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Role of Histone H3 Lysine 56 Acetylation in the Response to Replicative stress

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Abstract

In *Saccharomyces cerevisiae*, histone H3 lysine 56 acetylation (H3K56ac) occurs on all newly synthesized histones H3 that are deposited behind DNA replication forks. H3K56ac plays critical role in chromatin assembly during DNA replication and repair. H3K56ac is also required for genome stability and stabilization of stalled replication fork. Cells lacking H3K56ac are sensitive to methyl methane sulfonate and other drugs that cause replicative stress.

In this thesis, we investigated the links between the replisome protein Ctf4 and the H3K56 acetyltransferase Rtt109. Deletion of *CTF4* partially rescued the sensitivity of *rtt109Δ* cells to methyl methane sulfonate. Genetic analyses also showed that Ctf4, Rtt109, and the Rtt101-Mms1-Mms22 complex act in the same pathway to response to replicative stress. *ctf4Δ* and *rtt109Δ* cells displayed intense foci of the single-stranded DNA binding complex RPA during replicative stress, suggesting formation of excess single-stranded DNA regions at stalled replication forks, leading to hyper activation of DNA damage checkpoints. These mutants accumulated anaphase bridges and persistent foci of the homologous recombination proteins Rad51 and Rad52 in response to genotoxins, suggesting that abnormal DNA structure formed at stalled replisome may compromise their recovery. Deletion of HR genes (RAD51, RAD52, RAD54, RAD55 and MUS81) together with *ctf4Δ* and *rtt109Δ* presents synergistic sensitivity to MMS, suggesting that H3K56ac deficient cells use HR to repair the damages caused by replicative stress. Overall our results demonstrate that H3K56ac deficient cells cannot recover MMS- induced damages because HR is compromised in these mutants.

Keywords: Ctf4, Rtt109, MMS, histone H3 lysine 56 acetylation, chromatin structure, post-translational histone modifications, cell cycle, DNA replication, DNA replication coupled chromatin assembly, DNA damage response pathway, homologous recombination.

Résumé

Chez la levure *Saccharomyces cerevisiae*, l'acétylation de l'histone H3 sur la Lysine 56 (H3K56ac) a lieu sur toutes les histones H3 nouvellement synthétisées qui sont déposées derrière les fourches de réplication. L'acétylation de H3K56 joue un rôle primordial dans l'assemblage de l'ADN lors la réplication et la réparation. L'acétylation de H3K56 joue également un rôle important dans la stabilité génomique et la stabilisation des fourches de réplication bloquée. En effet, les cellules dépourvues de H3K56ac sont sensibles au méthane sulfonate de méthyle (MMS) et à d'autres agents génotoxiques qui causent du stress réplicatif. Notre projet visait à investiguer les liens entre la protéine du réplisome Ctf4 et l'acétyltransférase d'histone Rtt109. Dans un premier lieu, la délétion de *CTF4* a partiellement contré la sensibilité des cellules *rtt109Δ* au MMS. Notre analyse génétique a aussi montré que Ctf4, Rtt109, et le complexe Rtt101-Mms1-Mms22 agissent dans la même voie de réponse face à un stress réplicative. Nos résultats montrent que les cellules *ctf4Δ* et *rtt109Δ* présentent des foyers intenses du complexe de liaison à l'ADN simple-brin RPA en réponse au stress réplicatif, suggérant la formation excessive de régions d'ADN simple-brin aux fourches de réplication bloquées, ce qui conduit à une hyper activation des points de contrôle des dommages à l'ADN. Ces mutants présentent des ponts anaphase et des foyers persistants des protéines de recombinaison homologues Rad51 et Rad52 en réponse aux génotoxines, suggérant ainsi que la structure anormale des réplisomes bloqués peut compromettre leur récupération. Nos résultats indiquent également que la délétion des gènes de la RH (RAD51, RAD52, RAD54, RAD55 et MUS81) avec *ctf4Δ et rtt109Δ* respectivement, engendre une sensibilité synergique au MMS, suggérant que les cellules qui

sont déficientes en H3K56 acétylation utilisent la RH pour réparer les dommages causés suite à un stress réplicatif. En conclusion, nos résultats suggèrent que les cellules déficientes en H3K56ac présentent des défauts de RH en réponse aux dommages à l'ADN induits par le MMS durant la phase S.

Mots-clés:

Ctf4, Rtt109, MMS, acétylation de la lysine 56 de l'histone H3, structure de la chromatine, modifications post traductionnelles des histones, cycle cellulaire, réplication de l'ADN, assemblage de la chromatine couplée à la réplication de l'ADN, voie de réponse aux dommages à l'ADN, recombinaison homologue.

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$(H3\text{-}H4)_2$	H3-H4 Tetramer
3-mA	3-methyladenine
7-mG	7-methylguanine
A	Adenine
Ac	Acetylation
APC	Anaphase Promoting Complex
Asf1	Anti-Silencing Function 1
Asf1	Anti-Silencing Factor 1
CAF1	Chromatin Assembly Factor-1
Cdc6	Cell Division Cycle 6
CDK	Cycline-Dependent Kinase
Cdt1	Cdc10- Dependent Transcript 1
CPT	Camptothecin
CTAB	Cetyl Trimethyl Ammonium Bromide
CTD	Carboxy-Terminal Domain
Ctf4	Chromosome Transmission Fidelity 4
DDK	Dbf4-Dependent Kinase
DDR	DNA Damage Response
DDT	DNA Damage Tolerance
dHJ	Double Holliday Junction
Dpb11	DNA Polymerase B (II)

DSBs	Double Strand Breaks
FACT	Facilitates Chromatin Transcription
FHA	Fork Head Associated Domain
GINS	Go, Ichi, Ni, San
H3K56ac	Histone H3 Lysine 56 Acetylation
HAT	Histone Acetyltransferase
Hda1	Histone Deacetylase 1
HDAC	Histone Deacetylase enzyme
Hif1	Hat1p-Interacting Factor-1
HJ	Holiday Junction
HR	Homologous Recombination
K	Lysine
MCM	Mini Chromosome Maintenance
MMS	Methyl Methane Sulfonate
NAD^+	Nicotinamide Adenine Dinucleotide
NASP	Nuclear Autoantigenic Sperm Protein
ORC	0rigin Recognition Complex
PCNA	Proliferating Cell Nuclear Antigen
PH domain	Pleckstrin Homology domain
Pol α	DNA Polymerase Alpha
Pol δ	DNA Polymerase Delta
Pol ε	DNA Polymerase Epsilon
Pol ζ	DNA Polymerase Zeta

Pol η	DNA Polymerase Eta
Pre-IC	Pre Initiation
Pre-RC	Pre-Replicative Complex
PRR	Post Replication Repair
PTM	Post Translational Modification
RF	Replication Fork
Rfa1	Replication Factor A1
RFC	Replication Factor C
RNR	Ribonucleotide Reductase
RPA	Replication Factor A
RPA	Replication Protein A
Rpd3	Reduced Potassium Dependency 3
Rtt109	Regulator of Ty1 Transposition 109
SC	Synthetic Complete media
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Sir2	Silent Information Regulator 2
Sld	Synthetically Lethal with Dpb11
ss- DNA	Single Stranded DNA
SSBs	Single Strand Breaks
T	Thymine
TCA	Trichloroacetic
TLS	Transleison Synthesis
TS	Template Switching

Ub	Ubiquitination
UV	Ultraviolet
YFP	Yellow Fluorescent Protein
YPD	Yeast extract-Peptone-Dextrose media

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Introduction

1-1 Chromatin structure

The eukaryotic genome is packaged into a structure known as chromatin [2]. The basic subunit of chromatin is called a nucleosome, which is formed by wrapping 147 base pairs of DNA around a histone octamer [3] [4]. An octamer is formed of a histone tetramer $(H3+H4)_2$ and two histone dimers (H2A+H2B) [4]. Histones are classified into two main groups: core histones (H2A, H2B, H3, and H4) and linker histones (H1 and H5). Core histones are involved in the nucleosome structure, while the linker histones modulates higher levels of chromatin organization [4] [5] [6]. Linker DNA, approximately 20-90 base pairs length, connects nucleosomes together [5]. Histones permit DNA packaging by neutralizing the negative charge of the DNA backbone (formed of phosphodiester bonds), thereby reducing electrostatic repulsion between DNA molecules in the tiny confines of the eukaryotic nucleus [7]. Histones are rich in Arginine and Lysine amino acids that contain free amino group (NH_2). Because of this, histones are basic, positively charged, and are able to interact with the negatively charged DNA backbone [7]. These interactions permit approximatively two meters of human DNA to fit into a nucleus that is only a few micrometers wide [8].

Chromatin is a barrier for several cellular processes involving DNA, e.g. transcription, replication and DNA repair [9]. Eukaryotic cells have evolved different strategies to modify chromatin structure; these modifications facilitate the access of regulatory factors to the underlying DNA. Reversible covalent histone modification (PTM) is one of the major

mechanisms by which eukaryotic cells regulate chromatin structure and recruit remodeling enzymes[10].

In this thesis, we will discuss H3K56ac, a modification involved in DNA replication and repair. Therefore, before fruther describing histone PTMs, the following sections will provide an overview of the cell cycle, DNA replication, DNA repair and chromatin assembly.

1-2 Key stages of the cell cycle

In eukaryotes, the cell cycle is divided into four phases: G1 (G0), S, G2, M. Regulation of the cell cycle is critical for the survival of uni- or multicellular organisms and is highly controlled at a molecular level to prevent unregulated proliferation [11]. In the quiescent or senescent stage, the replication process is at rest and the cell status remains stable from a division standpoint. This stage is the "G0 phase"- or Gap 0 [11]. Actively proliferating cells usually do not present G0 phases. In the "G1 phase", cell volume increases significantly and the G1 restriction checkpoint (see 1-2-1), first identified in *S. cerevisiae* as START, must be met in order for the cell to proceed with DNA replication in the synthesis phase- or "S phase". Beyond the START switch, cells proceed to DNA synthesis, regardless of upstream signals or cell size [12]. A checkpoint, termed the "intra S checkpoint" can be activated to restrict S phase progression if DNA replication problems are encountered (see below). After DNA replication, cells enter G2, which occurs just prior to mitosis. In many cell types, an increase in cell volume and in protein synthesis takes place during G2. Mitosis- or "M phase" is the process by which a parent cell divides into two smaller daughter cells. Another checkpoint, the M checkpoint must be met in order to proceed with cell division [13].

In the case of actively dividing cells, the daughter cells can enter another interphase after the M phase. In other cases, like for some fully differentiated cells of multicellular organisms, the cells can also enter a prolonged period of quiescence (G0); this is the case for some highly differentiated neuron cells. Many other cell types in various tissues (e.g. lung, liver, kidneys) reach a transient period of quiescence and only enter another interphase in response to the detection of appropriate cell signals. In *Saccharomyces cerevisiae*, cells normally go through all stages every 90 minutes [11].

1-2-1 The G1-S transition

Despite evolutionary divergence in ortholog protein sequences, highly similar networks are present in *S. cerevisiae* and in mammalian cells to control the passage of START or the restriction point leading to the S phase [14]. This makes budding yeast cells a valid model to study these phenomena. The G1/S regulon, activating nearly 200 genes in *S. cerevisiae* [15], is under the control of 3 cyclins CLN1, -2 and -3 and several CDKs (cyclin-dependent kinases). CLN 1- and 2 exert a positive transcriptional feedback on each other in order to drive effective yeast cell budding, coherent G1/S regulon expression, and the activation of additional B-type cyclins [16]. The product of CLN3 initiates the transition to START by phosphorylating, in conjunction with the cyclin-dependent kinase (CDK) Cdc28, protein complexes bound to the promoter regions of transcription factors like MBF and SBF or repressor like Whi5, thereby modulating gene transcription [17]. Later in the cell cycle, mitotic cyclins will further inactivate SBF and MBF factors to effectively switch off the G1/S regulon and ensure a timely and sequentially regulated replication process [18]. S-phase includes an internal checkpoint to ensure the accurate replication of DNA and prevent stalling

of single strand DNA during replication that would also impair the progression of the replication fork (see section 1-3-1).

1-2-2 DNA replication in S phase

DNA replication takes place during S phase in several different steps. First, to initiate DNA replication, the two DNA strands should be unwound. This takes place at specific genomic regions called "origin of replication" [19]. The replication origins of *S. cerevisiae* are probably the best characterized eukaryotic origins. The 11 bp consensus initiation sequence, 5′-(A/T) TTTA (T/C) (A/G) TTT (A/T)-3′, is recognized by the hexameric origin recognition complex (ORC1-5) throughout the cell cycle. ORC acts as a platform to recruit other replication proteins [20]. During late M and early G1 phase, MCM2-7 are loaded to ORC-bound origins via the concerted action of Cdc6 and Cdt1, resulting in the formation of the "pre-replicative complex" (pre-RC) [21]. Assembly of pre-RC occurs only when cyclin-dependent kinase (CDK) activity is low. Indeed, at the onset of S phase, CDK becomes highly active thus leading to the degradation of Cdc6 and the nuclear export of Cdt1 [22] [23]. This degradation prevents DNA re-replication during a single cell cycle. The MCM hexamer has a globular shape with a central cavity through which DNA passes. Therefore, MCM remains bound to DNA even after degradation of Cdc6 and Cdt1 [24]. MCM complexes are not active during G1 phase; the helicase becomes active during the G1 to S phase transition (see below). Sld3, Sld7 and Cdc45 bind to the pre-RC in early-firing origins in G1 phase and with late firing origins in late S phase [25] [26]. These proteins need Dbf4/Cdc7 (Dbf4-dependent kinase or DDK) for their stable recruitment [25]. MCM (2-4-6) and Sld2-3 are phosphorylated in S phase by DDK and CDK, respectively [27, 28] which lead to recruitment of Dpb11/Top BP1 [29]. Dpb11 binds polymerase epsilon (Pol ε), the leading strand replicative DNA

polymerase, in *S. cerevisiae* [30]. Recruitment of a second helicase co-activator, the GINS complex (Go, Ichi, Ni, San, Japanese for 5, 1, 2, 3) which is composed of Psf1, Psf2, Psf3 and Sld5 to the pre-RC in early S phase results in the formation of the so-called CMG complex (Cdc45, MCM2-7, GINS) [31]. Formation of the active form of the CMG complex requires the addition of MCM10 which facilitates recruitment of the single stranded DNA (ssDNA) binding protein, RPA (replication factor A), precluding formation of ssDNA-containing secondary structure [32]. MCM10 also interacts with DNA polymerase α (Pol α) and Ctf4/And1 [33] [34]. Ctf4 (Chromosome transmission fidelity 4), one of replisome progression complex member [35], is required for efficient DNA synthesis, normal cell-cycle progression, genomic stability and sister chromatid cohesion. During replication, Ctf4 act as a scaffold to couple the CMG helicase with pol α within the replisome. The carboxy-terminal domain (CTD) of Ctf4 creates a trimer, which interacts with the amino-terminal tails of the catalytic subunit of Pol α and Sld5 from GINS complex [36].

After the helicase opens up the double stranded DNA at origins, and the replisome is assembled to the replication fork, a specific enzyme called primase together with Pol α catalyze the polymerisation of a short segment of RNA that acts as a primer for further DNA elongation by providing a free 3' OH to DNA polymerases [37]. Then Pol α switches to two conserved DNA polymerases to continue adding deoxyribonucleotides to the 3' end of the newly forming strand. This switching takes place with binding of replication factor C (RFC) to the 3' OH end of the nascent DNA strand, which loads the DNA processivity factor proliferating cell nuclear antigen (PCNA) at the junction between single-stranded and double-stranded DNA [38]. RFC dissociation allows binding of the replicative polymerases, DNA Polymerase delta (Pol δ) and Pol ε, which are the major lagging and leading strands DNA

polymerases in *S. cerevisiae*, respectively [39] [40] **(Figure 1)**. Once the replicative machinery is set on DNA, it moves forward to generate a DNA replication fork.

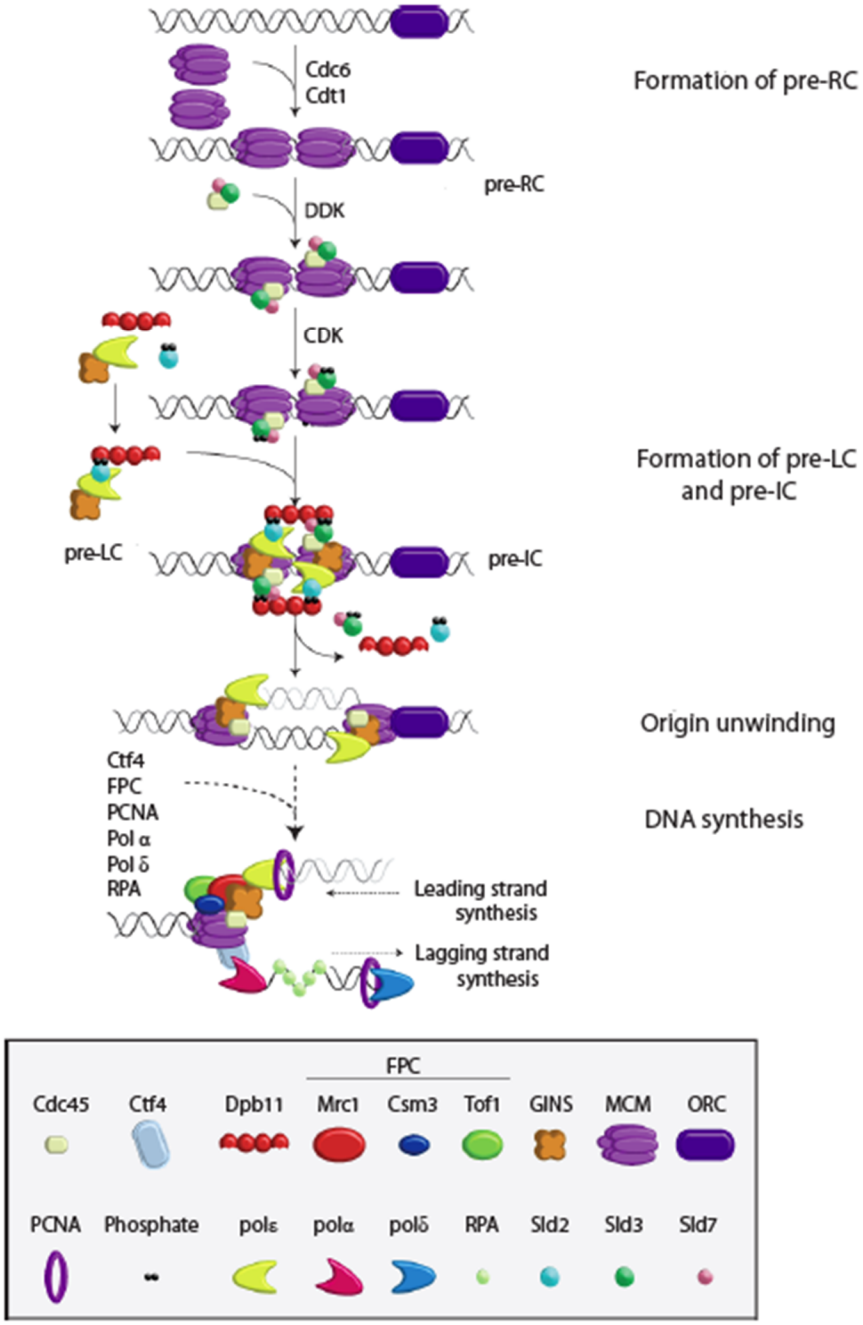


Figure 1: Schematic of DNA replication in budding yeast. Figure is presented with permission [1].

1-3 Response to DNA damage during replicative stress

DNA is constantly under different stresses which could affect its integrity. The sources of these damages can be endogenous (e.g. DNA replication, reactive oxygen species) or exogenous (e.g. UV radiation, mutagenic chemicals). Below is a discussion of various aspect of the DNA damages response, including DNA damage-induced checkpoint activation and DNA repair.

1-3-1 DNA damage and replication checkpoints

The recognition of DNA damage activates "checkpoints", leading to arrest in specific cell cycle phases. Checkpoints are well conserved between eukaryotes, indicating their significant role [41]. During of each cell cycle stages (G1, S and G2 phases), checkpoints can be activated to ensure that cells do not pass through the phase without repairing eventual DNA lesions. In S phase, the checkpoint action is most important because cells are replicating their DNA and each error might cause genomic instability and mutagenesis in a cell's progeny. DNA damage during DNA replication activates the intra-S checkpoint signaling cascade which is initiated through signals that emanate are from the apical kinases (Mec1, Tel1) and relayed via mediators (Rad9, Mrc1) and effectors (Rad53, Chk1) (details are described below). This signaling pathway promotes stabilization of stalled replication forks, inhibition of late firing origins, eventual cell cycle arrest in G2, induction and modification of DNA damage response proteins, and DNA repair processes [42] [43] [44]. Overall, the intra S phase checkpoint promotes eventual completion of replication after DNA damage has been repaired [45] [46]. Mediators of the intra S phase checkpoint will be discussed in the following subsections.

1-3-1-1 Apical checkpoint kinases: Mec1 and Tel1

Most DNA lesions occurring during replication produce ssDNA, which is generated by uncoupling of the MCM helicase and the replicative DNA polymerases, as well as by the 5'-3' resection of the end of a DSB [47, 48]. This ssDNA is recognized by replication protein A (RPA). ssDNA-bound RPA signals DNA damages and activate checkpoint sensors during S phase [47] [49]. RPA coated ssDNA recruits Ddc2, which interacts with and permits the activation of Mec1, a protein kinase of the PIKK3 family (phosphoinositol-3-kinase-related kinase). Ddc2 and Mec1 are conserved from yeast to human and their homologs in human respectively are ATRIP and ATR [50]. ATR/Mec1 is the apical kinase of the intra S checkpoint response. Mec1 activation also requires the PCNA-like 9-1-1 complex (Ddc1, Mec3 and Rad17) which is loaded at stalled forks by the checkpoint clamp loader Rad24/RFC2-5 [52]. Likewise Dpb11, a DNA replication initiation protein, binds to Ddc2 and 9-1-1 to stimulate Mec1 activity [53] [54]. Once activated, Mec1-dependent phosphorylation of downstream proteins (checkpoint mediators) initiates a signal transduction cascade which culminates in the effects of the S phase checkpoint response.

Tel1 is another kinase in yeast *S. cerevisiae* that presents similarities to Mec1 and human ATM. Tel1/ATM is recruited to DNA damage sites via a DNA end-binding complex formed of Mre11-Rad50 and Xrs2 in yeast (MRX complex) or its human homolog MRN, (Mre11-Rad50 and Nbs1) [55]. Like Mec1, Tel1 phosphorylates checkpoint mediators to initiate a signaling cascade [56]. Tel1 is mostly active in response to DNA double-strand breaks and does not play critical roles in the S phase checkpoint [57].

1-3-1-2 Checkpoint mediators: Rad9 and Mrc1

Rad9 and Mrc1 act as adaptors to promote phosphorylation of downstream proteins by the Mec1 kinase in yeast *S. cerevisiae*. Hyper-phosphorylation of Rad9 by Mec1 causes activation of this scaffold protein, which permits it to mediate Mec1-dependent phosphorylation/activation of Rad53 (see below, section 1-3-1-3) [58] [59] [60] [61]. During replication, the activation of Rad53 is also promoted by Mrc1 that is present at replisome to pair DNA pol Ɛ with Cdc45 and MCM helicase [62] [63] [64]. Mrc1 plays a mediator function in the presence of stalled fork via its phosphorylation by Mec1. Phosphorylation of Mrc1 in SCD domain causes it to lose its interaction with pol Ɛ, which makes it able to recruit Rad53 and to promote its phosphorylation by Mec1 [64] [65].

1-3-1-3 Checkpoint effector kinases: Rad53, Dun1, Chk1

Rad53 (Chk2 in human cells) is recruited and activated at DNA damage site, which permits phosphorylation of downstream target proteins [66]. One of these targets is Dun1 kinase which, after DNA damage and during S phase, physically binds to Sml1. Sml1 is an inhibitor of ribonucleotide reductase (RNR). Dun1 binding leads to phosphorylation and degradation of Sml1 and consequent increased RNR function. RNR catalyze the conversion of ribonucleoside 5'- diphosphate (NDP) to 2' deoxyribonucleoside 5'-diphosphate (dNDP). The activation of Dun1 and the decrease of Sml1 increase cellular pools of dNTP, presumably to facilitate DNA repair and replication [67]. Rad53 also phosphorylates Sld3 (CDK) and Dbf4 (DDK) which leads to inhibition of CDKs and DDKs-dependent pathways during replication. Phosphorylation of the C-terminal domain of Sld3 inhibits its interaction with Dbp11 and

Cdc45, whereas phosphorylated Dbf4 prevents the activation of the MCM helicase by Dbf4-Cdc7 complex [68].

Another checkpoint kinase, Chk1, was first identified in the fission yeast but is well conserved in different species including human. Chk1 functions mostly during G2/M in the presence of DNA damage; its activation leads to phosphorylation of Pds1 and inhibition of its ubiquitination. Pds1 ubiquitination causes its degradation via APC (anaphase promoting complex). Checkpoint-mediated accumulation of Psd1, in response to DNA damage, inhibits sister chromosomes segregation during mitosis, thereby preventing cell cycle progression [69].

1-3-2 MMS induced DNA Damages

In our work, we frequently use methyl methane sulfonate (MMS), a DNA alkylating agent, to impede DNA replication progression. It is therefore appropriate to describe its action here. MMS cause DNA methylation on N7-deoxyguanine (N7-methylguanine; 7-mG) and N3-deoxyadenine (N3-methyladenine; 3-mA). Although 90% of total adducts induced by MMS is 7-mG, this lesion is nontoxic and non-mutagenic. The remaining 10% of adducts are 3-mA that are both toxic and mutagenic **(Figure 2-a-b-c)**. 3-mA inhibit DNA replication fork progression **(Figure 2-b)**, and cells are most sensitive to MMS during replication [70] [71]. During S-phase exposure to MMS, cells try to rescue stalled replication fork by different strategies such as restarting replication by firing dormant origins, repriming replication, reversing the stalled fork or activating the DNA damage tolerance (DDT) pathways (see section 1-3-4).

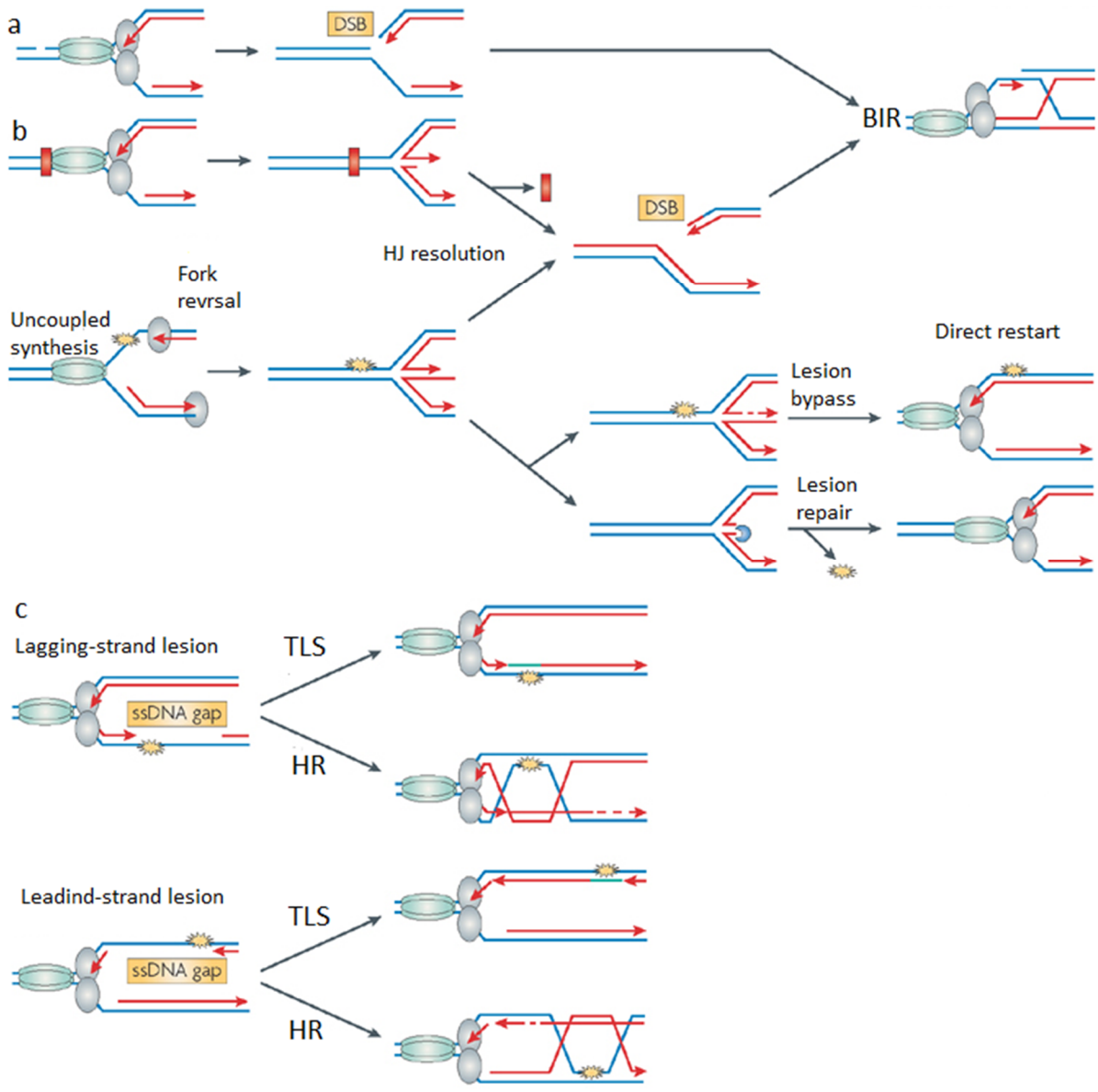


Figure 2: DNA damage and repair during replication. a) ssDNA nick in the leading strand template will directly lead to RF collapse and a one-ended DSB that could repair by BIR. **b)** DNA adducts can block replisome machinery which cause stalled fork or uncoupled synthesis. Then, fork reversal can take place leading to the formation of a chicken-foot structure. Restart can occur by HJ-cleavage followed by BIR but the RF can also undergo direct restart by HJ reversal to a fork after lesion bypass through template switching or lesion repair. **c)** Lesions can block the synthesis of only one DNA strand without inhibiting fork progression. In both cases, ssDNA gaps can be repaired by error-prone translesion synthesis (TLS) or by error-free HR [72]. The figure is presented with permission.

Repriming replication at the damage sites can leave ssDNA gaps, which could be a target for endonucleases, leading to DSBs [73]. Removal of the lesions by BER (base excision repair) can also generate single-strand breaks, which upon replication forms DSBs which can be repaired by HR **(Figure 2-a)** [74]. Stalled fork also could reverse to a chicken foot structure including Holiday Junction, again promoting HR-mediated repair **(Figure 2-b)** [73] [74] .

1-3-3 DNA repair: Homologous recombination

Impediments in replication forks progression can eventually lead to DNA double strand breaks (DSBs) [75]. DSBs, as its name indicate, occur when the phospho-sugar backbone of both complementary strands of DNA are broken at the same position or in sufficient proximity to allow detachment of the double helix into separate molecules [76]. Unrepaired DSBs lead to deletions, translocations and fusions in DNA; the resulting loss of genetic information or genomic rearrangements can cause cell death or diseases such as cancer.

DSBs are repaired mostly by two different mechanisms; non-homologous end joining (NHEJ) and homologous recombination (HR) [76, 77]; since NHEJ is not a dominant mechanism during S phase, it will not be discussed further here. Homologous recombination (HR) permits the repair of double-strand breaks by using the genetic information present in homologous DNA sequences (sister chromatids or homologous chromosomes) as a template to repair the damage in an error-free manner [78]. Despite its complexity, HR is very well conserved in evolution. HR occurs mainly after DNA replication (during the S and G2 phases) in both yeast and human to repair DSBs, and also permits the restart of stalled DNA replication forks (discussed above; **Figure 2**) [79].

In the case of DSB repair by HR, the MRX complex (Mre11, Rad50 and Xrs2) first detect damaged DNA, binds the broken DNA ends, and recruits Tel1. Next, DSB ends undergo 5´to

3′ resection **(Figure 3)**. MRX and Sae2 endonuclease activity generate a 3′ single stranded end. Then, additional proteins are recruited to the 3′ ends to promote more extensive resection, including the Exo1 and Dna2 exonucleases and the, Sgs1-Top3-Rmi1 helicase complex [80] [81, 82]. The newly-formed ssDNA is rapidly coated by heterotrimeric RPA to protect it from further degradation and to remove inhibitory secondary structures. RPA can at that point promote loading of Mec1-Ddc2 to trigger checkpoint activation and cell-cycle arrest [83]. RPA-coated ssDNA recruits recombinases to search and invade the homologous DNA sequences. Rad52 is loaded onto ssDNA which helps to recruit Rad51. Rad51 recombinase replaces RPA under the action of Rad52 [84], and forms a nucleoprotein filament on single-stranded DNA. This filament plays central roles in HR: the search for a homologous template DNA and the formation of a joint heteroduplex molecule between the damaged DNA and template [85]. Rad51 binds weakly to DNA; Rad55 and Rad57 which are Rad51 paralogs in *S. cerevisiae*, are required to stabilize and facilitate Rad51 binding and function [86] [87, 88]. Indeed, Rad55-Rad57 heterodimer stimulate the formation of Rad51nucleoprotein filament [87, 89]. Rad54 and Rdh54, which are DNA-dependent ATPase with translocase activity, then aid Rad51 filament to invade its homologous DNA either on its sister chromatid or, in diploid cells, on its homologous chromosome, forming a joint heteroduplex molecule [90]. At this point, the 3 'single stranded end can invade homologous DNA, hybridizes to the template strand and displaces the complementary strand. This forms a displacement loop (D-loop) **(Figure 3)**. After strand invasion, a DNA polymerase extends the end of the invading 3' strand by synthesizing new DNA. This changes the D-loop to a cross-shaped structure known as a Holliday junction (HJ). A second HJ forms when the second 3' overhang (which was not involved in strand invasion) is captured to the extended D loop **(Figure 3)**. After gap-repair

DNA synthesis and ligation, the structure is resolved at the HJs in a non-crossover or crossover mode **(Figure 3)**. Chromosomal crossover will occur if one Holliday junction is cut on the crossing strand and the other Holliday junction is cut on the non-crossing strand. Alternatively, if the two Holliday junctions are cut on the crossing strands, then gene conversion without crossover will be produced [91]. These recombination products are generated by endonucleolytic cleavage by specific nucleases during resolution, for example Mus81-Mms4 and Yen1 in *S. cerevisiae* [92] [93]. Another HR pathway is single-strand annealing (SSA), which occurs when a DSB is closely flanked by direct repeats. SSA is a simple process which does not require Rad51 nor strand invasion of an intact duplex DNA, but annealing of two complementary ss-DNAs.

Some DSBs, such as those that can occur at telomeres or at broken replication forks, are single-ended. Break-induced replication (BIR) pathway could repair theses DSBs [94] [95]. The resection step of BIR is similar to the traditional HR but the invasion step is different, with only one end of the DSB invading the homologous duplex. Like DSBR, the presynaptic filament often invades the sister chromatid or homolog chromosome, which then leads to extensive DNA synthesis. Therefore, all three major replicative DNA polymerases and all essential components of DNA replication machinery, with the exception of pre-RC assembly factors, are required for BIR. This mechanism can re-form replisome and restart replication following fork collapse and DSB formation **(Figure 2-a-b)** [96] [97].

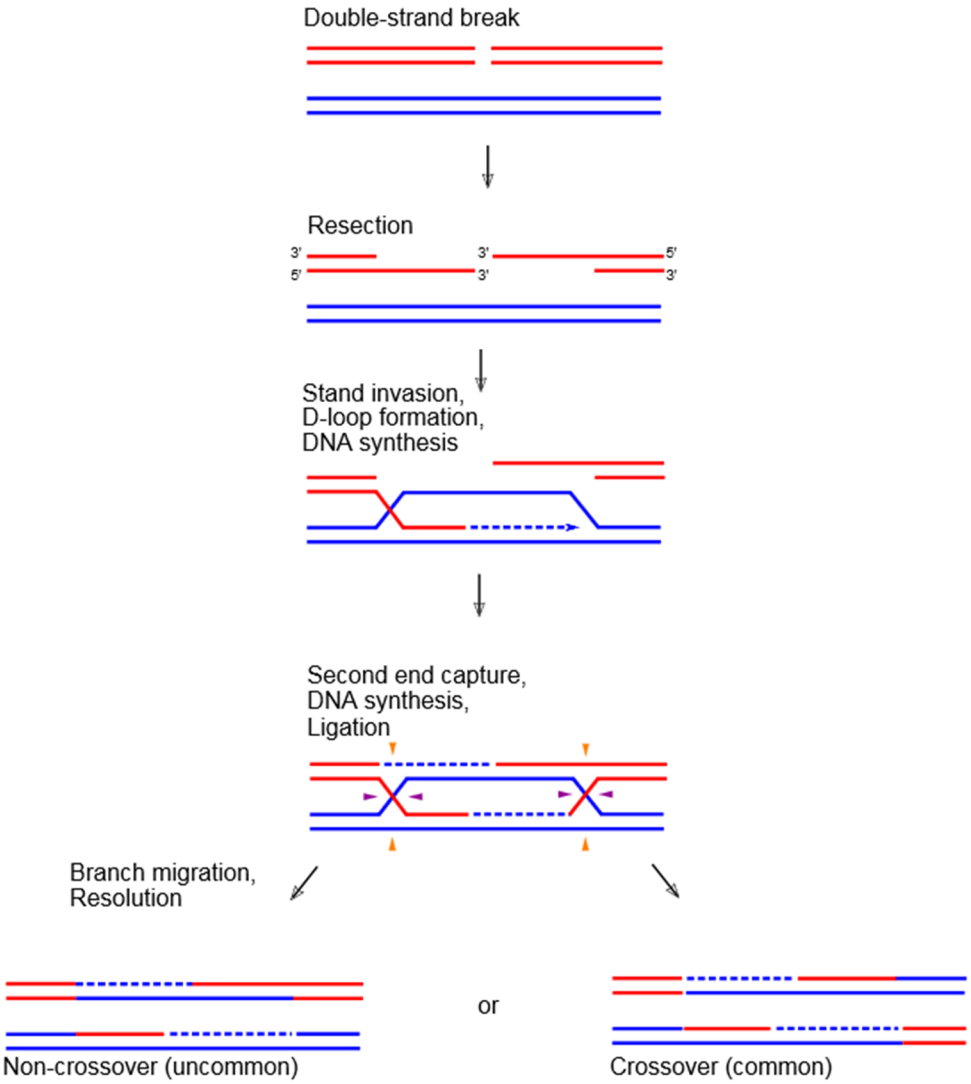


Figure 3: Diagram of the homologous recombination process. The figure is presented with permission [91].

1-3-3-1 HR proteins form foci at the site of DNA damage

Interestingly, many HR proteins accumulate into focal assemblies at the site of DNA damage.

Such repair foci form at DSBs, sites of DNA replication stress, shortened telomeres, and

regions of single-stranded DNA *in vivo*. Foci are useful marker to follow ongoing HR. To visualize foci, microscopy coupled with indirect immunofluorescence or fluorescent protein fusions is used. DNA repair foci are highly dynamic structures. They can assemble and disassemble rapidly, which could help to repair and swift recovery from cell cycle arrest after completion of the repair reaction [98] [99] [100].

In this thesis, the formation of Rfa1, Rad52 and Rad51foci in the absence of Ctf4 and Rtt109 proteins has been investigated. We will therefore describe these proteins in more details.

Rfa1: The Rfa1 protein is the large subunit of the yeast heterotrimeric RPA. Replication protein A (RPA) also known as replication factor A (RFA) is a highly conserved single-stranded DNA-binding protein. RPA from *S. cerevisiae* consists of three subunits; RFA1, RFA2 and RFA3 with approximately 70, 30, and 14 kDa molecular weight, respectively **(Figure 4)**. All three members are crucial for cell viability and bind to ssDNA. RPA is a critical protein in several processes, including DNA replication, DNA repair and recombination [101] [102].

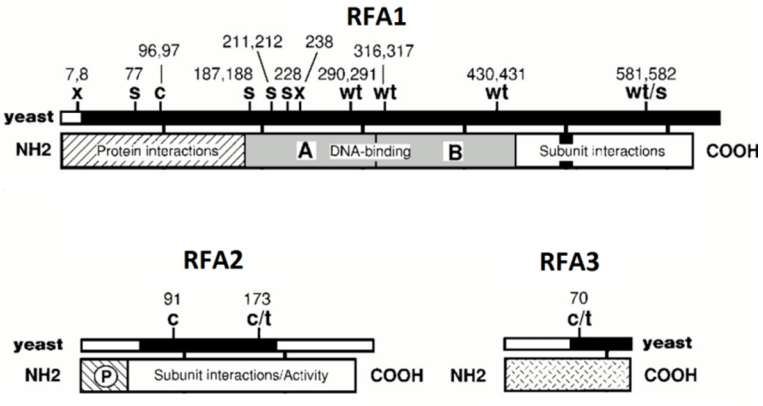


Figure 4: Schematic diagram of the structural and functional domains of the three subunits of RPA in *S. cerevisiae*. The figure is presented with permission [102].

Rad52: Rad52 is one of the most important members of the RAD52 epistasis group in *S. cerevisiae*. Rad52 is a ring-shaped oligomer with DNA binding activity. Also it interacts with both Rad51 and RPA, physically and genetically **(Figure 5)**. During HR, Rad52 promotes presynaptic filament assembly on RPA-covered ssDNA by recruiting of Rad51 **(Figure 5)**, and stimulates ssDNA annealing in second end capture and SSA. Therefore, deletion of *RAD52* (*rad52Δ*) in *S. cerevisiae* causes extreme sensitivity to a variety of DNA damaging agents (e.g. MMS) and also a general defect in HR pathways, such as DSBR, BIR and SSA [103] [104] [89].

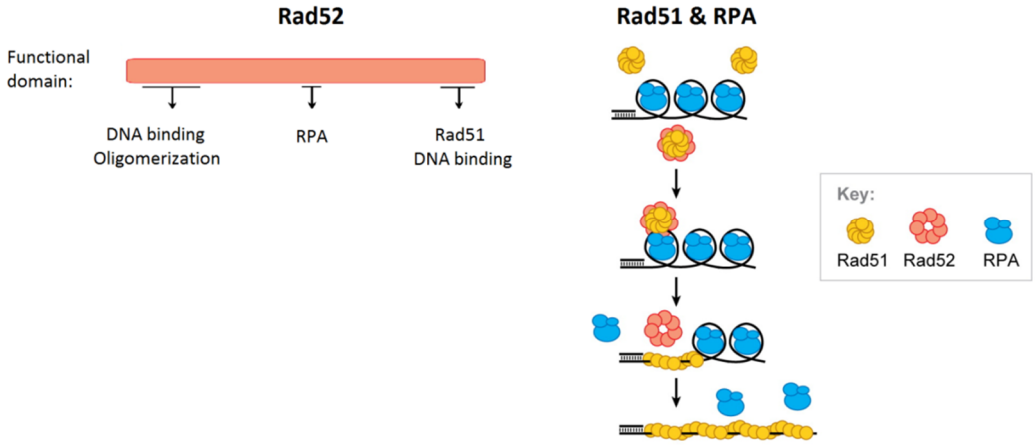


Figure 5: Rad52 and its role as a recombination mediator. The figure is presented with permission [94].

Rad51: Rad51 **(Figure 6)** is another member of the RAD52 epistasis group. As mentioned before, Rad51 is a recombinase that binds DNA through Rad52 mediator activity **(Figure 5)**. The formation of Rad51filament catalyzes homology search and strands pairing. Rad51 interacts with itself, with Rad52, and with other downstream proteins such as the Rad55-Rad57 heterodimer, Rad54 and Rdh54. Rad51 mutants are defective in the repair of DNA

damage induced by ionizing radiation and MMS, in the maintenance of telomere length, in mitotic and meiotic recombination, and in mating-type switching [105] [106].

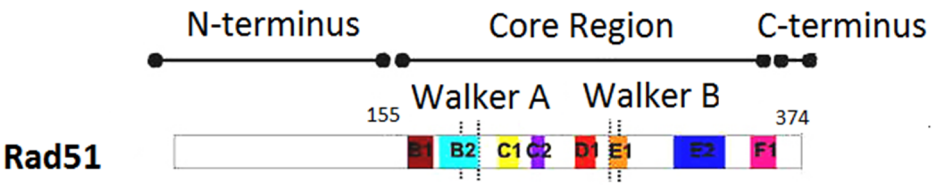


Figure 6: Rad51 protein. 8 sub-domains (B1, B2, C1, C2, D1, E1, E2, and F1) of Rad51 core region and ATP binding motifs A and B (dotted lines) are shown. The numbers immediately above sub-domains B1 and F1 are the first and last residues respectively, of the core domains of Rad51 protein. The figure is presented with permission [106].

1-3-4 DNA damage tolerance pathways: post-replication repair

In S phase, DNA lesions can be bypassed through DNA damage tolerance (DDT) pathways, in order to rapidly recharge the replicative polymerases on the DNA and to finalize the replication. DDT also is termed post replication repair (PRR). Cells via PRR pathways are allowed to continue DNA replication even in the presence of damage. PRR can occur in either an error-prone or error-free manner, which is regulated by posttranslational modifications of PCNA (ubiquitination is described below; section 1-3-4-1). Mono-ubiquitination of PCNA on lysine 164 triggers transleison synthesis (TLS) and its subsequent poly-ubiquitination leads to template switching (TS) [107].

During TLS, DNA is synthesized using damaged DNA template by translesion synthesis polymerases which can accommodate modified bases at their catalytic sites [108]. In yeast, TLS is initiated either by polymerase-η (Pol η), encoded by the RAD30 gene or the

polymerase-ζ (Pol ζ). The latter is a heterodimer composed of the Rev3 (the catalytic subunit) and Rev7 (the regulatory subunit) [109]. The replacement of these polymerases with the replicative ones is stimulated through mono-ubiquitination of PCNA. Rad18, an E3 ubiquitin ligase, is recruited to RPA coated ss-DNA and forms a complex with Rad6, an E2 ubiquitin conjugating enzyme, which mono-ubiquitinates PCNA on lysine 164 [110] [111]. Mono-ubiquitinated PCNA interacts with Rev1, an essential component of TLS [112]. Rev1 mediates the interaction of pol ζ with the mono-ubiquitinated PCNA [112] [113]. The structural subunit of pol ζ, Rev7, interacts with both Rev1 and Rev3; in yeast, Rev7 stimulates the polymerase activity of Rev3 [113] [114]. TLS is an error prone DDT pathway which can incorporate incorrect nucleotide and generate point mutations [115]. Conversely, template switching is an error free bypass which works similarly to homologous recombination. Template switching uses undamaged sister chromatid as the template, which does not induce mutations, but may lead to chromosomal rearrangements [116] [117]. TS signals are initiated by the mono-ubiquitination of PCNA mediated by Rad6-Rad18 complex [118] and subsequent recruitment of the E3 ligase Rad5 to mono-ubiquitinated PCNA, which stimulates the E2 ubiquitin conjugating activity of the Mms2–Ubc13 complex [119]. This complex adds additional ubiquitin to the mono-ubiquitinated K164 PCNA to synthesize K63-linked poly-ubiquitin chains [120]. These modifications lead to the recruitment of HR factors such as Rad51 and Rad52 which allows the formation of TS intermediates [121].

1-3-4-1 Ubiquitination

Ubiquitin is a highly conserved 76 amino acid polypeptide which is conjugated to lysine residues on protein substrates through the sequential action of three enzymes; E1-activating,

E2-conjugating and E3-ligating enzymes. E1, ubiquitin-activating enzyme, in an ATP dependent reaction binds to ubiquitin and produces an ubiquitin-adenylate intermediate which is then transferred to an active site cysteine residue. E2 ubiquitin-conjugating enzyme binds to both ubiquitin and E1 to transfer ubiquitin from the E1 enzyme to the E2. E3 is generally referred to as the ubiquitin ligase, which recognizes the substrate proteins and directly or indirectly catalyzes its ligation to the ubiquitin [122]. E3 enzymes are classified in two major groups; the HECT (Homologous to the E6-AP Carboxyl Terminus) family and the RING (Really Interesting New Gene) family. The mechanisms that these two families use to transfer the ubiquitin to substrates are different. In HECT ligases, E2 transfers ubiquitin to the E3 then, the attachment of ubiquitin to the substrate is catalyzed directly by E3. This is in contrast to RING ligases, in which the E2 transfers ubiquitin to the E3-bound substrate. Of particular interst for this thesis, the cullin family of proteins is considered as RING E3s. Cullins bind to small RING proteins (Rbx1/Roc1or Hrt1/Roc2) to promote their ubiquitin ligase activity and form cullin-RING E3 ligase (CRL) [123] [124]. The *S. cerevisiae* genome encodes three cullins: Cul1/Cdc53, Cul3, and Cul8/Rtt101 [123] (Rtt101 is described below; section 1-7).

A single lysine residue on the substrate protein can receive only one ubiquitin (mono-ubiquitination) or chains of ubiquitin (poly-ubiquitination) [125]. These modes of ubiquitination lead to different substrate fates. For example, mono- ubiquitination can regulate DNA damage bypass (discussed above; section 1-3-4) and poly-ubiquitination on lysine 48 residue often, but not always, promotes proteasomal degradation [126].

1-4 DNA replication coupled chromatin assembly

During DNA replication, parental histones are segregated on daughter chromatids, leading to the formation of nucleosome-free gaps that are filled in by assembly of new nucleosomes comprising newly synthesized histones [127]. Nucleosomes are assembled by two different pathways, replication-coupled assembly or replication-independent assembly; Replication-independent nucleosome assembly occurs during gene transcription and histone exchange [128]; this pathway is not relevant for this thesis and will not be discussed further here. Replication-coupled nucleosome assembly occurs during S phase and is closely coupled to passage of the replication fork [129]. We will focus on this latter pathway, specifically in *S. cerevisiae*.

During DNA replication, existing parental histones and newly synthesized histones areassembled into chromatin. Parental H3-H4 histones are segregated as tetramers $(H3\text{-}H4)_2$ and are transferred onto replicated DNA. However, the molecular mechanism by which they are transmitted behind replication forks remain poorly understood [130]. During DNA replication-coupled chromatin assembly, new histone H3/H4 dimers are bound by the Asf1 histone chaperone. Asf1 presents H3/H4 dimers to Rtt109, which permits acetylation on H3K56 [131, 132]. The Rtt101–Mms1–Mms22 ubiquitin ligase complex influences the dissociation of histones from Asf1 (see section 1-7), thereby modulating availability of histones for Caf1 and Rtt106 (chaperones downstream of Asf1) [133]. Rtt106 and CAF1 then deposit H3-H4 onto newly replicated DNA (**Figure 7**). Recent studies suggest that Rtt106 binds to $(H3\text{-}H4)_2$ tetramer [130] and CAF1 binds to two H3-H4 dimers or a single cross-linked $(H3\text{-}H4)_2$ tetramer with similar affinities [134]. Both Asf1 and CAF1 interact with

replication machinery, through their interactions with PCNA [135] [136], and in the case of Asf1, the PCNA loader RFC (replication factor C) [137].

After $(H3\text{-}H4)_2$ deposition, H2A-H2B dimers are incorporated on either side of $(H3\text{-}H4)_2$ tetramer, to form a histone octamer. FACT (facilitates chromatin transcription) and Nap1 chaperones both are important for the transfer and deposition of H2A-H2B dimers [138] [139]. In *S. cerevisiae*, FACT interacts with Nap1 and RPA. FACT consists of two conserved subunits; Spt16 (Suppressor of ty 16) and SSRP1 (structure-specific recognition protein 1). Through these subunits, FACT can bind to both $(H3\text{-}H4)_2$ tetramer and H2A-H2B dimer. In budding yeast, SSRP1 is separated into two proteins Pob3 and Nhp6 [140]. Pob3 contains tandem PH domains capable of binding histones, a motif also found in Rtt106. The N terminus of Spt16, in budding yeast, has also been shown to bind H3–H4 *in vitro* [141] [142].

The classic H2A–H2B chaperone, Nap1, shares sequence homology with a large class of histone chaperones, such as Vps75 in yeast. *In vitro*, Nap1 binds to $(H3\text{-}H4)_2$ tetramer and H2A-H2B dimer, with same affinity whereas *in vivo*, it appears to bind H2A-H2B dimer preferentially. Nap1 is a nucleocytoplasmic shuttling protein which enables H2A-H2B import. Nap1 also promotes nucleosome formation by disassembling nonproductive histone-DNA interactions, and directly deposits histones onto DNA [141].

Histone H1 is the last histone to be assembled onto the chromatin after replication. Hif1/NASP (nuclear auto antigenic sperm protein) chaperone could incorporate H1 into DNA. In *S. cerevisiae*, Hif1 (Hat1p-interacting factor-1) demonstrate H3-H4 and H1 chaperone activity. Hif1 physically interacts with Hat1 and Hat2 and functions in the acetylation of newly synthesized histone H4 [143].

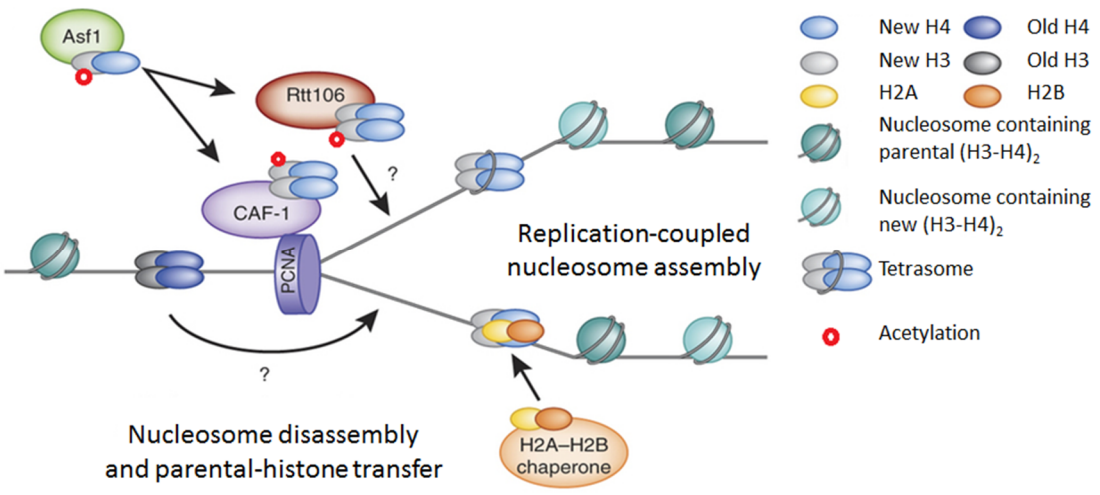


Figure 7: Replication- coupled chromatin assembly. The histone chaperones (Asf1, Rtt106 and CAF1), acetylated and deacetylated H3K56 are shown. The figure is presented with permission [141].

1-5 Histones post translational modifications

Histones are the most important proteins in the chromatin structure [144] and are remarkably well conserved throughout evolution [145]. Histones contain two domains; N-terminal tail and central domain (histone fold domain). The latter is formed from three α-helices which are separated by two loop regions, and is involved in the interactions of histone-histone and histone- DNA [146] [147] [148]. Both of these domains are subjected to post translational modifications by adding or removing small chemical groups, such as acetyl, methyl, phosphate or proteins like ubiquitin and SUMO. These enzymatic modifications alter the interactions between histones and DNA which leads to chromatin structural changes; dynamic PTMs can also modulate the recruitment of proteins and complexes with specific enzymatic activities [149] by increasing affinity for protein recognition modules. For example,

acetylated lysines are bound by bromodomains [150, 151] and methylated lysines or arginines by chromodomains, Tudor domains and PHD domains [152] [153]. Ongoing studies are describing an ever expanding list of histone PTMs **(Figure 8)** highlighting the important roles of these modifications in several vital cellular processes such as replication, transcription and DNA repair [133] [154] [155] [156].

Figure 8: Histones are subject to post-transcriptional modifications. Well characterized histone post-transcriptional modifications are depicted in this figure: acetylation (blue), methylation (red), phosphorylation (yellow) and ubiquitination (green). The number in gray under each amino acid represents its position in the sequence [157]. Figure is reproduced with permission.

1-5-1 Histone Acetylation

Addition of an acetyl function to the Ɛ-amino group of specific lysine residues on the histone tails or globular histone core is catalyzed by enzymes called Histone acetyltransferases (HATs) [158] and removed by histone deacetylases (HDACs) **(Figure 9)** [159].

Histone acetylation affects several nuclear processes **(Table 3)** through different mechanisms. By neutralizing the positive charge of Lysine, histone acetylation leads to a decrease in the affinity of histones for negatively charged DNA molecules. Therefore, acetylation at certain lysine residues causes a loosening of chromatin structure and promotes transcription [160, 161]. Lysine acetylation also promotes its interaction with certain protein recognition modules, including bromodomains [151]. For example, Gcn5 (Histone acetyltransferase belongs to GNATs family) in yeast and human contains bromodomains which mostly binds to acetylated histone H3 residues (e.g. H3K9/K14) [150] [162] [163] [164] [165] [166]. In addition, acetylated lysines are able to interact with other protein motif such as PH-like domain, e.g., *S. cerevisiae* Rtt106 interacts with K56 acetylated histone H3 through its PH-like domain [167].

Histone	Acetylation site in *S. cerevisiae*	Acetylation site in H. sapiens
H2A	K4, K7, k14 [168]	K5, K9
H2B	K3, K6, K11, K16, K21, K22, K49 [169]	K5, K11, K12, K15, K16, k20, K23,K46, K120 [170]
H3	K4, K9, K14, K18, K23, K27, K36, K56	K4, K9, K14, K18, K23, K27, k36, K56
H4	N-terminus, K5, K8, K12, K16	N-terminus, K5, K8, K12, K16, K91

Table 1: Acetylated modification sites found in *S. cerevisiae*, in comparison to those found in and H. sapiens. The most conserved sites are found in histone H3 [171]. H3K56 is well conserved from *S. cerevisiae* to human.

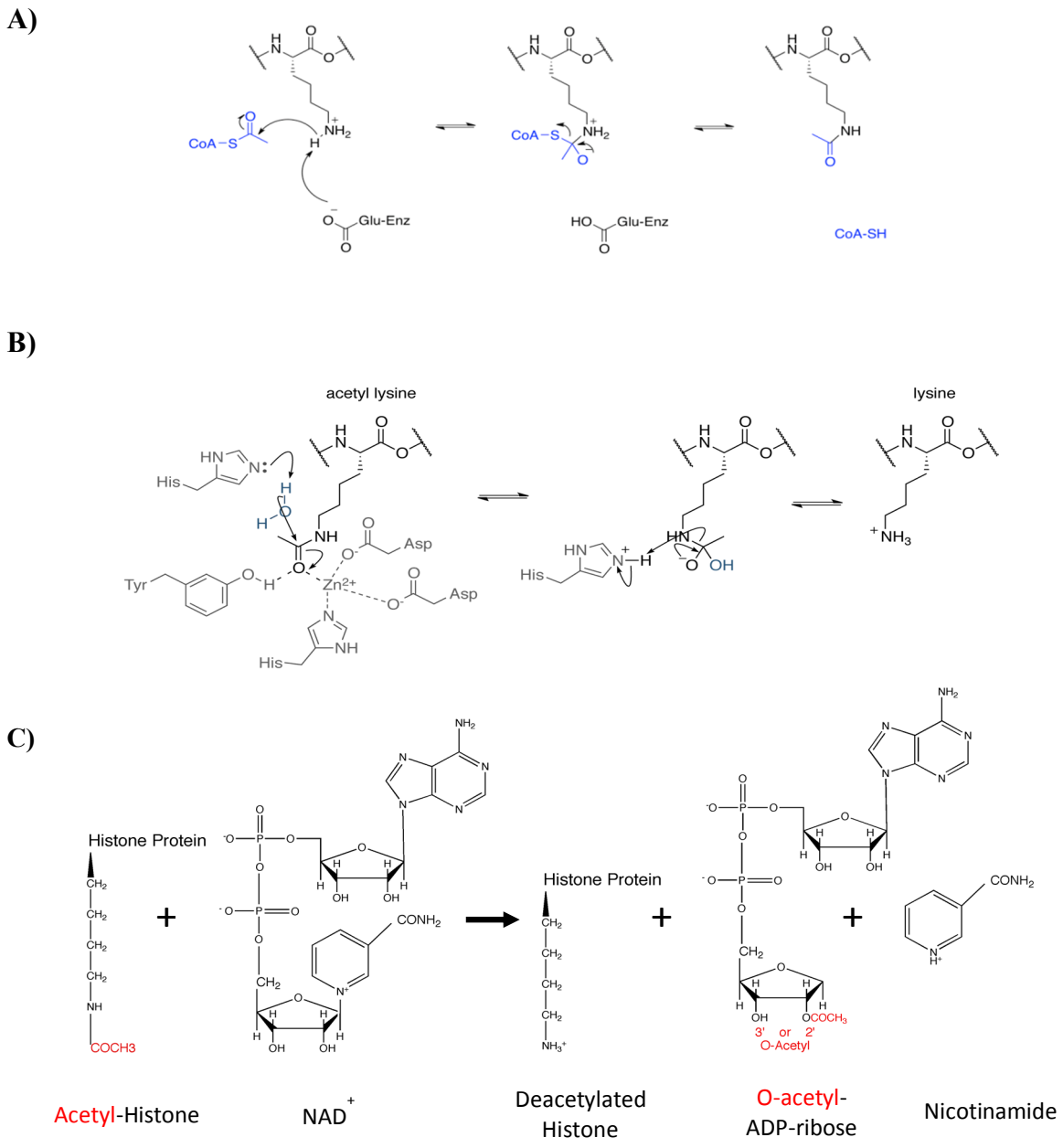


Figure 9: Lysine acetylation and deacetylation. A) HATs catalyze the transfer of the acetyl group (from Acetyl-CoA) to the Ɛ-amino group of specific lysine residues on the histone. Figure A is created by Trcum and is published in Wikipedia. Sharing the image does not require permission. **B)** HDACs remove the acetyl group from acetylated lysine in different mechanisms. This figure shows deacetylation of acetyl lysine by class I, II and IV histone deacetylases. Figure B is presented with a permission from Epigenetics (ATDBio).**C)** The global mechanism of sirtuins (class III HDACs). These HDACs are more relevant to H3K56ac. The figure is created by Dr Wurtele.

The role of histone acetylation in chromatin folding and transcriptional regulation is the focus of vigorous research. In addition, its role in DNA replication and repair remains poorly understood [172]. In this thesis, the acetylation of specific lysine residue on histone H3 (H3K56ac) in *Saccharomyces cerevisiae* and its role in the cellular response to DNA damage will be discussed. H3K56ac is conserved from yeast to human although it remains unclear whether this modification plays similar roles in both organisms **(Table 1)**.

1-5-1-1 Histone acetyltransferases

HATs come in two major classes based on their subcellular localization and substrate specificity; type-A and type-B. Type-A HATs are found in the nucleus and are able to acetylate histones in the context of nucleosomes, whereas type-B HATs are cytoplasmic and acetylate free histones before/during chromatin assembly [173]. In reality, HATs diversity does not allow them all to be strictly classified within these two groups; HAT can therefore be categorized in several families based on their sequence homology, structural features and functional roles; GNATs, MYST, HAT1, CBP/p300 and RTT109 **(Table 2)**.

Rtt109 catalyzes H3K56ac in yeast, and its structural homolog CBP/P300 does the same in human cells [174] [175]. Rtt109 (named for its initial identification as a yeast regulator of Ty1 transposition gene product 109), also known as KAT11, is a fungal-specific HAT [158]. Rtt109 associates with either of two histone chaperone proteins Asf1 or Vps75, which stimulates its acetyltransferase activity [176] [177] [131]. The interaction of Rtt109 with these chaperones is distinct; Rtt109-Vps75 forms an almost stoichiometric complex [178] which promotes the stability of Rtt109 *in vivo*, while Rtt109-Asf1 forms a rather loose and transient one [131]. Importantly, formation of these two complexes is an important determinant of Rtt109's substrate specificity. Rtt109 paired with the Vps75 chaperone associates with either

H3-H4 dimers or $(H3\text{-}H4)_2$ heterotetramers [179] [180] and acetylates histone H3 on its N-terminal lysine 9 and 27 residues. In contrast, Rtt109-Asf1 can only bind H3-H4 dimers and acetylates H3K56 [179] [181] [182] [183] [184] [185].

Family	Members	Substrate specifity	Protein domain	Possible function
GNATs	Gcn5	H2B/H3/H4	Bromodomain	transcriptional activation
	Elp3	H3		transcriptional elongation
	Hpa2	H3/H4		?
	Hpa3	H4		?
	ATF2	H4/H2B	Bromodomain	CRE-dependent transcriptional activation
	Nut1			RNA pol II transcription
MYST	Esa1	H2A/ H4/ H3	Chromodomain	transcriptional activation
	Sas2	H4	Zinc finger domain	anti-silencing
	Sas3	H3/H4/H2A	Zinc finger domain	transcriptional activation?
HAT1(KAT1)	Hat1	H4/H2A	Bromodomain	histone deposition
CBP/P300			Bromodomain Zinc finger domain	
RTT109 (KAT11)	Rtt109	H3		histone deposition

Table 2: Different families of histone acetyltransferase in *S. cerevisiae*. HAT families along with their associated members, histone substrates, and protein domains in *S. cerevisiae* [158] [173] [186] [187] [188] [185] [189] [190] [191] [192]. Protein domains are parts of a polypeptide chain with similar sequence and structure which can fold independently of the rest of the protein chain [193].

Site	Domain	Histone acetyltransferase	Histone deacetylase	Proposed function	Ref.
K4	N-terminal tail	Gcn5, Rtt109	Hst1, Sir2	Transcriptional activation	[194]
K9	N-terminal tail	Gcn5, Rtt109	Sir2, Rpd3	Transcriptional activation, Histone deposition	[195] [196, 197]
K14	N-terminal tail	Gcn5, Hat1, Esa1, NuA3	Sir2, Rpd3	Transcriptional activation, DNA repair	[196] [198, 199] [197, 200, 201]
K18	N-terminal tail	Gcn5	Hda1, Rpd3	Transcriptional activation	[202] [197, 203]
K23	N-terminal tail	Gcn5, Sas3,	Rpd3	DNA repair	[199, 204] [197, 205]
K27	N-terminal tail	Gcn5, Rtt109	Rpd3	Histone deposition, DNA repair	[197, 206]
K36	N-terminal tail	Gcn5	Rpd3	Transcriptional activation	[207, 208]
K56	Fold domain	Spt10, Rtt109	Hst3/Hst4	Transcriptional activation, DNA repair, Histone deposition	[209, 210]

Table 3: Histone H3 acetylation and associated functions in *S. cerevisiae*. H3K56ac occurs in the core domain of histone H3, most of histone H3 acetylation residues are situated on the N-terminal tail.

1-5-1-2 Histone deacetylases

Histone deacetylase enzymes (HDAC) reverse the effect of HATs and remove the acetyl group from lysine residues. HATs and HDAC act in concert to regulate the acetylation level which is important in numerous cell processes such as transcription, replication and DNA repair [159]. HDACs are broadly divided in two categories: so-called “classical” HDACs; (Class I, II, and IV) and Sir2-related (sirtuin; Class III). Based on sequence homology and cofactor dependency, these two families of HDAC are further subdivided in four different classes as indicated above. In humans, the classical HDAC family includes class I (HDAC 1, -

2, -3, -8) and class II (HDAC 4, -5, -6, -7, -9, -10) and class IV (HDAC 11). They share sequence similarity and require Zn^{2+} for deacetylase activity. The sirtuin family contains class III enzymes (human SIRT 1-7). Sirtuins present no sequence similarity to members of the classical family and use nicotinamide adenine dinucleotide (NAD+) as a cofactor for catalysis [211, 212].

Human HDACs are homolog to archetypal yeast enzymes: class I HDACs are most closely related to the yeast (*Saccharomyces cerevisiae*) transcription regulator (Rpd3); histone deacetylase 1 (Hda1), corresponds to Class II human enzymes, while silent information regulator 2 (Sir 2), corresponds, to Class III [213]. The *S. cerevisiae* genome encodes five sirtuins: Sir2p, Hst1p, Hst2p, Hst3p and Hst4p. Deacetylation of H3K56 in the chromatin of *S. cerevisiae* depends on Hst3 and Hst4 activity, and these enzymes appears at least partially redundant in this regard. Indeed, while single *hst3* or *hst4* mutants present mild phenotypes and relatively minor modulation of H3K56ac levels, lack of both of these sirtuins (in *hst3*Δ *hst4*Δ mutants) causes defects in cell cycle progression, chromosome missegregation, elevated rates of mitotic recombination, reduced viability, telomeric silencing, spontaneous DNA damage, also severe sensitivity to genotoxic agents, thermosensitivity and synthetic UV sensitivity [214] [215]. All these phenotypes appear to mostly results from constitutive H3K56 hyper acetylation throughout the cell cycle since mutations that abolish this modification, e.g. *H3K56R*, causes partial phenotypic rescue [216] [217].

Hst3 and Hst4 are expressed in G2/M and M/G1, respectively and they deacetylate H3K56. Deacetylation of H3K56 occurs largely in G2 when the expression of Hst3 is at the peak, suggesting the remarkable role of Hst3 in this act [218]. In the double mutant (*hst3Δhst4Δ*), virtually all H3K56 remain acetylated throughout cell cycle [215]. These deletions

(*hst3Δhst4Δ*) have no effect on the other H3/H4 residues, indicating that these enzymes display remarkable substrate specificity *in vivo* [219] [217].

1-5-1-3 Acetylation of newly synthesized free histones and their role in chromatin assembly and DNA damage response

New histones are synthesized in the cytoplasm and need to be transferred to the nucleus to perform their essential functions. In *S. cerevisiae*, histone H3-H4 associated with the chaperone Hif1 is acetylated at H4K5 and H4K12 [220] by Hat1 and Hat2 (histone acetyltransferases) [221] [222]. Asf1, in association with karyopherins, receive histones H3-H4 (K5ac, K12ac) and escort them toward the nucleus [223]. Rtt109 forms a complex with Asf1 to acetylate H3K56 [210] [44] [112], while Gcn5 mediates the acetylation of lysines on the N-terminus of H3, including H3K9ac and H3K27ac [165]. H3K56ac increases the affinity of both Rtt106 and CAF1 for H3-H4 [167]. While the reasons explaining increased affinity of CAF1 for acetylated H3K56 remain unclear, Rtt106 contains two tandem PH domains connected by a disordered loop, and interacts with dsDNA and H3K56ac-H4 via these PH domains [224] [225]. H3K9ac and H3K27ac also increase the binding affinity for CAF1 [206].

After deposition of $(H3\text{-}H4)_2$ tetramer on DNA, two H2A-H2B dimers incorporate on either sides of tetramer to form an octamer. Lysine 91 residue of histone H4 is a core domain acetylation site which controls the interaction between H2A/H2B dimers and H3/H4 tetramers [226]. Newly synthesized histone H2A and H2B have not been shown to present any particular pattern of acetylation [227]. Overall, the evidence indicates that PTMs on newly synthesized histones play an important role in the formation of nucleosomes on newly

replicated DNA. Furthermore, the acetylation of these sites can be critical in DNA damage response signaling and viability of cells [228].

In *Saccharomyces cerevisiae*, cells lacking H3K56 acetylation (*H3K56R* mutant) are sensitive to DNA-damaging agents (see section 1-6) [210]. Lack of H4K91ac also cause defects in silent chromatin formation and sensitivity to DNA-damaging agents in yeast [229]. In addition, mutations in all three sites of acetylation on newly synthesized histone H4 (triple mutants of *H4K91Q*, *H4K5R* and *H4K12R*) result in sensitivity to DNA- damaging agents and DNA replication stress; in addition, combining of these mutations together with *H3K56R* are synthetically lethal [228]. Therefore, H3K56ac, H4K5ac, H4K12ac, and H4K91ac appear at least partially functionally redundant in yeast [228].

Finally, after the repair of HO-mediated DNA double-strand breaks, *H3K56* mutants are not able to reassemble chromatin around the break with proper kinetics [230]. The same issue exists in triple mutants of *H4K91Q*, *H4K5R* and *H4K12R*. Thus H3K56ac and H4 acetylation sites on the newly synthesized histones can be important for chromatin assembly in DNA damage signaling [230] [228].

1-6 Cellular functions of H3K56ac

In *S. cerevisiae*, H3K56ac has been involved in several important cellular functions including replication-coupled nucleosome assembly, replication-independent histone deposition, regulation of gene expression and the DNA damage response [167] [231] [230]. This thesis examines the role of this modification in DNA replication and repair, which will be the focus of this section.

In S phase, H3K56ac promotes replication-coupled nucleosome assembly by increasing the affinity of Rtt106 and CAF-1 for histones [167], as described above (see section 1-4). After deposition of histones onto DNA and completion of replication, the acetylation is removed from H3K56. This deacetylation is critical for cell viability and the response to replicative stress; indeed, mutants that cannot deacetylate H3K56ac (for example those lacking both Hst3 and Hst4), present very severe phenotypes as mentioned in section 1-5-1-2. Indeed, proper regulation of the level of H3K56ac and by Rtt109 and Hst3/4 is important to maintain genomic stability. Unregulated level of H3K56ac gives rise to spontaneous DNA damage [232] [233]. Cells lacking H3K56ac, (i.e. *rtt109*Δ or *H3K56R* mutants) are sensitive to several genotoxic agents such as methyl methane sulfonate (MMS) and camptothecin (CPT), which induce replicative stress [234]. These mutants are unable to complete DNA replication and display persistent foci of the homologous recombination (HR) protein, Rad52, after MMS-induced replicative stress [235]. This persistence of DNA repair foci is consistent with published results indicating that HR-mediated sister-chromatid exchange is defective in cells lacking H3K56ac in response to MMS, and suggests that HR defects may account in part for the sensitivity to genotoxic stress of cells that are devoid of H3K56ac [236] [237]. Moreover, Lack of H3K56ac shows continual activation of the DNA damage checkpoint [230], which is consistent with deactivation of DNA damage checkpoints requiring proper chromatin reassembly in the presence of H3K56ac after completion of DNA repair [230] [238].

Finally, hyper-acetylation of H3K56 in *hst3*Δ *hst4*Δ deacetylases mutants generates exquisite sensitivity to genotoxic agents and causes cell death in the human fungal pathogen Candida albicans [239], and cause defects in sister chromatid cohesion and recombination in budding

[240]. However, the molecular mechanisms explaining the impact of mutations affecting H3K56ac on the DNA damage response remains in general poorly characterized.

1-7 Rtt101/Mms1/Mms22 ubiquitin ligase complex and its substrates

The proteins Rtt101/Mms1/Mms22 forms an E3 ubiquitin ligase complex similar to Cul4/Ddb1 in human (homologs of Rtt101/Mms1) which is involved in nucleotide excision repair pathway [241] [123]; the human homolog of Mms22 (MMS22L) promotes cell survival and HR after exposure to camptothecin [242] [243] [244].

Interestingly, genetic evidence indicates that Rtt101/Mms1/Mms22 complex is part of the H3K56ac pathway [245]. These factors are also involved in HR [246] since mutations that eliminate H3K56ac or Rtt101/Mms1/Mms22 inhibit HR between sister chromatids in response to MMS [234] [246] [236]. Similar to *rtt109Δ* or *asf1Δ* mutants, cells lacking *RTT101*, *MMS1* or *MMS22* are sensitive to replisome blocking agents such as MMS [247] [248] [249]. Deletion of these genes (*RTT101*, *MMS1* and *MMS22*) in *asf1Δ* or *rtt109*Δ mutants does not induce any additional sensitivity to DNA damaging agents [235] [245]. Moreover, deletion of *RTT101*, *MMS1* or *MMS22* suppresses the phenotypes of *hst3*Δ *hst4*Δ mutants without preventing H3K56 hyeracetylation [245] which strongly suggesting the activity of these proteins is modulated by H3K56ac levels in chromatin. Nevertheless, the functional links between genes of the H3K56ac genetic pathway remain unclear at the molecular level [234] [246] [236].

Rtt101 or Cul8 is one of four cullins in *S. cerevisiae* that act as a scaffold for cullin-based E3 ubiquitin ligases. Rtt101 directly binds to Mms1 which bridge Rtt101 and Mms22 [250]. Mms1 probably acts as an adaptor protein in Cul8 complexes, while Mms22 as a substrate

specific adaptor protein [250]. Deletion of *CUL8* (*cul8Δ*) does not cause cell lethality but leads to delay in anaphase progression. These cells are also sensitive to fork arrest induced by DNA alkylation, suggesting a function for Cul8 in maintaining genomic integrity [123]. Rtt101/Mms1/Mms22 also binds to other cellular factors including Rtt107 and Ctf4. It has been proposed that Ctf4 and Rtt107 might act as loading proteins for the Cul8 complex at DNA damage sites [250].

It is known that Rtt101/Mms1/Mms22 ubiquitinates Mms22. Proteasomal degradation of Mms22 is necessary for eventual cell cycle progression through a G2/M arrest induced by DNA damage [251]. Spt16 (subunit of FACT; see section 1-4) is also an ubiquitination target of Rtt101, which does not induce proteasomal degradation but is important for FACT functions during DNA replication [252]. Importantly, H3K56ac promotes the ubiquitination of H3K121, H3K122 and H3K125 by Rtt101/Mms1/Mms22 complex [133]. Mutation of these lysine residues (K) to arginine (R) or deletion of *RTT101* cause defect in the deposition of H3K56ac onto replicating DNA, leading to high level of H3 associated with Asf1. Ubiquitination helps to weaken the H3-Asf1 interaction, which may facilitate the transfer of H3-H4 from Asf1 to other chaperones involved in histone deposition including Rtt106 and CAF1. Altogether, H3K56ac and Rtt101/Mms1/Mms22 complex promote proper deposition of new H3 [133].

1-8 Rationale and objectives

In the yeast *S. cerevisiae*, H3K56ac occurs during replication-coupled chromatin assembly, in new histone molecules deposited behind replication forks. H3K56ac plays roles in the DNA damage response, since cells devoid of H3K56ac or hyper acetylated at this residue are

sensitive to genotoxic agents that impeded the progression of replication forks. Indeed, after genotoxin-induced replicative stress, mutants that misregulate H3K56ac cannot complete DNA replication and exhibit persistent foci of key HR proteins, revealing a role for H3K56ac in promoting the stability of damaged replication forks and their repair via HR. Additionally, during replicative stress, Rtt101/Mms1/Mms22 complex is thought to bind to Ctf4 (replisome machinery protein) to promote replication restart after DNA repair. In addition, the phenotypes of *hst3Δhst4Δ* cells are suppressed by deletion of *CTF4* [232]. However, the interplay between H3K56ac and other factors of its pathway remain poorly understood at the molecular level.

Based on these results, we hypothesize that:

H3K56Ac has a role in DNA repair by regulating Ctf4, MMS22, MMS1, and Rtt101.

To test this hypothesis our research aim is:

Characterize and compare the DNA damage response in cells with mutations in the genes encoding these proteins.

Material and methods

2-1 Yeast strains and media

Yeast strains used in this study are listed in **(Table 4)**. Strains were grown in yeast extract-peptone-dextrose media (YPD) or on synthetic complete media (SC), using standard conditions [253].

Table 4: Yeast strains used in the research project

Strain	Genotype	Source	Figure/table used
HWY19	BY4743 *MAT*a *his3Δ1 leu2Δ0 ura3Δ0 CAN1*	[235]	Fig. 10,11,12, 18-C, 21
HWY17	BY4743*MAT*a *his3Δ1 leu2Δ0 ura3Δ0 rtt109*Δ::*KanMX CAN1*	[235]	Fig. 10,11,12, 18-C
HWYR70	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 ctf4Δ::URA3MX*	This study	Fig. 10,11,12, 18-C, 21
HWAG42	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rtt109Δ::KanMX ctf4Δ::URA3MX*	This study	Fig. 10,11
HWAG43	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rtt109Δ::KanMX ctf4Δ::URA3MX*	This study	Fig. 10
HWAG44	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rtt109Δ::KanMX ctf4Δ::URA3MX*	This study	Fig. 10
HMY140	W303 *MAT*a *trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100 hht1-hhf1::LEU2 hht2-hhf2Δ::KanMX trp1::HHT1 K56R-HHF1::TRP1*	[210]	Fig.12
HMY152	W303 *MAT*a *trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100 hht1-hhf1::LEU2 hht2-hhf2Δ::KanMX trp1::HHT1-HHF1::TRP1*	[210]	Fig.12
HWYH1	BY4743 *MAT*a *ura3Δ0 rtt101Δ::URA3MX*	[235]	Fig.12
HWYH5	BY4743 *MAT*a *ura3Δ0 mms1Δ::URA3MX*	[235]	Fig.12
HWYH6	BY4743 *MAT*a *ura3Δ0 mms22Δ::URA3MX*	[235]	Fig.12
HWYG26	W303 *MATa ADE2 trp1-1 ura3-1 his3-11,15 leu2-3,112 LYS2 can1-100 RAD52-YFP RAD5*	This study	Fig. 13, 19-D
HWYM53	W303 *MATa ADE2 trp1-1 ura3-1 his3-11,15 leu2-3,112 LYS2 can1-100 RAD52-YFP RAD5 ctf4Δ::URA3MX*	This study	Fig. 13, 19-D

HWYM57	W303 *MATa ADE2 trp1-1 ura3-1 his3-11,15 leu2-3,112 LYS2 can1-100RAD52-YFP RAD5 rtt109Δ::URA3MX*	This study	Fig. 13, 19-D
HWYK25	W303 *MATa ADE2 trp1-1 LYS2 RAD5 YFP-RAD51 RAD52-CFP*	M. Lisby	Fig. 14
HWYS17	W303 *MATa ADE2 trp1-1 LYS2 RAD5 YFP-RAD51 RAD52-CFP rtt109d::URA3MX#3*	[235]	Fig. 14
HWYS21	W303 *MATa ADE2 trp1-1 LYS2 RAD5 YFP-RAD51 RAD52-CFP ctf4d::URA3MX#6*	This study	Fig. 14
HWYN59	W303 *MATa ADE2 trp1-1 ura3-1 his3-11,15 leu2-3, 112 LYS2 can1-100 bar1::LEU2 RFA1-8ala-YFP RAD5*	This study	Fig. 15,16,17, 19-E, table 4,5
HWYN60	W303 *MATa ADE2 trp1-1 ura3-1 his3-11,15 leu2-3, 112 LYS2 can1-100 bar1::LEU2 RFA1-8ala-YFP RAD5 rtt109Δ::URA3MX*	This study	Fig. 15,16,17, 19-E, table 4,5
HWYQC1	W303 *MATa ADE2 trp1-1 ura3-1 his3-11,15 leu2-3, 112 LYS2 can1-100 bar1::LEU2 RFA1-8ala-YFP RAD5 ctf4Δ::URA3MX*	This study	Fig. 15,16,17, 19-E, table 4,5
HWYV63	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 RAD53-V5-HIS::KANMX*	Open Biosystem	Fig. 18-B
HWYV75	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 RAD53-V5-HIS::KANMX rtt109Δ::URA3MX*	This study	Fig. 18-B
HWYV72	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 RAD53-V5-HIS::KANMX ctf4Δ::URA3MX*	This study	Fig. 18-B
HWYG18	W303 *MATa ade2-1 trp1-1 ura3-1 his3-11,15 leu2-3, 112 LYS2 can1-100 mec1Δ::TRP1 sml1Δ::HIS3*	This study	Fig. 18-C
HWYG19	W303 *MATa ade2-1 trp1-1 ura3-1 his3-11,15 leu2-3, 112 LYS2 can1-100 sml1Δ::URA3MX rad53Δ::LEU2*	This study	Fig. 18-C
ASYY76	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 slx4Δ::HPHMX*	This study	Fig. 18-C
HWYH2	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rtt107Δ::URA3MX*	This study	Fig. 18-C
HWYI17	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 sgs1Δ::KanMX*	[235]	Fig. 20
HWYI18	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 sgs1Δ::KanMX rtt109Δ::URA3MX*	[235]	Fig. 20
HWYR74	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 sgs1Δ::KanMX ctf4Δ::URA3MX*	This study	Fig. 20
ASYY75	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rad52Δ::KanMX*	This study	Fig. 21

HWYT27	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 ctf4Δ::URA3MX rad52Δ::HPHMX*	This study	Fig. 21
HWYI13	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rad51Δ::KanMX*	[235]	Fig. 22
HWYOB9	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rad54Δ::KanMX*	Eun-Hye Lee	Fig. 22
HWYOC1	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rad55Δ::KanMX*	Eun-Hye Lee	Fig. 22
HWYOB5	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rad59Δ::KanMX*	Eun-Hye Lee	Fig. 22
HWYS63	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rad51Δ::KanMX ctf4Δ::URA3MX*	This study	Fig. 22
HWYS66	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rad54Δ::KanMX ctf4Δ::URA3MX*	This study	Fig. 22
HWYS69	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rad55Δ::KanMX ctf4Δ::URA3MX*	This study	Fig. 22
HWYS72	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rad59Δ::KanMX ctf4Δ::URA3MX*	This study	Fig. 22
HWYOB11	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rad54Δ::KanMX rtt109Δ::URA3MX*	Eun-Hye Lee	Fig. 22
HWYOC3	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rad55Δ::KanMX rtt109Δ::URA3MX*	Eun-Hye Lee	Fig. 22
HWYOB7	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 rad59Δ::KanMX rtt109Δ::URA3MX*	Eun-Hye Lee	Fig. 22
HWYAI31	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 mus81Δ::KANMX*	This study	Fig. 22
HWYAI27	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 mus81Δ::LEU2 ctf4Δ::URA3MX*	This study	Fig. 22
HWYAI29	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1 mus81Δ::LEU2 rtt109Δ::URA3MX*	This study	Fig. 22

2-2 Cell synchronization and transient exposure to genotoxic agents

To synchronize cells in G1, exponentially growing cells were incubated in medium containing 5 μg/ml α-factor for 90 minutes at 30°C, followed by a second dose of 5 μg/ml of α-factor for 75 minutes. A single dose of 50 ng/ml α-factor was used in the case of *bar1Δ* cells. G1 arrested cells were released in YPD or SC medium containing 50 μg/mL pronase and appropriate genotoxic drugs (MMS or HU). After transient exposure to MMS, cells were

washed briefly in 2.5% sodium thiosulfate/medium to inactivate the MMS prior to release in pronase-containing medium. Indicated time points were taken and samples were flash-frozen on dry ice prior to being processed for subsequent manipulations. MMS, HU and pronase were purchased from Sigma.

2-3 Cell viability assays

For viability assays, appropriate dilutions of the cultures were plated on YPD before and after MMS exposure. Colonies were counted and viability was calculated as the ratio of colonies after treatment vs before treatment (in G1). For spot test experiments, 5-fold serial dilutions of saturated overnight cultures were spotted and incubated on indicated medium for 48 to 72 hours at 25° or 30° C [254].

2-4 Immunoblotting

Proteins were extracted using alkaline cell lysis (Kushnirov method) [255] or standard trichloroacetic (TCA)/glass beads procedures. Samples were loaded on SDS-polyacrylamide gel. Following gel electrophoresis, proteins were transferred on a PVDF (Polyvinylidene Difluoride) membrane through wet or semi-dry methods. Wet transferring was done at 100 volt for 1 hour or 30 volt for overnight in cold chamber at 90 mA. Semi-dry method was done at 15 volt for 2 hours and the amperage was determined by the size of the membrane and the number of the gel (1mA/cm^2). For example, transferring of two gels on the 9x7cm membranes occurred at 126 mA (0.13 Amp). Afterwards, the membrane was incubated with antibodies; primary antibody (specific antibody) and secondary antibody (anti-mouse or anti-rabbit). Proteins were detected by super signal ECL (Pierce Chemical, Rockford, IL). The antibodies used were: Anti-V5 (ab27671) from Abcam and anti-Rad53 (sc-6749) from Santa Cruz.

2-5 Fluorescence microscopy

Cells were grown in synthetic medium supplemented with 100 µg/ml adenine to reduce autofluorescence. Samples were taken at appropriate time points; cells were incubated in fixation solution (3.7% formaldehyde in 0.1 M potassium phosphate buffer pH 6.4) for 15 minutes and then resuspended in 50 mM Tris pH 7.5. Fixed cells were kept at 4°C in the dark. Multi-well glass slides surfaces were coated with 1 mg/ml concenavalin A, to immobilize yeast cells. DAPI (4', 6-diamidino-2-phenylindole) mounting medium was used to stain fixed cells. To visualize proteins of interest through Fluorescence microscopy, we used yeast strains expressing proteins tagged with fluorophores such as yellow fluorescent protein (YFP), green fluorescent protein (GFP), cyan fluorescent protein (CFP). Microscopy was performed on a Zeiss Z2 Imager fluorescence microscope equipped with AxioVision software using a 100× objective lens. Images were analyzed using the Image J version 1.46r.

2-6 Neutral 2-dimensional (2D) gel electrophoresis

DNA replication and recombination intermediates were studied using 2D gel electrophoresis. Cells are collected from time course as explained before (Cell synchronization and transient exposure to genotoxic agents). DNA samples were extracted and quantified DNA was digested using EcoRV and HindIII restriction enzymes (New England Biolabs).

For the first dimension electrophoresis, the samples containing 20x loading dye (0.83% bromophenol blue, 0.83% xylene cyanol FF, 50% glycerol) were loaded on the 0.35% agarose gel (agarose in TBE buffer). In this step, the fragments were separated in proportion to their mass. DNA fragments in sizes of ~ 4.5 to 12kb were cut under the UV light. After carefully arranging the slices, the second dimension gel (0.9%) in the presence of ethidium bromide

was poured. DNA fragments were transferred from gel to a nylon membrane by southern blotting. Transferred DNA was cross linked to the nylon using a UV-crosslinker. The presence of desired DNA sequence, on the membrane, was detected using a radioactive [α-^{32}P] hybridisation probe specific to the efficient early-firing replication origin *ARS305* as described [256]. The labeled DNA was purified from the enzymes and unincorporated dNTPs using a small Sephadex G-50 column. The hybridized probe was detected by storm scanner.

2-7 Automated evaluation of Rfa1-YFP foci intensity

Accurate and non-biased analysis of the data was done using an algorithm programmed on Matlab (Mathworks, Cambridge, MA) which allowed the detection of fluorescent foci and computation of their fluorescence intensity in images composed of multiple cells. The method used to detect cell and DNA foci were described in the paper published by our lab [217].

2-8 Measurement of DNA content by flow cytometry

Cellular DNA content was measured by fluorescence-activated cell sorting (FACS) using Sytox Green as nucleic acid stain [257]. Cells were diluted 1/10 in 70% ethanol, for 30 min at room temperature. After centrifugation, cells were resuspended in 500 µl of 50 mM Tris-HCl, pH 7.5 containing 20 µl of ribonuclease A (10 mg/ml) and incubated at 37°C overnight. Cell pellets were resuspended in 200 µl of 50 mM Tris-HCl, pH 7.5 buffer containing 20 µl proteinase K (10 mg/ml) and incubated for 30 min at 50°C. Then samples were prepared by adding 100 µl of processed cell suspension to 500 µl of 50 mM Tris-HCl, pH 7.5 containing 1 µM Sytox Green. FACS analysis was performed on FACScalibur cytometers using the FlowJo (Treestar) software.

Results

The results shown in this thesis have been generated before I left for an extended maternity leave. Therefore, during my absence, more research has been done on this subject by our lab members; these results are not shown here to respect other people's work and efforts. These issues are addressed in the Discussion section.

3-1 CTF4 acts via an RTT109- and H3K56ac-dependent genetic pathway.

Although *ctf4Δ* suppresses the phenotypes of *hst3Δ hst4Δ* cells [232], *rtt109Δ ctf4Δ* cells were reported to be more sensitive to MMS than either single mutant, suggesting that Rtt109 and Ctf4 may play non-redundant roles in response to MMS [250]. In contrast, our yeast growth assays clearly indicate that deletion of *CTF4* partially rescued the sensitivity of *rtt109Δ* mutants to chronic MMS exposure **(Figure 10)**.

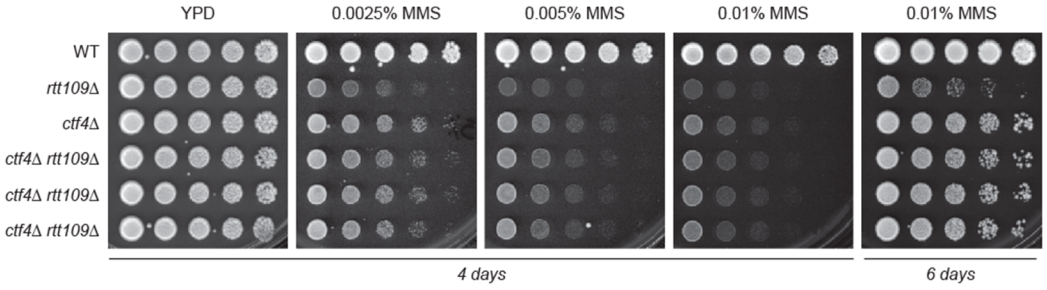


Figure 10: Ctf4 and Rtt109 act in a same pathway in response to replicative stress. Mutation of *CTF4* partially rescues sensitivity of *rtt109Δ* cells to chronic MMS exposure. Serial dilutions of saturated cells were spotted on the indicated medium and incubated at 25° or 30°C.

To better understand the link between Ctf4 and H3K56ac, we next performed viability assays for *ctf4Δ* and *rtt109Δ* mutants. G1-arrested cells were released into the cell cycle in YPD containing MMS for 90 min. In these conditions, a large majority of cells enter S-phase at the same time. In response to transient MMS exposure during S phase, mutants lose viability with the increase of MMS concentration compared to WT. 0.04% of MMS concentration is mostly lethal for them.

Interestingly, the viability of *rtt109Δ* and *ctf4Δ* double mutants is not worse than single gene deletion and they are epistatic (**Figure 11**), suggesting that the suppression observed in Figure 10 reflects cumulative effects sustained over several cell cycles. Also, these effects are perhaps too subtle to be readily observed during transient exposure to MMS in a single cell cycle (**Figure 11**) compared to chronic exposure to MMS which was 4-6 days (**Figure 10**). Overall, our data strongly suggest that RTT109 and CTF4 act in a same pathway in response to replicative stress.

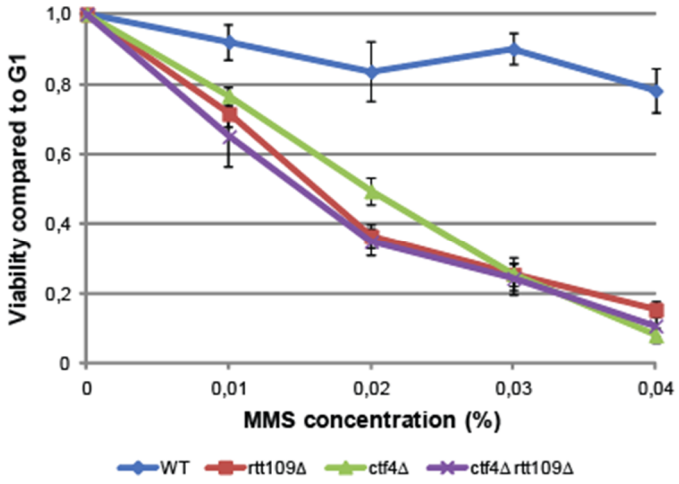


Figure 11: In response to MMS exposure during S, *ctf4Δ* and *rtt109Δ* mutations are epistatic. Cells were released from G1 in medium containing MMS for 90 min. MMS was inactivated with 2.5% sodium thiosulfate in YPD. Cells were plated on YPD before and after MMS; viability is the ratio of the number of colonies obtained after relative to before MMS exposure.

3-2 H3K56ac-deficient cells form persistent HR foci following transient exposure to MMS.

Published results indicate that *ctf4Δ* and *rtt109Δ* mutants are sensitive to MMS suggesting that they may not be able to repair MMS-induced damage. HR is a critical DNA damage repair pathway that operates during S-phase, and it necessary for cell survival in response to MMS-induced DNA damage. We therefore decided to investigate the behavior of HR proteins in *ctf4Δ* and *rtt109Δ* mutants in response to MMS. In order to test if HR is defective in H3K56ac- deficient cells, we first quantified spontaneously arising Rad52-YFP foci in asynchronously growing mutant cells of the H3K56ac pathway **(Figure 12)**. Rad52 is a key HR protein which acts early in this pathway, and it is known to form intranuclear foci upon DNA damage. We visualized these foci by fluorescence microscopy using cells expression a version of Rad52 fused to YFP (yellow fluorescent protein).

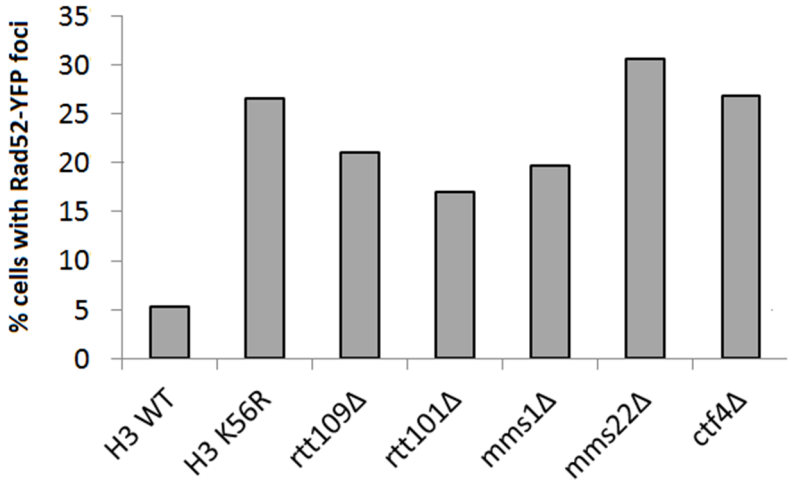


Figure 12: Mutants of the H3K56 acetylation pathway display frequent spontaneous Rad52-YFP foci. Asynchronous cells were examined by fluorescence microscopy. 200 to 300 cells were counted for each sample.

The result shows that mutants of the H3K56ac pathway present very frequent Rad52-YFP foci compared to WT cells, even in the absence of genotoxic agents. One possibility to explain these results is that these mutants may have defects in HR in response to spontaneously arising DNA lesions. In my thesis, we focused on *ctf4Δ* and *rtt109Δ* mutants. Published data of our lab indicates that transient exposure of *H3K56R* cells to MMS results in persistent Rad51 and Rad52 foci in cells lack in H3K56ac (*H3K56R* mutants) [234]. We decided to check if this also holds true for *ctf4Δ* and *rtt109Δ* mutants. Cells were arrested in G1 using alpha factor **(Figure 13-A, α-factor),** and were then released into MMS toward S phase. Afterwards the removal of MMS **(Figure 13-A, 0 min)**, WT cells are able to continue the cell cycle **(Figure 13-A, 60-120-240 min)** and initiate a new one, but *ctf4*Δ and *rtt109*Δ mutants are blocked in G2 **(Figure 13-A, 300 min)**. Cells from this experiment were fixed with formaldehyde and visualized by fluorescence microscopy. At least 200 cells were counted for each time point. As in Figure 12, asynchronously growing *ctf4Δ* and *rtt109Δ* mutants arise spontaneously Rad52-YFP foci compared with that in WT cells (30-36% versus 2%, respectively) **(Figure 13-B, Asynch)**. Likewise, a high frequency of spontaneous Rad52-YFP foci is observed in α-factor-arrested *ctf4Δ* and *rtt109Δ* mutants compared to WT **(Figure 13-B, α-factor).** 60 min after release from MMS treatment, WT cells present foci frequencies that are similar to mutants **(Figure 13-B, 0, 60 min)**. However**,** after 120 min of MMS removal, the fraction of WT cells containing Rad52-YFP foci began to decline, whereas foci continued to accumulate in *ctf4Δ* and *rtt109Δ* mutants until they were present in 70-80% of the cells compared to 5% in WT cells **(Figure 13-B, 120, 240, 300 min)**. These results indicate that resolution of Rad52-containing HR foci is abnormal in cells lacking either Rtt109 or Ctf4.

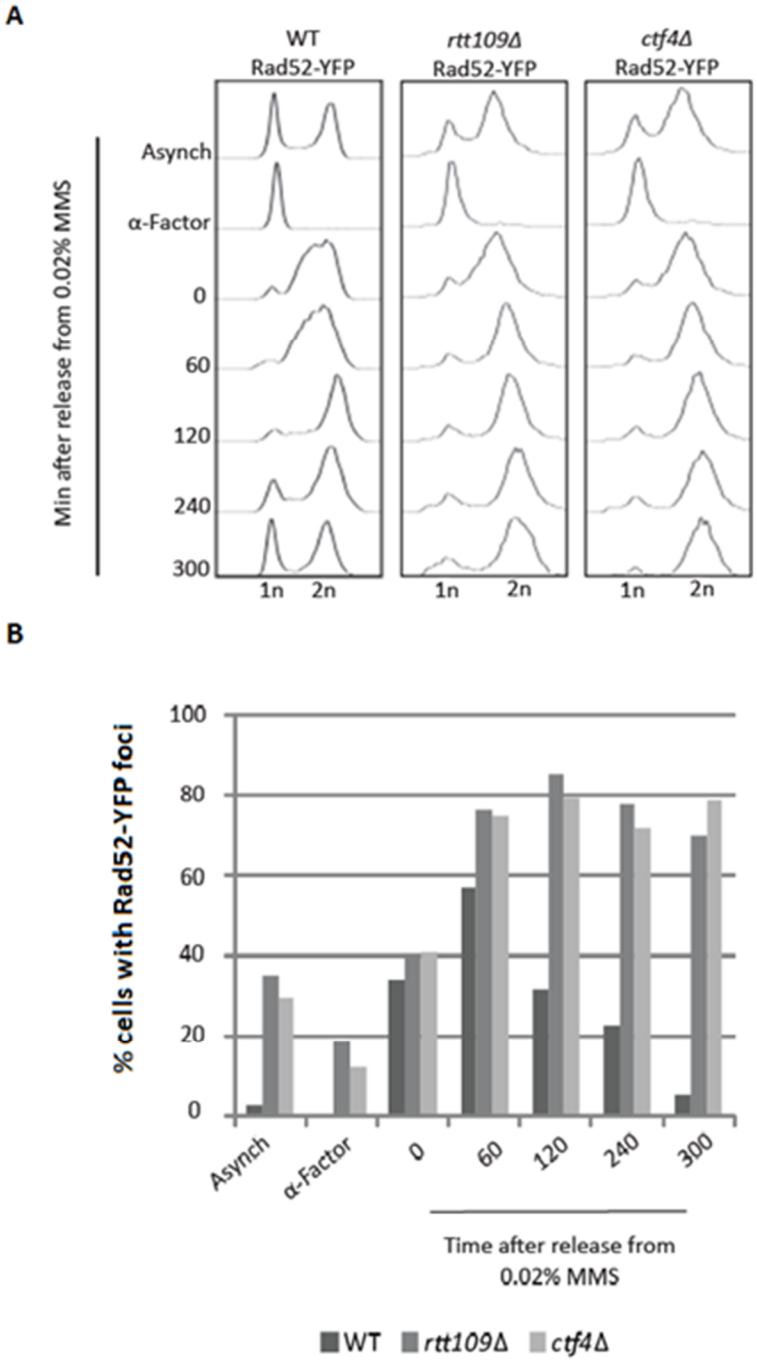


Figure 13: Transient exposure of ctf4Δ and *rtt109Δ* mutants to MMS results in persistent Rad52 foci. Cells arrested in G_1 were released into the cell cycle in the presence of 0.02% MMS for 1 h 30 min. MMS was inactivated with 2.5% sodium thiosulfate in synthetic complete medium, and cells were incubated in fresh medium. **A)** Samples were processed to determine DNA content by flow cytometry. **B)** Fraction of cells containing Rad52-YFP foci as assessed by fluorescence microscopy.

We next sought to characterize the behavior of Rad51-YFP foci in *ctf4Δ* and *rtt109Δ* mutants. Rad51 is recruited to DNA by Rad52. Similar to the situation observed using Rad52-YFP-expressing cells, the fraction of spontaneous Rad51-YFP foci in asynchronously growing *ctf4Δ* and *rtt109Δ* mutants also exhibits enormous difference compared to WT (24% and 28% versus 1%, respectively) **(Figure 14, Asynch)**. The differences between WT and mutant cells are also obvious in α-factor-arrested cells **(Figure 14, α-factor)**.

After the quenching of MMS **(Figure 14, 0 min)**, in contrast to mutant cells presenting persistent foci at 300 min (78%), WT cells initially accumulate foci but those appear to be resolved at later time points (e.g. at 120 min the fraction of Rad51-YFP foci decreases: 39% in WT compared to 78% in mutant cells) **(Figure 14, 0-60-120-240-300 min)**. These results suggest that MMS-induced damage is being repaired by HR in WT cells, but that lack of Ctf4 and Rtt109 precludes appropriate HR-mediated repair of MMS-induced damage presence. Our results further confirm that *CTF4* deletion induce persistent Rad52-YFP and Rad51-YFP foci accumulation, similar to *H3K56R* and *rtt109Δ* mutants [234].

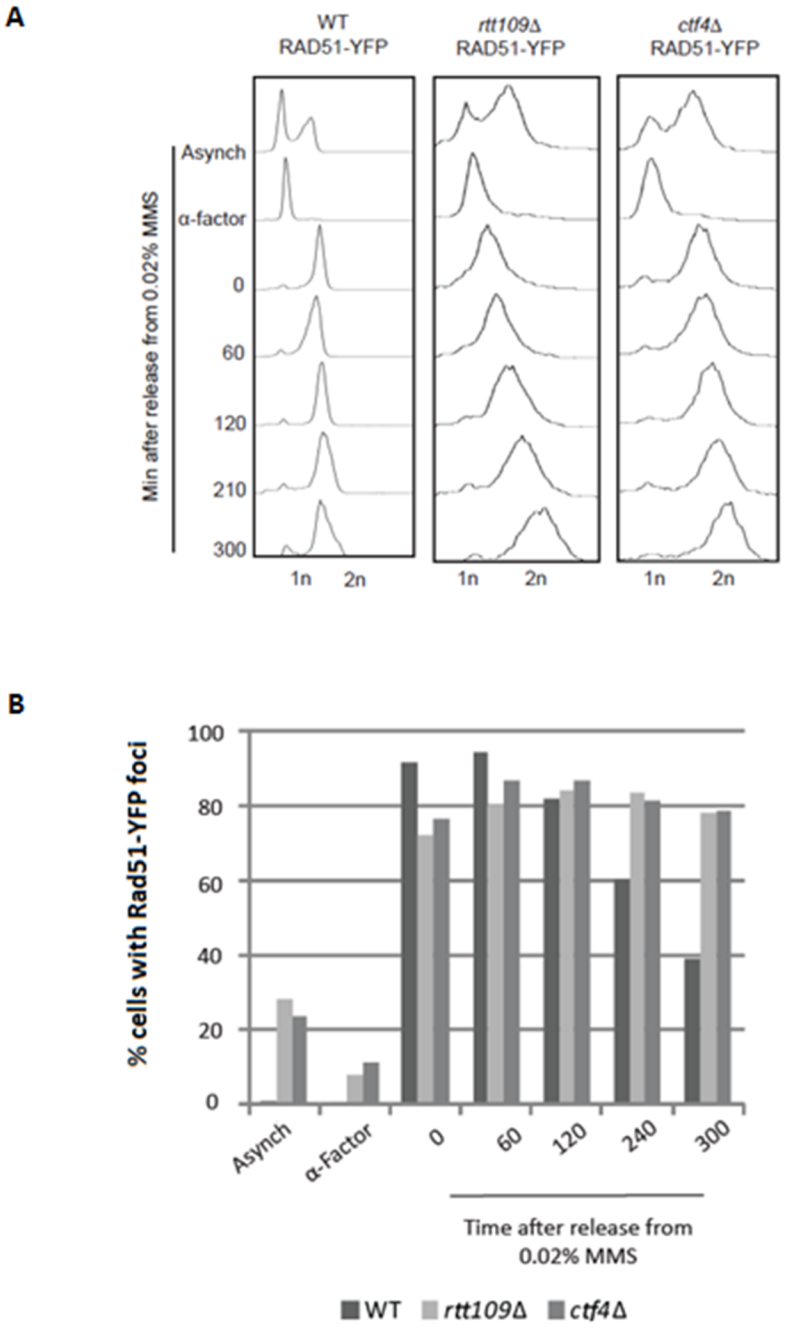


Figure 14: Transient exposure of *ctf4Δ* and *rtt109Δ* mutants to MMS results in persistent Rad51 foci. Cells arrested in G_1 were released into the cell cycle in the presence of 0.02% MMS for 1 h 30 min. MMS was inactivated with 2.5% sodium thiosulfate in synthetic complete medium, and cells were incubated in fresh medium. **A)** Samples were processed to determine DNA content by flow cytometry. **B)** Fraction of cells containing Rad51-YFP foci as assessed by fluorescence microscopy.

We reasoned that as is the case with Rad51 and Rad52, Rfa1 (a critical HR initiation protein) would present abnormal behavior in response to replicative stress in H3K56ac mutants. To verify this, we performed experiments with Rfa1-YFP-expressing cells. As expected, the fraction of Rfa1-YFP foci in WT and mutant cells is very similar to those in Rad51 and Rad52 **(Figure 15)**. One significant difference is the brightness of Rfa1-YFP foci compared to Rad51-YFP and Rad52-YFP ones. Visual inspection of microscopy images indicated that Rfa1-YFP foci in *ctf4Δ* and *rtt109Δ* mutants were brighter and more intense **(Figure 16-A)**. Microscopy images were analysed in an unbiased manner using a *in house*-developed software which is able to recognize and measure pixel intensity **(Figure 16-B)**. This software was developped in collaboration with Dr. Santiago Constantino (Maisonneuve-Rosemont hospital/Université de Montréal; see Methods). The result indicates that persistent Rfa1-YFP foci in *ctf4Δ* and *rtt109Δ* cells are more intense than in WT **(Figure 16-C** and **Table 5**). This result could be explained by the presence of excess RPA-bound ssDNA at MMS-stalled replication forks.

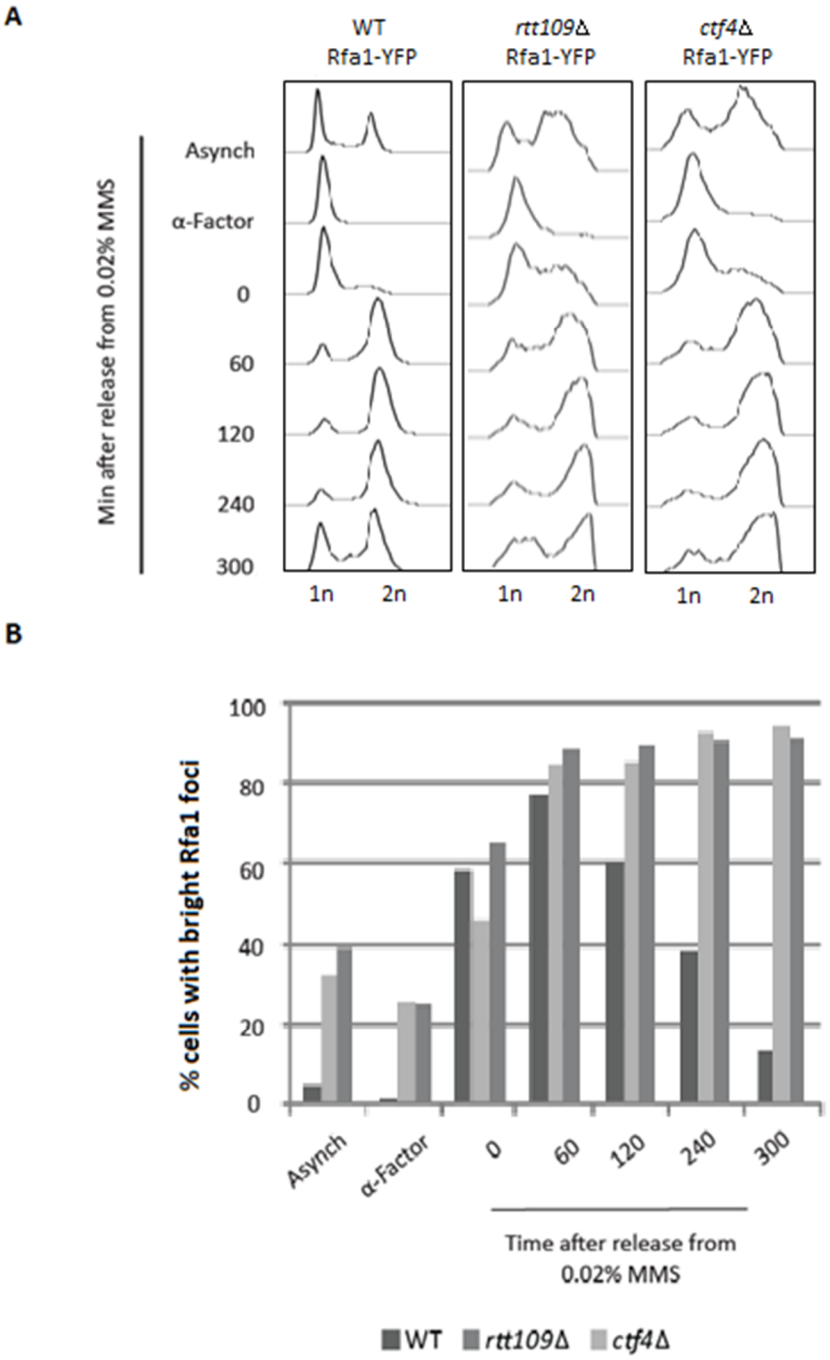


Figure 15: Transient exposure of *ctf4Δ* and *rtt109Δ* mutants to MMS results in persistent Rfa1 foci. Cells arrested in G_1 were released into the cell cycle in the presence of 0.02% MMS for 1 h 30 min. MMS was inactivated with 2.5% sodium thiosulfate in synthetic complete medium, and cells were incubated in fresh medium. **A)** Samples were processed to determine DNA content by flow cytometry. **B)** Fraction of cells containing Rfa1-YFP foci as assessed by fluorescence microscopy.

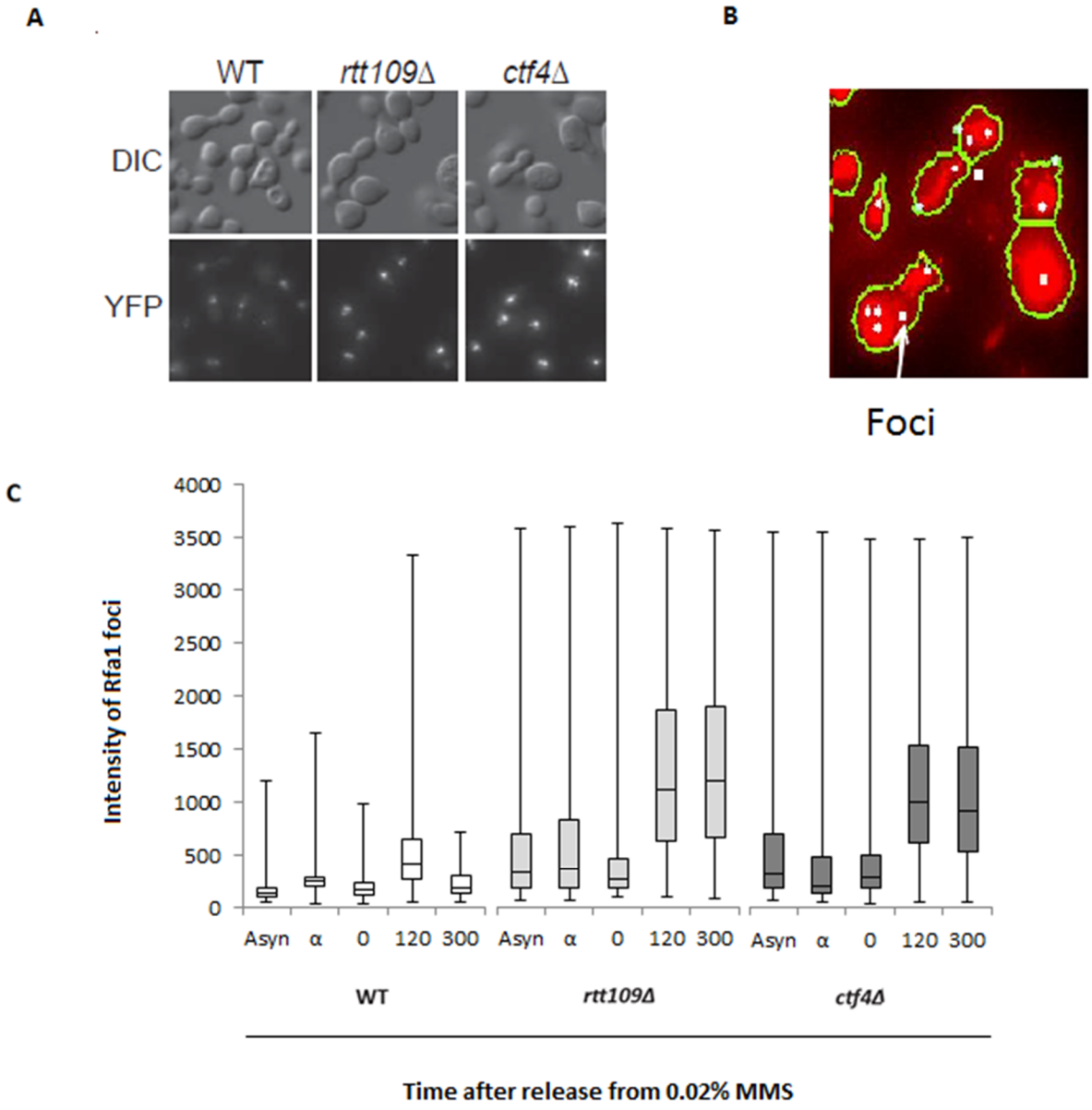


Figure 16: The intensity of Rfa1 foci. A) Transient exposure of *ctf4Δ* and *rtt109Δ* mutants to MMS results in the formation of intense Rfa1 foci. **B)** Software recognises all the foci and measures the brightness level which represents the intensity of foci. **C)** The intensity of Rfa1-YFP foci are measured in different time points before and after MMS exposure, using a software (see material and methods). The box-and-whiskers plot shows the distribution of data, using the five number summary (Minimum, first quartile, median, third quartile and maximum). The line between boxes represents median. Ends of the box show first and third quartiles. Maximum and minimum of the data is displayed through whiskers. The statistical analysis of data is shown in (**Table 5**).

Strain	Time (min)	Mean Rfa1-YFP intensity	P-value vs WT
Wild-type	Asyn	299	N/A
	A	332	N/A
	0	328	N/A
	120	683	N/A
	300	389	N/A
rtt109Δ	Asyn	689	7.3E-17
	A	790	4.4E-03
	0	549	7.8E-21
	120	1618	2.0E-69
	300	1680	3.2E-103
ctf4Δ	Asyn	700	3.3E-15
	A	572	5.6E-02
	0	624	5.6E-62
	120	1485	3.6E-52
	300	1436	3.5E-85

Table 5: Rfa1-YFP intensity of cells treated with MMS. Mean is the average intensity of systematically analyzed Rfa1-YFP foci. *P*-value was calculated using an unpaired two-tailed Student's *T*-test.

We reasoned that it was possible that dying cells would present very strong RPA foci, and that such accumulation of Rfa1 at stalled forks could represent non-specific responses to lethal doses of MMS. We therefore investigated the behavior of Rfa1 foci in WT cells exposed to a highly lethal dose of MMS (**Figure 17, Table 6**). Our results indicate that, in response to high doses of MMS, dying WT cells do not present Rfa1 foci that are as intense as those from dying *rtt109*Δ and *ctf4*Δ mutants. This suggest that accumulation of excess RPA at MMS-stalled replication forks in H3K56ac mutants does not represent a non-specific response to cell death, but instead may be the result of active mitigation of RPA accumulation at stalled forks in response to replicative stress by Rtt109 and Ctf4. However, the mechanisms remain poorly understood at this point. Figure 17 shows the viability of WT cells treated with 0.06% MMS compared to WT, *ctf4Δ* and *rtt109Δ* mutants treated with 0.02% MMS (**Figure 17**). Then, the foci intensity of these cells is verified. For each cell the intensity of 200-300 foci is measured and *p*-value is calculated using an unpaired two-tailed Student's T-test. The *p*-value is used to determine statistical significance in a hypothesis test (**Table 5** and **Table 6**).

Strain	Time (min)	Mean Rfa1-YFP intensity	P-value vs WT
Control	360	945	N/A
Wild-type	360	371	6.8E-13
rtt109Δ	360	1904	5.6E-53
ctf4Δ	360	1670	6.1E-30

Table 6: Mean Rfa1-YFP intensity of cells treated with 0.02% MMS compared to WT treated with 0.06% MMS (Control).

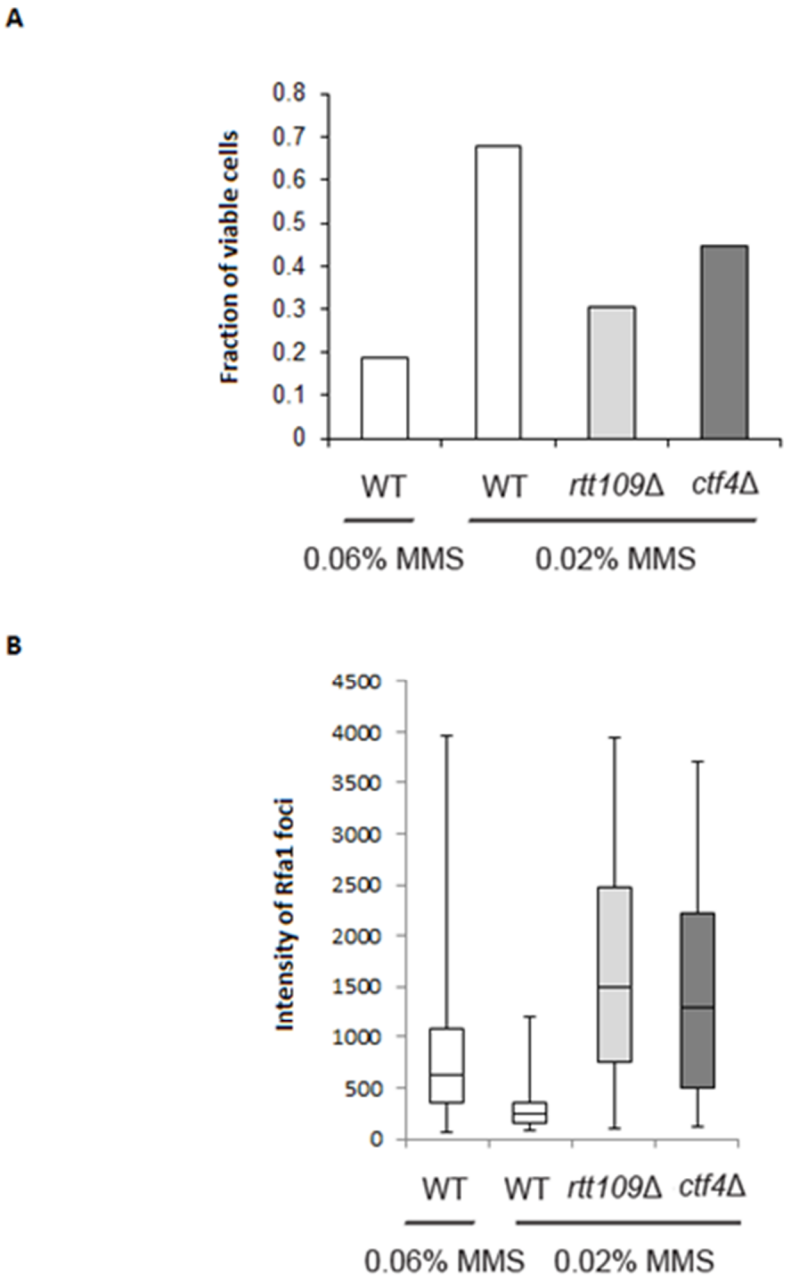


Figure 17: Rfa1-YFP foci intensity depends on *ctf4Δ* and *rtt109Δ* mutants. A) The viability of WT cells treated with 0.06% MMS compared to WT, *ctf4Δ* and *rtt109Δ* treated with 0.02% MMS. **B)** The intensity of Rfa1-YFP foci in WT, *ctf4Δ* and *rtt109Δ* treated with 0.02% MMS are measured and compared with WT cells treated with 0.06% MMS. The statistical analysis is shown in **(Table 6)**.

3-3 Cells lacking *Ctf4* or *Rtt109* present hyperactivation of the DNA damage checkpoint kinase Rad53

One of the probable consequense of persistent and intense Rfa1 foci could be their effect on DNA damage checkpoint signaling cascade. Rfa1 interacts with Ddc2 to promote activation of the checkpoint kinase, Mec1, and subsequent activation of mediators and checkpoint effectors including Rad53 **(Figure 18-A)** [47] **[**51**]** [258].

We verified whether persistent and intense Rfa1 foci in *ctf4Δ* and *rtt109Δ* mutants could affect DNA damage-induced signaling. WT, *ctf4Δ* and *rtt109Δ* strains containing Rad53 tagged with a V5 epitope and six histidines (6×HIS) were arrested in G1 **(Figure 18-B, α-factor)**, and then released in YPD medium containing 0.02% MMS for 90 minutes **(Figure 18-B, 0).** MMS was inactivated, and cells were incubated in fresh YPD for 120 minutes **(Figure 18-B, 60-120).** We used Anti-V5 antibody to detect Rad53-6His-V5 by immunoblot.

The result shows that *ctf4Δ* and *rtt109Δ* mutants displayed persistent phosphorylation of Rad53 after transient exposure to MMS during S-phase **(Figure 18-B)**. This is concordant with reported hyperactivation of Rad53 in cells lacking H3K56ac in response to DSBs [234]. Rtt107 and Slx4 are two other proteins which prevent Rad53 hyperactivation in response to replication stress. Rtt107-Slx4 complex physically interacts with Dpb11 and histone H2A serine 128 which counteracts Rad9-dependent Rad53 activation in response to MMS [258]. We compared the extent of Rad53 phosphorylation in *ctf4Δ* and *rtt109Δ* with *rtt107Δ* and *slx4Δ*. At low MMS concentration (0.01% MMS), the result show that compared to WT cells, *ctf4Δ* and *rtt109Δ* mutants present high levels of Rad53 phosphorylation that are similar to *rtt107Δ* and *slx4Δ* **(Figure 18-C)**. These results are consistent with a model in which persistent Rfa1 foci cause persistent hyperactivation of checkpoint kinases such as Rad53.

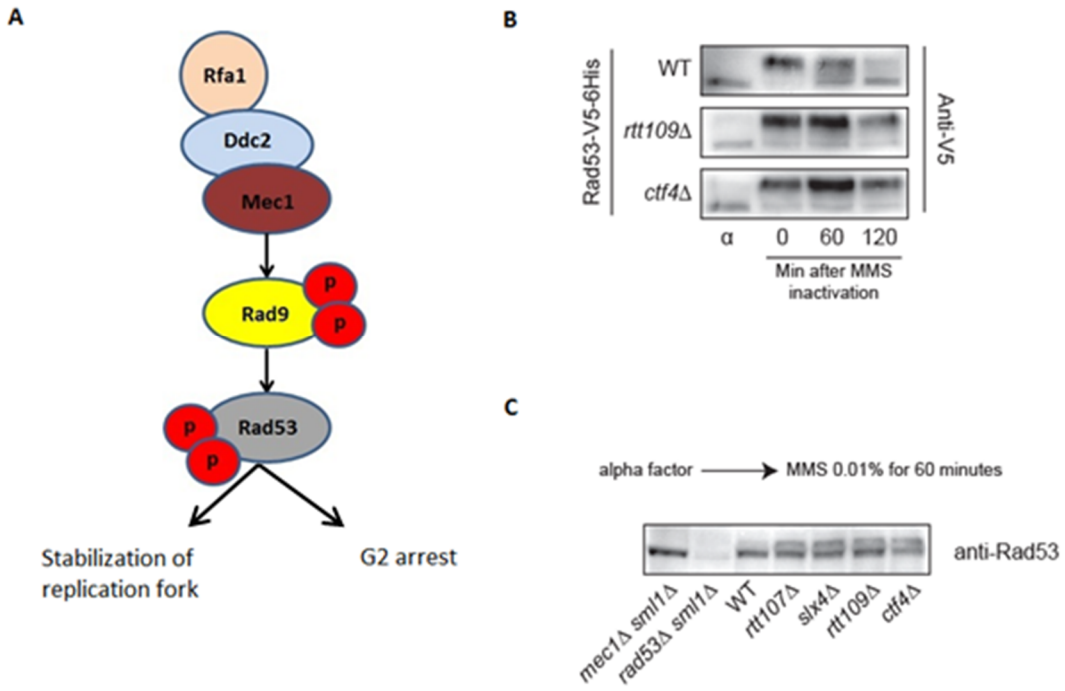


Figure 18: Cells lacking *Rtt109* or *Ctf4* present hyperactivation of Rad53 in response to MMS exposure during S. A) Rfa1 induces activation of Rad53. **B)** Cells released from G1in medium containing 0.02% MMS for 90 minutes. MMS was inactivated and cells were released in fresh medium. Samples were analyzed by immunoblot. **C)** Cells were released from G1 in medium containing 0.01% MMS for 60 minutes. Samples were analyzed by immunoblot.

3-4 Cells lacking *Ctf4* or *Rtt109* present anaphase bridge formation

The presence of intense and persistent HR initiation foci (Rfa1-YFP, Rad52-YFP and Rad51-YFP) suggest compromised HR may cause the formation of abnormal DNA structures at stalled replication forks in *rtt109Δ* and *ctf4Δ* mutants. One possibility is that HR fails during later step such as formation or resolution of Holliday junctions. It was suggested that consequence of HR defects during S-phase could be anaphase bridge formation [259]. Anaphase bridge is formed when two chromosomes that are connected together are not able to separate (**Figure 19-A)**.

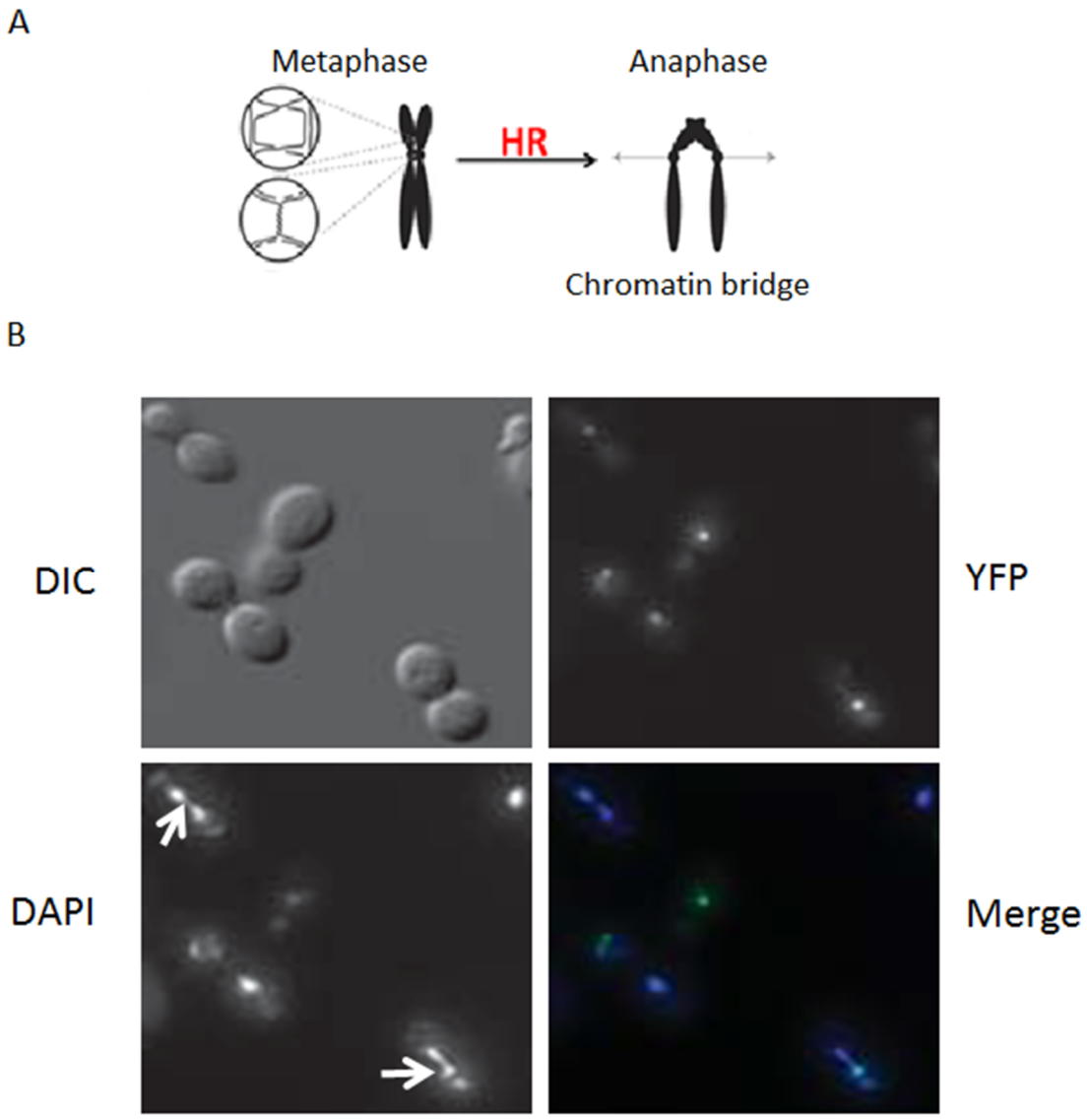


Figure 19: Anaphase bridges are formed in *rtt109Δ and ctf4Δ* cells treated with MMS during replication. A) HR have been suggested to promote the formation of anaphase bridges in response to replicative stress. Sharing the image does not require permission [259]. **B)** Anaphase bridges and foci are analysed in YFP and DAPI-stained samples; arrow: anaphase bridges.

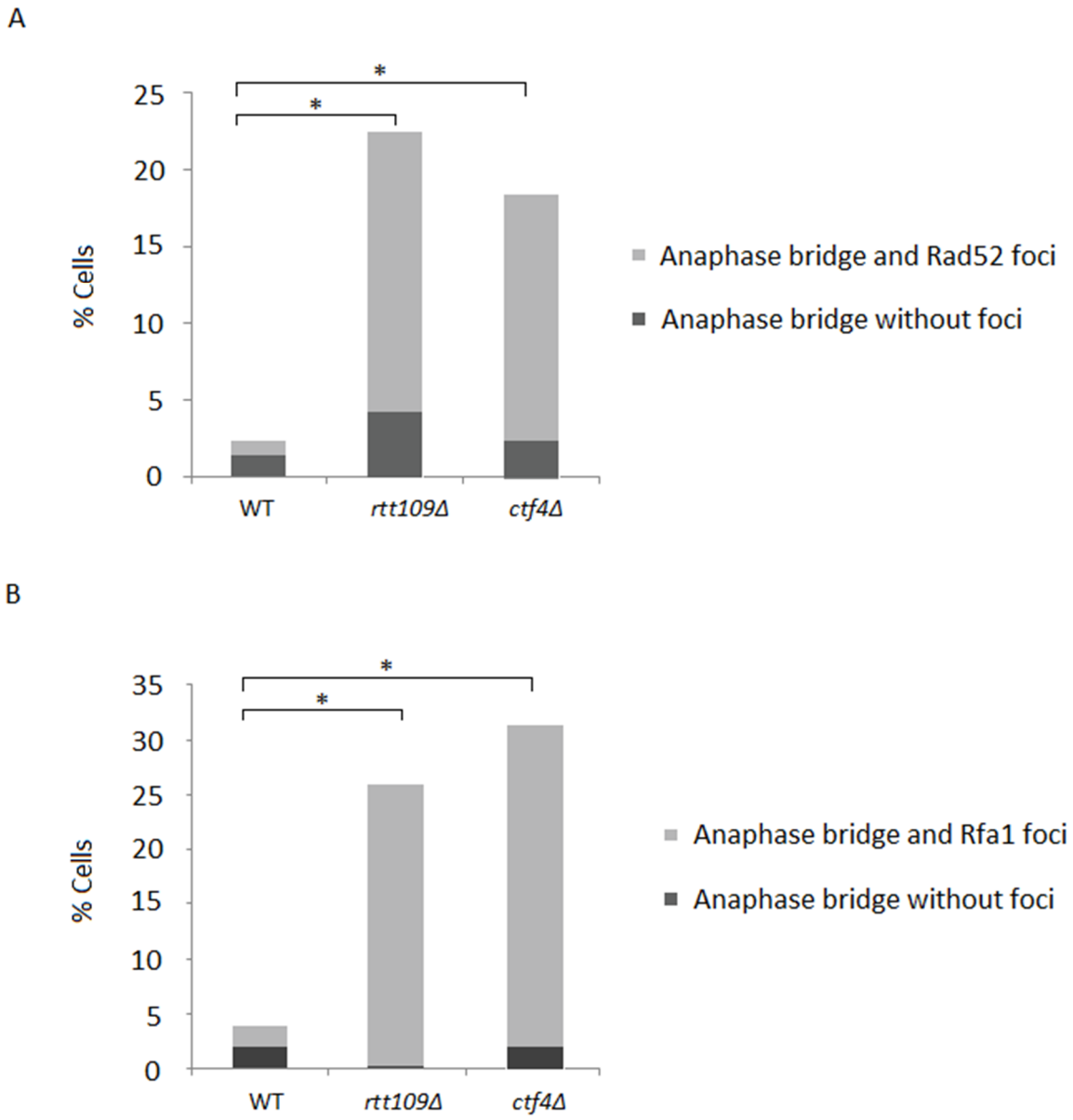


Figure 20: Cells lacking *Ctf4* or *Rtt109* present anaphase bridge formation after MMS exposure during S. A) Comparing cells with anaphase bridge and Rad52-YFP foci with cells with anaphase bridge without Rad52-YFP foci in 300 min time point. * The results are significant at $p < 0.05$ (**Table 7**). B) Comparing cells with anaphase bridge and Rfa1-YFP foci with cells with anaphase bridge without Rfa1-YFP foci in 300 min time point. * The results are significant at $p < 0.05$ (**Table 8**).

Strain	Time (min)	Cells with AB	Cells w/o AB	P-value vs WT
Wild-type	300	5	208	N/A
rtt109Δ	300	57	195	0
ctf4Δ	300	30	132	0

Table 7: Anaphase bridges formation in *rtt109Δ* and *ctf4Δ* cells compared to WT in the presence of MMS. The *p*-value was calculated using Fisher exact test. A 2 x 2 contingency table was used to compare mutants vs WT. AB; Anaphase Bridge. The results are significant at $p < 0.05$.

Strain	Time (min)	Cells with AB	Cells w/o AB	P-value vs WT
Wild-type	300	14	342	N/A
rtt109Δ	300	95	271	0
ctf4Δ	300	100	218	0

Table 8: Anaphase bridges formation in *rtt109Δ* and *ctf4Δ* cells compared to WT in the presence of MMS. As explained before (**Table 7**). The results are significant at $p < 0.05$.

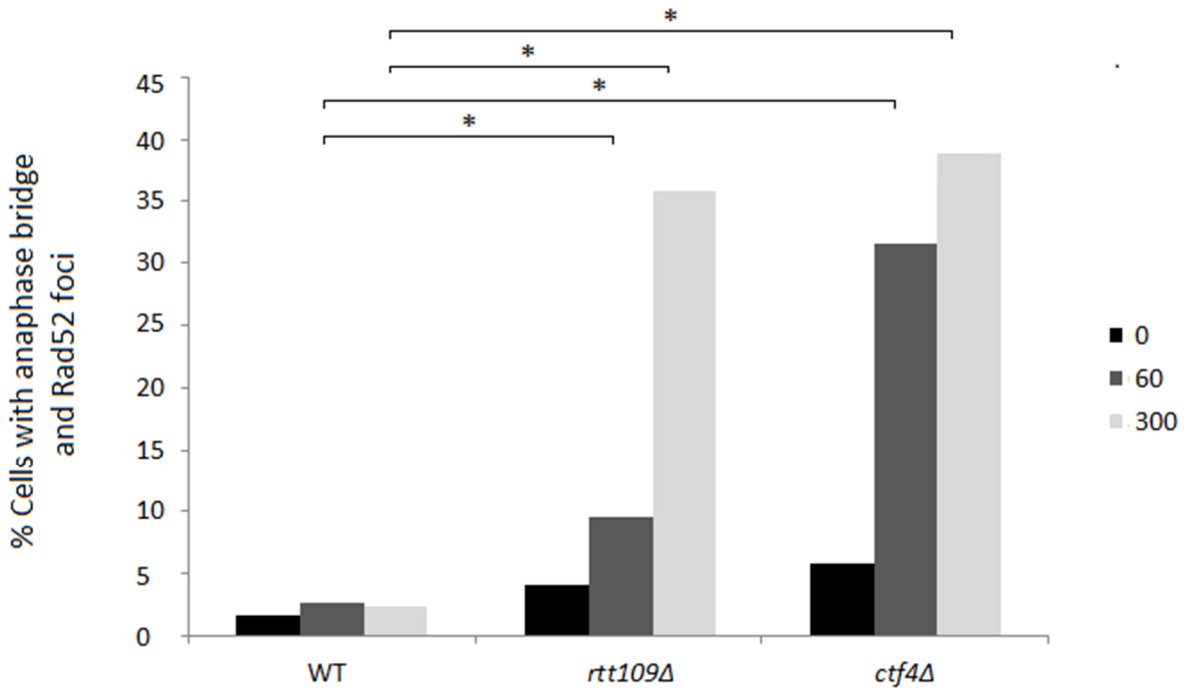


Figure 21: Cells lacking Ctf4 or Rtt109 present persistent anaphase bridges and Rad52 foci after MMS exposure during S. Cells arrested in G_1 were released into the cell cycle in the presence of 0.02% MMS for 1 h 30 min. MMS was inactivated with 2.5% sodium thiosulfate in synthetic complete medium, and cells were incubated in fresh medium. Fraction of cells containing anaphase bridge and Rad52-YFP foci as assessed by fluorescence microscopy. * The results are significant at $p < 0.05$ (**Table 9**).

We investigated the formation of anaphase chromatin bridges in *rtt109Δ* and *ctf4Δ* mutants. To do so, we collected cells from time course experiments (similar to those presented in **Figure 13**), stained them with DAPI to visualize DNA, and counted anaphase bridges until 300 min after repair (**Figure 19-B**). The result shows that after MMS exposure during S-phase, *rtt109Δ* and *ctf4Δ* mutants form more anaphase bridges compared to WT (**Figure 20**). The *p*-value were calculated using a Fisher exact test to determine whether this result is statistically significant (**Table 7** and **Table 8**).

Interestingly a large fraction of cells with anaphase bridge also contained HR foci (**Figure 20, Figure 21** and **Table 9**), suggesting that defective HR may prevent proper resolution and

segregation of sister chromatids after S phase. This result is concordant with the fact that *H3K56R* mutants also forms anaphase bridge in similar condition [234]. Persistent foci of HR initial steps proteins and anaphase bridges in *rtt109Δ* and *ctf4Δ* mutants after MMS exposure during S-phase are consistent with the notion that HR is deficient in these mutants.

Strain	Time (min)	Cells with AB and Rad52-YFP foci	Cells w/o AB and Rad52-YFP foci	P-value vs WT
Wild-type	60	6	227	N/A
	300	3	123	N/A
rtt109Δ	60	21	198	0.002309
	300	63	113	0
ctf4Δ	60	69	150	0
	300	42	66	0

Table 9: Anaphase bridges and Rad52-YFP foci formation in *rtt109Δ* and *ctf4Δ* cells compared to WT in the presence of MMS. The *p*-value was calculated using two-tailed Fisher exact test. A 2x2 contingency table was used to compare mutants vs WT. AB; Anaphase Bridge. The results are significant at $p\ 0 < 0.05$.

3-5 Are Ctf4 and Rtt109 required for resolution of HR intermediate structures?

During HR, after invasion and the priming of DNA synthesis, HR intermediates such as Holliday junctions can be generated. To separate the two recombining molecules another step called "resolution" is required. Two different mechanisms are identified to resolve the HR intermediates [260] .

One mechanism is termed "double Holliday junction dissolution" which is processed by the activity of RecQ helicases in concert with other group of proteins [261]. Sgs1 is the unique RecQ helicase present in *S. cerevisiae* and like other RecQ family members is involved in the replication, recombination and DNA repair. Sgs1 is recruited to sites of DNA damage following by a fork stalling or DSB, and participates in stabilizing of arrested replication forks [45] [262]. In concert with Top3 (topoisomerase III) and Rmi1, Sgs1 contributes in resolving of dHJ [261] [263] [264]. Lack of *Sgs1* leads to the accumulation of abnormal replication structures following exposure to MMS in a HR-dependent manner as evaluated by two-dimensional gel electrophoresis [265]. *sgs1*Δ mutants are therefore an interesting model to investigate the role of other factors in the formation of HR-dependent template switching intermediates. Wurtele et al. showed that deletion of *RTT109* in *sgs1Δ* cells did not inhibit formation of X-shaped intermediates [234]. Therefore, we were interested to see whether deletion of *CTF4* in *sgs1Δ* cells could prevent the formation of these Rad51-dependent X-shaped molecules.

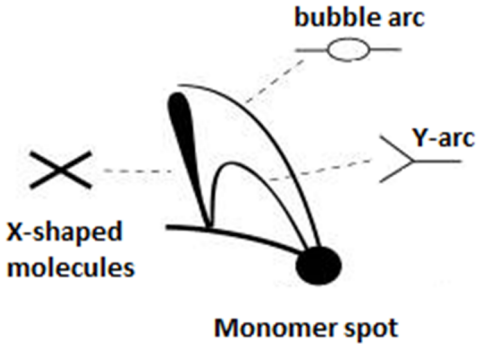
To investigate this, we have verified DNA structures at MMS-damaged DNA replication forks by 2D gel electrophoresis. Cells (*sgs1Δ*, *sgs1Δ rtt109Δ* and *sgs1Δ ctf4Δ*), were synchronized in G1 and were released into the cell cycle in the presence of 0.03% MMS for

90 min. Then, DNA was extracted and pursuant to the protocol described in the material and methods processed for 2D gel electrophoresis. Southern blotting was performed using a probe specific to the *ARS305* replication origin [234] [264] [265] [266]. As shown in figure 20, likewise *sgs1Δ*, X-shaped intermediates accumulate in the two-dimensional gel electrophoresis in the absence of both *sgs1Δ rtt109Δ* and *sgs1Δ ctf4Δ*, when cells are treated with MMS for 90 min (**Figure 22**).

Previous studies showed that unlike *sgs1Δ*, *rtt109Δ* mutants do not accumulate X-shaped structures [234]. Consistent with this, our result could suggest that Ctf4 and Rtt109 do not prevent the invasion step of HR, which is dependent on Rad52, Rad51 and RPA. However, a recent study demonstrates that lack of *Ctf4* in both WT and *sgs1Δ* cells reduces, but does not abolish the accumulation of X-shaped structure [267]. In contrast to our result, this could suggest a role for Ctf4 in template switching, although the reasons for these discrepancies remain unclear. The classical resolution pathway is another mechanism known to resolve the HR intermediates through resolvase enzyme [261] [268]. Mus81 is a crossover junction endonuclease which is involved in DNA repair, replication fork stability, and joint molecule resolution during meiotic recombination in eukaryotes [269] [270] [271] [272].

Interestingly, we found that deletion of *RTT109* and *CTF4* in *mus81Δ* mutants cause synthetic sensitivity to MMS which is similar to the lack of *Rtt109* and *Ctf4* in Rad51 paralog genes, with the exception of *rad59Δ* (*rad51Δrtt109Δ* and *rad52Δctf4Δ* is previously published [234]) **(Figure 23)**. Base on later studies and the effect that all these genes (Rad51 paralog genes and Mus81) are involved in HR-mediated DSB repair, it is obvious that lack of them cause MMS sensitivity [234] [270]. But the fact that double mutants (deletion of *CTF4* and *RTT109*

conjunction with them) have synergistic effect suggest that cells lacking *Ctf4* and *Rtt109* need HR to resolve the abnormal structures generated at stalled replisomes.



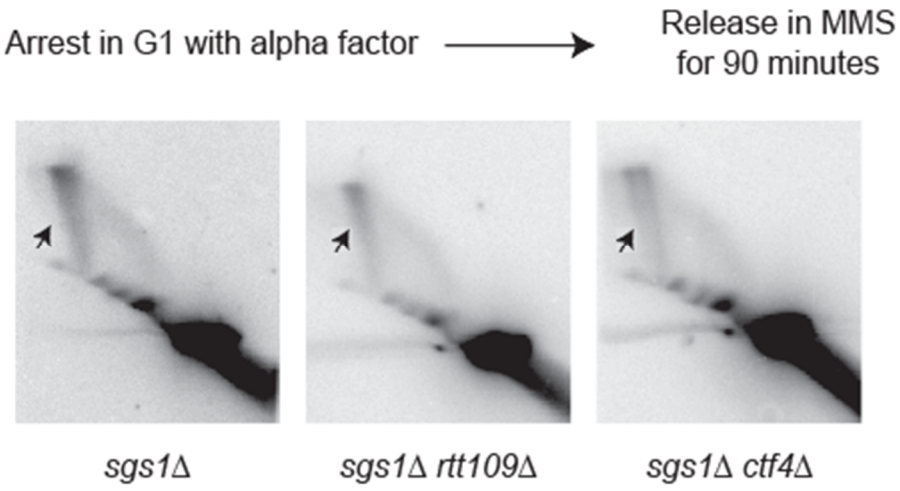


Figure 22: H3K56ac deficient cells do not prevent X-structure accumulation in *sgs1Δ* mutants. Cells were released from G_1 into the cell cycle in the presence of 0.03% MMS for 90 min. DNA was extracted, digested with HindIII and EcoRV, and subjected to neutral 2D gel electrophoresis. Southern blots were hybridized to a probe that detects *ARS305*. X-structures are shown with arrow by two-dimensional Southern analysis (see material and methods).

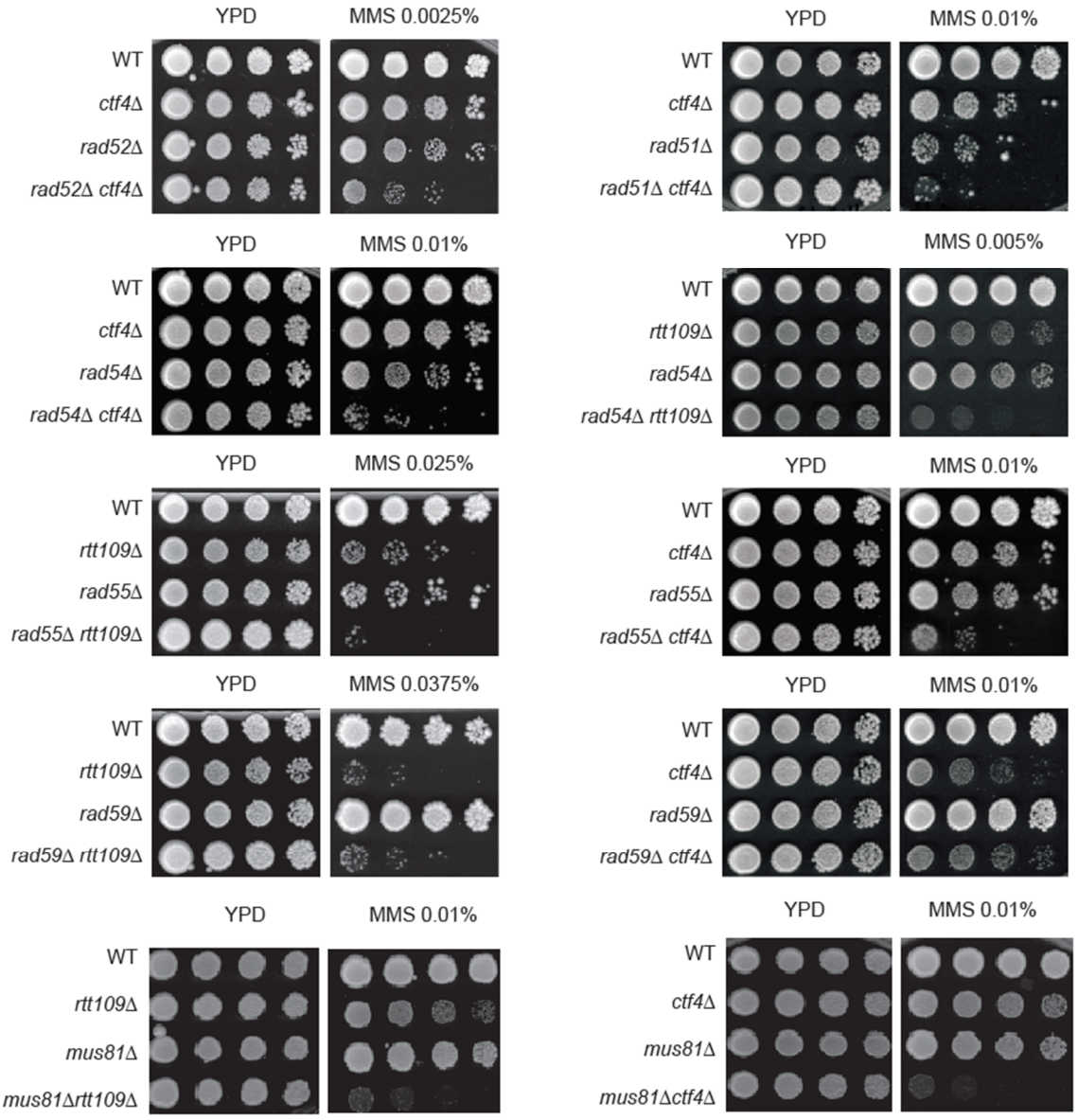


Figure 23: Deletion of HR genes causes synthetic sensitivity to MMS in conjunction with *rtt109Δ* and *ctf4Δ*. Yeast growth assay is performed with serial dilutions of cells in 96 well plates. After saturation, cells were spotted on the indicated medium and incubated at 25° or 30°C.

Discussion

Ctf4 is a replication machinery protein which from its C-terminal binds to the replisome and links polymerase α to the helicase. On the other hand, from its N-terminal, Ctf4 physically interacts with Mms22. Ctf4 via its interaction with ubiquitin ligase complex Rtt101/Mms1/Mms22, is expected to be present at damaged replication forks [250]. Since this latter complex act downstream of H3K56ac pathway [245] and these genes are involved in HR [246], there was a high possibility that Ctf4 also functioned in H3K56ac pathway. In addition, like *rtt101Δ*, *mms1Δ* or *mms22Δ* deletion of *CTF4* suppresses the phenotypes of *hst3*Δ *hst4*Δ [232] [245], and also slightly affects MMS sensitivity of *H3K56R* cells [232].

Based on the preliminary data described above, herein we verified the links between the H3K56ac acetyltransferase Rtt109 and Ctf4. In response to genotoxins, we demonstrated that Ctf4 acts via Rtt109- and H3K56ac-dependent genetic pathway **(Figure 10** and **Figure 11)**. Our results further demonstrate that deletion of *CTF4* partially rescue the viability of *rtt109Δ* cells in MMS exposure (**Figure 10**) but not HU (data not shown). Translesion DNA synthesis (TLS) is an error-prone DNA damage tolerance pathway which allows cells to replicate across damaged DNA through polymerase ζ activity. Rev3 and Rev7 encode subunits of pol ζ and lack of these genes reduces mutagenesis [273] [274]. Previously, it has been shown that Rev3-dependent spontaneous mutation frequency is increased in *rtt109Δ* as compared to WT [275]. Our lab verified the rate of MMS-induced mutagenesis in *rtt109Δ* cells at the *CAN1* locus [235]. To do so, cells were grown in the presence of MMS and then they plated on canavanine containing medium. Only *can1* mutant can survive [276]. Interestingly, our results indicate that the rate of MMS-induced CAN1 mutations is equal in WT and *rtt109Δ* cells, and it depends on Rev3 not gross chromosomal rearrangements [235].

However, our recent unpublished results show that deletion of *CTF4* causes elevated MMS-induced mutagenesis (data not shown). It is therefore possible that *ctf4*Δ may rescue the sensitivity of cells lacking Rtt109 to MMS by increasing TLS and reducing reliance on other cellular pathways such as HR-dependent template switching which appear defective in *rtt109*Δ mutants.

Based on our 2D gel results (**Figure 22**), we found that Ctf4 does not strongly influence TLS via template switching. In contrast, Fumasoni et al shows that in the absence of *Sgs1*, lack of *Ctf4* reduces the accumulation of X-shaped structure, suggesting a role for Ctf4 in template switching [267]. While the reasons for these discrepancies remain unclear, our fluorescence microscopy data clearly shows that HR cannot progress beyond early steps (including invasion which is measured by 2D gel electrophoresis). This may provide a rationale for increased resistance to MMS when TLS is favored (as is the case when *CTF4* is deleted) over HR-dependent processes in H3K56ac mutants.

During replicative stress when replication fork is blocked, the replicative helicase and DNA polymerase can be uncoupled, which generates ssDNA [48] [277] [278]. Likewise, collapsed RF can reveal DSBs which leads to the formation of ssDNA by end resectioin [73]. ssDNA is rapidly bound by Rfa1. Rfa1 is a single stranded DNA binding protein which is involved in DNA replication, repair, and recombination [101] [102]. We found that H3K56ac deficient cells (*rtt109Δ* and *ctf4Δ* mutants) demonstrate persistent and intense Rfa1 foci in response to replicative stress **(Figure 15** and **Figure 16)**. This result suggests the presence of excess ssDNA which have an important role in DNA damage-induced signaling [73]. The persistence of ssDNA, bound by Rfa1 promotes the activation of DNA damage checkpoint signaling cascade [48], consequently inhibiting cell cycle progression [279]. Indeed, Rfa1 interacts with

Ddc2 to promote Mec1 activation [50] [51]. Mec1- Ddc2 complexes activate Rad53, which delays further progression in the cell cycle [279] [280]. The persistent hyper-activation of Rad53 in cells lacking *Ctf4* and *Rtt109* **(Figure 18)** is consistent with the fact that cells lacking H3K56ac are defective in DNA damage checkpoint deactivation after the repair of a DSB by single-strand annealing even after the completion of DNA repair [230] [238]. Therefore, we hypothesize that persistent phosphorylation of Rad53 in cells lacking H3K56ac is a consequence of excess ssDNA-bound Rfa1. Further experiments, including downregulation of RPA levels in H3K56ac, will be required to verify the validity of such a model.

As mentioned above, our result demonstrate that lack of *Ctf4*, as is the case for *rtt109*Δ and *H3K56R* mutants, induces formation of persistent and intense Rad51 and Rad52 foci after transient exposure to genotoxic agents **(Figure 13** and **Figure 14)** [234]. This result, together with formation of Rfa1 foci **(Figure 15** and **Figure 16)** suggest that H3K56ac-deficient cells are able to initiate HR to repair the damages caused by replicative stress, but present defects in eventual resolution or subsequent HR steps. We also found that deletion of HR genes including *RAD51*, *RAD52*, *RAD54, RAD55* and *MUS81* generates synergistic sensitivity to MMS when combined with *ctf4Δ* or *rtt109Δ* **(Figure 23)**. This may reflect formation of DSBs in *ctf4*Δ and *rtt109*Δ mutants and subsequent requirement of HR to recover such collapsed replication fork [281]. In this sense, presenting partial HR defects as a result of lack of proper H3K56ac pathway activity appears to be better than having no HR at all in response to MMS-induced replicative stress.

The presence of persistent HR mediated foci (Rfa1, Rad52 and Rad51) in H3K56ac pathway mutants indicate that these proteins can initiate the first steps of HR at damaged sites (as also

evidenced by our 2D gel results presented in (**Figure 22**), but that downstream HR steps are compromised and do not proceed normally. Previous studies have shown that H3K56ac deficient cells manifest defective sister-chromatid exchange, which also reveals the existence of abnormal HR in these mutants [237]. The formation of anaphase bridges in H3K56ac mutants **(Figure 19, Figure 20** and **Figure 21)** is also consistent with the hypothesis that these cells are able to invade homologous DNA, but fail to correctly resolve HR structures. However, we are not sure if really these non-resolvable anaphase bridges have derived from HR intermediates. To test this possibility, future studies should assess the presence of anaphase bridges during HR prevention by deletion of *RAD52* or *RAD51* genes in *ctf4Δ* or *rtt109Δ* mutants. In addition, deletion of the HR resolvase-encoding gene *MUS81* with *CTF4* or *RTT109* cause synergistic sensitivity to MMS **(Figure 23)**. This could suggest that Ctf4 and Rtt109 are involved in resolution step in a pathway that acts in parallel to Mus81. Overall, our results and previous studies support a model in which H3K56ac deficient cells cannot recover from MMS- induced damages because of HR defects.

Even though more studies are needed to understand the mechanisms through which H3K56ac pathway genes promote HR mediated recovery of damaged DNA replication forks, our results highlight the importance of nascent chromatin structure in these phenomena. Further experiments will be required to evaluate the validity of the models presented in this thesis, and to verify whether similar mechanisms operate in human cells or contribute to human diseases.

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