 2004. **168**(3): p. 1205-18.