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Le rôle de la structure de la chromatine naissante dans la réponse au stress réplicatif

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Résumé

La viabilité de chaque organisme dépend de sa capacité à répliquer son génome en conservant son intégrité. Paradoxalement, la réplication de l'ADN est un processus pendant lequel le génome est particulièrement vulnérable à une multitude de génotoxines issues de l'environnement et du métabolisme cellulaire qui peuvent endommager l'ADN. Chez plusieurs espèces, les nucléosomes nouvellement synthétisés durant la phase S comportent des modifications cycliques qui régulent, entre autres, la réponse au stress réplicatif. Notamment, chez *Saccharomyces cerevisiae*, l'acétylation de la lysine 56 de l'histone H3 (H3K56ac) est ajouté par Rtt109 pendant la réplication et enlevée suite à la duplication du génome par les histones désacétylases de la famille des sirtuines, Hst3 et Hst4. La perturbation du cycle d'acétylation/désacétylation d'H3K56ac mène à des défauts de croissance accompagnés d'hypersensibilité aux génotoxines qui induisent du stress réplicatif, mais les mécanismes sous-jacents à H3K56ac pouvant expliquer ces phénotypes demeurent mal compris.

Dans le but d'élucider le rôle d'H3K56ac, nous avons examiné les causes des phénotypes engendrés par une situation où H3K56 est constitutivement acétylé dans un mutant *hst3*Δ *hst4*Δ. Nous avons initialement caractérisé les effets sur la réponse aux dommages à l'ADN dans ce contexte et découvert que l'hyperacétylation d'H3K56ac provoque l'apparition de foyers de protéines de réparation ainsi qu'une hyperactivation de la signalisation en réponse aux dommages à l'ADN (SRDA). Nous avons ensuite fait un crible pour identifier des mutants d'histones pouvant supprimer les défauts de croissance d'*hst3*Δ *hst4*Δ, ce qui a permis de découvrir que l'acétylation d'H4K16 et la méthylation d'H3K79 sont toxiques en présence d'hyperacétylation d'H3K56. Nos résultats suggèrent que prévenir ces deux modifications améliore la croissance du mutant *hst3*Δ *hst4*Δ en réduisant l'activation de Rad53, une kinase clé dans la SRDA. Nous avons subséquemment effectué un crible chimiogénétique pour identifier des gènes dont la mutation sensibilise les cellules à l'hyperacétylation d'H3K56 induite par le nicotinamide (NAM), un inhibiteur pan-spécifique des sirtuines. De cette manière, nous avons identifié que les cellules mutantes pour *SLX4* et *PPH3*, deux gènes impliqués dans la réduction de l'activité de Rad53, sont inviables en présence d'hyperactétylation d'H3K56. Une caractérisation plus poussée a révélé que l'hyperactivation de Rad53 dans le mutant *hst3*Δ *hst4*Δ

cause des défauts de croissance en inhibant l'activation d'origines de réplication tardives et en perturbant la réponse au stress oxydatif. Le crible a également mené à la découverte que le raccourcissement des télomères sensibilise les cellules au NAM et au stress réplicatif en provoquant la redistribution de l'activation des origines de réplication à travers le génome.

En résumé, les résultats présentés dans cette thèse indiquent qu'une composante majeure des phénotypes associés à l'hyperacétylation d'H3K56 provient de l'hyperactivation de la signalisation en réponse aux dommages à l'ADN. Collectivement, nos travaux révèlent de nouveaux mécanismes par lesquels la structure de la chromatine influence la capacité des cellules à répondre au stress réplicatif.

Mots-clés : H3K56ac, Structure de la chromatine, réponse aux dommages à l'ADN, stress réplicatif, réplication de l'ADN, assemblage de la chromatine, télomères

Abstract

The information necessary for every cellular process is encoded in DNA, and the survival of any species requires that it faithfully and successfully replicates this molecule. Paradoxically, it is when cells undergo DNA replication that the integrity of their genome is the most vulnerable to agents that cause DNA damage. As such, cells possess a wide variety of mechanisms that protect genomic integrity, which includes regulation of chromatin structure. In all eukaryotic species studied so far, newly synthesized nucleosomes in S-phase harbor transient modifications that influence, amongst other things, the response to replicative stress. Particularly, in *Saccharomyces cerevisiae*, histone H3 lysine 56 acetylation (H3K56ac) is added by Rtt109 during DNA replication, and is removed after completion of genome duplication by members of the sirtuin family of histone deacetylases, Hst3 and Hst4. Interestingly, any perturbation of acetylation/deacetylation cycle causes severe growth defects accompanied by an extreme sensitivity to replicative stress-inducing genotoxins. However, the H3K56ac functions underlying these phenotypes are poorly understood.

To further understand the role of H3K56ac in maintaining genomic integrity, we examined the phenotypes caused by constitutive hyperacetylation of H3K56, such as in the *hst3*Δ *hst4*Δ mutant. We first characterized the effects of H3K56 hyperacetylation on the DNA damage response (DDR), and found that it induces spontaneous and aberrant appearance of DNA repair protein foci, as well as hyperactive DDR signaling. We next performed a screen to identity histone mutations that can suppress the growth defects of *hst3*Δ *hst4*Δ mutants, and found that H3K79 methylation and H4K16 acetylation are toxic for cells harboring hyperacetylated H3K56. Our results suggest that preventing the addition of these two modifications improves the growth of *hst3*Δ *hst4*Δ mutants by reducing the activity of Rad53, a key kinase for DDR signaling. Hst3 and Hst4 can be inhibited by nicotinamide (NAM), a pan-sirtuin inhibitor, which induces H3K56 hyperacetylation. We performed a chemogenetic screen to identify genes whose mutation confers fitness defects in the presence of nicotinamide (NAM)-induced H3K56 hyperacetylation. Interestingly, amongst the tops hits were mutants for *SLX4* and *PPH3*, two genes encoding proteins involved in the dampening of DDR signaling. By using *slx4*Δ and *pph3*Δ cells to probe for the root causes of sensitivity to DDR signaling in cells with constitutive H3K56ac, we found that the defects of *hst3*Δ *hst4*Δ mutants are instigated by Rad53

hyperactivity-mediated inhibition of late replication origin firing and crippling of the response to oxidative stress. Intriguingly, this last screen led to the surprising finding that telomere length influences replication origin firing genome-wide. Moreover, our results suggest that cells with short telomeres are sensitive to NAM and replicative stress because of sequestration of origin firing factors to telomeres.

In summary, results presented herein indicate that hyperactive DDR signaling is a major component of the extreme phenotypes observed in cells harboring constitutive H3K56ac. Collectively, our work unveils novel mechanisms by which chromatin structure influences cells' ability to cope with replicative stress.

Keywords : H3K56ac, DNA damage response, replicative stress, DNA replication, chromatin structure, telomeres, chromatin assembly

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A : Adénine

ADN : Acide DéoxyriboNucléique

APC : Anaphase Promoting Complex

ARN: Acide RiboNucléique

ARS : Autonomous Replicating Sequence

ATP : Adénosine TriPhosphate

BER : Base Excision Repair, Réparation par excision de base

BRCT : BRCA C-Terminus

CAF-I : Chromatin Assembly Factor I, facteur d'assemblage de la chromatine I

CDK : Cyclin-Dependent Kinase

CPD : Cyclobutane Pyrimidine Dimers, Dimères cyclobutyliques de pyrimidine

CS : Cockayne syndrome

CST : Cdc13-Stn1-Ten1

dNTP : déoxyNucléotide TriPhosphate

DSB : Double-Strand Break, bris double-brin

DSBR : Double-Strand Break Repaire, réparation des bris double-brins

dsDNA : ADN double-brin

ERC : Extrachromosomal rDNA Circles, Cercles extrachromosomiques de rDNA

FACT : FAcilitaites Chromatin Transcription

FHA : ForkHead Associated

GG-NER : Global Genome NER

GINS : Go-Ichi-Ni-San (5-1-2-3 en japonais)

HU : Hydroxyurée

HR : Recombinaison Homologue

K : lysine

MCM : MiniChromosome Maintenance

MRX : Mre11-Rad50-Xrs2

NAM : Nicotinamide

NER : Nucleotide Excision Repair Réparation par excision de nucléotide

NHEJ: Non-Homologous End-Joining, Jonction d'extrémités non-homologues

NTS : Non-Transcribed Sequence, Séquence non-transcrite

ORC : Origin Recognition Complex

PCNA : Proliferating Cell Nuclear Antigen

PRR : Post-Replicative Repair, Réparation post réplication

RFB : Replication Fork Barrier

RNR : RiboNucléotide Réductase

ROS : Reactive Oxygen Species, espèces oxygénées réactives

RPA: Replication Protein A

S : Sérine

SIR : Silent Information Regulator, régulateur d'information silencieuse

S-CDK : S-phase Cyclin-Dependent-Kinase, kinase dépandante des cyclines de phase S

SSB : Single-Strand Break, bris simple-brin

SDSA : Synthesis-Dependent Strand Annealing

ssDNA : ADN simple-brin

T : Thymidine

TC-NER : Transcription-coupled NER, NER couplé à la transcription

TLS : TransLesion Synthesis, Synthèse translésion

TS : Template switching, permutation de matrice

UV : UltraViolet

XP : Xeroderma pigmentusum

Liste des abréviations

i.e. : *id est* (C'est-à-dire ou en d'autres mots)

e.g. : *exempli gratia* (par exemple)

À toutes les levures sacrifiées au cours de la rédaction de cette thèse

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Chapitre 1. Introduction

La vie est un phénomène biologique fascinant. À la base, celle-ci est issue d'une multitude de réactions chimiques et enzymatiques individuellement simples, mais qui ensemble forment un système d'une formidable complexité. C'est l'extrême coordination de toutes ces réactions qui permet à la vie d'émerger et qui résulte en organismes capables de se reproduire et de s'adapter à leur environnement immédiat. Il est remarquable que toute l'information nécessaire à cette coordination, et donc à la vie, soit encodée dans des molécules d'ADN qui peuvent être fidèlement répliquées à chaque division cellulaire.

La compréhension du rôle de l'ADN en tant que matrice contenant l'information génétique est relativement récente. Le concept d'hérédité génétique a été initialement découvert par Gregor Mendel au 19^{e} siècle, puis oublié, pour ensuite être redécouvert par Hugo de Vries au début du 20^{e} siècle. C'est ce dernier qui a formulé pour la première fois les notions de gènes et de mutations. Or, jusqu'au milieu du 20^{e} siècle, la théorie la plus répandue était que les protéines des chromosomes, plutôt que l'ADN, comportaient l'information génique. Plusieurs chercheurs, notamment Oswald Avery, Alfred Hershey, Martha Chase et Alexander Hollaender (1–3), ont fourni des évidences allant à l'encontre de cette interprétation, mais c'est l'élucidation de la structure de l'ADN par James Watson et Francis Crick à partir des travaux de Rosalind Franklin qui a permis de saisir son importance dans la conservation et passation de l'information génétique (4). Leurs travaux ont été indispensables pour comprendre comment cette macromolécule, par sa structure intrinsèque, est à la base de deux mécanismes essentiels à la vie : l'établissement d'un code génétique et la réplication de celui-ci. En effet, l'appariement antiparallèle de deux chaînes de nucléotides admet un mécanisme semi-conservatif de réplication de l'ADN (4). Ensuite, la réplication et le maintien d'un ordre précis de nucléotides (Guanosine, Cytosine, Adénosine et Thymidine) permet la formation d'un code génétique conservé de cellule en cellule, sous-jacent au dogme de la biologie moléculaire (ADN-ARN-protéines) (5, 6).

L'ADN est une merveille de la nature, mais il n'est pas parfait. Les bases peuvent être chimiquement modifiées et altérées, ce qui peut endommager l'ADN et causer des mutations

potentiellement fatales. En fait, à chaque instant, l'ADN de toute cellule est sujet à une quantité importante de lésions. En considérant que les cellules d'un organisme ne possèdent qu'un nombre limité de précieuses copies de chacun de leurs chromosomes, il est formidable que les cellules réussissent à maintenir et répliquer fidèlement leur ADN pour que la vie puisse prospérer. C'est d'ailleurs un constat qui a rapidement été fait suite aux travaux de Watson et Crick, et depuis, une multitude de mécanismes de réparation et de protection du génome permettant aux cellules de faire face à l'énorme diversité de lésions à l'ADN rencontrées ont été découverts.

Une composante importante de ces mécanismes provient de la structure adoptée par les chromosomes. L'ADN n'est pas libre dans le noyau des eucaryotes et il est enroulé autour d'octamères d'histones, formant ainsi l'unité de base de la chromatine : le nucléosome. Bien qu'initialement les histones étaient considérées comme des protéines inertes, il a été découvert au début des années 90 que celles-ci peuvent être modifiées post-traductionnellement ce qui module en contrepartie la structure de la chromatine (7, 8). Ceci a des répercussions majeures sur notre compréhension de processus essentiels à la biologie de l'ADN, en particulier sur la régulation de l'expression des gènes. Or, au cours des deux dernières décennies, une multitude d'enzymes qui influencent la capacité des cellules à réparer les lésions à l'ADN par la modification post-traductionnelle d'histones ont été identifiées, et l'élucidation de leurs fonctions est présentement le sujet de recherches intensives.

Au cours de mon doctorat, je me suis ainsi intéressé à comprendre comment la structure de la chromatine peut influencer les processus de réparations des dommages à l'ADN. J'ai porté une attention particulière à un type intriguant de modifications transitoires d'histones ayant lieu au cours de la réplication de l'ADN. Plus spécifiquement, je me suis penché sur le cycle d'acétylation de la lysine 56 de l'histone H3 (H3K56ac) en utilisant *Saccharomyces cerevisiae* comme modèle. La régulation de cette modification est extrêmement importante pour la viabilité des cellules faisant face à des dommages à l'ADN spécifiquement pendant la réplication de l'ADN, une phase du cycle cellulaire où l'ADN est particulièrement vulnérable aux lésions. Les fonctions d'H3K56ac combinent trois concepts fondamentaux; la réplication de l'ADN, la réponse aux dommages à l'ADN et la modulation de la structure de la chromatine. Ainsi, pour mieux cerner le sujet, les prochaines sections traiteront sommairement des connaissances accumulées à ce jour sur ces trois piliers du maintien de l'intégrité génomique.

1.1 La réplication de l'ADN

1.1.1 Principes généraux

Tel que mentionné plus haut, la réplication de l'ADN est un élément fondamental pour la vie et chaque cellule doit s'assurer que celui-ci ait lieu sans anicroche. Suite aux travaux de Watson et Crick sur sa structure, il a rapidement été démontré que l'ADN est répliqué de manière semi-conservative, c'est-à-dire que chaque double hélice nouvellement formée est composée d'un brin parental et d'un brin nouvellement synthétisé (9). La réplication est initiée durant la phase S, ou de synthèse, à plusieurs positions du génome eucaryote que l'on nomme origines de réplication (Figure 1.1A). Les brins complémentaires de la double-hélice sont d'abord dénaturés à cette origine, puis l'ADN est déroulé de manière bidirectionelle de part et d'autre de l'origine. Ceci entraîne la formation d'une bulle de réplication cernée de structures en Y, que l'on nomme fourches de réplication, qui s'éloignent de l'origine en générant de l'ADN simple-brin (Figure 1.1B). C'est à ces fourches de réplication que la synthèse de l'ADN en tant que tel a lieu par le biais des polymérases à ADN. Ces enzymes ajoutent les bases complémentaires au brin parental selon une polarité 5' vers 3'. Or, les deux brins de l'ADN sont antiparallèles et ont donc une polarité inverse ce qui fait en sorte que seule la branche 3' vers 5' à chaque fourche de réplication peut être synthétisé en continu par les polymérases. La synthèse du second brin a ainsi plutôt lieu selon un mode discontinu qui implique le réamorçage répétitif des polymérases au fil de la progression des fourches de réplication, générant ainsi des courts fragments d'ADN que l'on appelle fragments d'Okazaki (Figure 1.1C) (10, 11). Les « trous » entre les fragments d'Okazaki sont ensuite remplis indépendamment de la progression des fourches de réplication. La duplication du génome est complétée lorsque les fourches de réplications ont parcouru tous les chromosomes. Bien que les principes de base de la réplication de l'ADN soient assez simples, celle-ci nécessite une panoplie de facteurs qui, ensemble, forment une chorégraphie étroitement régulée assurant non seulement la réplication du génome en entier, mais aussi le maintien du nombre de copies de chaque chromosome. Les prochaines sections traiteront plus en détail des connaissances acquises au cours des dernière décennies des processus entourant la réplication de l'ADN chez les eucaryotes.

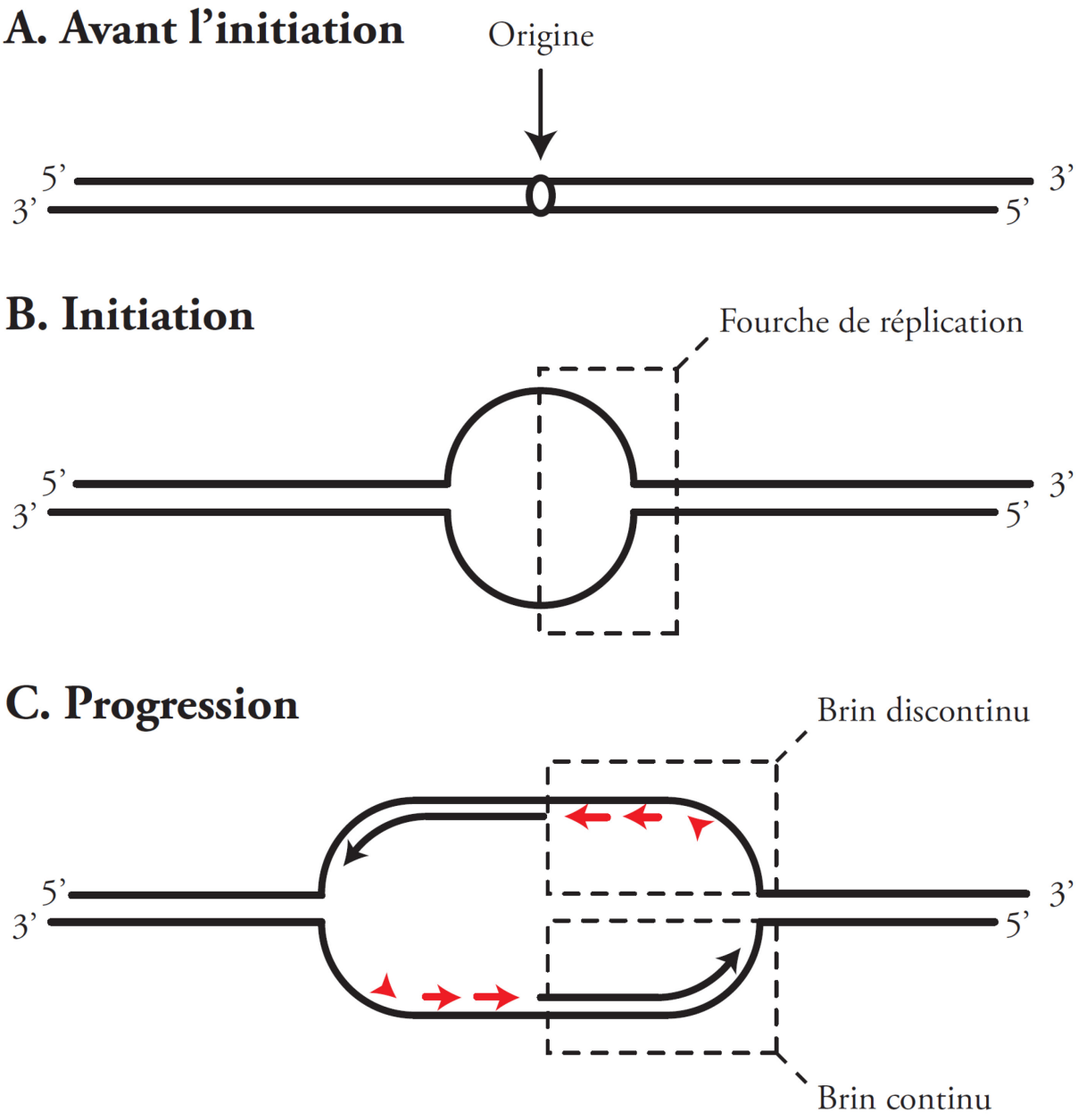

Figure 1.1. Principes de bases de la réplication de l'ADN

1.1.2 Où débute la réplication de l'ADN?

La réplication de l'ADN est initiée à partir de plusieurs sites sur chaque chromosome que l'on nomme origines de réplication. La première étape de la réplication de l'ADN consiste à déterminer la position de ces origines. Chez la plupart des eucaryotes, celles-ci sont définies par le contexte de la chromatine, c'est-à-dire qu'elles se retrouvent en général dans des régions riches en euchromatine et près de gènes fortement exprimés. L'emplacement exact des origines est assez variable d'une cellule à l'autre, ce qui complique l'étude de la réplication chez la plupart des systèmes modèles. Or, chez la levure *Saccharomyces cerevisiae*, bien que le contexte chromosomique ait un rôle que l'on décrira plus loin, les origines de réplication sont plutôt définies par des séquences d'ADN spécifiques que l'on nomme « Autonomously Replicating Sequence » (ARS) (12, 13). Cette particularité a permis une avancée spectaculaire de notre compréhension des processus entourant la réplication et ainsi on se concentrera sur ce modèle dans les prochaines sections. Il est cependant important de noter que les mécanismes de réplication de l'ADN sont fortement conservés au cours de l'évolution et on retrouve des homologues chez les eucaryotes supérieurs de la plupart des facteurs décrits ci-dessous.

1.1.3 La séquence des ARS

Chez *S. cerevisiae*, chaque ARS comprend de 100 à 150 paires de bases avec une forte prédominance d'adénines (A) et de thymidines (T). Une ARS typique contient une séquence consensus ACS (ARS consensus sequence) ainsi qu'un élément B1 (14–17). Deux autres éléments, B2 et B3, sont aussi présents sur la plupart des origines, mais leur séquence est plus divergente (18–20). À cause de la prévalence de bases A-T qui réduisent l'affinité des nucléosomes pour l'ADN, les ARS sont pauvres en nucléosomes, ce qui a en contrepartie pour effet de favoriser l'association des facteurs de réplication (21–24).

1.1.4 Définir une origine de réplication

Avant que la réplication ne puisse être initiée à partir d'une ARS, il est nécessaire que celle-ci soit préalablement « autorisée » en tant qu'origine de réplication, c'est-à-dire que deux complexes hélicases MCM composés de six sous-unités (Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, Mcm7) doivent d'abord y s'y lier en orientation opposée (25, 26). Ce processus débute par le recrutement du complexe ORC (Origin Recognition Complex) à une région comprenant l'ACS et l'élément B1 de l'ARS (Figure 1.2A). Le complexe ORC est formé des ATPases AAA+ Orc1-5 ainsi que d'Orc6 et dépend de l'ATP pour son association aux ARS (27). Le complexe ORC lié à l'ARS recrute ensuite l'ATPase Cdc6 (Figure 1.2B) (28). L'interaction entre la sous-unité Mcm3 de MCM et Cdt1 permet leur import nucléaire mutuel et MCM-Cdt1 s'associe à ORC-Cdc6 aux origines de réplication (Figure 1.2C) (26, 28–31). Le complexe MCM forme un anneau qui doit entourer l'ADN double-brin pour ses fonctions subséquentes et doit être remodelé afin de pouvoir incorporer l'ADN à l'intérieur de son anneau. Cette étape requiert l'action concertée des ATPases de Cdc6, Mcm2-7 et la stabilisation d'une forme ouverte de MCM par Cdt1 (Figure 1.2C) (32–34). La relâche de Cdc6 et Cdt1 permet à l'anneau MCM de se refermer autour de l'ADN double-brin (Figure 1.2D) (35). Un second anneau MCM doit ensuite être lié en direction opposée, ce qui requiert cette fois l'activité ATPase du complexe ORC ainsi que Cdc6 et Cdt1 (36, 37). Lorsque deux complexes MCM sont chargés sur l'ARS, ORC est délocalisé de l'origine (Figure 1.2E) (35, 38). Les deux complexes MCM demeurent ainsi inactifs jusqu'à l'initiation de la réplication pendant la phase S (38).

1.1.5 Régulation temporelle de l'autorisation des origines de réplication

Une origine autorisée, i.e. liée par deux hexamères de MCM, pourrait théoriquement initier la réplication à n'importe quel moment durant la réplication. Il est donc extrêmement important pour la stabilité du génome de restreindre temporellement le processus d'autorisation des origines, car la ré-autorisation d'une origine au cours de la phase S pourrait résulter en un nombre aberrant de chromosomes (39). La restriction temporelle du recrutement des MCM aux

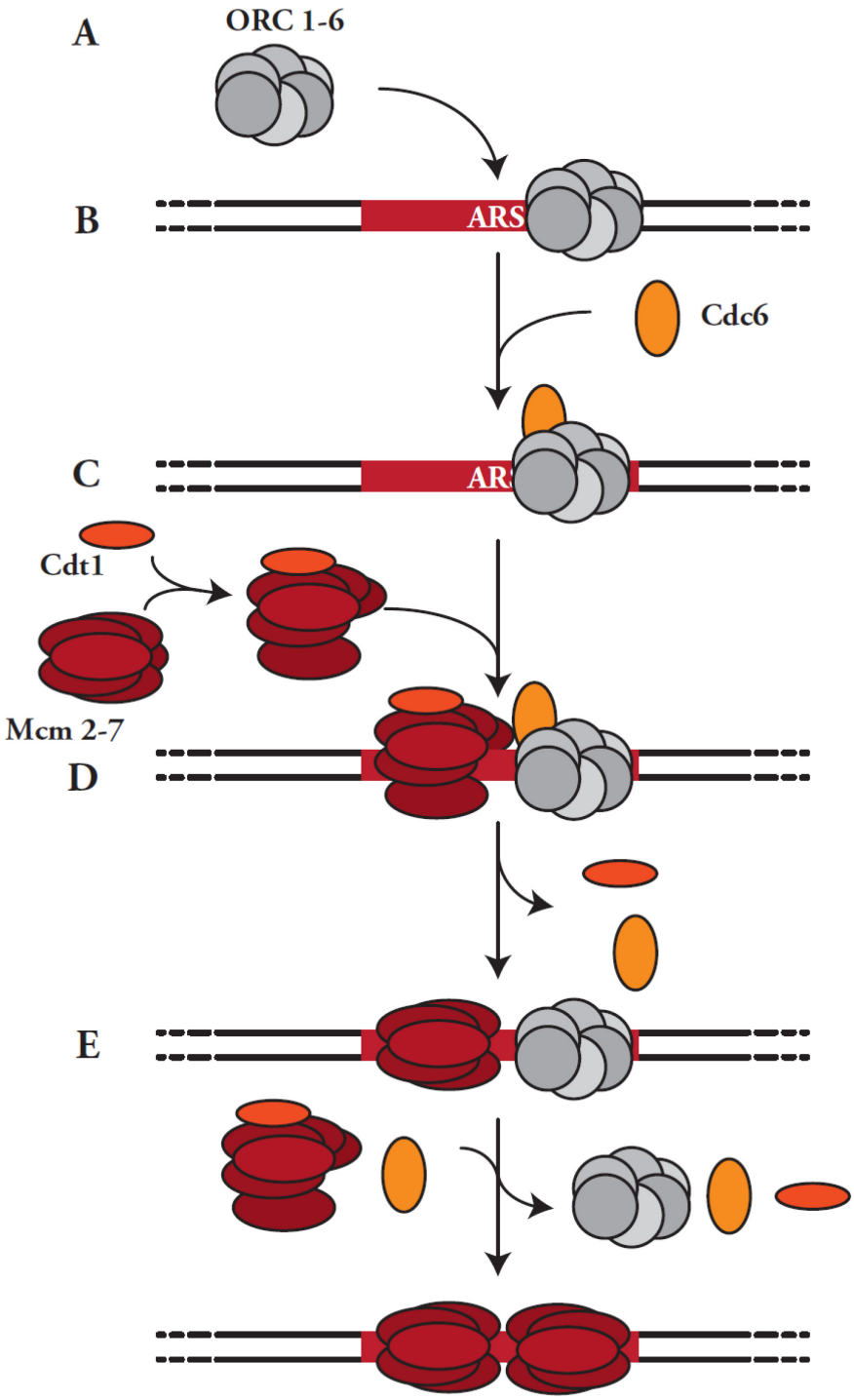

Figure 1.2. Modèle de l'autorisation des origines

origines est majoritairement médiée par l'action de la kinase clé du cycle cellulaire, Cdk1 (Cdc28 chez la levure) (40). Celle-ci phosphoryle Mcm3, Cdc6 et certaines sous-unités de ORC, ce qui a pour effet d'inhiber leur association aux MCM ou aux origines de réplication (29, 41–43). L'autorisation des origines est donc limitée à la phase G1 lorsque l'activité de la kinase Cdk1 est pratiquement nulle (44).

1.1.6 Initiation de la réplication

L'initiation de la réplication a lieu en fin de G1 au moment où l'expression des cyclines de transition G1/S augmente et entraîne la hausse de l'activité de la kinase CDK (Cdc28 en complexe avec Cln1 ou Cln2). Celle-ci phosphoryle plusieurs substrats ayant pour effet, entre autres, l'inhibition de l'APC (Anaphase Promoting Complex), ce qui résulte en l'arrêt la dégradation de la protéine Dbf4 (45). Cette dernière peut ensuite s'associer avec la kinase Cdc7 pour former le complexe DDK (Dbf4-Dependent Kinase). DDK permet l'assemblage de la machinerie de réplication, i.e. le réplisome, sur les origines préalablement autorisées par la phosphorylation de plusieurs cibles. À ce titre, bien que d'autres facteurs soient phosphorylés, Mcm4 et Mcm6 semblent être les seuls substrats de DDK essentiels à l'initiation de la réplication (46, 47) et leur phosphorylation permet le recrutement de Cdc45, Sld3 et Sld7 (Figure 1.3B) (48–52). Sld3 et une autre protéine, Sld2, sont ensuite phosphorylés par S-CDK (Cdc28 en complexe avec les cyclines Clb5 ou Clb6), ce qui favorise le recrutement additionnel de Sld2 au réplisome (Figure 1.3C) (53–55). Puis, Dpb11 se lie aux sites phosphorylés de Sld2 et Sld3 par l'entremise de ses domaines BRCT (BRCA1 C-Terminus) (Figure 1.3C) (53, 54, 56). Le complexe GINS (Sld5, Psf1, Psf2, Psf3) est ensuite recruté au réplisome par l'interaction de ses sous-unités avec Cdc45 et Dpb11 (57–61). La polymérase du brin continu, Pol ε (epsilon, Pol2, Dpb2, Dpb3, Dpb4) s'associe aussi au réplisome à cette étape (53).

Mcm10 s'associe ensuite simultanément avec les deux complexes MCM, provoquant alors l'activation de leur fonction d'hélicase et le déroulement bidirectionnel de l'ADN (Figure 1.3D-E) (38, 48, 62–66). Les sous-unités Sld3, Sld7, Dpb11 et Mcm10 ne font pas partie du réplisome actif et sont dissociées à cette étape (38, 50, 52, 54, 56).

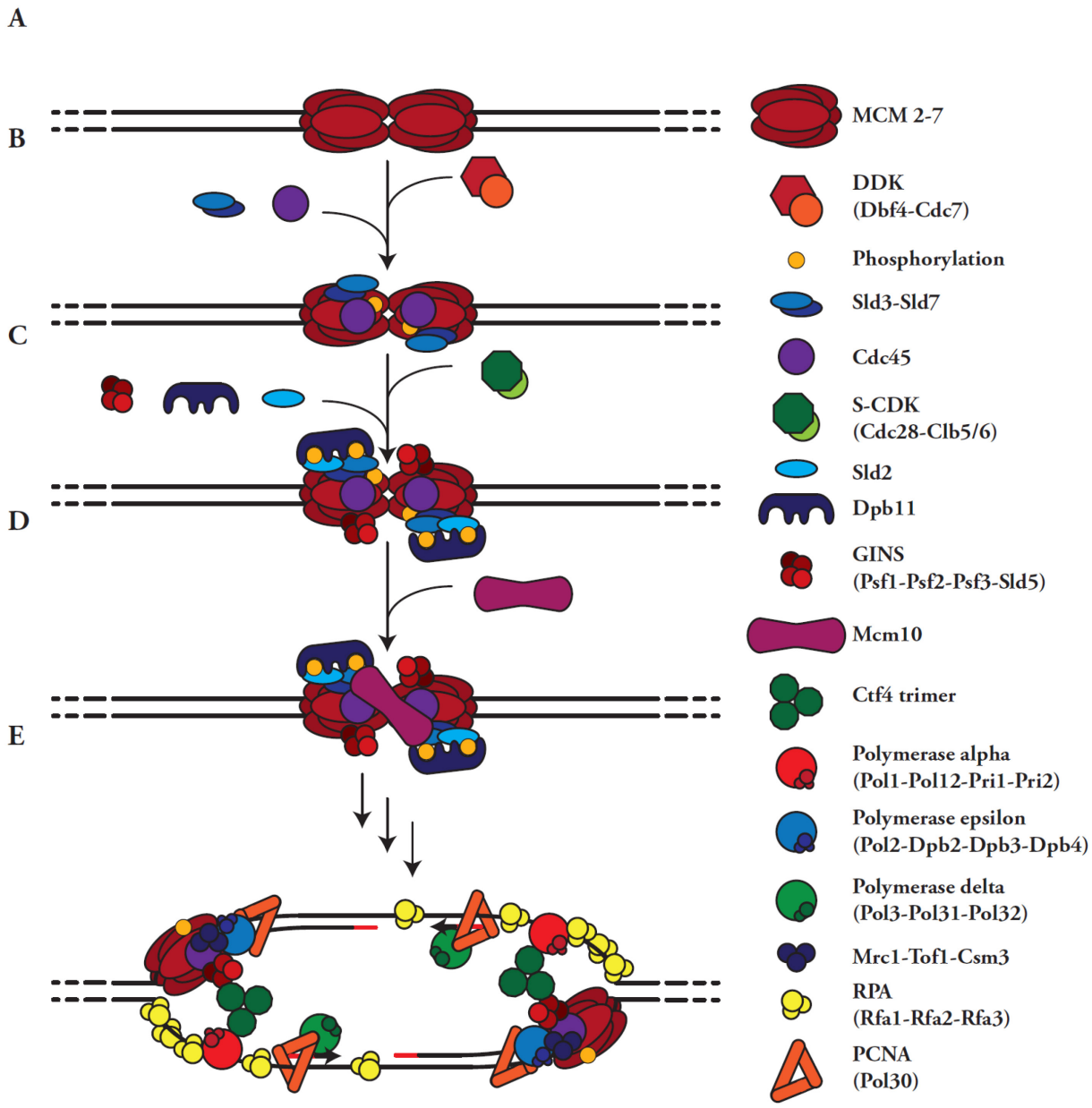

Figure 1.3. Modèle de l'initiation de la réplication

Plusieurs autres facteurs s'associent au réplisome, mais la séquence de leur recrutement demeure nébuleuse (38, 55, 67). Premièrement, la réplication requiert l'activité de deux polymérases supplémentaires : Polymérase α (alpha, Pol1, Pol12, Pri1, Pri2) et polymérase δ (delta, Pol3, Pol31, Pol32). Polα est responsable de la synthèse des amorces d'ARN-ADN nécessaires pour l'extension d'ADN par les deux autres polymérases, Polδ et Polε (68). Alors que la synthèse d'ADN selon le mode continu ne requiert virtuellement qu'une seule amorce d'ARN-ADN à l'origine de réplication, le mode discontinu nécessite une amorce pour chaque fragment d'Okazaki. Polα est donc reliée au réplisome par l'entremise de Ctf4 (69, 70). Ce dernier forme un homotrimère qui coordonne également plusieurs autres facteurs essentiels à la réplication, notamment, Tof2 et Chl1 ainsi que Dna2 (71–75).

Polδ est responsable de la synthèse d'ADN sur le brin discontinu et n'est pas directement reliée au réplisome (38). Son recrutement aux amorces ARN-ADN dépend de l'homotrimère PCNA (Proliferating Cell Nuclear Antigen, Pol30 chez *S. cerevisiae*). Ce dernier est chargé aux jonctions double-brins/simple-brins de chaque fragment d'Okazaki par le complexe RFC (Replication Factor C), composé de Rfc1 à 5. PCNA est essentiel à l'activité de Polδ et y demeure associé pendant l'extension de l'ADN (67). Bien que PCNA ne soit pas nécessaire au recrutement de Polε à l'ADN, il est chargé sur le brin continu lors de l'initiation de la réplication, demeure associé au réplisome et augmente la processivité de Polε (67, 76).

Le déroulement de l'ADN par le complexe MCM engendre nécessairement la formation d'ADN simple-brin (ssDNA). Or, le ssDNA peut former des structures secondaires potentiellement toxiques menant à des évènements de réarrangements chromosomiques majeurs (77). Du côté du brin continu, ceci ne pose pas de problème, car Polε est directement associée au réplisome et synthétise le brin complémentaire dès la sortie de l'ADN simple-brin du canal de MCM. Par contre, il est estimé que Polα synthétise une amorce toutes les 100 -10000 paires de bases et il y a ainsi présence d'ADN simple-brin sur le brin discontinu pendant la réplication entre chaque fragment d'Okazaki. Pour pallier ce problème, tout ssDNA formé durant la réplication est couvert et protégé par le complexe RPA (Replication Protein A) composé de trois sous-unités fortement conservées: Rfa1, Rfa2 et Rfa3 (78–81). Mis à part son rôle de protection de l'ADN simple-brin, RPA contribue à la progression du réplisome (55, 81).

Le complexe Mrc1-Tof1-Csm3 relie Polε au reste du réplisome, stabilise ce dernier et est nécessaire pour la progression adéquate des fourches de réplication (67, 82–85). Mrc1 joue

aussi un rôle dans la réponse aux dommages à l'ADN au cours de la phase S, tel que décrit plus loin.

1.1.7 Régions nécessitant un mode de réplication particulier

Plusieurs régions chromosomiques possèdent des particularités nécessitant une régulation spécifique de la réplication de l'ADN pour assurer la stabilité du génome, notamment l'ADN ribosomique, les télomères et les sites fragiles.

1.1.7.1 L'ADN ribosomique

Chez la levure, l'ADN ribosomique (rDNA) est localisé sur le chromosome XII et consiste en 100 à 200 répétitions en tandem d'une séquence de 9.1kb qui encode les ARN ribosomiques et contient une ARS (86–88). La nature répétitive du rDNA fait en sorte que des dommages issus de rencontres entre le réplisome et les polymérases à ARN pourraient avoir des effets catastrophiques et causer des événements de recombinaison potentiellement toxiques, en plus de bloquer la progression des fourches de réplication. Ainsi, la majorité des répétitions de rDNA sont inactives (voir la section Sir2 plus bas concernant le mécanisme) et la réplication au rDNA est principalement unidirectionnelle, dans le même sens que la transcription des ARN ribosomaux, possiblement pour limiter les collisions entre le réplisome et les polymérases d'ARN. Ceci est dû à l'action et l'association de Fob1 à la séquence RFB (Replication Fork Barrier) qui forme une barrière ne permettant qu'aux fourches de réplication provenant d'une seule direction de la traverser (89, 90).

1.1.7.2 Les télomères

Les extrémités des chromosomes linéaires sont des substrats idéaux pour des nucléases et les mécanismes de réparation des bris double-brins (décrits en détails plus loin), ce qui

pourrait mener à des réarrangements catastrophiques pour l'intégrité du génome. Ainsi, les terminaisons chromosomiques forment elles-mêmes des structures nucléoprotéiques spécialisées, que l'on nomme télomères. Les différents facteurs composant les télomères varient d'une espèce à l'autre, mais ceux-ci ont entre autres pour fonction de protéger les terminaisons chromosomiques et éviter que la cellule ne les reconnaisse en tant que bris double-brin. Or, les chromosomes linéaires posent un autre problème conceptuel : le mode discontinu de synthèse de l'ADN élucidé par Okazaki sous-entend qu'il y a perte d'ADN aux extrémités des chromosomes à chaque division cellulaire en raison de l'ablation de l'amorce d'ARN (10, 11, 91). Il a été découvert que les cellules remédient à ce problème à l'aide d'une réverse transcriptase spécialisée, la télomérase, qui prévient la perte d'ADN en assurant l'extension des chromosomes à l'aide d'une matrice d'ARN (92–95). En l'absence de celle-ci, les extrémités des chromosomes raccourcissent graduellement jusqu'à atteindre une taille critique, ce qui cause l'entrée en sénescence réplicative. À travers les eucaryotes, la télomérase est formée de sous-unités régulatrices et catalytiques et inclue une matrice d'ARN. Chez la levure, l'holoenzyme de la télomérase est composée de la sous-unité catalytique Est2, de la matrice d'ARN TLC1 et des sous-unités régulatrices Est1 et Est3 (96–100). Pour assurer le maintien d'une taille optimale des télomères, le recrutement de la télomérase est régulé par les protéines des télomères chez tous les eucaryotes.

Chez la plupart des organismes, les télomères possèdent deux caractéristiques structurelles importantes : ils sont formés de séquences répétées et ont une extrémité 3' simple-brin de quelques bases. Les télomères de la levure ont une taille d'environ 300 paires de bases de séquences $TG_{1\text{-}3}$ répétées et sont normalement séquestrés à la membrane nucléaire. Rap1 s'associe à ces répétitions par intervalle de 20 paires de base et il est estimé qu'environ 20 molécules de Rap1 recouvrent chaque télomère (101–103). Rap1 est nécessaire au maintien de la longueur des télomères (104) et prévient les fusions chromosomiques (101, 105). Cette protéine recrute Rif1/2 et Sir3/4 qui s'associent à un même site de liaison sur Rap1 de manière compétitive (106–110). Rif1 et Rif2 inhibent l'action de la télomérase par deux mécanismes indépendant qui semblent dépendre du nombre de molécules Rap1 liées aux télomères (110–112). Lorsque les télomères sont longs, l'élongation par la télomérase est inhibée par Rif1 et Rif2, tandis cet effet est dissipé aux télomères courts. Ainsi, des mutants pour *RIF1* ou *RIF2* ont des télomères anormalement longs (110, 111). Sir3 et Sir4 sont impliqués dans la répression

transcriptionnelle aux télomères (Voir le mécanisme dans la section Sir2) et la localisation de ceux-ci à la périphérie du noyau (113).

Le complexe Yku70/80 forme un anneau autour de l'ADN double-brin aux extrémités des télomères et les protège contre la dégradation nucléolytique ainsi que les réarrangements chromosomiques. En son absence, les cellules présentent des télomères courts avec de longues protrusions 3' simple-brins générées par Exo1 (114–118). Yku80 interagit avec une région de l'ARN matrice de la télomérase, *TLC1*, et contribue à l'import nucléaire et au recrutement de celle-ci aux télomères (119–124). De plus, Yku70/80 stabilise l'association aux télomères de la sous-unité régulatrice Est1 (125).

L'extrémité 3' simple-brin des télomères est liée par le complexe CST, composé des trois sous-unités Cdc13, Ten1 et Stn1, (126–131). Cdc13 est nécessaire pour le recrutement de la télomérase (130, 132) et sa mutation provoque le raccourcissement graduel des télomères au fil des générations jusqu'à la sénescence réplicative de façon similaire aux mutants de la télomérase (97). Le complexe CST prévient la dégradation des télomères par des nucléases et l'absence d'une des trois sous-unités est suffisante pour causer l'activation de la signalisation en réponse aux dommages à l'ADN et la mort cellulaire (133–135).

La dégradation d'Est1 par le protéasome restreint l'assemblage de la télomérase et l'extension des télomères à la fin de phase S, après que ceux-ci aient été répliqués par les polymérases conventionnelles (100, 136). Or, à la fin de la réplication, des mécanismes distincts aux brins continus et discontinus assurent le maintien de la longueur des télomères lorsque les fourches de réplication atteignent l'extrémité des chromosomes. Du fait que les polymérases ont une activité 5' vers 3', le brin comportant l'extrémité 3' simple-brin est toujours synthétisé selon le mode discontinu. Dans ce contexte, la taille de la matrice demeure identique lors du passage des fourches et ne nécessite pas d'extension (Figure 1.4A-B). L'emplacement de la dernière amorce d'ARN est régulé par l'association de Polα à Cdc13 et Stn1, ce qui établit la longueur de l'ADN 3' simple-brin à environ 10-14 bases après l'ablation de l'amorce d'ARN (137–139). Ainsi, en l'absence d'activité de télomérase, le brin synthétisé selon le mode discontinu génère un télomère identique en longueur et structure au parental (140, 141).

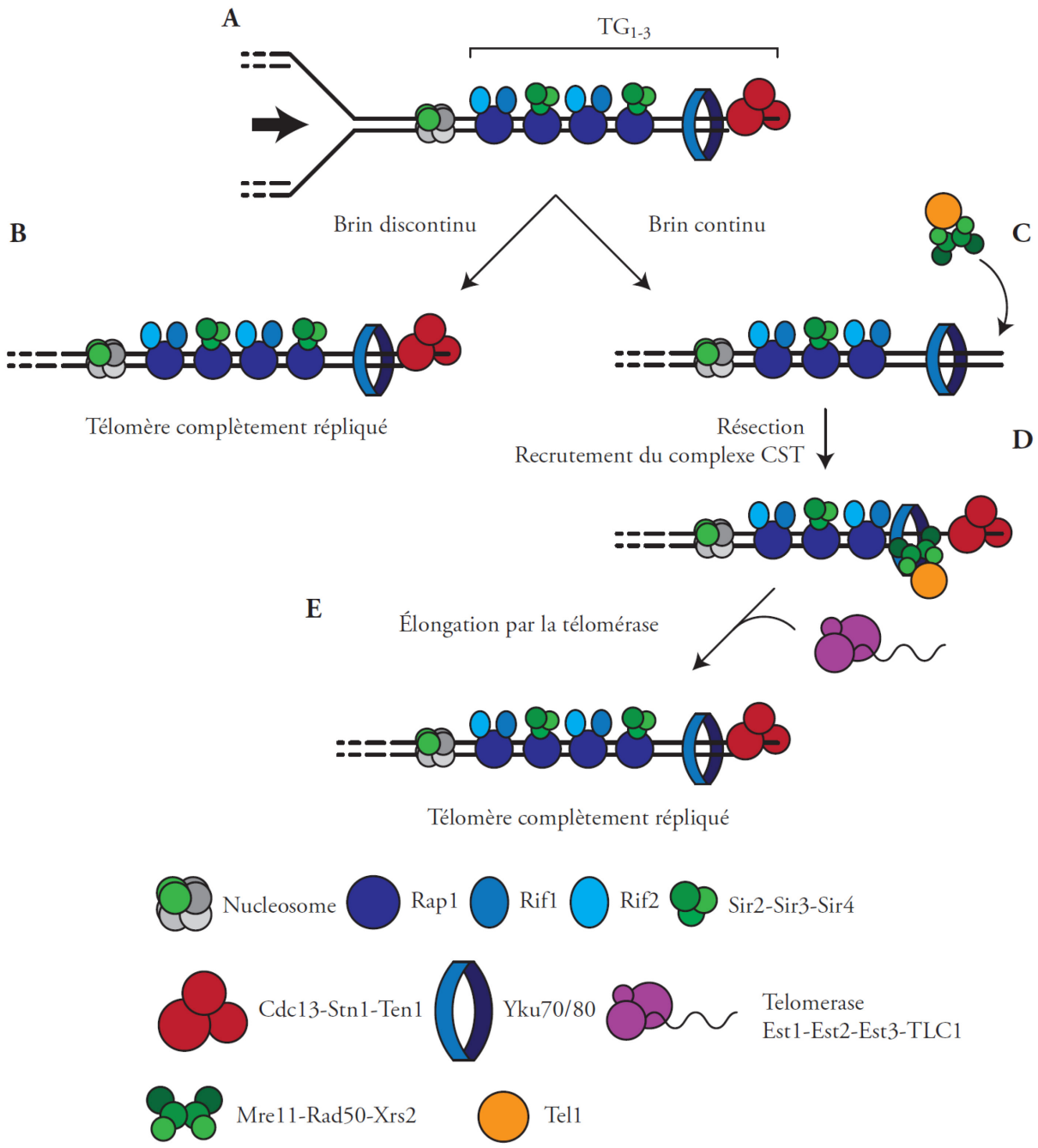

Figure 1.4. Structure et réplication des télomères de levure

À l'inverse, la synthèse selon le mode continu génère une extrémité franche de même longueur que l'extrémité 5' de l'ADN parental et implique la perte de l'extrémité 3' simple-brin (Figure 1.4C). La stabilité du télomère et l'association de la télomérase nécessite donc la résection 5' vers 3' de l'ADN au brin continu pour reformer une extrémité 3' simple brin (142). Cette tâche est accomplie par le complexe MRX (Mre11-Rad50-Xrs2) qui s'associe seulement aux télomères répliqués selon le mode continu où il initie la résection par la nucléase Mre11 (143, 144). Le complexe CST peut ensuite se lier à l'extrémité 3' simple-brin nouvellement formé et favoriser le recrutement de la télomérase qui ne peut catalyser l'extension qu'à partir d'ADN simple-brin (Figure 1.4D-E) (143). L'association aux télomères de MRX permet le recrutement additionnel de Tel1, une kinase de la famille des PI3K, par une région en C-terminal de Xrs2 (145, 146). L'activité kinase de Tel1 favorise l'élongation des télomères mais ses substrats dans ce contexte demeurent inconnus (147). MRX-Tel1 s'associe spécifiquement aux télomères courts au moins en partie par l'inhibition de leur association aux télomères longs par Rif2 (148). Des mutants pour *TEL1*, *XRS2*, *MRE11* et *RAD50* présentent d'ailleurs tous des télomères stablement courts (115, 149, 150). Ainsi, contrairement à l'assomption initiale et généralement acceptée, la perte d'ADN sans l'action de la télomérase est issu de brin synthétisé par le mode continu (140, 141, 151).

1.1.7.5 Les sites fragiles

Certaines régions du génome, de par leur structure spécifique, posent obstacle à la progression des fourches de réplication. Deux hélicases spécialisées sont responsables de la résolution de ces structures : Rrm3 et Pif1. Rrm3 possède une activité hélicase 5' à 3' et enlève les complexes protéiques autres que les nucléosomes rencontrés par les fourches de réplication notamment aux télomères et au rDNA. Son absence provoque le ralentissement du réplisome et même l'accumulation ou le bris des fourches à ces régions (152–154). Pif1 favorise la progression des fourches en catalysant la résolution des G-quadruplex et en y prévenant la formation de lésions à l'ADN (155–157).

1.1.8 Régulation temporelle de l'activation des origines de réplication

Plus de 600 origines de réplication ont été identifiées dans le génome de levure (158, 159), mais seulement une fraction de celles-ci initient la réplication lors d'une phase S. En fait, la plupart des origines sont répliquées passivement par une fourche de réplication convergente (160). Ceci est dû à la paucité des facteurs d'initiation de la réplication (Cdc45, Dpb11, Sld2, Sld3, Sld7 et Dbf4) qui sont recyclés d'origine en origine jusqu'à complétion de la réplication (51, 161). Bien que les premières études sur le sujet proposaient un programme réplicatif prédéterminé, l'initiation des origines semble plutôt suivre un modèle stochastique à travers le génome; chaque origine possède une certaine efficacité intrinsèque qui détermine sa probabilité d'initier la réplication à tout moment durant la phase S (162–164). Ainsi, une origine extrêmement efficace, telle ARS305 ou ARS607 chez la levure, va initier la réplication tôt dans la majorité des cycles cellulaires, tandis qu'une origine inefficace sera fréquemment passivement répliquée plus tard en phase S (158).

1.1.9 Qu'est-ce qui détermine l'efficacité d'une origine?

L'activité d'une origine peut être influencée par une multitude de facteurs. Le premier de ceux-ci est la séquence spécifique de l'ARS qui peut en faire varier l'efficacité. Par exemple, la présence des éléments B2 et B3 facilite l'association d'ORC et des MCM aux origines ce qui augmente la probabilité de leur autorisation et de leur activation subséquente (15, 16, 165–169). Ensuite, l'environnement chromosomique module l'efficacité des origines. Notamment, les ARS près d'un télomère ou d'un centromère sont activées en fin et début de phase S, respectivement (158, 170–173). Relocaliser une ARS à ces régions est suffisant pour modifier son efficacité, i.e. une ARS normalement activée tôt le sera beaucoup plus tardivement lorsque transposée près d'un télomère (170). Deux complexes protéiques contribuent à ce phénomène aux télomères. D'une part, l'association télomérique du complexe SIR limite l'activation des origines télomérique (Sir2-Sir3-Sir4) possiblement en favorisant la formation de chromatine silencieuse et diminuant de ce fait l'accessibilité aux ARS des facteurs d'initiation (38, 174,

175). D'autre part, Rif1 recrute la phosphatase PP1 aux télomères et celle-ci contrecarre l'action de DDK dans l'initiation de la réplication en déphosphorylant les sous-unités MCM (176–179). Rif1-PP1 est aussi recruté à un sous-ensemble d'origines et réduit également l'activité de celles-ci. À l'inverse, la phosphorylation des MCM par DDK est favorisée aux centromères par le recrutement de Dbf4 au centrosome par l'intermédiaire de Ctf19 (180).

Outre ces régions spécifiques, d'autres facteurs influencent l'activation des origines à travers le génome. Notamment, Rpd3 est une histone desacétylase (HDAC) de classe I qui diminue l'efficacité d'un sous-ensemble d'origines (181–183). Les facteurs de transcription forkhead (Fkh1 et Fkh2) quant à eux, favorisent l'initiation de la réplication. La proximité de Fkh1/2 à certaines origines, notamment ARS305 et ARS607, augmente leur efficacité et la probabilité qu'elles s'activent tôt (184, 185). Il a été proposé que l'effet de Fkh1/2 ne découle pas directement de leurs fonctions de facteurs de transcription, mais plutôt de la juxtaposition des origines efficaces dans l'espace tridimensionnelle du noyau, ce qui augmenterait la concentration effective des facteurs d'initiation à ces origines (184).

1.1.9.1 Régulation de l'activation des origines aux télomères courts

Lorsque les télomères sont raccourcis, par exemple chez un mutant *yku70*Δ ou à l'aide d'un système inductible, il a été observé que les origines télomériques et sous-télomériques s'activent plus tôt durant la réplication (186, 187). L'activation précoce des origines à un télomère court augmente vraisemblablement le temps d'occupation de la télomérase ainsi que son activité à l'extrémité du chromosome. Cet effet semble indépendant du statut d'acétylation des nucléosomes sous-télomériques et des fonctions du complexe SIR (188). L'activation des origines aux télomères courts requiert plutôt l'inactivation des fonctions de Rif1 aux télomères par la kinase Tel1 (189, 190). Plusieurs sites de phosphorylation par Tel1 ont été identifiés sur Rif1, mais la phosphorylation de ceux-ci n'est pas suffisante ni essentielle à l'inhibition de l'activité de Rif1-Glc7. Il y a ainsi probablement d'autres substrats de Tel1 encore non-identifiés impliqués dans ce processus (189, 190).

1.2 La chromatine pendant la réplication

L'ADN ne se retrouve pas nu dans le noyau des eucaryotes ; il est présent sous forme de complexe nucléoprotéique que l'on nomme chromatine. L'unité de base de celle-ci est le nucléosome qui est formé d'environ 146 paires de bases d'ADN enroulées autour d'un octamère d'histones composé de deux molécules de chacune des histones H2A, H2B, H3 et H4 (191–193). L'enroulement autour des histones, qui sont chargées positivement, est important pour permettre à l'ADN d'entrer dans le noyau; la répulsion électrostatique des longues molécules d'ADN négativement chargées (plus de 2 mètres pour le génome humain) ne permettrait pas leur entrée dans un noyau de quelques micromètres de diamètre. La formation de nucléosomes est donc essentielle chez les eucaryotes et le maintien du ratio histones/ADN doit être strictement régulé pour assurer la viabilité (194). À ce titre, la réplication de l'ADN pose deux problèmes ; d'une part, la réplication implique la duplication graduelle du génome et ainsi la synthèse d'ADN doit être étroitement liée à l'assemblage de nucléosomes sur l'ADN naissant ; d'autre part afin que le réplisome puisse progresser, les nucléosomes parentaux en aval du réplisome doivent être désassemblés et réassemblés derrière les fourches de réplication.

1.2.1 Assemblage *de novo* de la chromatine

La nature alcaline des histones leur confère une toxicité accrue, car elles peuvent interagir non-spécifiquement avec l'ADN. Ainsi, leur expression est régulée à tous les niveaux, i.e. transcriptionnel, traductionnel et post-traductionnel (195). De plus, les histones se retrouvent rarement libres et sont associées à des facteurs spécialisés de leur traduction jusqu'à leur déposition sur la chromatine. Dès leur synthèse dans le cytoplasme, les histones forment rapidement des dimères H2A-H2B et H3-H4 et sont importés au noyau par des karyophérines ou chaperones d'histones : Kap121 et Kap123 pour H3-H4 et Kap114 et Nap1 pour H2A-H2B (196–200). Les dimères sont ensuites pris en charge dans le noyau par des chaperones d'histones (Asf1, CAF-I, Rtt106, Nap1 et Vps75) qui assurent l'assemblage séquentiel des dimères en nucléosomes.

Chez tous les eucaryotes, les histones H3-H4 nouvellement synthétisées sont acétylées sur une multitude de résidus sur leur queue N-terminale (H3K9, 14, 18, 23, 27 et H4K5, 8, 12) et leurs domaines globulaires (H4K91 et H3K56) par les acétyltransférase Gcn5, Hat1 et Rtt109 (200–207). Ces modifications transitoires sont enlevées suite à l'incorporation des histones en nucléosomes (208) et semblent jouer un rôle au moins partiellement redondant dans l'import nucléaire et l'assemblage des nucléosomes sur l'ADN (196, 203, 206, 209–211).

L'acétylation d'H3K56 (H3K56ac) est l'une des modifications des histones nouvellement synthétisées dont les fonctions dans l'assemblage *de novo* des nucléosomes sont les mieux caractérisées. Chez *S. cerevisiae*, cette modification est catalysée par l'association à la chaperone Asf1 de l'acétyltransférase Rtt109 (Figure 1.5A-B) (212–217). Un complexe ubiquitine-ligase formé de la E3 Rtt101, la E2 Cdc34, la protéine à domaine RING-finger Hrt1 et les sous-unités adaptatrices Mms1 et Mms22, catalyse ensuite l'ubiquitination d'H3 sur plusieurs lysines, provoquant le transfert d'H3-H4 d'Asf1 vers l'une de deux autres chaperones, CAF-I (composé de Cac1, Cac2 et Cac3 chez *S. cerevisiae*) et Rtt106 qui ont une préférence marquée pour les dimères acétylés sur H3K56 (Figure 1.5C-D) (218–225). CAF-I et Rtt106 interagissent ensembles (226) et combinent deux dimères H3-H4 pour former un tétramère $(H3\text{-}H4)_2$ qui est ensuite déposé sur la chromatine (Figure 1.5E) (221, 224, 226–229). L'interaction de CAF-I avec PCNA assure sa localisation derrière les fourches de réplication et favorise le chargement de tétramères $(H3\text{-}H4)_2$ sur l'ADN naissant (230–232).

L'ajout des dimères H2A-H2B est ensuite complété par Nap1 et possiblement le complexe FACT, formé des sous-unités Spt16, Pob3 et Nhp6 chez *S. cerevisiae*. Ceux-ci peuvent chacun lier un dimère H2A-H2B nouvellement synthétisé et le déposer sur le tétramère $(H3\text{-}H4)_2$ préalablement associé à l'ADN naissant, formant ainsi un nucléosome complet (197, 200, 233–236). Malgré la présence de défauts d'assemblage de la chromatine, un double mutant *rtt106*Δ *cac1*Δ n'est étonnamment pas létal (221). De récentes évidence suggèrent que le complexe FACT peut aussi assembler les dimères H3-H4 sur la chromatine, ce qui laisse supposer que les chaperones Rtt106 et CAF-I et le complexe FACT sont au moins partiellement redondants dans l'assemblage des tétramères $(H3\text{-}H4)_2$ (236–238).

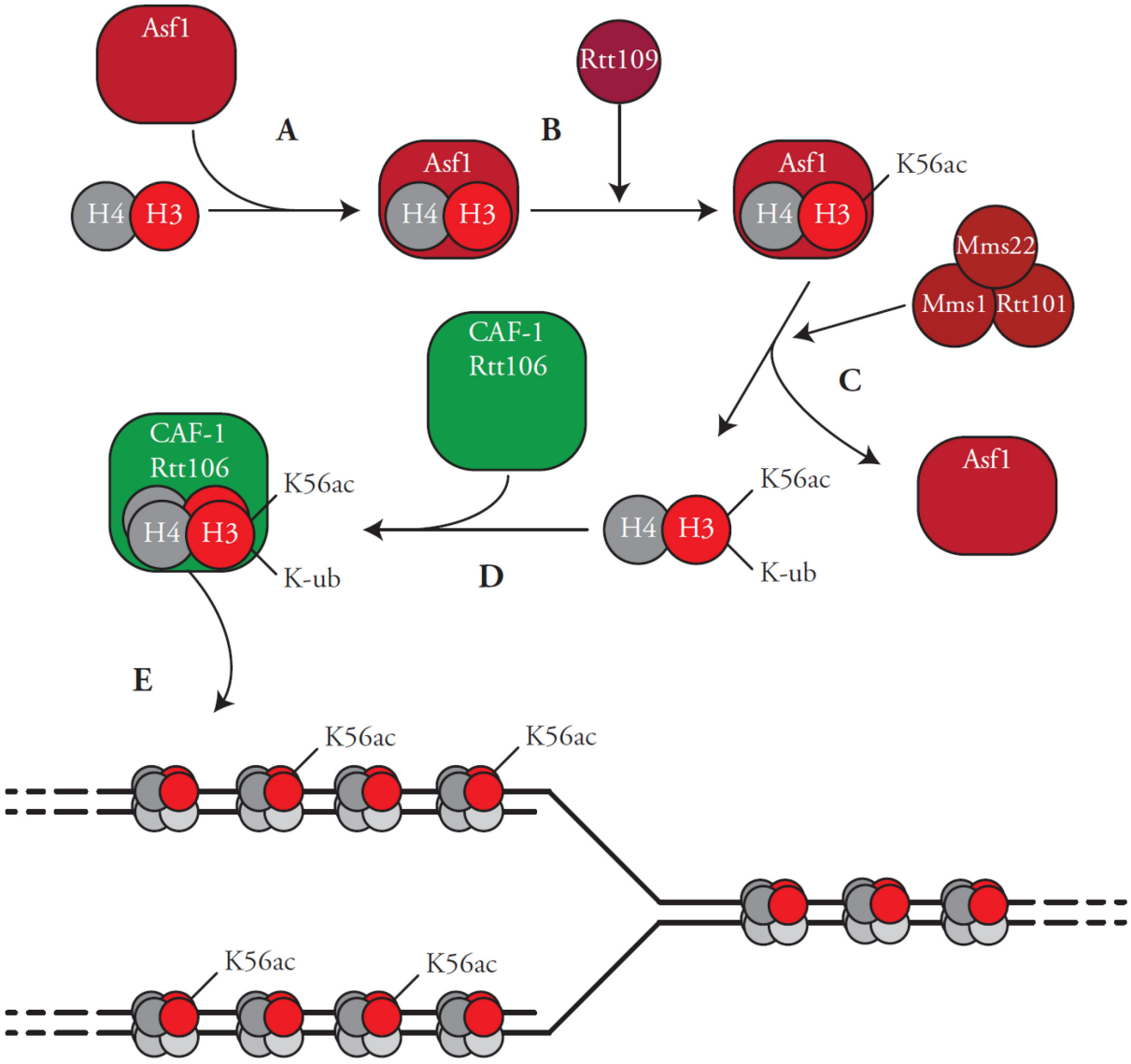

Figure 1.5. Assemblage *de novo* de la chromatine

1.2.2 Récupération des nucléosomes

En raison de la complexité technique de l'étude de la récupération des nucléosomes durant la réplication, ce mécanisme demeure encore mal compris, mais certains éléments clés ont néanmoins pu être élucidés. Ce processus nécessite au moins deux facteurs : le complexe FACT et Mcm2. FACT se lie aux dimères H2A-H2B sur une surface de contact à l'ADN et les dissocie du nucléosome en face des fourches de réplication (236–239). Le tétramère $(H3\text{-}H4)_2$ est ensuite transféré vers l'arrière des fourches de réplications, selon un mécanisme encore mal compris, mais qui semble nécessiter Mcm2 et le complexe FACT (234, 240–242). Il est proposé que deux dimères H2A-H2B sont ensuite ajoutés par le complexe FACT au tétramère $(H3\text{-}H4)_2$ pour ainsi former un octamère d'histone complet derrière les fourches de réplication (234, 236).

1.3 La réponse aux dommages à l'ADN

L'ADN peut être chimiquement modifiée par différents groupements moléculaires pouvant endommager son intégrité et nuire aux processus reliés au métabolisme de l'ADN, e.g. obstruer la réplication et la transcription (243). Les modifications de l'ADN peuvent être issues de l'environnement immédiat d'une cellule (radiations ultraviolettes (UV) et ionisantes) ou de son métabolisme basal (espèces oxygénées réactives, ROS). Ainsi, malgré tous les efforts déployés par la cellule pour répliquer son information génétique et compte tenu de l'importance de l'information contenue sur l'ADN, la présence de mécanismes assurant la réparation de l'ADN est cruciale pour la survie. Claude S. Rupert a fourni les premières évidences en 1958 de l'existence de tels processus en montrant que la survie de bactéries exposées à la radiation UV peut être améliorée en les exposant subséquemment à de la lumière bleue, ce qui a mené à l'identification de la première enzyme de réparation de l'ADN : la photolyase (244). Depuis, il y a eu une réelle effervescence de l'étude des processus de réponse aux dommages à l'ADN, notamment parce qu'il a vite été compris que les mutations issues de dommages mal réparés sont généralement les principaux éléments instigateurs du cancer. Une panoplie de mécanismes qui permettent de répondre à une grande variété de dommages dans tous les contextes cellulaires

(e.g. toute phase du cycle cellulaire) ont ainsi été identifiés et caractérisés au cours des dernières décennies. Ceux-ci impliquent en général trois processus intrinsèquement liés : la détection des dommages et la signalisation qui en découle, la réparation des lésions et le retour à la normale. Les prochaines sections traiteront de ces trois aspects en mettant l'accent sur ceux en lien avec la réplication de l'ADN.

1.3.1 La signalisation en réponse aux dommages à l'ADN

La phase S est une période pendant laquelle le génome est particulièrement vulnérable aux dommages à l'ADN. La rencontre d'une fourche de réplication avec certains types de dommages peut provoquer l'effondrement de celle-ci, ce qui a des conséquences catastrophiques sur l'intégrité génomique. Le terme « stress réplicatif » fait référence à tout ce qui est susceptible d'affecter la progression des fourches de réplication durant la phase S. Il existe plusieurs sources de stress réplicatif : des modifications des bases en amont des fourches de réplication peuvent provoquer un encombrement stérique ne permettant pas l'entrée de la base dans le site catalytique des polymérases à ADN, ce qui empêche les polymérases à ADN de progresser (e.g. 3-méthyladenine, 8-oxoguanine, dimères cyclobutilique de pyrimidines (CPD) et photoproduits 6,4 induits par le rayonnement UV) ; la réduction de la quantité de dNTP par l'inhibition de la ribonucléotide réductase (RNR) par l'hydroxyurée (HU) ralentit considérablement la progression des fourches de réplication. Pour parvenir à compléter la réplication malgré ces obstacles, la cellule emploie plusieurs mécanismes qui assurent la détection et la gestion des dommages à l'ADN pendant cette phase.

Lorsqu'une fourche de réplication fait face à du stress réplicatif issu de lésions simple-brins, il y a découplage entre Polε et le reste du réplisome (Figure 1.6A-B). Les MCM continuent donc à dérouler l'ADN, ce qui provoque la formation de longs filaments de ssDNA auxquels RPA s'associe (245–248). L'effet du stress réplicatif sur la formation de ssDNA au brin discontinu dépend du type de lésion : par exemple, la réduction de dNTP entraînera la formation de longs filaments de ssDNA, mais dû au mécanisme discontinu qui implique l'amorçage répétitif des polymérases, une lésion causant un encombrement stérique avec la polymérase aura un effet plus modeste. Mec1, l'homologue de la kinase ATR chez la levure, s'associe ensuite

aux filaments de ssDNA couverts par RPA par l'intermédiaire de Ddc2 (Figure 1.6B) (249–251). Mec1 est une kinase clé de la réponse aux dommages à l'ADN et bien que son recrutement à RPA soit requis, il n'est pas suffisant pour son activation et la phosphorylation subséquente de la plupart de ses substrats. À cet effet, au moins trois facteurs présents ou recrutés aux fourches endommagées participent à l'activation de Mec1 : Dna2, la serre moléculaire 9-1-1 et Dpb11. Tel que décrit plus haut, Dna2 fait partie du réplisome, mais par un mécanisme impliquant des résidus aromatiques de son domaine N-terminal, Dna2 favorise l'activation de Mec1 aux fourches assujetties au stress réplicatif (252). La serre moléculaire 9-1-1 (Rad17, Ddc1, Mec3) est chargée sur la jonction double-brin/simple-brin par un complexe RFC alternatif dans lequel Rfc1 est remplacé par Rad24 (Figure 1.6B) (253, 254). Similairement à Dna2, l'activation de Mec1 dépend de résidus aromatiques dans la région C-terminale de Ddc1 (255). Dpb11 requiert l'action et la phosphorylation préalable d'au moins trois substrats : H2A-S128, Rad9-S462, -T474 et Ddc1-T602. Suite à son recrutement, Mec1 phosphoryle les histones H2A près des fourches de réplication sur la sérine 128 chez la levure (256), ce qui entraîne le recrutement de Rad9 à la chromatine entourant la lésion (Figure 1.6C) (257). La phosphorylation de Rad9 par S-CDK sur plusieurs résidus, dont la sérine 462 et la thréonine 474, permet à Dpb11 de se lier à Rad9 via l'un de ses domaines BRCT en tandem (BRCT1/2) (Figure 1.6C) (258, 259). Mec1 phosphoryle aussi Ddc1 sur la thréonine 602, ce qui mène à la liaison à Ddc1 de Dpb11 par deux autres domaines BRCT en tandem (BRCT 3/4) (252, 260, 261). L'activation de Mec1 par Dpb11 implique la formation d'un complexe ternaire qui inclue Rad9 (259).

En plus d'H2A-S128-P, l'association de Rad9 au nucléosome nécessite sa dimérisation et l'interaction de son domaine tudor avec H3K79me, une modification d'histone presque ubiquitaire sur la chromatine (257, 262–265). Rad9 agit comme sous-unité adaptatrice et d'échafaudage dans la transduction du signal par Mec1. La phosphorylation de Rad9 par Mec1 permet le recrutement aux fourches de Rad53, une autre kinase clé dans la signalisation en réponse aux dommages à l'ADN. Cette dernière se lie à Rad9 via ses domaines FHA (forkhead associated) (266–268), ce qui entraîne sa phosphorylation par Mec1 (267, 269, 270). La phosphorylation de Rad53 induit son autophosphorylation en trans, i.e. l'autophosphorylation de Rad53 provient d'une autre molécule de Rad53, ce qui lui permet d'atteindre son activation maximale (271). Mrc1 agit aussi comme sous-unité adaptatrice et est nécessaire pour

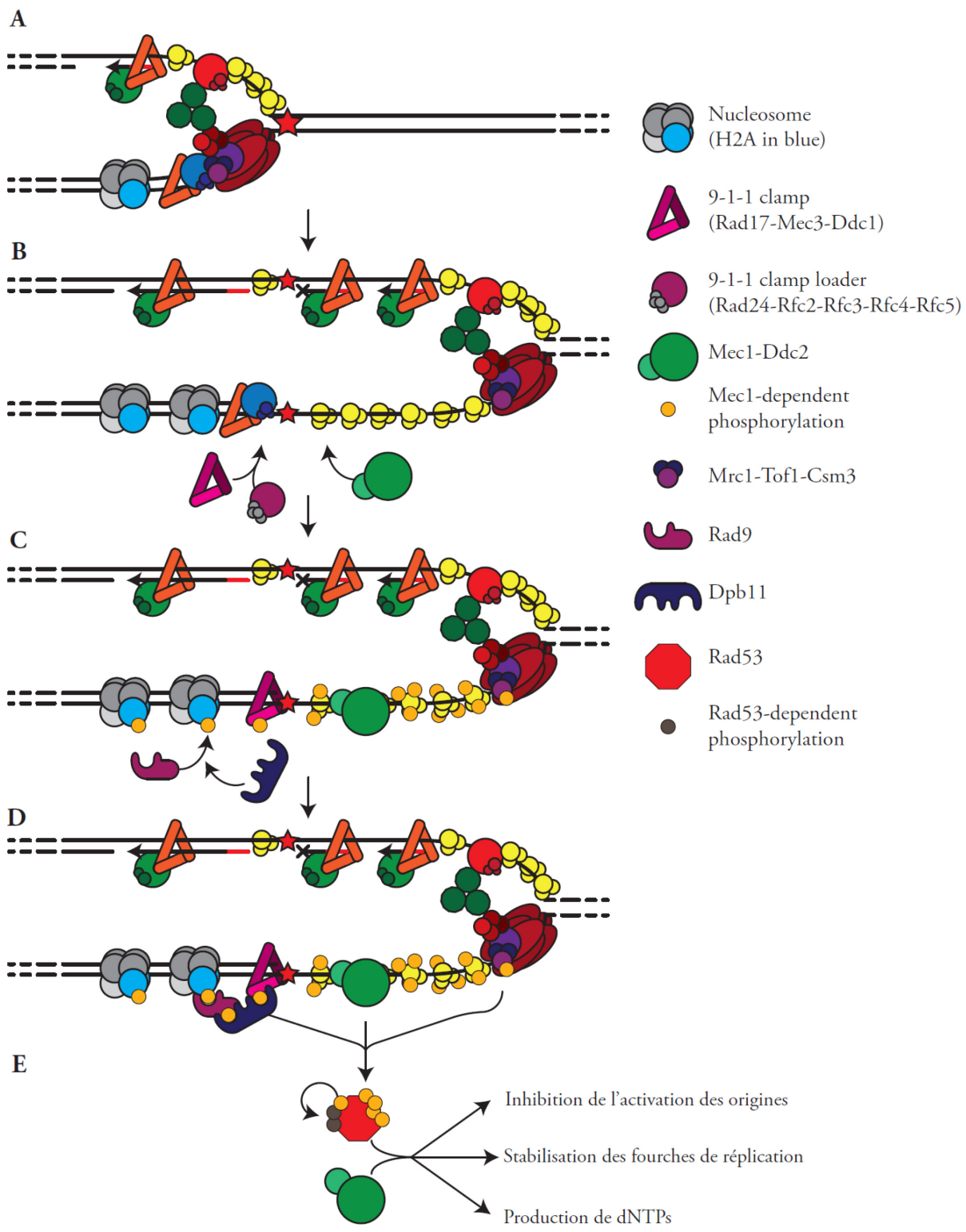

Figure 1.6. La signalisation en réponse au stress réplicatif

l'amplification du signal d'activation de Rad53 et de la signalisation en réponse au stress réplicatif dans certains contextes, notamment lorsque les niveaux de dNTP sont réduits en présence d'hydroxyurée. (272). La phosphorylation par Mec1 stabilise Mrc1 aux fourches de réplication et induit une boucle de rétroaction positive sur la phosphorylation et l'activation de Rad53 (273, 274).

Une fois activés, Rad53 et Mec1 catalysent la phosphorylation d'une multitude de protéines. Plusieurs études à large échelle de spectrométrie de masse se sont penchées sur l'indentification de leurs substrats (275–278), mais vu l'étendue des cibles de Mec1 et Rad53, les fonctions spécifiques de la plupart de ces modifications demeurent nébuleuses. Néanmoins, ces deux kinases protègent l'intégrité du génome en augmentant la quantité de dNTP disponibles, en induisant l'expression des protéines de réparation, en stabilisant le réplisome et en régulant l'activité des origines de réplication.

Une kinase additionnelle, Chk1, est aussi activée en réponse au stress réplicatif. Celle-ci stabilise sécurine (Pds1) en la phosphorylant, ce qui inhibe l'activité de séparase (Esp1), empêchant le clivage de la sous-unité Mcd1 du complexe cohesin et l'entrée en anaphase (279–282). L'activation de Chk1 force ainsi l'arrêt du cycle cellulaire en mitose avant l'anaphase.

La signalisation en réponse à d'autres types de dommages à l'ADN qui n'ont pas lieu strictement durant la réplication suit un processus similaire. Dans la plupart des cas, il y a génération de ssDNA rapidement recouvert par RPA, ce qui provoque la cascade de signalisation décrite ci-haut. Dans le cas des bris double-brins (DSB), MRX, qui est l'un des premiers complexes à s'associer aux extrémités du DSB, recrute initialement la kinase Tel1 qui peut amorcer la cascade de signalisation similairement à Mec1 (283–286). Du ssDNA est néanmoins généré suite à la résection (voir plus bas), ce qui favorise l'activation de la réponse aux dommages à l'ADN par Mec1. En fait, chez *S. cerevisiae*, Tel1 ne joue qu'un rôle mineur et ne devient important pour la réponse aux dommages à l'ADN qu'en l'absence de Mec1 (287). L'homologue de Tel1 chez les mammifères, ATM, occupe cependant des fonctions plus importantes au sein de la signalisation suite à la détection de DSBs (288).

1.3.1.1 Augmentation de la quantité de dNTP intracellulaire

L'une de fonctions majeures de Rad53 et Mec1 en réponse au stress réplicatif est d'augmenter la quantité de dNTP intracellulaire. Les raisons sous-jacentes à ce phénomène sont encore mal comprises, mais des évidences suggèrent que ceci favorise l'activité de certaines polymérases et leur permet de contourner des lésions, ce qui réduit la génération de ssDNA (289–291). À cette fin, Rad53 et Mec1 induisent l'activité de la Ribonucléotide Réductase (RNR), l'enzyme catalysant l'étape limitante dans la production de dNTP, par au moins deux mécanismes : la dégradation de Sml1, un inhibiteur de la RNR et l'induction de l'expression des sous-unités de la RNR. Dans les deux cas, Mec1 et Rad53 phosphorylent Dun1 qui agit comme kinase effectrice (292, 293). L'association de Sml1 au complexe RNR limite son activité et réduit la production de dNTP (294, 295). En présence de stress réplicatif, Dun1, suite à son activation par Rad53 et Mec1, phosphoryle Sml1, ce qui prévient son association à la RNR et provoque sa dégradation subséquente (296). L'activation de Dun1 conduit également à la phosphorylation et l'inhibition de Crt1, un répresseur transcriptionnel de trois sous-unités de la RNR, *RNR2*, *RNR3* et *RNR4*, menant ainsi à l'augmentation de leur expression (297, 298). Il est proposé que Crt1 régule de plus l'expression d'autres facteurs de réparation des dommages à l'ADN (297). Même en l'absence de stress réplicatif, la régulation des niveaux de dNTP par Mec1/Rad53/Dun1 est importante pour la complétion de la réplication, car *dun1*Δ et des mutants hypomorphes de *MEC1* et *RAD53* ont une plus faible concentration de dNTP intracellulaire et progressent plus lentement à travers la phase S (293, 296). Soulignant leur importance à cet effet, la létalité des mutants *mec1*Δ et *rad53*Δ peut être supprimée par la mutation de *SML1* (295).

1.3.1.2 Stabilisation du réplisome et régulation des origines de réplication

Tel que mentionné plus haut, Mec1 et Rad53 phosphorylent plusieurs protéines ayant un rôle dans la réplication, suggérant que ceux-ci régulent certaines fonctions du réplisome en présence de stress réplicatif. Plusieurs groupes ont observé que l'absence de Mec1 ou Rad53 provoque l'apparition de structures de réplication inhabituelles (299, 300). De plus, les mutants

*rad53*Δ et *mec1*Δ sont incapables de compléter la réplication suite à l'arrêt des fourches, et ce, même après l'élimination des sources exogènes de stress réplicatif (301). Il a initialement été suggéré que l'effondrement des fourches de réplication et la dissociation des polymérases sont responsables de ces phénotypes (302, 303). Or, une récente étude démontre que le réplisome demeure intact en l'absence d'activité de Rad53 et Mec1, et suggère que les phénomènes observés découlent du dysfonctionnement du réplisome et de sa progression aberrante (304). Ceci est au moins en partie dû à la perte d'activité du complexe Mrc1-Tof1-Csm3, une cible de la voie Mec1-Rad53, qui est nécessaire pour l'arrêt et la stabilisation des fourches de réplication en présence de stress réplicatif (305). Rad53 est de plus requis pour l'inhibition de la formation de ssDNA aux fourches réplication par l'inhibition de l'exonucléase Exo1 (306, 307). La perte de cette dernière fonction est l'une des causes de la létalité du mutant *rad53*Δ et celle-ci peut d'ailleurs être supprimée par la mutation supplémentaire d'*EXO1* (308).

1.3.1.3 Inhibition de l'activation des origines

Il a observé qu'en présence de stress réplicatif, l'initiation de la réplication à partir des origines inefficaces et tardives est inhibée par les kinase Mec1 et Rad53 (309). La réduction du nombre d'origines activées a en contrepartie pour effet de ralentir la progression à travers la phase S, vraisemblablement pour permettre la réparation des lésions en présence de stress réplicatif. Rad53 cible Dbf4 et Sld3 dans ce contexte, ce qui limite leur activité au niveau de l'initiation de la réplication. Ainsi, des mutants non-phosphorylables de ces deux protéines sont insensibles à l'activité de Mec1 et Rad53 et contournent l'inhibition de l'activation des origines en réponse au stress réplicatif (310–312).

1.3.2 La réparation des dommages à l'ADN

Une fois les dommages à l'ADN détectés, il est évidemment important que ceux-ci soient réparés. Ainsi, les cellules utilisent une multitude de mécanismes pour pouvoir répondre à presque tous les types de lésions pouvant compromettre l'intégrité du génome.

1.3.2.1 Les bris double-brins

Les bris doubles-brins (DSB) figurent parmi les lésions les plus dangereuses pour l'intégrité génomique, car ils impliquent la scission d'un chromosome, ce qui peut entraîner sa perte partielle. La réparation des DSBs est majoritairement assurée par deux mécanismes conservés, mais conceptuellement très différents : la jonction d'extrémités non-homologues (NHEJ) et la recombinaison homologue (HR).

Le NHEJ consiste en la simple ligation des extrémités d'un DSB. Ce mécanisme a un fort potentiel mutagénique, car la perte de nucléotides de part et d'autre d'un DSB entraînera forcément la perte d'information génétique. Chez la levure, le complexe MRX et Yku70/80 sont les premiers arrivés au DSB. Le complexe MRX maintient les extrémités du DSB à proximité l'une de l'autre tandis que le complexe Yku70/80 forme un anneau encerclant l'ADN de chacun des bouts, de façon similaire à son recrutement aux télomères. Yku70/80 et MRX agissent ensuite comme plateforme de recrutement pour Lif1, Nej1 et la ligase IV (Dnl4) (313–318). S'ensuit la ligation du DSB par Dnl4. Ce processus a lieu surtout en phase G1, mais est relativement inefficace et peu utilisé chez la levure comparativement à d'autres espèces. Les eucaryotes supérieurs possèdent d'ailleurs des facteurs additionnels tels Artemis et DNA-PKc qui assurent le traitement des bases endommagées et améliorent l'efficacité du NHEJ (319, 320).

La recombinaison homologue repose sur l'utilisation de la chromatide sœur comme gabarit pour réparer un DSB et est donc restreinte à la phase G2 du cycle cellulaire. De façon similaire au NHEJ, le complexe MRX est le premier complexe à être recruté au DSB, mais initie en ces circonstances le processus de résection des extrémités du DSB. Celui-ci consiste en la dégradation 5' vers 3' de l'ADN des deux côtés du DSB et est catalysée par Mre11, Sae2, Exo1 et Dna2-Sgs1 pour former des filaments de ssDNA rapidement couverts par RPA (Figure 1.7C) (321–323). Les nucléosomes autour du DSB forment une barrière à la résection et doivent être enlevés pour que celle-ci ait lieu. À cette fin, trois remodeleurs de la chromatine se chargent de l'éviction des histones autour du DSB : Ino80, le complexe RSC et Fun30 (324–328). RPA est ensuite remplacé par Rad51 sur le ssDNA, ce qui est catalysé par un homoheptamère de Rad52 (321, 329, 330). Un des filaments de ssDNA-Rad51 envahit la chromatide sœur et

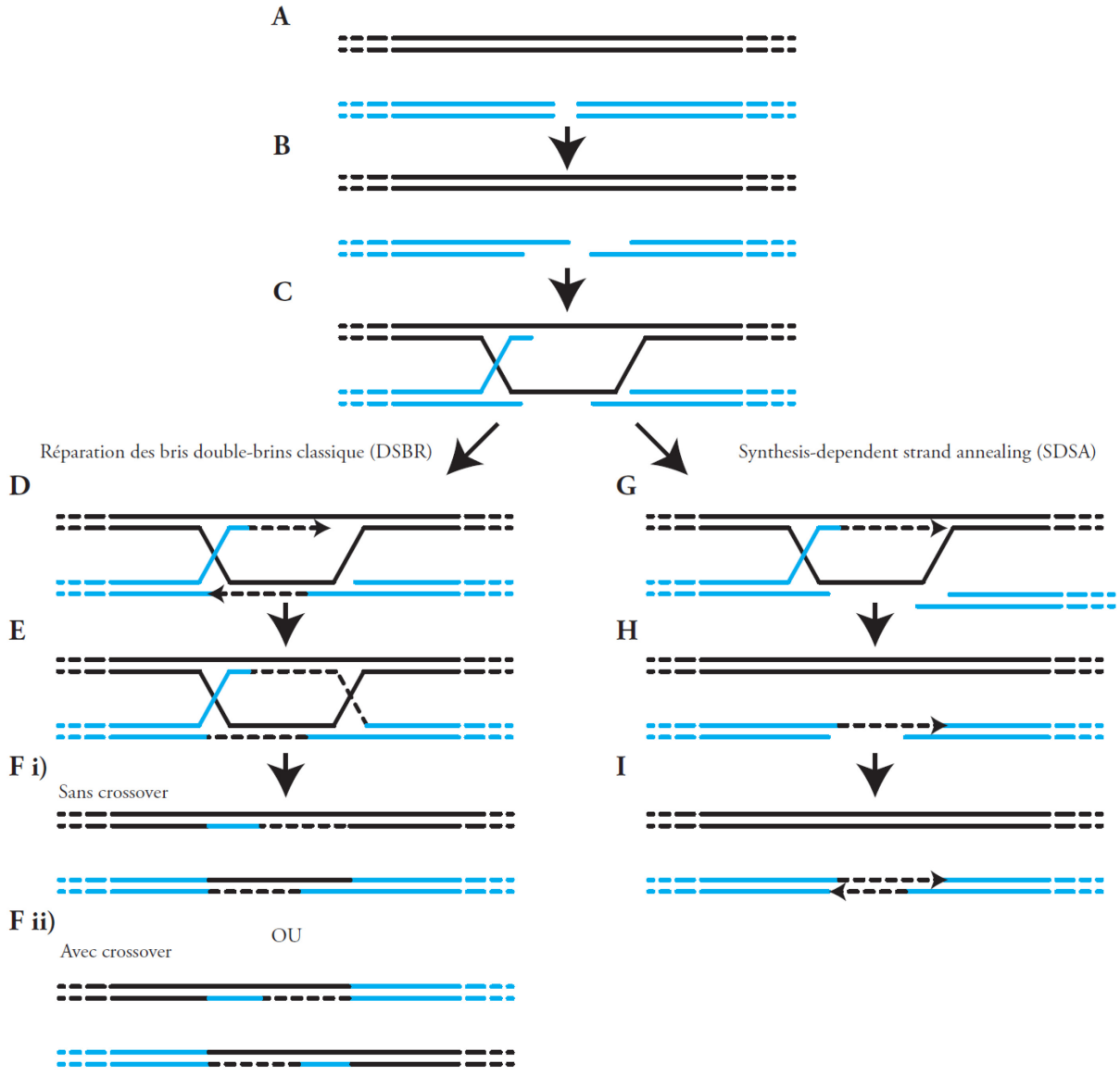

Figure 1.7. Mécanismes de réparation des bris double-brins par recombinaison homologue

recherche une séquence homologue. Encore ici, la présence de nucléosomes limite l'étape d'invasion et nécessite un autre remodeleur de la chromatine, Rad54, qui fait partie de la famille SWI-SNF. Ce dernier facilite la recherche d'homologie et l'invasion par les filaments Rad51 en faisant glisser les nucléosomes pour découvrir l'ADN de la chromatide sœur (331–333). L'invasion est de plus régulée par les actions opposées de stabilisation et de dislocation des filaments de Rad51 par l'hétérodimère Rad55/Rad57 et l'hélicase Srs2, respectivement (334–339). Ce mécanisme améliore l'efficacité de la recherche d'homologie en réduisant la fréquence d'invasions non-productives. La complétion de la recombinaison homologue peut ensuite procéder par deux voies : la voie canonique de réparation des DSB (DSBR) ou la voie de SDSA (synthesis-dependent strand annealing). Dans les deux cas, le filament envahisseur est allongé par Polδ-PCNA en utilisant le brin complémentaire comme matrice, ce qui forme une boucle D (Figure 1.7C). Dans la voie de DSBR, la boucle D est utilisée comme gabarit pour l'extension du filament de ssDNA produit de l'autre extrémité du DSB (Figure 1.7D). Il y a ensuite formation de jonctions doubles d'Holliday qui sont résolues par l'endonucléase Mus81-Mms4 (Figure 1.7E) (340–343). Dépendamment du sens des incisions faites par Mus81-Mms4, il peut y avoir croisement (Figure 1.7F i) (crossover) ou non (Figure 1.7F ii) entre les chromatides sœurs. Dans la voie du SDSA, seul le filament envahisseur est rallongé à partir de la chromatide sœur et il est subséquemment déplacé par Sgs1 vers sa chromatide originelle (Figure 1.7G). Il sert ensuite de gabarit pour synthétiser le brin complémentaire (Figure 1.7H) (344). Il existe d'autres variantes de la réparation par homologie, notamment au cours de la réplication comme on le verra plus loin.

L'étude de la recombinaison homologue, et des processus qui y sont liés, est largement facilitée par le fait que la plupart des protéines impliquées dans cette voie forme des foyers de réparation en présence de dommages à l'ADN. Ceux-ci peuvent être visualisés par microscopie à fluorescence lorsque les différents facteurs affichent des épitopes fluorescents. Ceci permet entre autres de corréler l'ordre temporel d'apparition et de disparition des foyers avec des défauts de recombinaison homologue (345).

1.3.2.1 Réparation des bases endommagées

Des modifications de bases n'impliquant pas de distorsion de l'hélice d'ADN peuvent être réparées par la réparation par excision de base (BER). Les bases endommagées sont reconnues par des glycosylases spécifiques qui clivent leur lien N-glycosidique, créant des sites abasiques (apuriniques ou apyrimidiques) (346). Apn1 (Apurinique/apyrimidique endonucléase 1) fait ensuite une incision en 5' du site endommagé (347, 348), ce qui permet le recrutement d'une polymérase, Polβ chez les mammifères et Polε chez *S. cerevisiae*, qui catalyse le remplacement du site abasique par une nouvelle base non-endommagée (349–351).

Les adduits d'ADN provoquant des distorsions de l'hélice d'ADN, tels que les dommages induits par le rayonnement UV, sont quant à eux réparés par le NER (réparation par excision de nucléotide). Ce mécanisme a été caractérisé chez l'humain par l'investigation des causes moléculaires de deux maladies génétiques, Xeroderma pigmentusum (XP) et le syndrome de Cockayne (CS). Il existe deux voies de NER; le GG-NER (global genome-NER) qui a lieu partout dans le génome et le NER couplé à la transcription (TC-NER). XP et CS sont causés par la mutation de facteurs agissant spécifiquement dans le GG-NER et le TC-NER, respectivement. Au niveau global, l'hétérodimère XPC^{Rad4}-$Rad23B^{Rad23}$ (Rad4-Rad23 chez la levure) surveille le génome et reconnaît les distorsions de l'hélice (352–356) avec l'aide de UV-DDB pour certaines lésions dont les CPDs (cyclobutylique pyrimidine dimers) (357, 358). TFIIH s'associe ensuite à XPC^{Rad4}-$Rad23B^{Rad23}$ et déroule l'ADN autour de la lésion, principalement par l'activité hélicase de ses deux sous-unités XPB^{Rad25} et XPD^{Rad3} (352, 356, 359–361). L'ADN simple-brin ainsi généré est couvert par RPA et trois autres sous-unités, XPA^{Rad14}, XPG^{Rad2} et $ERCC1^{Rad1}$-XPF^{Rad10}, sont recrutés et provoquent la dissociation de XPC^{Rad4}-$Rad23B^{Rad23}$ (362). XPG^{Rad2} et $ERCC1^{Rad1}$-XPF^{Rad10} sont des endonucléases qui font des incisions, en 3' et 5' de la lésion respectivement, ce qui permet la relâche d'un oligonucléotide de 25 à 30 bases (363–365). PCNA est ensuite chargé par le complexe RFC et l'interstice simple-brin est complémenté par Polδ ou Polε (366, 367). Le TC-NER ne requiert pas XPC-Rad23 ou UV-DDB pour la reconnaissance des lésions mais dépend plutôt de la rencontre de la polymérase à ARN II (RNAPII). Cette voie nécessite initialement des facteurs

supplémentaires (CSA^{Rad28}, CSB^{Rad26}, XAB2 et HMGN1), mais le mécanisme subséquent est identique à celui décrit plus haut pour le GG-NER (368).

1.3.3 La gestion des lésions au cours de la réplication

Les lésions rencontrées par les fourches de réplication au cours de la phase S sont préférentiellement contournées plutôt que réparées sur le champ, vraisemblablement pour éviter la formation de structures d'ADN potentiellement toxiques lorsque combinées avec les fourches de réplication. Tel que mentionné précédemment, certaines lésions paralysent complètement les polymérases à ADN, ce qui compromet évidemment la complétion de la réplication et ce, particulièrement pour le brin continu. En effet, la synthèse selon le mode discontinu implique la déposition d'amorces au fur et à mesure de la progression des fourches de réplication et ainsi Polδ peut être chargé en aval d'une lésion et compléter la synthèse de l'ADN. À l'inverse, la synthèse selon le mode continu est normalement initiée à partir d'une seule amorce à chaque fourche de réplication. Ceci implique que l'obstruction de Polε condamne la complétion de la réplication du brin continu. Or, chez la bactérie Polα peut poser une nouvelle amorce d'ARN en aval d'une lésion et permettre la reprise de la synthèse d'ADN sur le brin continu (369–371). Bien qu'il ne soit pas clair si ce mécanisme existe chez *S. cerevisiae* (371), certaines évidences suggèrent que la synthèse du brin continu puisse contourner des lésions dans l'ADN chez les eucaryotes supérieurs par une primase alternative, PrimPol, suggérant que c'est un mécanisme conservé dans l'évolution (371–375). L'initiation de la réplication à partir de plusieurs origines sur les chromosomes à travers le génome chez les eucaryotes fournit une voie alternative. En effet, les fourches arrêtées à cause de lésions sur le brin continu peuvent être secourues par d'autres provenant d'origines distales.

Les eucaryotes possèdent un autre mécanisme permettant de contourner les lésions durant la réplication : la réparation post-réplicative (PRR). Comme son nom l'indique, le PRR peut opérer après la complétion de la réplication, mais aussi pendant la phase S. Le PRR est divisé en deux sous-voies, la synthèse de translésion (TLS) qui est propice aux erreurs et la permutation de matrice (TS), qui est peu mutagénique. PCNA est le régulateur central du TLS

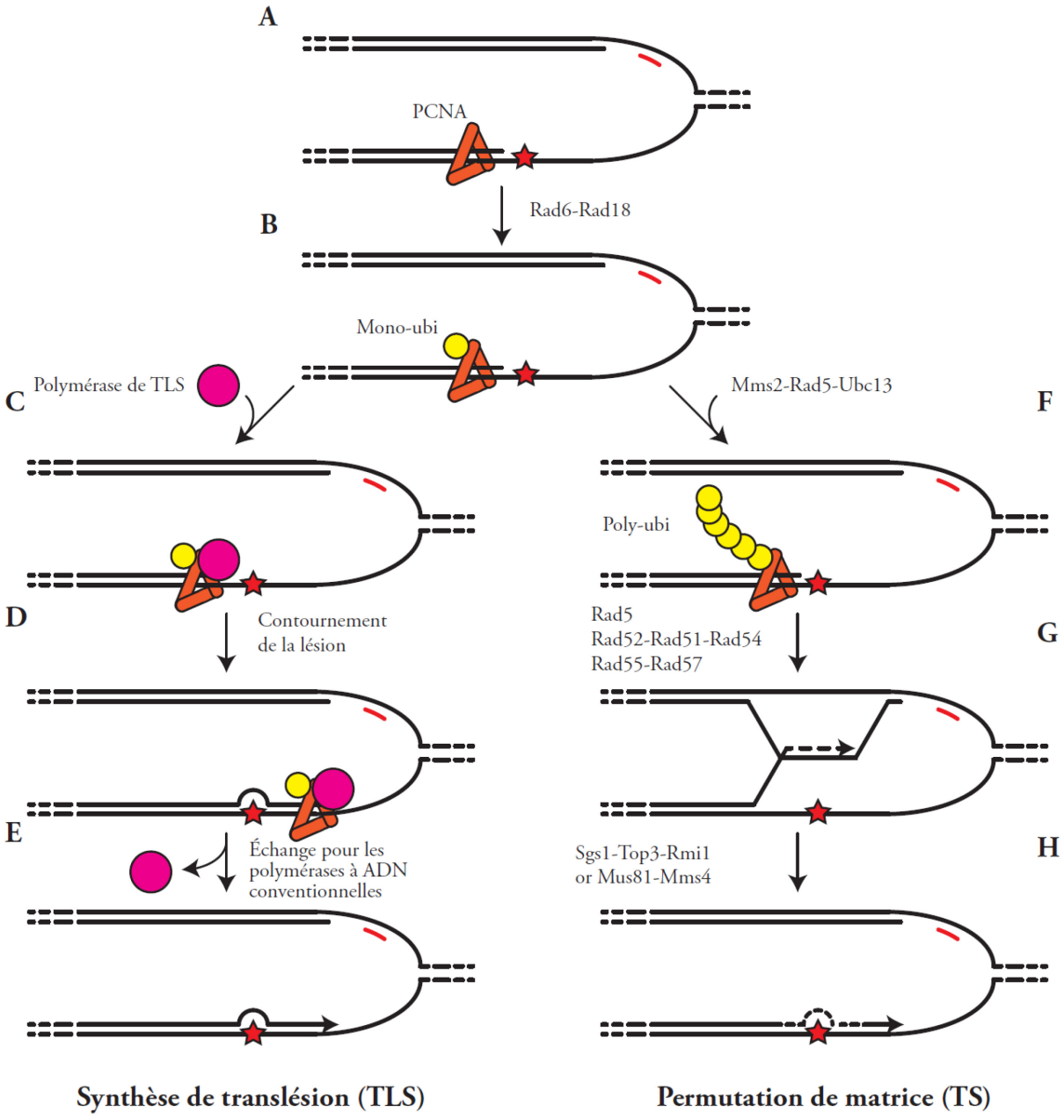

Figure 1.8. Mécanismes de réparation post-réplicative (PRR)

et du TS et ces deux processus reposent sur la modification de PCNA par la mono- et la poly-ubiquitination, respectivement (376). Rad6-Rad18 et Rad5-Mms2-Ubc13 sont les enzymes responsables de ces modifications; Rad5 et Rad18 sont des ligases E3 (377) tandis que Rad6 et Mms2-Ubc13 occupent chacun des fonctions de E2 (376, 378, 379). Lorsqu'une polymérase (ε ou δ) rencontre une lésion, la formation de filaments d'ADN simple brin couverts par RPA permet le recrutement de Rad6-Rad18 par l'interaction entre Rad18 et RPA (380). Rad6-Rad18 catalyse ensuite la mono-ubiquitination de PCNA sur la lysine 164 (figure 1.8A-B), ce qui permet l'échange des polymérases δ et ε pour des polymérases de TLS qui ont une plus grande affinité pour PCNA-mono-ubiquitiné (Figure 1.8C) (381). Les polymérases de TLS ont un site catalytique plus vaste y permettant l'entrée de lésions trop encombrantes pour les polymérases conventionnelles. Il existe trois polymérases de TLS chez *S. cerevisiae* : Polζ (Rad30), Polη (Rev3 et Rev7) et Rev1 (382). Chacune d'entre elles catalyse la synthèse d'ADN outre certains types de lésions (Figure 1.8D); Polη (Rad30) permet entre autres d'outrepasser les CPD induits par les UV en insérant deux adénines vis-à-vis de cette lésion; Polζ (Rev3-Rev7) s'associe avec les sous-unités Pol31-Pol32 de Polδ et synthétise le brin complémentaire à l'opposé d'une variété de lésions (383); Rev1 est une deoxycytidyl synthase et ajoute des cytosines en face de sites abasiques et de bases endommagées (384). L'imprécision et la flexibilité intrinsèques des polymérases de TLS fait en sorte que cette voie comporte un grand potentiel mutagénique (382). Après avoir contourné la lésion (Figure 1.8E), les polymérases de TLS sont rééchangées pour les polymérases à ADN conventionnelles.

L'association entre Rad18 et Rad5 favorise le recrutement additionnel de Mms2-Ubc13 et le complexe Rad5-Mms2-Ubc13 catalyse la poly-ubiquitination de PCNA par des liens K63 sur l'ubiquitine (Figure 1.8F) (376, 377). PCNA poly-ubiquitiné canalise ensuite le PRR vers le TS, une voie similaire à la recombinaison homologue qui utilise le brin complémentaire de la chromatide sœur pour la complétion de la synthèse d'ADN. Bien que les mécanismes moléculaires sous-jacents au choix de cette voie et aux fonctions de la poly-ubiquitination demeurent mal compris (385), les principaux acteurs et leurs fonctions sont connus. L'hélicase Rad5 déplace le brin en synthèse (386) et tout comme lors de la recombinaison homologue, Rad51 forme un filament nucléoprotéique avec l'aide de Rad52, Rad54, Rad55 et Rad57 (387). Une jonction avec la chromatide sœur est ensuite formée et le brin nouvellement synthétisé est utilisé comme matrice par Polδ (Figure 1.8G) (388–391). La résolution de cette structure est

accomplie par l'hélicase Sgs1-Top3-Rmi1 pendant la phase S et par Mus81-Mms4 en phase G2/M (Figure 8H) (390, 392–394).

Les lysines 164 et plus faiblement 127 de PCNA sont aussi la cible de la sumoylation par Siz1-Ubc9 et Mms21 indépendamment de la présence de stress réplicatif (376, 395). PCNA sumoylé est reconnu par un domaine PIP et une région C-terminale de Srs2, ce qui engendre son recrutement aux fourches de réplication (396–398). Comme lors de la recombinaison homologue, Srs2 défait les filaments Rad51 et prévient la formation d'intermédiaires de recombinaison toxiques pouvant avoir lieu durant la réplication (397, 398). Srs2 et PCNA-SUMO ont des fonctions opposées au TS, mais en présence de stress réplicatif, Srs2 est déplacé des fourches de réplication par Esc2, permettant ainsi la tolérance des dommages par le TS (399).

Les lésions simple-brins sont réparées à travers toutes les phases du cycle cellulaire par le BER et le NER, et ces deux processus impliquent la formation d'intermédiaires de ssDNA. Or, la rencontre d'une fourche de réplication avec du ssDNA est nécessairement convertie en DSB à une seule extrémité. Ce type de lésion est évidemment extrêmement délétère pour l'intégrité du génome puisqu'une partie de chromosome peut demeurer non répliqués. Ces dommages sont canalisés vers la réplication induite par les bris (BIR, Break-induced replication), un processus initialement similaire à la recombinaison homologue et qui dépend de MRX-Sae2, Rad51, Rad52, Rad54, Rad55, Rad57 et Pol32. L'extrémité du DSB est d'abord résectée par MRX-Sae2 et le ssDNA ainsi généré est couvert par RPA. RPA est ensuite déplacé par Rad51, avec l'aide de Rad52, Rad55-Rad57, pour par la suite envahir la chromatide sœur par l'intermédiaire de Rad54. Une nouvelle fourche de réplication, qui inclue tous les facteurs de réplication conventionnels, est subséquemment formée par un mécanisme encore mal compris, mais qui dépend de Pol32, la sous-unité de Polδ (400–402). Certaines formes de BIR ne nécessitent pas la formation de filaments Rad51, mais requièrent tout de même Rad52, MRX et Pol32. Bien que le BIR permette de remédier à une terminaison DSB avec la machinerie conventionnelle de réplication, cette voie de réparation est très mutagénique (403).

1.3.5 Inactivation de la réponse aux dommages à l'ADN après la réparation

Pour qu'elle puisse continuer à proliférer, la cellule doit inactiver la signalisation en réponse aux dommages à l'ADN suite de la réparation des lésions. L'activité et le recrutement aux fourches de Mec1-Ddc2 diminue nécessairement avec la disparition d'ADN simple-brin couvert par RPA, mais Rad53 demeure activé et la phosphorylation de certaines cibles de Rad53 et Mec1 persistent. Au moins quatre phosphatases participent à la récupération après la réponse aux dommages à l'ADN : Glc7 (PP1), Ptc2, Ptc3 (PP2C) et Pph3-Psy2 (PP4). L'inactivation et la déphosphorylation de Rad53 est réalisée de manière redondante par Pph3-Psy2, Ptc2 et Ptc3. Ce n'est qu'en l'absence de ces trois phosphatases que l'on observe une perte notable de viabilité en réponse au stress réplicatif et une inhabileté à poursuivre la réplication de l'ADN après sa réparation (404–409). Pph3-Psy2 en complexe avec Psy4 déphosphoryle de plus γ-H2AX, mais seulement la fraction de H2A dissociée de l'ADN au cours la réparation (410). La déphosphorylation de H2A après la réparation des dommages est plutôt accomplie par Glc7 (411). La déphosphorylation d'autres protéines dans le contexte la récupération des dommages à l'ADN a été peu étudiée et l'implication de Ptc2, Ptc3, Glc7 et Pph3 à ce titre est incertaine.

Le dimère Rtt107-Slx4 fournit un mécanisme supplémentaire limitant l'amplitude de la signalisation en réponse au stress réplicatif. Rtt107 et Slx4 sont des protéines d'échafaudage qui coordonnent plusieurs complexes impliqués dans la réponse aux dommages à l'ADN; Rtt107 interagit avec les complexes Rtt101-Mms1-Mms22 et Smc5-Smc6-Nse5-Nse6-Mms21 (223, 412); Slx4 peut s'associer avec Slx1 ou Rad1-Rad10 qui sont des endonucléases nécessaires pour résoudre certaines structures d'ADN (413–415). Pour assurer leurs fonctions, Rtt107 et Slx4 doivent être recrutés aux fourches de réplication endommagées via deux interactions; la phosphorylation par Mec1 permet à Slx4 d'interagir avec les domaines tandem BRCT1/2 de Dpb11 (412, 416–418) et Rtt107-Slx4 est recruté à chromatine par l'interaction entre les domaines BRCT5/6 de Rtt107 et H2A-S128-P (419, 420). Tel que mentionné précédemment, Rad9 possède les mêmes sites d'interaction pour induire l'activation de Mec1 et Rad53 et ainsi, il compétitionne avec Rtt107-Slx4 pour la liaison à H2A-S128P et Dpb11. La présence de Rtt107-Slx4 limite donc la signalisation en réponse au stress réplicatif par Rad9 (420) et des

mutants pour *SLX4* ou *RTT107* présentent une activation plus forte de Rad53 qui peut être partiellement supprimée par la prévention de la phosphorylation d'H2A (420).

Ces mécanismes employés par les levures pour atténuer l'activation de la signalisation en réponse aux dommages à l'ADN sont au moins partiellement redondants et l'élimination de deux de ces voies, en mutant *SLX4* et *PPH3* par exemple, mène à une hypersensibilité aux toxines générant du stress réplicatif accompagnés d'une inhabilité à compléter la réplication (421).

1.4 Les régulateurs de la structure de la chromatine impliqués dans la réponse au stress réplicatif

La réponse aux dommages à l'ADN a lieu dans le contexte de la chromatine et la structure adoptée par celle-ci, par le biais de différentes modifications d'histones, influence évidemment la signalisation et les mécanismes de réparation. Les prochaines sections traiteront de certaines des modifications impliquées dans la réponse au stress réplicatif en mettant l'accent sur une famille de désacétylases, les sirtuines, qui régule une multitude de mécanismes dont plusieurs sont impliqués dans le maintien de l'intégrité génomique.

1.4.1 Phosphorylation de H2A sur la sérine 128 (γ-H2AX)

γ-H2AX est probablement la marque d'histone la plus connue ayant un rôle dans la réponse aux dommages à l'ADN. La phosphorylation sur la sérine 139 de la variante d'histone H2A, H2AX, a initialement été découverte suite à l'exposition de cellules humaines et de souris à des radiations ionisantes (422). Des homologues fonctionnels ont depuis été identifiés chez toutes les espèces et cette modification est maintenant couramment utilisée comme marqueur de dommages à l'ADN. Chez la levure, c'est la sérine 128 de l'histone H2A qui est phosphorylée en réponse aux dommages à l'ADN par Mec1 et Tel1 (256) et comme chez d'autres organismes cette modification s'étend rapidement sur plusieurs kilobases de part et d'autre de chaque lésion

(423). Tel que décrit plus haut, H2A-S128-P agit comme plateforme de recrutement pour Rad9 ou Rtt107 en réponse aux dommages à l'ADN (257, 419), mais le rôle de H2A-S128-P au-delà de la région locale de la lésion demeure mal compris. En l'absence de sources de dommages exogènes, cette modification est présente constitutivement dans des populations de cellules aux régions réprimées transcriptionnellement. H2A-S128-P n'est vraisemblablement pas présente dans toutes les cellules d'une population, et sa présence témoigne probablement de la fréquence accrue d'effondrement de fourches à ces régions (424, 425). Cependant, son enrichissement aux télomères semble jouer un rôle dans le maintien de leur longueur, car l'absence d'H2A-S128-P se traduit en un léger raccourcissement des télomères (424). Tel1 est la kinase majoritairement responsable de la phosphorylation d'H2A aux télomères, mais le mécanisme par lequel cette modification affecte l'élongation des télomères demeure nébuleux (424).

1.4.2 La méthylation d'H3K79 par Dot1

La méthylation d'H3K79 (H3K79me) est une modification conservée au cours de l'évolution qui est présente sur environ 90% des nucléosomes chez *S. cerevisiae* (426). Dot1 est la seule méthyltransférase responsable de cette modification et catalyse la mono-, di- et tri-méthylation d'H3K79 (426–429). Le recrutement de Dot1 aux nucléosomes requiert son association à la queue N-terminale de l'histone H4 ainsi que la monoubiquitination de l'histone H2B sur la lysine 123 par l'E2 Rad6 et l'E3 ligase Bre1 (430–436). Dot1 a initialement été caractérisé dans un crible visant l'identification de facteurs dont la surexpression élimine la répression transcriptionnelle aux télomères (437). Incidemment, la présence d'H3K79me est fortement corrélée avec l'état de transcription des gènes, i.e. H3K79me est présente aux gènes activement transcrits et absente de la chromatine silencieuse (438). Ceci est le résultat de l'exclusion mutuelle entre Dot1 et le complexe SIR dont le mécanisme sera discuté dans la section concernant Sir2.

Une des fonctions majeures d'H3K79me dans la réponse aux dommages à l'ADN est le recrutement de Rad9 à la chromatine. Rad9 s'associe à H3K79me par son domaine tudor pour poursuivre la cascade de signalisation en réponse aux dommages à l'ADN. Ainsi, des mutations qui éliminent H3K79me (Lysine en arginine (H3K79R), *bre1*Δ, *rad6*Δ, *dot1*Δ) résultent en

réduction de l'activation de Rad53 sans toutefois inactiver Mec1 (264, 265, 439, 440). Dépendamment du type de dommages à l'ADN, la perte de H3K79me et la diminution subséquente de l'activation de Rad53 ont des effets différents sur la viabilité cellulaire. L'absence de *DOT1* ou d'H3K79me favorise la survie des cellules lorsqu'elles sont exposées au méthylmétane sulfonate (MMS), un agent qui alkyle les bases (441). Ceci résulte d'une augmentation de la dépendance sur la tolérance des dommages par TLS lorsque l'activité de Rad53 est partiellement diminuée, mais le mécanisme sous-jacent demeure encore mal compris (442, 443). Cependant, la signalisation en réponse aux dommages à l'ADN, quoique partielle, demeure nécessaire pour cet effet, car son élimination complète, en combinant des mutations pour *RAD24* (nécessaire au chargement du complexe 9-1-1) et *DOT1* résulte en hypersensibilité au MMS (441). À l'inverse, la mutation de *DOT1* réduit la capacité des cellules à survivre aux dommages issus de radiations UV et ionisantes en conséquence de la dérégulation des voies de recombinaisons homologues, TS et de NER (439, 444–446). Encore une fois, la base mécanistique de ces effets demeure nébuleuse, mais l'absence d'H3K79me semble du moins déréguler l'étape de résection de la recombinaison homologue et réduire la fréquence de croisements entre chromatides sœurs (446, 447).

1.4.3 Les sirtuines

Les sirtuines forment une famille d'histone désacétylases (classe III) qui est fortement conservée au cours de l'évolution. Le génome de la levure code pour 5 sirtuines (Sir2 et Hst1-4, Homologue of Sir Two) (448) tandis que l'humain en possède 7, (SIRT1-7). Chez toutes les espèces, ces enzymes occupent une multitude de fonctions, de la répression transcriptionnelle au maintien de l'intégrité génomique en passant par la régulation de la longévité.

1.4.3.1 L'activité catalytique des sirtuines dépend du métabolisme du NAD

Différemment des autres familles de désacétylases, l'activité catalytique des sirtuines nécessite le nicotinamide adenine dinucléotide (NAD+). Lors de la réaction de désacétylation, les sirtuines transfèrent le groupement acétyle au NAD pour le scinder en nicotinamide (NAM) et O-ADP-Ribose (449). Il existe deux voies moléculaires conservées de synthèse du NAD dont les enzymes et réactions impliquées diffèrent entre les espèces; la biosynthèse *de novo* et la récupération des produits de dégradation du NAD. Lors de la synthèse *de novo* du NAD chez *S. cerevisiae*, le tryptophane est converti en acide nicotinic mononucléotide (NaMN) par une série de réactions enzymatiques nécessitant les protéines Bna1-2 et Bna4-7 (450). Dans la voie de récupération, le NAM est d'abord converti en acide nicotinique (NA) par Pnc1 et est ensuite transformé en Acide Nicotinique MonoNucléotide (NaMN) par Npt1. Le NaMN, produit par les voies *de novo* et de récupération, est ensuite adénylé par Nma1-2 en acide nicotinique adenine dinucleotide (NaAD) (451, 452), puis finalement amidé par Qns1 pour former du NAD (453). Le NAM, produit de la réaction de désacétylation et précurseur du NAD, est un inhibiteur non-compétitif de l'activité enzymatique des sirtuines (454). Bien qu'il n'empêche pas la liaison du NAD aux sirtuines, le nicotinamide s'insère dans la pochette où le groupement nicotinamide du NAD s'engage normalement, laissant le NAD sous une forme inactive (455). Ainsi, les fonctions des sirtuines sont intrinsèquement liées au métabolisme du NAD. Curieusement, il a été montré que dans certaines circonstances, le NAM peut protéger le génome du stress réplicatif indépendamment son action sur l'activité des sirtuines, mais les mécanismes sous-jacents à cet effet sont encore inconnus (456). Dans d'autres situations, comme nous le verrons plus loins, le NAM cause du stress réplicatif.

1.4.3.2 Sir2 et ses multiples fonctions

Sir2, pour Silent Information Regulator 2, est le membre fondateur de la famille des sirtuines et il a été initialement identifié, en plus de Sir1, Sir3 et Sir4, dans un crible visant l'identification de gènes influençant la répression transcriptionnelle aux loci silencieux modèles HMR et HML chez la levure (457, 458). Il a subséquemment été observé que Sir2 est aussi

nécessaire pour la répression transcriptionnelle aux télomères et la prévention de la recombinaison au rDNA (459–461). Le mécanisme de répression par Sir2 ne repose pas sur la suppression transcriptionnelle spécifique aux promoteurs de gènes, mais dépend plutôt de la formation de chromatine silencieuse. Ce processus a lieu en deux parties : l'initiation à un point d'origine, le E-silencer aux loci HMR et HML par exemple, et l'étalement de l'hétérochromatine à partir de ce point. ORC, Rap1 et Abf1 s'associent au E-silencer et agissent comme plateforme de recrutement pour Sir1, Sir2, Sir3 et Sir4 par le biais de plusieurs interactions protéine-protéine (462–465); Sir2 forme le complexe SIR avec Sir3 et Sir4 (109, 466–468); Sir1 interagit avec Orc1 et Sir4 (469, 470), et Sir3 s'associe avec Rap1 (107).

Sir1, Rap1, ORC et Abf1 ne sont nécessaires que pour l'initiation de la formation d'hétérochromatine au E-silencer (463, 471). L'étalement de l'hétérochromatine est plutôt régulé par l'action opposée entre les complexes SIR, SAS-I et Dot1 (Figure 1.9A). Au cœur de la formation de l'hétérochromatine repose deux marques d'histones, l'acétylation d'H4K16 et la méthylation d'H3K79 ; ces deux marques sont absentes de l'hétérochromatine, mais abondantes aux régions d'euchromatine (426, 472). Le complexe SAS-I (composé de Sas2, Sas4 et Sas5) est responsable en grande partie de l'acétylation d'H4K16 (472–475), tandis que Sir2 possède une spécificité pour la désacétylation d'H4K16ac (476–478) et tel que discuté plus haut, Dot1 catalyse la mono-di- et tri-méthylation d'H3K79. L'association à la chromatine du complexe SIR outre le E-silencer dépend de la liaison de Sir3 à une région alcaline de la queue N-terminale de l'histone H4 et à une région entourant la lysine 79 de l'histone H3. Ces deux interactions sont respectivement sensibles à l'acétylation d'H4K16 et à la méthylation d'H3K79 (430, 479, 480) et de plus Sir3 compétitionne avec Dot1 pour le même site de liaison sur l'histone H4 (430). Ainsi, le modèle actuel de l'étalement de l'hétérochromatine propose que suite à la liaison du complexe SIR à son point d'origine, Sir2 désacétyle H4K16ac sur les nucléosomes adjacents, ce qui permet à Sir3 de s'y lier et d'y recruter Sir2-Sir4. S'ensuit la désacétylation progressive d'H4K16ac et l'étalement de l'hétérochromatine le long des chromosomes. Sir2 est présent en quantité limitante dans les cellules (481) et l'étalement s'arrête lorsqu'un équilibre entre l'activité de Sir2 et SAS-I/Dot1 est atteint, établissant ainsi une frontière euchromatine/hétérochromatine (472). Le maintien de cette frontière est essentiel à la formation de chromatine silencieuse et un étalement plus extensif du complexe SIR, en mutant

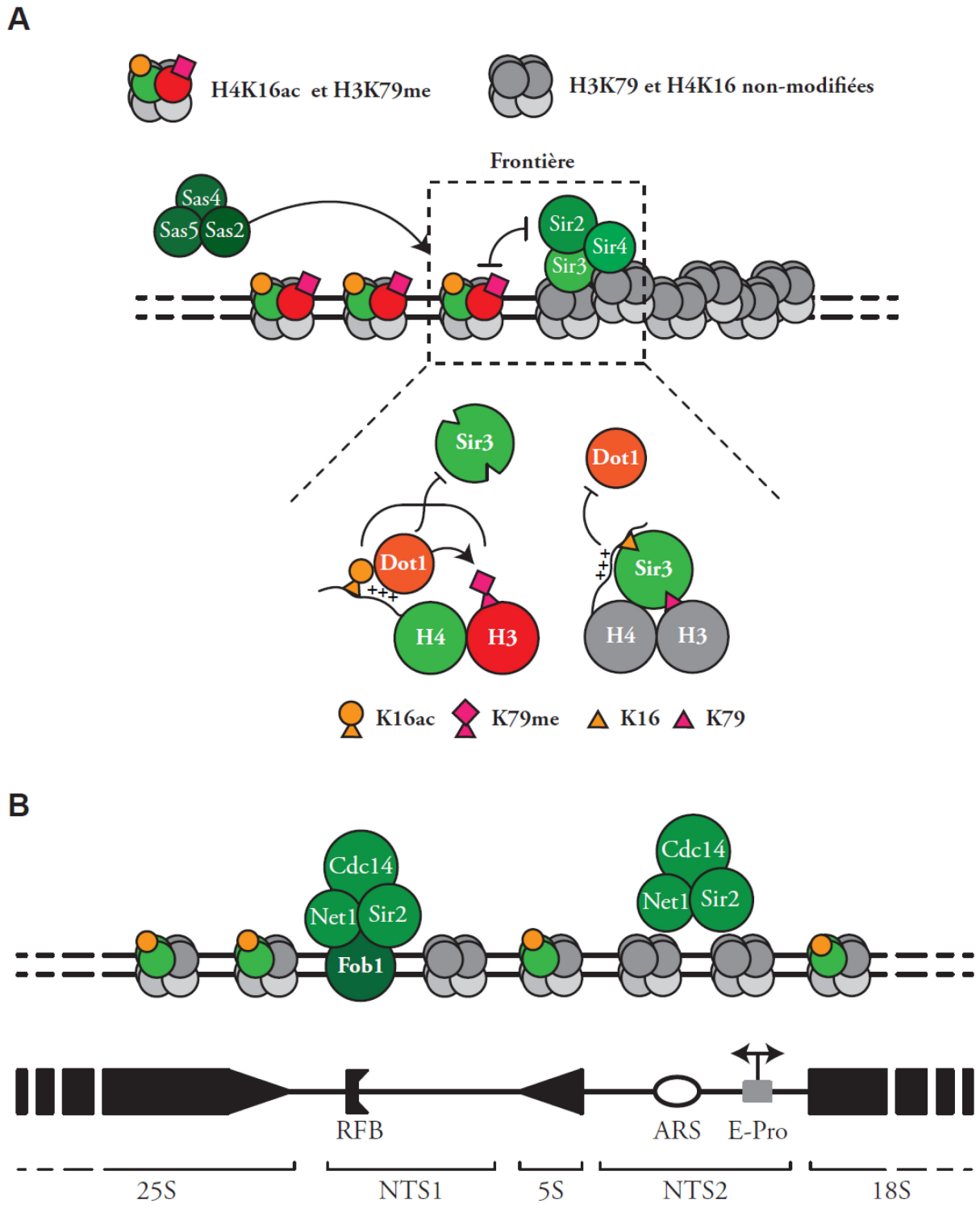

Figure 1.9. Répression transcriptionnelle par les complexes impliquant Sir2

les gènes codant pour Sas2 ou Dot1 par exemple, résulte en réduction de la répression transcriptionnelle aux loci HML et HMR (426, 482). La répression transcriptionnelle aux télomères et aux régions sous-télomériques nécessite essentiellement les mêmes facteurs, à l'exception de Sir1 dont les fonctions sont remplacées par le complexe Yku70/80 qui recrute Sir4 aux télomères (115, 426, 459, 483).

La répression transcriptionnelle au rDNA par Sir2 est accomplie par un mécanisme différent de la formation d'hétérochromatine et ne nécessite pas Sir3 et Sir4 (Figure 1.9B) (460, 461). Sir2 est plutôt recruté au rDNA par Net1 pour former le complexe RENT (REgulator of Nucleolar silencing and Telophase exit) qui inclut aussi Cdc14 (484–487). Le rDNA est composé d'environ 100-200 répétitions en tandem d'une séquence de 9.1Kb. Chacune de celles-ci contient les séquences codantes pour l'ARN ribosomal précurseur 35S, les ARN ribosomaux 5S et 25S ainsi que deux régions non-transcrites (NTS1/2, Non-Transcribed Sequence) contenant le RFB et la rARS. Le complexe RENT s'associe avec le NTS1 et une région comprenant le NTS2 ainsi que le promoteur et une partie du précurseur 35S (488). Encore ici, la désacétylation d'H4K16ac par Sir2 semble également nécessaire à la répression transcriptionnelle à ces régions, car d'une part la dérépression chez un mutant *sir2*Δ y est accompagnée d'une augmentation d'H4K16ac et d'autre part, la délétion de *SAS2* réduit l'expression des gènes du rDNA (489–491). Ainsi, la répression médiée par Sir2 fait en sorte qu'environ le tiers des répétitions de rDNA est utilisé à tout moment (492).

La nature répétée du rDNA en fait un substrat idéal pour la recombinaison et le nombre de copies de rDNA peut ainsi être modulé pour subvenir aux besoins en ribosomes des cellules. Les fonctions opposées de Sir2 et Fob1 au NTS1 sont nécessaires à ce processus. Fob1 se lie au RFB ainsi q'un élément recombinogène *cis* au NTS1 et est nécessaire au recrutement de Sir2 à cette région (90, 488, 493). Fob1 favorise la recombinaison au rDNA par conversion génique, ce qui requiert également Rad52 et RNA Pol1, tandis que le complexe RENT supprime l'expansion du rDNA. Les fonctions de Sir2 à ce titre régulent la longévité réplicative, c'est-à-dire le nombre de divisions cellulaires qu'une cellule peut accomplir avant d'atteindre la sénescence réplicative. En effet, le phénomène de recombinaison au rDNA produit intrinsèquement des cercle extrachromosomiques de rDNA (ERCs) (493–496) et l'accumulation de ceux-ci est toxique et cause le vieillissement cellulaire (497). Ainsi, en l'absence de Sir2, les ERCs s'accumulent da façon aberrante, ce qui se traduit en réduction

notable de la longévité (497, 498). La longévité d'un mutant *sir2*Δ peut cependant être restaurée en inhibant la recombinaison au rDNA par la mutation de *FOB1* (499). Ce sont au moins en partie ces fonctions de Sir2 qui régulent l'extension de la longévité par la restriction calorique (500); la réduction de nutriments stabilise l'interaction entre Sir2 et le NTS1, réduisant ainsi la fréquence de recombinaison et l'accumulation d'ERCs (501). Le complexe SIR module aussi la longévité réplicative par la désacétylation d'H4K16ac aux télomères, mais les mécanismes sous-jacents sont encore mal compris (498, 502).

1.4.3.3 Hst1, le paralogue de Sir2

Hst1 forme un complexe avec Sum1 et Rfm1 et régule l'expression de plusieurs gènes reliés au métabolisme et à la méiose. Hst1-Sum1-Rfm1 réprime l'expression de gènes de la biosynthèse *de novo* du NAD+ (503), régule les gènes de métabolisme de la thiamine, un cofacteur essentiel aux fonctions de plusieurs enzymes du métabolisme des acides aminés et des sucres (504), et inhibe l'expression des gènes de sporulation intermédiaires (505, 506). Certaines études suggèrent qu'Hst1 désacétyle H4K5ac (507) et H4K16ac (508), mais il n'y a encore pas d'évidences directes à ce sujet. Hst1 est un paralogue de Sir2 qui est issu de la duplication ancestrale du génome de la levure (509–511). En l'absence d'Hst1, Sir2 peut prendre la relève au sein du complexe Sum1-Rfm1 et assurer la répression des gènes cibles d'Hst1 (510). Il n'est néanmoins pas clair si Sir2 accomplie normalement des fonctions précises en complexe avec Sum1-Rfm1.

Bien que des mutants du complexe Hst1-Sum1-Rfm1 ne démontrent pas de sensibilité aux agents génotoxiques qui entravent la réplication, celui-ci joue un rôle dans le maintien de l'intégrité génomique. En effet, le complexe Hst1-Sum1-Rfm1 module positivement l'activité de plusieurs origines de réplication (507, 512) et la mutation de *SUM1* cause la létalité synthétique avec des mutants hypomorphes de facteurs essentiels à la réplication (512, 513). De plus, Hst1 et Sum1 sont localisés aux télomères (514) et leur absence induit un raccourcissement modeste des télomères. Il a aussi été observé qu'Hst1 influence le niveau de recombinaison à l'ADN ribosomal (515). Néanmoins, les mécanismes sous-jacents aux fonctions d'Hst1-Sum1-Rfm1 aux télomères, au rDNA et dans l'activation des origines demeurent nébuleux.

1.4.3.4 Hst2 la sirtuine cytoplasmique

Le troisième membre de la famille, Hst2 est probablement le moins bien caractérisé. Cette enzyme est majoritairement cytoplasmique dû à un signal d'export nucléaire (516, 517). Comme Sir2, Hst2 possède une activité enzymatique ayant une préférence marquée pour la déacétylation d'H4K16ac *in vitro* et *in vivo* (518) et malgré son export, Hst2 régule l'expression de certains gènes (519). De plus, il a été montré qu'Hst2 régule l'extension de la longévité réplicative en réponse à la restriction calorique selon un mécanisme indépendant de Sir2 (515). D'autres particularités d'Hst2 sont plus mytérieuses : sa surexpression et son import nucléaire ectopique provoquent des effets opposés à Sir2 au niveau de la répression transcriptionnelle aux télomères et à l'ADN ribosomal : ceci réduit l'expression des gènes aux télomères et l'augmente au rDNA. (516, 517). Encore un fois, comment l'action d'Hst2 résulte en ces effets est mal compris.

1.4.3.5 Hst3, Hst4 et les fonctions d'H3K56ac sur la chromatine

Les deux derniers membres de la famille des sirtuines, Hst3 et Hst4 ont une préférence marquée pour la désacétylation d'H3K56ac (520–522), bien qu'Hst4 semble posséder des cibles additionnelles dans les mitochondries dont le rôle est moins bien caractérisé (523). L'expression et l'activité d'Hst3 et Hst4 sont restreintes à la fin de S-G2/M et en G2/M-G1 respectivement, où elles enlèvent complètement, ou presque, H3K56ac du génome (520, 524). L'acétylation d'H3K56 est donc cyclique. Au début de la phase S, aucun nucléosome n'est acétylé sur H3K56, puis tout au long de la réplication, les histones nouvellement synthétisées sont acétylées sur H3K56 par Rtt109 avant leur déposition sur la chromatine (212, 213, 525). H3K56ac s'accumule ainsi sur la chromatine derrière les fourches de réplication jusqu'à ce que 50% des histones H3 soient acétylées sur K56 en fin de phase S. Enfin, H3K56ac est ensuite désacétylé par Hst3 et Hst4 complétant alors le cycle (Figure 1.10). Les nucléosomes nouvellement synthétisés et déposés sur l'ADN à l'extérieur de la phase S, sont aussi acétylés sur H3K56, mais la stœchiométrie de ce processus n'atteint pas les niveaux observés en phase S (526). Les sirtuines Hst3 et Hst4 sont largement redondantes et ce n'est qu'en l'absence des deux que l'on

observe l'accumulation généralisée d'H3K56ac jusqu'à ce que cette marque soit présente sur virtuellement 100% des nucléosomes (520, 525, 527).

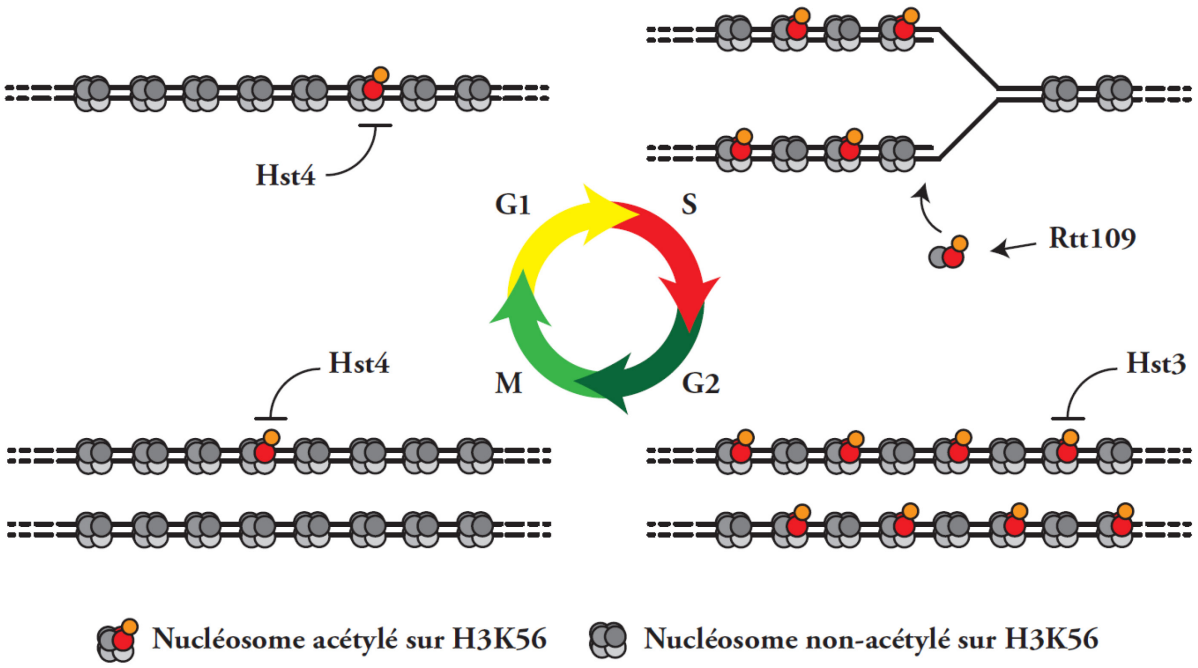

Figure 1.10. Le cycle d'acétylation d'H3K56

La régulation cyclique des niveaux d'H3K56ac est extrêmement importante pour la capacité des cellules à répondre au stress réplicatif. L'inhibition de l'acétylation d'H3K56, en mutant *RTT109* ou la lysine 56 en arginine (H3K56R) résulte en sensibilité aux agents génotoxiques causant du stress réplicatif et en défauts à compléter la réplication (213, 215, 216, 528, 529). Plusieurs groupes ont proposé que cet effet découle des défauts d'assemblage *de novo* de la chromatine en l'absence d'H3K56ac (219, 221, 530). En effet, l'absence de Rtt106 et CAFI, les deux facteurs responsables de la déposition des tétramères (H3-H4)2 sur l'ADN naissant et qui ont une préférence marquée pour H3K56ac, cause également une sensibilité accrue au stress réplicatif (221, 224). Or, les phénotypes causés par l'hyperacétylation constitutive d'H3K56 chez les mutants *hst3*Δ *hst4*Δ sont d'autant plus drastiques et leur hypersensibilité au stress réplicatif est accompagnée de thermosensibilité, de dommages spontanés à l'ADN, de réduction significative de la viabilité cellulaire et de défaut de ségrégation des chromosomes (448, 520, 531, 532). De plus, le mutant *hst3*Δ *hst4*Δ est aussi

extrêmement sensible à toute perturbation de la réplication et simplement ajouter un épitope aux facteurs de réplication, ce qui les déstabilise légèrement, confère des défauts de croissance majeurs chez ce mutant (531). Ces défauts d'*hst3*Δ *hst4*Δ sont inconsistants avec de simples problèmes à assembler la chromatine, puisqu'ils découlent nécessairement d'H3K56ac déjà présents sur la chromatine. De plus, un mutant *rtt109*Δ est beaucoup plus sensible aux agents génotoxiques que le double mutant *rtt106*Δ *cac1*Δ, ce qui soutient la notion qu'H3K56ac occupe des fonctions supplémentaires à la promotion de l'assemblage des nucléosomes nouvellement synthétisés.

Plusieurs évidences soutiennent l'idée selon laquelle H3K56ac occupe des fonctions en réponse aux dommages à l'ADN après sa déposition sur l'ADN. Premièrement, suite à la détection de lésions, Hst3 est dégradé après la phosphorylation d'un phosphodégron sur sa queue C-terminale, ce qui prévient la désacétylation d'H3K56ac jusqu'à ce que les dommages soient réparés (528, 533, 534). Deuxièmement, la mutation d'*HST3*, qui ne perturbe pas le cycle d'H3K56ac mais qui allonge le temps de résidence d'H3K56ac sur la chromatine, résulte en l'augmentation aberrante de la fréquence d'apparition spontanée de foyers de la protéine de réparation Rad52 et de perte d'un chromosome disomique comportant peu d'origines de réplication (535, 536). Finalement, plusieurs des phénotypes du mutant *hst3*Δ *hst4*Δ peuvent être supprimés par des mutations qui n'affectent pas la proportion de nucléosomes comportant H3K56ac et qui n'ont à priori pas d'effet sur l'assemblage de la chromatine (531). Par exemple, *rtt107*Δ supprime partiellement la thermosensibilité d'*hst3*Δ *hst4*Δ (537) sans perturber l'assemblage des nucléosomes (219).

H3K56 se retrouve dans le domaine globulaire de l'histone H3, au niveau de l'entrée et de la sortie de l'ADN du nucléosome. L'acétylation de ce résidu élimine sa charge positive et affaiblit l'interaction du nucléosome avec l'ADN (528). Or, à l'exception des chaperones CAF-I et Rtt106, aucune protéine ayant une préférence pour la liaison à H3K56ac n'a encore été identifiée (221, 224). Néanmoins, comme lors de l'assemblage des nucléosomes, les fonctions d'H3K56ac sur la chromatine semblent nécessiter le complexe ubiquitine-ligase Rtt101-Mms1-Mms22. Des mutants pour chacune de ces sous-unités sont épistatiques avec la mutation de *RTT109* et suppriment partiellement la sensibilité à la température et au stress réplicatif du mutant *hst3*Δ *hst4*Δ (212, 529). En l'absence de Rtt101-Mms1-Mms22 ou Rtt109, les cellules éprouvent des difficultés à compléter la réplication suite à l'exposition transitoire à des agents

génotoxiques qui induisent du stress réplicatif (225, 529, 538, 539). Ceci ne semble pas découler de défauts au niveau des voies de BER et TLS, car la sensibilité au stress réplicatif de *rtt109*Δ est additive avec celle de mutants des membres des voies de BER et TLS (529). Toutefois, la prévention d'H3K56ac provoque des défauts dans les processus de réparation par homologie et les mutant *rtt109*Δ, *mms22*Δ, *mms1*Δ et *rtt101*Δ sont inaptes à compléter la recombinaison avec la chromatide sœur (540, 541). De plus, en l'absence d'H3K56ac, les foyers de réparation de Rad52 et Rad51 persistent de façon aberrante suite à l'exposition transitoire au MMS durant la réplication (529).

Similairement, l'instabilité génomique et la réduction drastique de l'espérance de vie réplicative du mutant *hst3*Δ *hst4*Δ sont accompagnés de défauts dans la réparation des dommages à l'ADN par recombinaisons homologue et par BIR (532, 541, 542). La survie du mutant *hst3*Δ *hst4*Δ nécessite, même en l'absence de dommages exogènes, la présence de Rad52 et du complexe MRX, mais non de Rad51, Rad54, Rad55 ou Rad57 (531). Ceci suggère que la recombinaison homologue classique, i.e. qui dépend de la formation de filament Rad51, n'est pas nécessaire en présence d'H3K56 hyperacétylé, mais que d'autres processus impliquant Rad52 et le complexe MRX deviennent essentiels (531). Les mutants *hst3*Δ *hst4*Δ affichent aussi une activation chronique de la réponse aux dommages à l'ADN (520). Il n'est cependant pas clair si ceci découle de lésions causées par la présence aberrante d'H3K56ac ou bien de dérégulation de la réponse aux dommages à l'ADN. Étonnamment, la combinaison des mutations de *RAD9*, *MEC3*, *DDC1*, *RAD17*, *RAD53*, avec *hst3*Δ *hst4*Δ ne cause pas de défauts synthétiques de croissance, indiquant que l'activation de la signalisation par Rad53 en réponse aux dommages à l'ADN n'est en soit pas nécessaire pour la viabilité de ces cellules (531). Mec1 est cependant essentiel au mutant *hst3*Δ *hst4*Δ, mais ceci est probablement plutôt dû à son rôle dans la stabilisation de la machinerie de réplication (531).

Il est intéressant de noter que l'absence de la sirtuine Sir2 exacerbe sévèrement les défauts de croissance d'un mutant *hst3*Δ *hst4*Δ (448, 532). Il a été proposé que ceci est la conséquence de la combinaison de l'hyper-recombinaison au rDNA causé par *sir2*Δ et des défauts de réparation par homologie issus d'*hst3*Δ *hst4*Δ. Ainsi, la croissance d'*hst3*Δ *hst4*Δ *sir2*Δ peut être partiellement améliorée en prévenant la recombinaison au rDNA par la mutation de *FOB1* (532). Or, ceci soulève la possibilité qu'H3K56ac agit en de pair avec H4K16ac dans la réponse au stress réplicatif. Le cycle d'acétylation d'H3K56 semble donc être requis pour

maintenir la stabilité génomique en régulant certaines voies de réparation reposant sur l'homologie, mais les mécanismes sous-jacents demeurent toutefois nébuleux.

1.5 Rationnelle et objectif de la thèse

Le cycle d'acétylation d'H3K56 est un mécanisme intriguant permettant aux cellules de répondre adéquatement au stress réplicatif. Or, tel que souligné plus haut, les fonctions d'H3K56ac sur la chromatine, sous-jacentes à la sévérité des phénotypes associés à la dérégulation de son cycle, demeurent mal comprises. Dans le cadre de cette thèse, nous avons donc cherché à élucider les processus moléculaires liés à la réponse au stress réplicatif régis par cette modification. À cette fin, nous avons utilisé des systèmes dans lesquels H3K56 est constitutivement acétylé sur la chromatine, en mutant *HST3* et *HST4* ou en exposant les cellules au NAM. L'ampleur de d'hypersensibilité aux hautes températures et au stress réplicatif lorsque H3K56 est hyperacétylé offre un puissant outil génétique permettant l'identification de mutations supprimant ou amplifiant ces phénotypes, et nous avons tiré profit de ceci pour sonder les fonctions d'H3K56ac à l'aide de cribles.

Dans un premier temps, nous avons raisonné que la létalité synthétique des mutants *hst3*Δ *hst4*Δ et *sir2*Δ sous-entend qu'H3K56ac agit en conjonction avec H4K16ac et peut-être aussi d'autres modifications d'histones. Ainsi, dans l'article présenté dans le chapitre 2, nous avons fait un crible de mutants d'histones H3 et H4 visant à l'identification de modifications dont les niveaux influencent la viabilité des mutants *hst3*Δ *hst4*Δ exposés au stress réplicatif.

Ensuite, pour déceler les voies moléculaires régulées par H3K56ac, nous avons exploité le fait qu'Hst3 et Hst4 peuvent être inhibés par le NAM pour faire un crible chimiogénétique dans le but d'identifier des gènes dont la mutation influence la croissance cellulaire en présence de NAM. Les résultats de ce crible, ainsi que la caractérisation de certains gènes qui y ont été retrouvés, sont rapportés dans le chapitre 3.

Ce dernier crible a permis de découvrir que des mutants pour *YKU70* et *YKU80* sont incapables de croître en présence de NAM, ce qui suggère le concept intrigant que le maintien de la longueur des télomères est important pour la viabilité en réponse au stress réplicatif induit

par l'hyperacétylation d'H3K56ac. Dans l'article présenté au chapitre 4, nous avons tenté d'élucider les mécanismes moléculaires sous-jacents à ce phénomène.

Introduction au chapitre 2

Dans le premier article paru dans la revue *Genetics* en 2015 (543), nous avons tout d'abord voulu mieux caractériser les défauts replicatifs résultant e l'hyperacétylation d'H3K56. Nous avons ainsi trouvé qu'après l'induction de stress réplicatif, les mutants *hst3*Δ *hst4*Δ sont incapables de progresser à travers la phase S, ce qui est accompagné d'une persistance aberrante de la signalisation en réponse aux dommages à l'ADN.

Ensuite, tel que mentionné plus haut, nous avons fait un crible pour identifier des mutants d'histones pouvant supprimer les défauts du mutant *hst3*Δ *hst4*Δ. Ceci nous a permis d'identifier et de caractériser une interaction intrigante entre trois modifications d'histones, H4K16ac, H3K79me et H3K56ac. Nous avons trouvé que les niveaux d'H4K16ac influencent ceux d'H3K79me3 et que l'activation de la signalisation en réponse aux dommages à l'ADN, favorisée par cette dernière modification, est délétère pour les cellules comportant H3K56 constitutivement acétylé.

J'ai participé à la conception et l'élaboration de la rationnelle derrière cet article (30%). La majorité des expériences et analyses présentées dans cet article ont été réalisées par mes soins (60%), à l'exception du crible de mutants d'histones qui a été fait par Junbiao Dai du laboratoire de Dr Jef Boeke. J'ai participé à la rédaction de l'article (20%) et monté toutes les figures.

Delgoshaie N : 10% expériences et analyses

Celic I, Dai J, Boeke JD : 20% expériences et analyses, 20% conceptualisation

Ashiru N, Thibault P : 5% expériences et analyses

Costantino S : 5% expériences et analyses

Verreault A : 20% rédaction, 20% conceptualisation

Wurtele H : 60% rédaction 30% conceptualisation

Chapitre 2. Interplay between histone H3 lysine 56 deacetylation and chromatin modifiers in response to DNA damage

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2.1 Abstract

In *Saccharomyces cerevisiae*, histone H3 lysine 56 acetylation (H3K56Ac) is present in newly synthesized histones deposited throughout the genome during DNA replication. The sirtuins Hst3 and Hst4 deacetylate H3K56 after S-phase, and virtually all histone H3 molecules are K56-acetylated throughout the cell cycle in *hst3*Δ *hst4*Δ mutants. Failure to deacetylate H3K56 causes thermosensitivity, spontaneous DNA damage, and sensitivity to replicative stress via molecular mechanisms that remain unclear. Here we demonstrate that, unlike wild-type cells, *hst3*Δ *hst4*Δ cells are unable to complete genome duplication and accumulate persistent foci containing the homologous recombination protein Rad52 after exposure to genotoxic drugs during S-phase. In response to replicative stress, cells lacking Hst3 and Hst4 also displayed intense foci containing the Rfa1 subunit of the single-stranded DNA binding protein complex RPA, as well as persistent activation of DNA damage-induced kinases. To investigate the basis of these phenotypes, we identified histone point mutations that modulate the temperature and genotoxic drug sensitivity of *hst3*Δ *hst4*Δ cells. We found that reducing the levels of histone H4 lysine 16 acetylation or H3 lysine 79 methylation partially suppresses these sensitivities and reduces spontaneous and genotoxin-induced activation of the DNA damage response kinase Rad53 in *hst3*Δ *hst4*Δ cells. Our data further suggest that elevated DNA damage-induced signalling significantly contributes to the phenotypes of *hst3*Δ *hst4*Δ cells. Overall, these results outline a novel interplay between H3K56Ac, H3K79 methylation and H4K16 acetylation in the cellular response to DNA damage.

2.2 Introduction

Chromatin structure influences major DNA metabolic processes such as transcription, DNA replication and DNA repair (1, 2). The basic building block of chromatin is the nucleosome core particle, composed of 147 base pairs of DNA wrapped around the surface of a protein octamer consisting of two molecules each of histones H2A, H2B, H3 and H4. During DNA replication, pre-existing (old) histones are segregated onto sister chromatids, while new histones are deposited onto replicated DNA in order to restore normal nucleosome density on nascent sister chromatids (3, 4). In humans, newly synthesized histones H3 and H4 are acetylated on multiple residues within their N-terminal tails (5–7) and then deacetylated following their incorporation into chromatin (8, 9). In the yeast *Saccharomyces cerevisiae* and other fungi, new H3 and H4 molecules are acetylated on their N-terminal tails (10, 11), as well as within their globular domains, notably at histone H4 lysine 91 and H3 lysine 56 (H3K56Ac) (12–16). In yeast, H3K56Ac is present in virtually all newly synthesized H3 molecules deposited throughout the genome during S-phase (17) but is much less abundant in pre-existing histones (12). H3K56Ac is catalyzed by the Rtt109 acetyltransferase in concert with the histone-binding protein Asf1 (17–22), while deacetylation of this residue depends, in a largely redundant manner, on the sirtuins Hst3 and Hst4 (17, 23–25). Hst3 and Hst4 are absent during S-phase and, as a result, H3K56Ac progressively accumulates in nascent chromatin during replication and reaches maximal levels after completion of DNA synthesis (12, 23, 26). In the absence of DNA damage, H3K56Ac is then removed genome-wide upon induction of Hst3 and Hst4 expression during subsequent G2/M and G1 phases (23).

Hst3 and Hst4 are homologs of Sir2 (27), the founding member of the sirtuin family of nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases (28–31). Deletion of *HST3* causes mild phenotypes such as elevated frequencies of Rad52 foci and reduced replicative lifespan (32, 33). In striking contrast, cells lacking both *HST3* and *HST4* (*hst3*Δ *hst4*Δ mutants) display extreme sensitivity to genotoxic agents and severe phenotypes that may be related to their inability to appropriately respond to spontaneous DNA damage including thermosensitivity, reduced viability, mitotic instability, and dramatically reduced replicative lifespan (17, 27, 34). In contrast to *hst3*Δ or *hst4*Δ single mutants, essentially all H3 molecules

are K56-acetylated throughout the genome and during the entire cell cycle in the double mutant (17). Remarkably, many of the aforementioned *hst3*Δ *hst4*Δ-associated phenotypes are strongly attenuated by mutating H3K56 to a non-acetylable arginine residue (17, 23). This suggests that H3K56 hyperacetylation and/or the constitutive presence of H3K56Ac throughout the cell cycle is the root cause of the severe phenotypes observed in *hst3*Δ *hst4*Δ mutants. In support of this, our previously published mass spectrometry data indicate that, among several sites of acetylation in H3/H4, only H3K56Ac exhibited a striking increase in acetylation stoichiometry in *hst3*Δ *hst4*Δ mutants (35), illustrating the remarkable *in vivo* substrate selectivity of Hst3 and Hst4.

Accumulating evidence indicates that the yeast chromosome acetylation/deacetylation cycle is critical for efficient cellular responses to DNA damage. Indeed, both acetylation and, to an even greater extent, deacetylation of H3K56 promote cell survival in response to spontaneous or genotoxic agent-induced DNA lesions (12, 15–17, 23, 32, 36–39). The molecular mechanisms by which lack or excess H3K56Ac causes cellular sensitivity to DNA damage are poorly understood. H3K56Ac promotes efficient chromatin assembly during DNA replication at least in part by enhancing the affinity of nucleosome assembly factors for newly synthesized H3 molecules (40, 41). H3K56Ac also promotes efficient flow of newly synthesized histones between histone chaperones by facilitating transient ubiquitination of histone H3 by the Rtt101-Mms1-Mms22 ubiquitin ligase complex (42). Such ubiquitination events are believed to release new histones from Asf1, thereby increasing the availability of free histones for downstream chaperones (42). On the other hand, as a result of DNA damage-induced Hst3 degradation (24, 25), K56-acetylated H3 molecules incorporated into chromatin retain their acetylation until DNA damage has been repaired (12). In addition, several distinct mutations suppress the phenotypes of *hst3*Δ *hst4*Δ cells without modulating H3K56Ac levels, suggesting that abnormal persistence of H3K56ac throughout the cell cycle may cause defects in processes linked to DNA replication and repair (43, 44). However, the putative functions of K56-acetylated H3 molecules incorporated in chromatin remain poorly characterized. Here we further investigated the basis of the phenotypes caused by H3K56 hyperacetylation in yeast, and identified a novel feature of the yeast DNA damage response, namely a functional cross-talk between H3K56Ac and two other abundant histone post-translational modifications: histone H3 lysine 79 methylation and H4 lysine 16 acetylation.

2.3 Materials and methods

2.3.1 Strains, plasmids and growth conditions

Plasmids pJP11 [p*CEN LYS2 HHT1-HHF1*] and [p*CEN-URA3-HST3*] (pRS416-based) were previously described (17, 45) . The pEMH-based plasmids encoding *HHT2-HHF2* gene mutations [p*CEN TRP1 HHT2-HHF2*] were previously described (15). Tagging of the *CDC45* gene with a C-terminal triple HA epitope was achieved by transformation of *Nco*I-linearized pRS405-CDC45-HA/C (46) and selection of Leu^+ colonies where the epitope tagging vector was integrated at the *CDC45* locus. MATa- and MATalpha-expressing plasmids were previously described (47).

All the strains used in this work are described in Table 2.I. They were generated by standard methods and grown under standard conditions unless otherwise stated. Strain ICY1345 was used to assess the phenotypes caused by introducing histone H3/H4 gene mutations in cells carrying *HST3* and *HST4* gene deletions (Tables 2.III and 2.IV). pEMH7-based plasmids (*CEN TRP1 HHT2-HHF2*) that carried H3 or H4 mutations were transformed into ICY1345 and Ura^+ Lys^+ Trp^+ transformants were selected. The Lys^+ pJP11 plasmid encoding WT H3 and H4 was selected against on α-aminoadipic acid plates, resulting in Lys^- strains lacking the plasmid encoding WT H3 and H4 genes (48). To test whether specific H3 or H4 gene mutations were able to suppress the phenotypes of *hst3*Δ *hst4*Δ mutants, the aforementioned strains were plated on SC-Trp medium containing 5-FOA at different temperatures. 5-FOA was used to select against the [p*CEN-URA3-HST3*] plasmid (17). Selection against the *HST3* plasmid to uncover *hst3*Δ *hst4*Δ phenotypes was performed immediately before phenotypic analysis because long-term propagation of *hst3*Δ *hst4*Δ mutants leads to the emergence of spontaneous suppressors and genome rearrangements (27).

Table 2.I. Yeast strains used in this study

Strain	Genotype	Reference
HWY294	BY4743 *MATa ura3Δ0 leu2Δ0 his3Δ1*	This study
FY833	*MATa his3Δ200 leu2Δ1 lys2-202 trp1Δ63 ura3-52*	(49)
ICY703	FY833 *hst3Δ::HIS3 hst4Δ::TRP1* [p*CEN URA3 HST3*]	(17)
ICY918	FY833 *hst3Δ::HIS3 hst4Δ::TRP1* [p*CEN URA3 HST3*] *sas2Δ::kanMX*	This study
ICY1081	FY833 *hst3Δ::HIS3 hst4Δ::TRP1* [p*CEN URA3 HST3*] *rsc2Δ::kanMX*	This study
ICY1345	FY833 *hst3Δ::HIS3 hst4Δ::kanMX4 hht1-hhf1Δ:: natMX4 hht2-hhf2Δ:: hygMX4* [p*CEN URA3 HST3*] [p*CEN LYS2 HHT1-HHF1*]	This study
HWY51	FY833 *hst3Δ::HIS3 hst4Δ::kanMX hht1-hhf1::natMX hht2-hhf2::hygMX* [p*CEN TRP1 HHT2-hhf2 K16R*] [p*CEN URA3 HST3*]	This study
HWY200	FY833 *hst3Δ::HIS3 hst4Δ::TRP1* [p*CEN URA3 HST3*] *yta7Δ::LEU2*	This study
HWY186	FY833 *hst3Δ::HIS3 hst4Δ::TRP1* [p*CEN URA3 HST3*] *sir2Δ::LEU2*	This study
HWY190	FY833 *hst3Δ::HIS3 hst4Δ::TRP1* [p*CEN URA3 HST3*] *sir2Δ::LEU2 sas2Δ::KanMX*	This study
HWY192	FY833 *hst3Δ::HIS3 hst4Δ::TRP1* [p*CEN URA3 HST3*] *sir2Δ::LEU2 rsc2Δ::KanMX*	This study
HWY193	FY833 *hst3Δ::HIS3 hst4Δ::KanMX hht1-hhf1::natMX hht2-hhf2::hygMX sir2Δ::LEU2* [p*CEN TRP1 HHT2-hhf2 K16R*] [p*CEN URA3 HST3*]	This study
HWY385	FY833 *hst3Δ::HIS3 hst4Δ::TRP1* [p*CEN URA3 HST3*] *cdc45::CDC45-HA::LEU2*	This study
HWY387	FY833 *hst3Δ::HIS3 hst4Δ::TRP1* [p*CEN URA3 HST3*] *sas2Δ::kanMX cdc45::CDC45-HA::LEU2*	This study
HWY406	FY833 *hst3Δ::HIS3 hst4Δ::KanMX hht1-hhf1::natMX hht2-hhf2::hygMX* [p*CEN TRP1 HHT2-hhf2 K16R*] [p*CEN URA3 HST3*] *cdc45::CDC45-HA::LEU2*	This study
Tr1	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 tr1*	This study
Tr2	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 tr2*	This study
Tr3	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 tr3*	This study
Tr4	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 tr4*	This study
Tr5	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 tr5*	This study
Tr6	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 tr6*	This study
Tr7	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 tr7*	This study
Tr8	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 tr8*	This study
Tr9	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 tr9*	This study
Tr10	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 tr10*	This study
Tr11	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 tr11*	This study
Tr12	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 tr12*	This study
DWY1	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 rtt109::RTT109-Flag::His3MX6*	This study
DWY2	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 rtt109::RTT109-Flag::His3MX6 tr4*	This study
DWY3	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 rtt109::RTT109-Flag::His3MX6 tr6*	This study
DWY4	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 rtt109::RTT109-Flag::His3MX6 tr9*	This study
DWY5	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 rtt109::RTT109-Flag::His3MX6 tr11*	This study
DWY6	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 rtt109::RTT109-Flag::His3MX6 tr18*	This study
ASY2368	W303 *ADE2 RAD52-YFP*	This study
ASY2369	W303 *ADE2 RAD52-YFP hst3Δ::HIS5 hst4Δ ::KanMX6*	This study
HWY2493	W303 *RFA1-YFP RAD5 ADE2*	This study
ASY2391	W303 *RFA1-YFP RAD5 ADE2 hst3Δ::HIS5 hst4Δ ::KanMX6*	This study
ASY2737	FY833 *hst3Δ::HIS3 hst4Δ::kanMX4 hht1-hhf1Δ:: natMX4 hht2-hhf2Δ:: hygMX4* [p*CEN URA3 HST3*] [p*CEN TRP1 HHT1-HHF1*]	This study
ASY2741	FY833 *hst3Δ::HIS3 hst4Δ::kanMX4 hht1-hhf1Δ:: natMX4 hht2-hhf2Δ:: hygMX4* [p*CEN URA3 HST3*] [p*CEN TRP1 hht1K79A-HHF1*]	This study
ASY2745	FY833 *hst3Δ::HIS3 hst4Δ::kanMX4 hht1-hhf1Δ:: natMX4 hht2-hhf2Δ:: hygMX4* [p*CEN URA3 HST3*] [p*CEN TRP1 HHT1-hhf1K16R*]	This study
ASY2749	FY833 *hst3Δ::HIS3 hst4Δ::kanMX4 hht1-hhf1Δ:: natMX4 hht2-hhf2Δ:: hygMX4* [p*CEN URA3 HST3*] [p*CEN TRP1 hht1K79R-HHF1*]	This study
ASY2755	FY833 *hst3Δ::HIS3 hst4Δ::kanMX4 hht1-hhf1Δ:: natMX4 hht2-hhf2Δ:: hygMX4* [p*CEN URA3 HST3*] [p*CEN LYS2 HHT1-HHF1*] *CDC45-3HA::LEU2*	This study
ASY2758	FY833 *hst3Δ::HIS3 hst4Δ::kanMX4 hht1-hhf1Δ:: natMX4 hht2-hhf2Δ:: hygMX4* [p*CEN URA3 HST3*] [p*CEN TRP1 hht1K79A-HHF1*] *CDC45-3HA::LEU2*	This study
ASY2761	FY833 *hst3Δ::HIS3 hst4Δ::TRP1* [p*CEN URA3 HST3*] *dot1Δ::KanMX CDC45-3HA::LEU2*	This study
HWY2550	FY833 *hst3Δ::HIS3 hst4Δ::TRP1* [p*CEN URA3 HST3*] *rad9Δ::KanMX*	This study

Table 2.I. (continued)

Strain	Genotype	Reference
ASY2392	FY833 *hst3Δ::HIS3 hst4Δ::TRP1* [*pCEN-URA3-HST3*] *dot1Δ::KanMX*	This study
ASY3111	YBL574 *hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3* [*pCEN TRP1 HHT1-HHF1*]	(50)
ASY3112	YBL574 *hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3* [*pCEN TRP1 hht1K79A-HHF1*]	(50)
ASY3113	YBL574 *hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3* [*pCEN TRP1 HHT-hhf1K16A*]	(50)
ASY3169	FY833 *hst3Δ::HIS3 hst4Δ::TRP1* [*pCEN-URA3-HST3*] *rev3Δ::HPHMX*	This study
ASY3171	FY833 *hst3Δ::HIS3 hst4Δ::TRP1* [*pCEN-URA3-HST3*] *dot1Δ::KanMX rev3Δ::HPHMX*	This study
ASY3176	FY833 *hst3Δ::HIS3 hst4Δ::kanMX4 hht1-hhf1Δ:: natMX4 hht2-hhf2Δ:: hygMX4* [p*CEN URA3 HST3*] [p*CEN TRP1 HHT1-hhf1K16R*]	This study
ASY3178	FY833 *hst3Δ::HIS3 hst4Δ::kanMX4 hht1-hhf1Δ:: natMX4 hht2-hhf2Δ:: hygMX4* [p*CEN URA3 HST3*] [p*CEN TRP1 hht1K79R-HHF1*]	This study

2.3.2 Isolation of independent spontaneous suppressors of the temperature sensitivity phenotype of *hst3*Δ *hst4*Δ cells

A similar strategy was used to isolate spontaneous suppressors of *hst3*Δ *hst4*Δ mutants. Strain ICY703 (Table 2.I) was used as a starting point to identify spontaneous suppressors of the Ts- phenotype. ICY703 contains chromosomal deletions of the *HST3* and *HST4* genes that are covered by a [p*CEN-URA3-HST3*] plasmid. Independent cultures of ICY703 were plated on 5-FOA plates at 37°C. One temperature-resistant colony per independent culture of ICY703 was streaked onto a second 5-FOA plate at 37°C to isolate single colonies that were temperature- and 5-FOA-resistant. Those independent suppressor strains were tested by PCR to verify that the *HST3* gene was absent from the thermoresistant strains. The PCR primers chosen for this test amplify a 670-bp DNA fragment derived from the 3'-end of *HST3*. The forward primer was Hst3-C (5'- GTCACATTTCTTGAATCCCAAATAC) and the reverse primer was Hst3-D (5'-TTTGTAGACTGTTAAAGAGCCATCC).

2.3.3 Cell synchronization, transient treatment with genotoxic agents and cell viability assays

Cells were grown overnight in YPD medium at 25°C and arrested in G1 using 5μg/ml α-factor for 90 minutes, followed by the addition of a second dose of α-factor at 5μg/ml for 75 minutes. Cells were then released into the cell cycle by resuspending them in fresh YPD medium containing 50μg/ml pronase and methyl methane sulfonate (MMS) or hydroxyurea (HU). After transient MMS treatment, cells were washed with 2.5% sodium thiosulfate (a chemical that inactivates MMS) and released into fresh YPD medium. Aliquots of cells were collected as a function of time and flash frozen on dry ice before being processed for immunoblotting or pulsed field gel electrophoresis. Where applicable, appropriate dilutions of cells were plated on YPD to measure viability by colony formation assays.

2.3.4 Measurement of DNA content by flow cytometry

Cells were fixed with 70% ethanol prior to FACS flow cytometry analysis. DNA content was determined using Sytox Green as previously described (Invitrogen) (51). Flow cytometry was performed on a Becton-Dickinson LSR II instrument using the FACS Diva software, and on a FACS Calibur instrument using the Cell Quest software. Histograms were generated using FlowJo 7.6.5.

2.3.5 Pulsed field gel electrophoresis

10^7 cells were embedded in agarose plugs and treated for pulsed field gel electrophoresis as described previously (52). Electrophoresis was performed using a Bio-Rad CHEF DRIII instrument using manufacturer's protocols (Bio-Rad Laboratories).

2.3.6 Immunoblots

Whole-cell lysates were prepared for SDS–polyacrylamide gel electrophoresis using an alkaline cell lysis (53) or standard glass beads/trichloroacetic acid precipitation methods. SDS-PAGE and protein transfers were performed using standard molecular biology protocols. Our rabbit polyclonal antibodies against H3K56Ac (AV105) and H2A phosphorylated at S128 (AV137) were previously described (12). Anti- yeast H2A was purchased from Active Motif (Cat. No 39236). Our rabbit polyclonal antibody (AV94) raised against recombinant yeast histone H4 expressed in E. coli (which is devoid of H4 modifications) was previously described (54). Our rabbit polyclonal antibody (AV100) raised against a C-terminal peptide of H3 that is devoid of known modifications was also previously described (55). 12CA5 monoclonal antibodies were used to detect the HA epitope, and anti-Flag M2 antibodies were purchased from Sigma. Anti-acetyl histone H4 (Lys16Ac; Cat. No 07-329), and Anti-trimethylated histone H3 (Lys79Me3; ab2621) were purchased from Abcam.

2.3.7 Rad53 autophosphorylation assays

Protein samples were prepared by the glass beads/tricholoroacetic acid precipitation method, resolved by SDS-PAGE and transferred to PVDF membranes using standard Towbin buffer (25mM Tris and 192mM glycine) without methanol or SDS at 0.8 mA/cm^2 for 2h on a Bio-Rad SD semi-dry transfer apparatus. Membranes were then processed as previously described (56).

2.3.8 Densitometry analysis

Densitometry analyses of immunoblot and Rad53 *in situ* autophosphorylation assays were performed using Image J 1.46E. Signal obtained for histone modifications were normalized relative to the corresponding non-modified total histone signal (i.e. H4K16ac on H4,

H3K56ac on H3, etc.). Rad53 autophosphorylation signals were normalized between samples using several bands from Ponceau S staining. To facilitate comparison between assays, normalized signal from every lane was set as a ratio of the isogenic *hst3Δ hst4Δ* strain for each experiment. Average band intensity was calculated using this relative ratio from at least 3 independent experiments.

2.3.9 Drug susceptibility assays

Colony formation assays were performed as described previously (38). Colony formation was monitored after 3 to 5 days of incubation at the indicated temperature. Genotoxic drugs (methyl methane sulfonate and hydroxyurea) were purchased from Sigma.

2.3.10 Fluorescence microscopy

Cell samples were fixated using formaldehyde as previously described (38) and examined using a Zeiss Z2 Imager fluorescence microscope equipped with the AxioVision software. Images were analyzed using Image J 1.46E.

2.3.11 Automated evaluation of Rfa1-YFP foci intensity

DNA foci were assumed to be fluorescent puncta, most of them of subdiffraction-limit size. To accurately analyze the data in a non-biased way, an algorithm was programmed using Matlab (Mathworks, MA), which automatically detects puncta and computes their fluorescence intensity in images composed of several cells. The method used to detect cell and DNA foci were distinct and outlined below. Fluorescent puncta were detected using linear band-pass filters that preserved objects of a size window and suppressed noise and large structures. These filters were applied by performing two 2-dimensional convolutions of the image matrix with a Gaussian and a boxcar kernel. Firstly, the image was convolved with a Gaussian kernel of the

characteristic length of the noise. Secondly, the image matrix was convolved with a boxcar kernel twice as big as the point spread function. This last operation is a low-pass filter for near-diffraction limit objects. Finally, the subtraction of the boxcar image from the Gaussian images becomes a band-pass filter to choose elements bigger than noise up to twice the diffraction limit. In order to limit the puncta considered in the quantifications to only those inside cells, the algorithm combined an intensity threshold and a watershed approach. The intensity threshold was established using Otsu's method. The cell fluorescence was enough to use this automatic thresholding approach to assign foreground pixels to cells and background pixels to empty space. This coarse estimation of the foreground pixels was further refined by first cleaning the mask removing isolated objects of less than 50 pixels. Next, a morphological opening of the mask using a 4-pixel radius disc was performed. Finally, a watershed algorithm was used to identify individual cells within the mask and objects of size lower than 10% of the average size were removed. Only foci detected within cells were considered for statistical purposes and plots were created, clustering the intensity of all individual foci found with all images of the same condition.

2.3.12 Histone purification, derivatization and mass spectrometry.

Core histones were purified from yeast strains as previously described (57), except that 10 mM nicotinamide and 30 mM sodium butyrate was added to the lysis and wash buffers. Intact core histones were then fractionated by HPLC and analyzed by mass spectrometry.

2.4 Results

2.4.1 Transient exposure to genotoxic drugs during S-phase delays completion of DNA replication in *hst3*Δ *hst4*Δ cells.

S. cerevisiae cells lacking *HST3* and *HST4* are extremely sensitive to chronic exposure to genotoxic drugs (17, 24, 44). Although a number of these drugs, *e.g.* methyl methane sulfonate (MMS) and hydroxyurea (HU), interfere with DNA replication in wild-type cells, S-phase progression and cell survival of *hst3*Δ *hst4*Δ mutants transiently exposed to MMS or HU has not been studied in detail. We first determined whether DNA damage caused by transient exposure to MMS or HU during S-phase led to loss of viability of *hst3*Δ *hst4*Δ cells. Cells were synchronized in G1 and released towards S-phase in medium containing MMS, and viability before and after transient exposure to MMS was determined by counting colonies that formed on rich medium (YPD) plates lacking MMS. In contrast to wild-type (WT) cells, transient exposure to very low concentrations of MMS during S-phase led to significant loss of viability of *hst3*Δ *hst4*Δ cells (Figure 2.1A, left panel). HU exposure during DNA replication similarly caused loss of cell viability (Figure 2.1A, right panel). These results are consistent with the hypothesis that impeding DNA replication fork progression during a single S-phase is sufficient to kill *hst3*Δ *hst4*Δ mutant cells.

MMS interferes with DNA synthesis by inducing 3-methyladenine which strongly blocks the progression of replicative DNA polymerases (58, 59), whereas HU acts via depletion of deoxyribonucleotide pools thereby stalling replication fork progression (60). We monitored the extent of chromosome replication in *hst3*Δ *hst4*Δ cells transiently exposed to MMS by flow cytometry (FACS) to measure DNA content, and by pulsed field gel electrophoresis (PFGE) as an indicator of chromosome integrity. Incompletely replicated chromosomes cannot migrate through pulsed field gels resulting in decreased intensity of intact chromosome bands stained with ethidium bromide (52). After removal of MMS, WT cells completed chromosome duplication as judged by the emergence of chromosome bands in pulsed field gels, and the fact

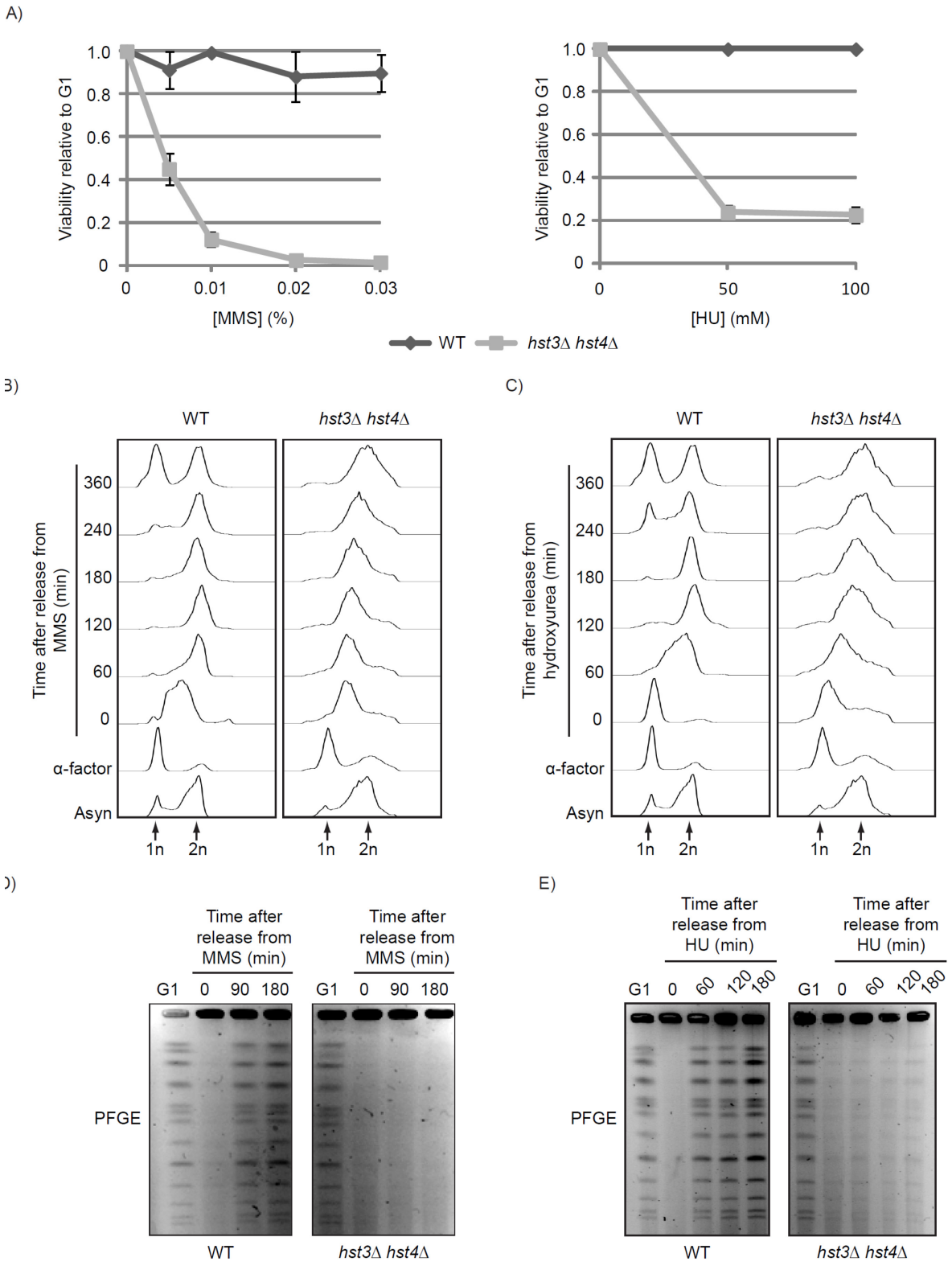

Figure 2.1. Transient exposure of *hst3*Δ *hst4*Δ cells to MMS or HU causes loss of viability and prevents the completion of DNA replication

Legend to figure 2.1: A) *hst3*Δ *hst4*Δ cells are sensitive to transient exposure to MMS and HU during S phase. Cells were arrested in G1 and released into the cell cycle in the presence of increasing concentrations of MMS (Left panel) or HU (Right panel) at 25°C. Appropriate dilutions of cells were plated on YPD during G1 arrest and after 90 min of MMS exposure. Viability was defined as the ratio of colonies that arose after MMS or HU treatment to colonies formed by G1-synchronized cells (see Material and Methods). B-C) Transient exposure to MMS or HU delays the completion of DNA replication in *hst3*Δ *hst4*Δ mutants. Cells were synchronized in G1 with □-factor and released toward S-phase in medium containing 0.03% MMS or 200 mM HU for 90 minutes. Genotoxic agents were then washed away and inactivated using 2.5% sodium thiosulfate in the case of MMS, and cells were released into fresh medium lacking genotoxins. Samples were processed for cell cycle analysis by FACS at the indicated time points. Asyn: Asynchronous cells. D-E) *hst3*Δ *hst4*Δ mutants cannot complete chromosome duplication after transient exposure to MMS. Cells were arrested in G1 and released into the cell cycle in the presence of 0.03% MMS or 200 mM HU for 1.5h. They were washed with YPD (containing 2.5% sodium thiosulfate in the case of MMS) and resuspended in fresh medium lacking genotoxins. Samples were taken at the indicated time points and processed for pulse-field gel electrophoresis.

that cells eventually completed mitosis as demonstrated by FACS (Figure 2.1B, D). In striking contrast to WT cells, FACS analysis of *hst3*Δ *hst4*Δ cells indicated that DNA content increased very slowly after removal of MMS from the medium, with most cells exhibiting sub-G2 DNA content after 6 hours following MMS removal (Figure 2.1B). Concordant with this, none of the chromosomes entered pulsed field gels for at least 3 hours following MMS removal from *hst3*Δ *hst4*Δ cells (Figure 2.1D). Similar results were obtained for *hst3*Δ *hst4*Δ cells treated with HU (Figure 2.1C, E). These data indicate that replicative stress strongly delays completion of chromosome duplication in *hst3*Δ *hst4*Δ cells, which holds true for all chromosomes regardless of size.

2.4.2 Exposure to genotoxins causes accumulation of homologous recombination protein foci and persistent activation of DNA damage checkpoint kinases in *hst3*Δ *hst4*Δ cells.

Repair of damaged DNA replication forks by homologous recombination (HR) in yeast depends on the Rad52 protein (59, 61). Importantly, in both yeast and humans HR proteins form nuclear foci in response to certain DNA damaging agents including MMS (61, 62). We hypothesized that defective replication fork recovery after DNA damage in *hst3*Δ *hst4*Δ mutant cells could engender the formation of abnormal HR structures. To test this, we generated *hst3*Δ *hst4*Δ strains expressing Rad52-YFP from its endogenous locus. Exponentially growing asynchronous *hst3*Δ *hst4*Δ cell populations presented a higher frequency of spontaneously arising Rad52-YFP foci as compared to WT cells (Figure 2.2A). Our results also indicated that, immediately after treatment with MMS during S-phase, a larger fraction of *hst3*Δ *hst4*Δ cells displayed Rad52-YFP foci (up to 60%) as compared to WT cells (Figure 2.2A; time 0). This behavior of *hst3*Δ *hst4*Δ mutants was unexpected because, in WT cells, activation of DNA damage response kinases has been reported to inhibit the formation of Rad52 foci during S-phase, at least until MMS is removed from the medium (63). The frequency of *hst3*Δ *hst4*Δ cells containing MMS-induced Rad52-YFP foci progressively increased to reach 80% of cells at 6

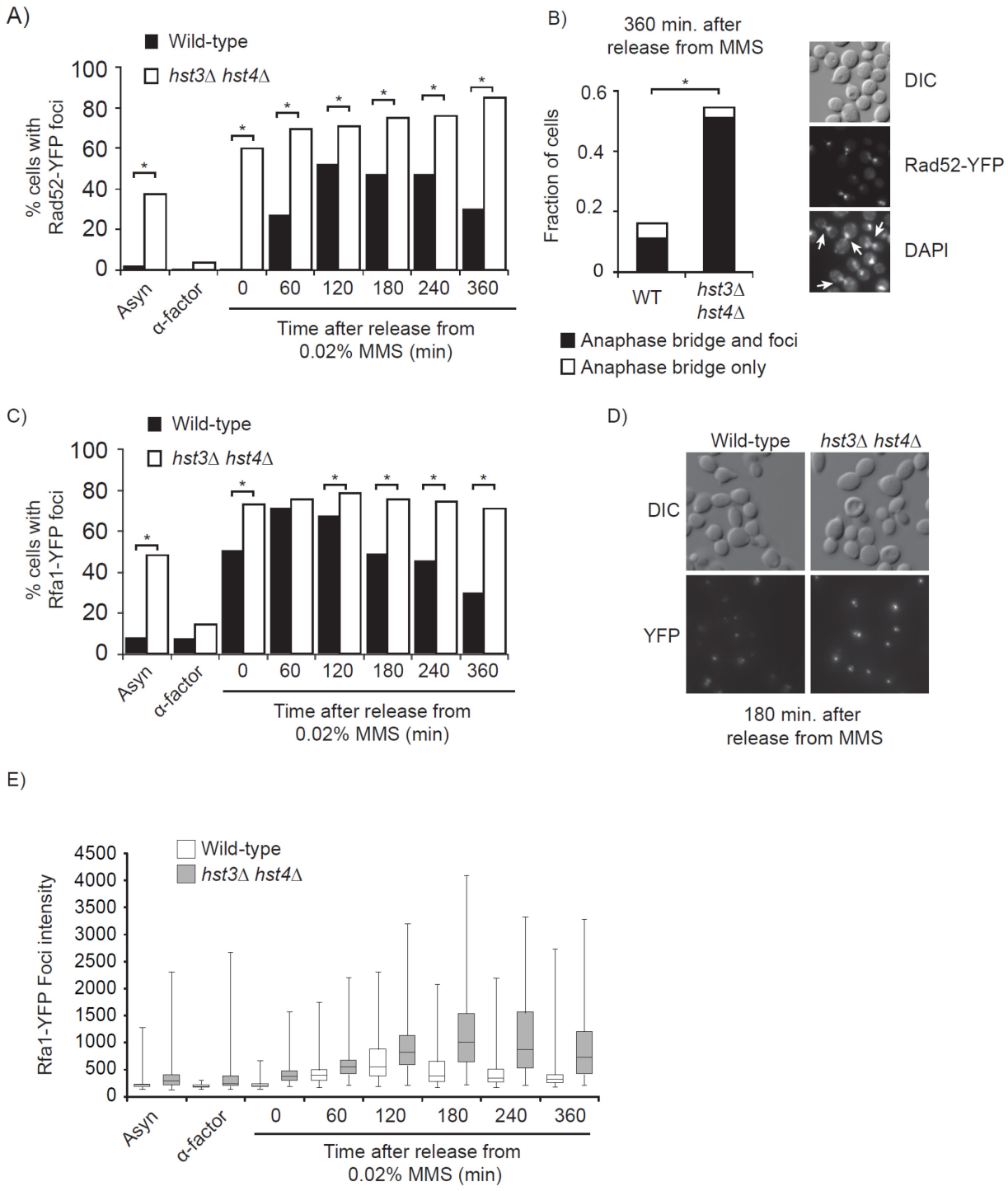

Figure 2.2. *hst3*Δ *hst4*Δ cells present abnormal frequencies of spontaneous and MMS-induced Rad52 and Rfa1 foci

Legend to Figure 2.2. A) Formation of persistent Rad52-YFP foci in *hst3*Δ *hst4*Δ mutants transiently exposed to MMS during S-phase. Cells were synchronized in G1 and released toward S-phase in the presence of 0.02% MMS for 90 min at 25°C. MMS was inactivated using sodium thiosulfate-containing medium, and cells were incubated in fresh medium without MMS. Samples were taken at the indicated time points and Rad52-YFP foci were detected by fluorescence microscopy. At least 300 cells were analyzed for each time point; results from a representative experiment are shown. (*) p-value <0.0001; Khi square test. B) *hst3*Δ *hst4*Δ mutants display anaphase bridges after transient exposure to MMS during S-phase. Images of DAPI staining and Rad52-YFP foci from the "360 min" sample in A were analyzed for the presence of anaphase bridges. Left panel: Fraction of cells containing anaphase bridge with or without Rad52-YFP foci. Right Panel: representative image of anaphase bridges (indicated by arrows). >350 cells were analyzed. (*) p-value <0.0001; Khi square test. C-D) Transient MMS-exposure during S-phase causes the formation of persistent Rfa1-YFP foci in *hst3*Δ *hst4*Δ mutants. C) Cells were treated as in A, except that samples were analyzed for the presence of Rfa1-YFP foci by fluorescence microscopy. A representative experiment is shown. >300 cells were analyzed for each time point. (*) p-value <0.0001 as determined by Fisher's exact test. D) Representative images of the "180 min" time point from panel C. E) Box The intensity of Rfa1-YFP foci was analyzed using a custom-made software (see Materials and Methods for details). Whiskers of the Box-and-whiskers plot represent the 1st and 4th quartiles of the distribution. Statistical analysis of this data is presented in Table 2.II.

hours following removal of MMS from the growth medium (Figure 2.2A). In contrast, the fraction of WT cells with Rad52-YFP foci peaked at 120 minutes after removal of MMS and then decreased. Overall, these data suggest that a DNA replication-coupled Rad52-dependent process fails to proceed normally after cells lacking Hst3 and Hst4 are transiently exposed to MMS. HR defects at sites of MMS-induced DNA lesions may cause persistent DNA strand exchange intermediates and/or regions of incompletely replicated DNA. Upon entry into anaphase, these aberrant structures would be expected to result in chromatin bridges between sister chromatids (64). We analyzed anaphase chromatin bridges by visualizing DAPI-stained cells derived from the experiment shown in Figure 2.2A. Compared to WT cells, a significantly larger fraction of cells devoid of Hst3 and Hst4 presented anaphase bridges after transient exposure to MMS during S-phase (Figure 2.2B). Moreover, a large fraction of both WT and *hst3*Δ *hst4*Δ cells containing anaphase bridges were marked by Rad52-YFP foci. This suggests that at least some MMS-induced lesions may lead to incompletely replicated chromosomes and/or accumulation of HR intermediates, which in turn may generate mitotic anomalies and the high incidence of mitotic chromosome segregation defects observed in *hst3*Δ *hst4*Δ mutants (17, 27).

Table 2.II. Rfa1-YFP foci intensity values in *hst3Δ hst4Δ* and WT cells

			Min. after release from 0.02% MMS					
	Asyn	Alpha	0	60	120	180	240	360
WT Mean[a]	267	208	230	433	656	517	463	405
hst3Δ hst4Δ Mean[a]	391	411	421	585	908	1152	1095	939
P-value[b]	1.3E-02	1.4E-04	5.7E-31	2.8E-23	3.7E-18	1.5E-47	2.1E-38	3.3E-20

a. Mean intensities of systematically analyzed Rfa-YFP Foci (see materials and methods for details)

b. *P*-values were calculated using an unpaired two-tailed Student's t test

RPA (replication protein A) is a three-subunit single stranded DNA-binding protein complex that is essential for DNA replication in both yeast and humans (65). In yeast, the subunits of RPA are encoded by the *RFA1, RFA2* and *RFA3* genes (66). RPA plays essential roles in DNA replication, HR, and activation of DNA damage checkpoints (67, 68), and binds single-stranded DNA generated at sites of DNA lesions (67). As was the case for Rad52-YFP,

we found that Rfa1-YFP formed persistent foci after transient exposure of *hst3*Δ *hst4*Δ mutants to MMS during S-phase (Figure 2.2C). Upon visual inspection of microscopy images, we noted that Rfa1-YFP foci in *hst3*Δ *hst4*Δ cells appeared brighter than in WT cells (Figure 2.2D). To verify this in an unbiased manner, we developed a software capable of analysing the intensity of individual foci (see Materials and Methods). This analysis indicated that, at every time point examined, Rfa1-YFP foci were significantly brighter in *hst3*Δ *hst4*Δ mutants than in WT cells, with a statistically significant increase of 1.4-fold in the absence of damage and as much as 2.2-fold after 180 minutes following removal of MMS (Figure 2.2D-E and Table 2.II). This suggests that, when H3K56Ac is present throughout the genome, as is the case in *hst3*Δ *hst4*Δ mutants released from G1 towards S-phase, abnormally long regions of RPA-bound single-stranded DNA may be formed at sites where DNA synthesis is impeded by MMS-induced lesions.

RPA-coated single-stranded DNA generated at blocked replication forks is critical for activation of the intra S-phase DNA damage checkpoint (68). Current models propose that the apical DNA damage response (DDR) kinase Mec1 is activated at sites of DNA lesions through its interaction with the Ddc2 adapter protein, which binds to RPA-coated single-stranded DNA (69). Extensive single-stranded DNA regions formed at damaged DNA replication forks in *hst3*Δ *hst4*Δ mutants exposed to MMS should lead to robust DDR kinases activity. Phosphorylation of *S. cerevisiae* histone H2A on serine 128 (H2AP), the functional counterpart of H2AX serine 139 phosphorylation (γ-H2AX) in vertebrates, is a well-established marker of DNA damage. H2AP formation is catalyzed by the DDR kinases Mec1 and Tel1 (70). After transient exposure to MMS, both WT and *hst3*Δ *hst4*Δ cells showed increased H2AP (Figure 2.3A). In WT cells, the H2AP signal declined as a function of time after removal of MMS, suggesting progressive repair of DNA damage as well as inactivation of Mec1/Tel1 (Figure 2.3A). In contrast, H2AP levels remained high in *hst3*Δ *hst4*Δ cells for at least 4.5h after removal of MMS (Figure 2.3A). The persistence of high levels of H2AP in *hst3*Δ *hst4*Δ cells transiently exposed to MMS during S-phase is consistent with robust and long-lasting DDR kinase activity in response to unrepaired DNA lesions. We assessed the activity of Rad53, a DDR kinase that is phosphorylated and activated by Mec1 following DNA damage (56, 71). As measured by *in situ* autophosphorylation assays, the kinase activity of Rad53 was inactivated following MMS removal from the medium in WT cells (Figure 2.3B). In contrast, Rad53 activity remained

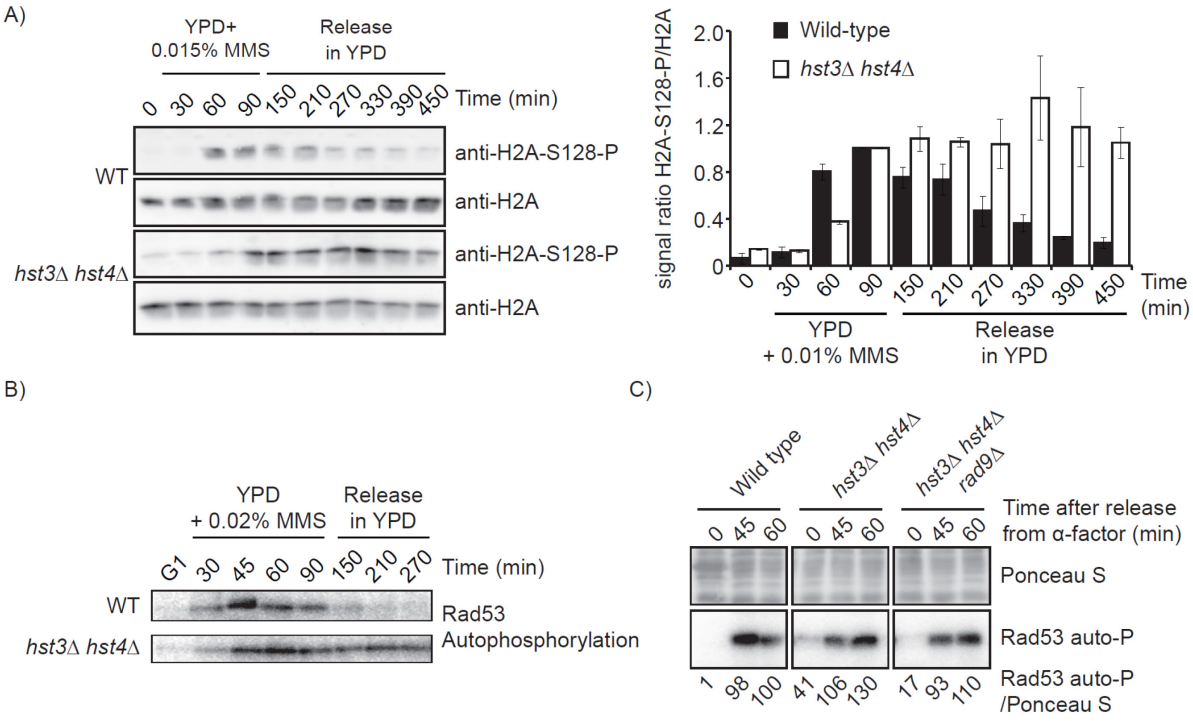

Figure 2.3. Persistent activation of DNA damage induced signalling in *hst3*Δ *hst4*Δ mutants exposed to MMS

A) *hst3*Δ *hst4*Δ mutants display persistent phosphorylation of histone H2A serine 128 following transient exposure to MMS. Cells were arrested in G1 and released into the cell cycle in the presence of 0.015% MMS for up to 90min. Cells were washed in YPD medium containing 2.5% sodium thiosulfate to inactivate MMS, and resuspended in fresh YPD without MMS. Left panel: Aliquots of cells were collected and whole-cell lysates analyzed by immunoblotting to detect histone H2A S128 phosphorylation (H2A-S128-P) and non-modified H2A. Right panel: H2A-S128-P signals were quantified by densitometry and normalized relative to H2A levels. For both strains, the value of time point "90 min" (end of MMS exposure) was set to 1 and values for other samples were normalized relative to this point. Error bars: standard error of the mean of densitometry values (3 loadings of the immunoblot samples). B) Cells were treated as in A) except that 0.02% MMS was used, and autophosphorylation of Rad53 was detected using *in situ* kinase assay (see Materials and Methods). C) *hst3Δ hst4Δ rad9*Δ triple mutantsdo not display Rad53 activation defects in response to HU-induced replication block. Cells were synchronized in G1 using α-factor and released into YPD medium containing 200 mM HU. Samples were taken at the indicated time points and Rad53 activity was monitored by *in situ* Rad53 autophosphorylation assay (Rad53 auto-P). Equal amounts of total protein were loaded for each sample. Rad53 autophosphorylation signals signals were quantified by densitometry relative to Ponceau S staining. Values were normalized to the "60 min" sample of the wild-type strain.

elevated for at least 4.5h in *hst3*Δ *hst4*Δ mutants (Figure 2.3B). This indicates that, in contrast to WT cells, DDR kinases remain active for long periods after transient exposure of *hst3*Δ *hst4*Δ cells to genotoxic agents. We further note that activated Rad53 and phosphorylated H2A are detectable in asynchronous *hst3*Δ *hst4*Δ cells even in the absence of genotoxic stress (Figure 2.7B-C), indicating constitutive activation of DDR kinases in these mutants. Overall, our results indicate that cells lacking Hst3 and Hst4 manifest persistent DNA damage-induced signalling in response to MMS-induced DNA lesions.

Previously published results suggested that cells lacking Hst3 and Hst4 may present defects in activation of the intra-S phase branch of the DNA damage checkpoint (24). Mrc1 is an important component of the intra-S phase checkpoint that promotes rapid activation of Rad53 in response to HU (72, 73). Rad9 is partially redundant with Mrc1 in this regard and, because of this, *mrc1*Δ *rad9*Δ cells are defective in Rad53 activation after exposure to HU. Deletion of *MRC1* also permits formation of Rad52 foci during MMS exposure, which form only after MMS has been removed from the growth medium in WT cells (63). The HU sensitivity (Figure 2.1A) and abnormal formation of Rad52 foci during MMS exposure (Figure 2.2A) observed in *hst3*Δ *hst4*Δ mutants are consistent with defects in intra-S phase checkpoint activity. We sought to determine whether Rad53 activation was indeed defective in response to HU in *hst3*Δ *hst4*Δ cells. Our *in situ* autophosphorylation assays indicate that Rad53 activation after HU treatment is comparable or perhaps even slightly higher in *hst3*Δ *hst4*Δ mutants than in WT cells (Figure 2.3C). In addition, we did not observe significant reduction in Rad53 autophosphorylation in *hst3*Δ *hst4*Δ *rad9*Δ triple mutants relative to *hst3*Δ *hst4*Δ cells after exposure to HU, indicating that the Mrc1 branch of the intra-S phase DNA damage checkpoint is most likely active in these mutants (Figure 2.3C). Overall, our results suggest that the HU sensitivity of *hst3*Δ *hst4*Δ cells (Figure 2.1A) or the untimely formation of Rad52 foci when this mutant is treated with MMS (Figure 2.2A) cannot be accounted for by complete loss-of-function of the intra-S branch of the DNA damage checkpoint.

2.4.3 Mutations that perturb chromatin structure suppress *hst3*Δ *hst4*Δ phenotypes.

The phenotypes of *hst3*Δ *hst4*Δ mutants appear to depend upon the fact that, unlike in WT cells, the vast majority of H3 molecules are K56-acetylated and/or that H3K56 hyperacetylation is present continuously throughout the cell cycle. Consistent with this, an *H3K56R* mutation that abolishes H3K56Ac suppresses many of these phenotypes (17, 44, 74). We sought to determine whether other histone gene mutations suppress the temperature- and/or genotoxic agent sensitivity of *hst3*Δ *hst4*Δ cells by screening a collection of histone H3/H4 mutants (15). To this end, we generated *hst3*Δ *hst4* strains expressing histone point mutants from a low copy centromeric *TRP1* plasmid. These strains also harbored a p*CEN-URA3-HST3* plasmid to prevent the emergence of spontaneous suppressors that arise during long-term propagation of *hst3*Δ *hst4*Δ mutants (see Material and Methods). To test their genotoxic drug and temperature sensitivity (Ts-), cells were grown on medium containing 5-FOA and genotoxins at different temperatures to select against the p*CEN-URA3-HST3* plasmid (17). As expected, we found that mutations at H3K56 partially suppressed the temperature, HU, and MMS sensitivity of *hst3*Δ *hst4*Δ mutants (Table 2.III), thus validating the conditions under which the screen was conducted. We note that this suppression is only partial since the triple mutants *hst3*Δ *hst4*Δ *H3K56R* retain the genotoxic agent sensitivity of cells lacking H3K56Ac (17).

Most H3/H4 point mutations, including those involving basic residues near H3K56 (H3R52 and R53), did not noticeably modulate *hst3*Δ *hst4*Δ phenotypes (Tables III and IV). We also found that certain mutations of H4K20 (H4K20A or Q) slightly rescued the Ts- phenotype (Table IV and data not shown). In contrast, mutation of histone H4 lysine 16 and H3 lysine 79 to either arginine or alanine strongly suppressed temperature sensitivity, as well as sensitivity to either chronic or transient MMS exposure (Table III-IV, Figure 2.4A-C). Densitometry analyses of immunoblots indicated that the H4K16R or H3K79R mutations did not reduce H3K56ac levels (Figure 2.4D, Figure 2.5A). We confirmed these results in a more precise manner using quantitative mass spectrometry (Table V), which revealed that the stoichiometry of H3K56ac was indeed not reduced in *hst3*Δ *hst4*Δ cells harboring either H4K16R or H3K79R

mutations as compared to *hst3*Δ *hst4*Δ mutants. Overall, the results of our screen suggest a previously unreported interplay between H3K56, H3K79 and H4K16 in the DNA damage response.

Table 2.III. Histone H3 gene mutations and phenotypes of *hst3*Δ *hst4*Δ mutant cells

Histone mutant	Sensitivity at 37°C	MMS sensitivity	HU sensitivity
hst3Δ hst4Δ			
H3 WT	Ts-	S	S
H3 R2A	Ts-	S	S
H3 R2K	Ts-	S	S
H3 T6A	Ts-	S	S
H3 T6E	Ts-	S	S
H3 K9A	Ts-	S	S
H3 K9R	Ts-	S	S
H3 K9Q	Ts-	S	S
H3 S10A	Ts-	S	S
H3 S10E	Ts-	S	S
H3 T11A	Ts-	S	S
H3 T11E	Ts-	S	S
H3 K14A	Ts-	S	S
H3 K14R	Ts-	S	S
H3 K14Q	Ts-	S	S
H3 R17A	Ts-	S	S
H3 R17K	Ts-	S	S
H3 K18A	Ts-	S	S
H3 K18R	Ts-	S	S
H3 K18Q	Ts-	S	S
H3 K23A	Ts-	S	S
H3 K23R	Ts-	S	S
H3 K23Q	Ts-	S	S
H3 R26A	Ts-	S	S
H3 K26K	Ts-	S	S
H3 K27A	Ts-	S	S
H3 K27R	Ts-	S	S
H3 K27Q	Ts-	S	S

Table 2.III. (continued)

Histone mutant	Sensitivity at 37°C	MMS sensitivity	HU sensitivity
hst3Δ hst4Δ			
H3 WT	Ts-	S	S
H3 S28A	Ts-	S	S
H3 S28E	Ts-	S	S
H3 R52A	Ts-	S	S
H3 R52K	Ts-	S	S
H3 R52Q	Ts-	S	S
H3 R53A	Ts-	S	S
H3 R53K	Ts-	S	S
H3 R53Q	Ts-	S	S
H3 K56R	Tr	R	R
H3 K56Q	Tr	R	R
H3 K79A	Tr	R	R
H3K79R	Tr	R	R
H3 K91A	Ts-	S	S
H3 K91R	Ts-	S	S
H3 K91Q	Ts-	S	S
H3 K115A	Ts-	S	S
H3 K115R	Ts-	S	S
H3 K115Q	Ts-	S	S
H3 T118A	Ts-	S	S
H3 T118E	Ts-	S	S
H3 K122A	Ts-	S	S
H3 K122R	Ts-	S	S
H3 K122Q	Ts-	S	S

Ts- : Thermo-sensitive (fails to grow at 37°C)
Tr : Thermo-resistant (grows at 37°C)
S : Growth compromised on plates containing either 0.01% MMS or 100mM HU
R : Histone gene mutations that rescue, at least partially, the MMS or HU sensitivity of *hst3*Δ *hst4*Δ cells

Table 2.IV. Histone H4 gene mutations and phenotypes of *hst3Δ hst4Δ* mutant cells

Histone mutant	Sensitivity at 37°C	MMS sensitivity	HU sensitivity
hst3Δ hst4Δ			
H4 WT	Ts-	S	S
H4 S1A	Ts-	S	S
H4 S1E	Ts-	S	S
H4 R3A	Ts-	S	S
H4 R3K	Ts-	S	S
H4 K5A	Ts-	S	S
H4 K5R	Ts-	S	S
H4 K5Q	Ts-	S	S
H4 K8A	Ts-	S	S
H4 K8R	Ts-	S	S
H4 K8Q	Ts-	S	S
H4 K12A	Ts-	S	S
H4 K12R	Ts-	S	S
H4 K12Q	Ts-	S	S
H4 K16R	Tr	R	S
H4 K16A	Tr	R	S
H4 K20A	Tr	R	S
H4 K20R	Ts-	S	S
H4 K20Q	Tr	R	S
H4 K31A	Ts-	S	S
H4 K31R	Ts-	S	S
H4 K31Q	Ts-	S	S
H4 S47A	Ts-	S	S
H4 S47E	Ts-	S	S
H4 K59A	Ts-	S	S
H4 K59R	Ts-	S	S
H4 K59Q	Ts-	S	S
H4 K77A	Ts-	S	S
H4 K77R	Ts-	S	S
H4 K77Q	Ts-	S	S
H4 K79A	Ts-	S	S
H4 K79R	Ts-	S	S
H4 K79Q	Ts-	S	S
H4 K91A	Ts-	S	S
H4 R91R	Ts-	S	S
H4 K91Q	Ts-	S	S
H4 R92A	Ts-	S	S
H4 R92K	Ts-	S	S

Ts- : Thermo-sensitive (fails to grow at 37°C)
Tr : Thermo-resistant (grows at 37°C)
S : Growth compromised on plates containing either 0.01% MMS or 100mM HU
R : Histone gene mutations that rescue, at least partially, the MMS or HU sensitivity of *hst3*Δ *hst4*Δ cells.

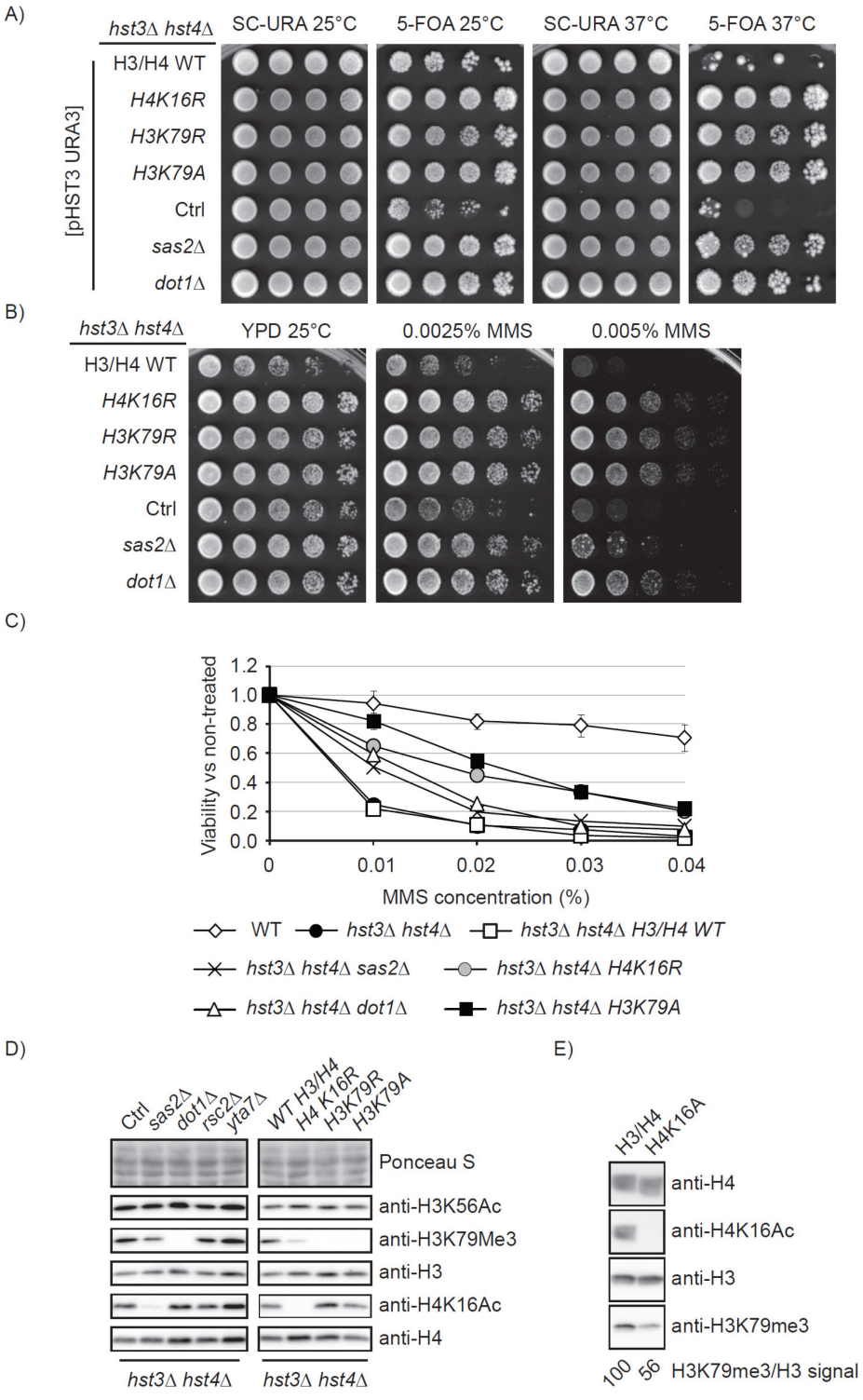

Figure 2.4. Mutations that prevent modifications of H3 lysine 79 or H4 lysine 16 suppress the MMS sensitivity and Ts- phenotypes of *hst3*Δ *hst4*Δ mutants

Legend to Figure 2.4. A) Mutations of enzymes that methylate H3K79 or acetylate H4K16, or point mutations of these residues, suppress the Ts- phenotype of *hst3*Δ *hst4*Δ mutants. Five-fold serial dilutions of cells carrying a centromeric plasmid expressing *URA3* and *HST3* were spotted on the indicated media and grown at either 25°C or 37°C. B-C) The sensitivity of *hst3*Δ *hst4*Δ mutants to MMS is rescued by mutations that reduce H3K79 methylation or H4K16Ac. B) Five-fold serial dilutions of cells were spotted onto YPD medium containing MMS and incubated at 25°C. C) Exponentially growing cells were exposed to increasing concentrations of MMS for 90 minutes at 25°C. Viability was defined as the ratio of the number of colonies that arose after MMS treatment to the number colonies formed by cells that were not exposed to MMS. Error bars: standard error of the mean from at least 3 independent experiments for each strain. D-E) Whole-cell lysates of exponentially growing cells were probed by immunoblotting using the indicated antibodies (see Figure 15 for densitometry analysis). Ctrl: *hst3*Δ *hst4*Δ cells.

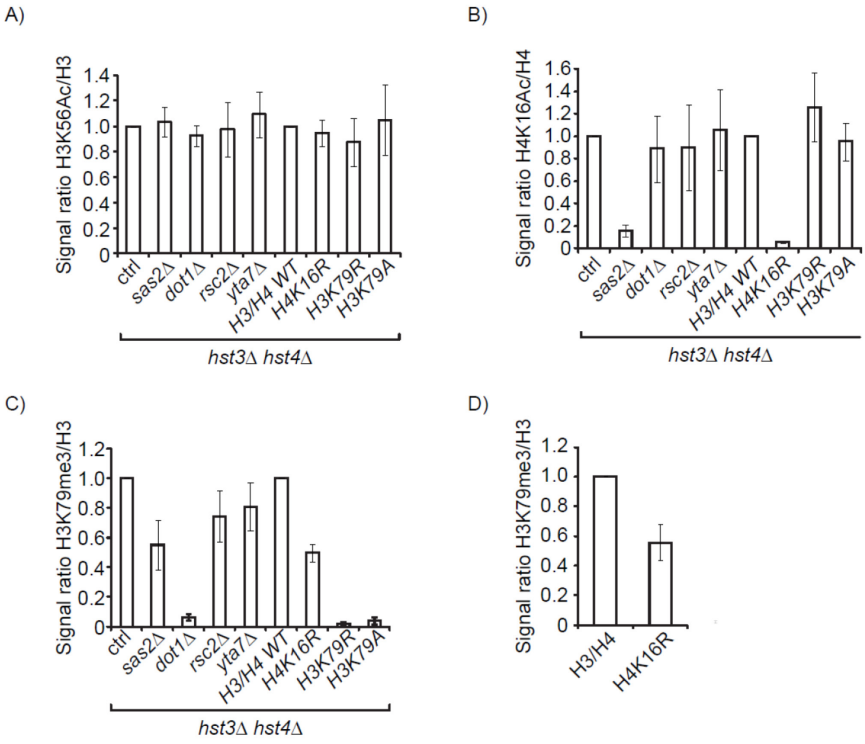

Figure 2.5. Densitometry analysis of immunoblots shown in figure 14D and E
A-C) Immunoblot images from Figure 14D were analyzed by densitometry. Error bars represent the standard error of the mean of between 3 to 12 independent loadings of each sample. D) H3K79me3 immunoblot images from Figure 14E were analyzed by densitometry. Average values relative to the control signal derived from total histone H3 are shown and error bars indicate the standard error of the mean (from 4 independent loading of each sample).

H4K16 acetylation (H4K16Ac) and H3K79 methylation (H3K79Me) are very abundant histone modifications in both yeast and human (75, 76). We evaluated whether H4K16Ac and H3K79Me contribute to the severe phenotypes of *hst3*Δ *hst4*Δ cells. In *S. cerevisiae*, the SAS-I acetyltransferase complex (composed of Sas2, Sas4 and Sas5) is primarily responsible for histone H4 lysine 16 acetylation (77–79). We found that deletion of *SAS2*, which encodes the catalytic subunit of the SAS-I complex (79), resulted in partial suppression of the temperature and MMS sensitivity of *hst3*Δ *hst4*Δ mutants (Figure 2.4A-C). The degree of suppression imparted by *sas2*Δ was not as pronounced as that conferred by an *H4K16R* mutation, possibly reflecting the fact that H4K16Ac is completely abolished in *H4K16R* mutants, but detectable amounts of H4K16Ac persist in *sas2*Δ cells (Figure 2.4D). We cannot exclude that, in addition to abolishing H4K16ac, mutation of H4K16 to either arginine or alanine may also in itself contribute to the phenotypic suppression of *hst3*Δ *hst4*Δ cells. The methyltransferase Dot1 is responsible for H3K79 mono-, di- and tri-methylation in yeast (75, 80). We found that deletion of *DOT1* in *hst3*Δ *hst4*Δ cells strongly suppressed their temperature and MMS sensitivity (Figure 2.4A-C). Importantly, deletion of either *DOT1* or *SAS2* did not reduce the level of H3K56Ac (Figure 2.4D, Figure 2.5A), indicating that both H3K79 methylation and H4K16 acetylation contribute to the phenotypes of *hst3*Δ *hst4*Δ via other mechanisms.

We did not detect elevated levels of H3K79 tri-methylation or H4K16 acetylation when *hst3*Δ *hst4*Δ mutants were compared to WT cells (data not shown). However, densitometry analyses of immunoblots revealed a reproducible, albeit modest reduction in H3K79 tri-methylation levels in *hst3*Δ *hst4*Δ *sas2*Δ and *hst3*Δ *hst4*Δ *H4K16R* cells (Figure 2.4D, Figure 2.5C). This result was unexpected because previous publications examining the relationship between H4K16 acetylation and H3K79 tri-methylation did not report reduced H3K79 tri-methylation in cells where H4K16 cannot be acetylated (81, 82). Interestingly, we obtained similar immunoblotting results (*i.e.* reduced H3K79 tri-methylation) in *HST3 HST4* cells expressing *H4K16A* suggesting that this effect is not restricted to cells that present abnormally high levels of H3K56ac (Figure 2.4E, Figure 2.5D). To validate these results, we evaluated the relative abundance of mono-, di- and tri-methylated histone H3K79 using quantitative mass spectrometry (Table 2.V). The data indicates a modest reduction in H3K79 tri-methylation in *hst3*Δ *hst4*Δ *H4K16R* as compared to isogenic *hst3*Δ *hst4*Δ H3/H4 WT cells (compare strains

ASY2737 with ASY2745 in Table 2.V). Consistent with such a decrease in H3K79 tri-methylation, levels of both mono- and di-methylated H3K79 in *hst3*Δ *hst4*Δ *H4K16R* mutants were increased in comparison to those observed in *hst3*Δ *hst4*Δ H3/H4 WT cells. For example, the ratio of tri- to di-methylation of H3K79 is approximately three times higher in *hst3*Δ *hst4*Δ H3/H4 WT than in *hst3*Δ *hst4*Δ *H4K16R* cells (ratios of 6.1 versus 2.2, respectively). This is consistent with immunoblotting data from Evans *et al*. who reported increased levels of H3K79 mono- and di-methylation in yeast strains harboring the *H4K16R* mutation (82). Overall, our data indicate that H4K16 acetylation and H3K79 methylation both contribute to the severe phenotypes caused by H3K56 hyperacetylation, and raise the intriguing possibility that suppression of *hst3*Δ *hst4*Δ mutant phenotypes by the *H4K16R* mutation may be, at least in part, due to a decrease in H3K79 tri-methylation.

Table 2.V. Calculated relative abundance (% of total histone H3) for H3 K79 methylation and H3K56ac[a]

	Yeast genetic background[b]		
	*hst3*Δ *hst4*Δ H3/H4 WT (ASY2737)	*hst3*Δ *hst4*Δ H3/H4K16R (ASY2745)	*hst3*Δ *hst4*Δ H3K79R/H4 (ASY2749)
H3K79Me0 ± SEM[c,d]	13.1 ± 0.4	10 ± 1	NA[f]
H3K79Me1 ± SEM[c,d]	4.9 ± 0.1	8 ± 1	NA[f]
H3K79Me2 ± SEM[c,d]	11.6 ± 0.4	26 ± 2	NA[f]
H3K79Me3 ± SEM[c,d]	70.4 ± 0.1	56 ± 4	NA[f]
H3K56ac ± SEM[c,e]	80.3 ± 0.7	92.4 ± 0,5	88.3 ± 0.3

a: The abundance of each peptide was assessed by mass spectrometry of total histone H3 purified from each strain in buffers containing a cocktail of deacetylase inhibitors (see Materials and Methods).
b: Strains ASY2737, ASY2745, and ASY2749 are in the same genetic background (see table 2.I).
c: Standard error of the mean of two mass spectrometry technical replicates.
d: The values for the different forms of H3K79 reflect the relative abundance of a given isoform (e.g.H3K79me0) expressed as a percentage of the abundance of all H3K79 isoforms (K79me0+me1+me2+me3). For technical reasons, these values should not be equated stoichiometries. See Materials and Methods for a more detailed explanation.
e: The values for H3K56ac reflect stoichiometries, i.e. the fraction of all H3 molecules that are K56-acetylated. This is expressed as percentages obtained as follows. Abundance of K56ac divided by abundance of K56ac+K56pr. See Materials and Methods for a more detailed explanation.
f: NA: not applicable

H4K16Ac and H3K79Me are involved in preventing heterochromatin from invading euchromatic regions (75, 77, 78, 80, 83, 84). Hence, we tested whether suppression of *hst3*Δ *hst4*Δ phenotypes resulting from decreased H4K16Ac or H3K79Me might also be observed in

other mutants in which chromatin boundary functions are impaired. Rsc2 is a subunit of one of the two forms of the RSC ATP-dependent chromatin remodeling complex (85, 86). RSC exerts a number of cellular functions (85, 87, 88), but is critical for restricting the spread of silencing factors from heterochromatin into euchromatin (83). Likewise, Yta7 contributes to the function of chromatin boundaries (83, 84, 89). We found that deletion of either *RSC2* or *YTA7* partially suppressed the phenotypes of *hst3*Δ *hst4*Δ cells, although to a lesser extent than *H4K16R* or H3K79R mutations (Figure 2.6A, C). H4K16Ac, H3K79Me3 and H3K56Ac levels were not affected in mutants lacking either Rsc2 or Yta7 (Figure 2.4D, Figure 2.5A-C), indicating that *rsc2*Δ and *yta7*Δ mutations do not suppress the phenotypes of *hst3*Δ *hst4*Δ cells by influencing these histone modifications. We also found that deletion of *RSC1*, which forms an alternative RSC complex playing a less important role in restricting the spread of silencing in yeast (83), causes synthetic growth defect in combination with *hst3*Δ *hst4*Δ mutations (Figure 2.6B). Overall, these results support a model in which modulation of chromatin boundary function may partly account for the suppressor effect of H3K79 and H4K16 mutations on *hst3*Δ *hst4*Δ phenotypes.

Sir2 deacetylates both H3K56 and H4K16 *in vitro* (28, 31, 90). *In vivo*, the importance of Sir2 for H4K16 deacetylation in heterochromatic regions is undisputed (77, 78), but whether Sir2 plays a role in H3K56 deacetylation within heterochromatic domains is controversial (90, 91). Indeed, there is substantial evidence that, even within heterochromatic domains, Hst3 and Hst4 are needed for deacetylation of H3K56 while Sir2 is not (91). Despite the aforementioned controversy, one model to explain how perturbation of chromatin boundaries suppresses *hst3*Δ *hst4*Δ phenotypes would be that, when boundaries are defective, Sir2 might spread from heterochromatic domains and remove H3K56Ac and/or H4K16Ac within euchromatin leading to partial phenotypic suppression. This model predicts that Sir2 should be essential for the suppressor effect of *H4K16R* or *sas2*Δ mutations on the phenotypes of cells lacking Hst3 and Hst4. Our results and those of BRACHMANN *et al.*, show that a *sir2*Δ mutation exacerbates the phenotypes of *hst3*Δ *hst4*Δ cells (27) (Figure 2.6D). Interestingly, we found that the *sas2*Δ and *H4K16R* mutations suppress the growth defects of *hst3*Δ *hst4*Δ *sir2*Δ cells (Figure 2.6D), which strongly argues that the suppression of *hst3*Δ *hst4*Δ mutant phenotypes caused by *SAS2* deletion or H4K16 mutations does not depend on deregulated Sir2-mediated histone deacetylation.

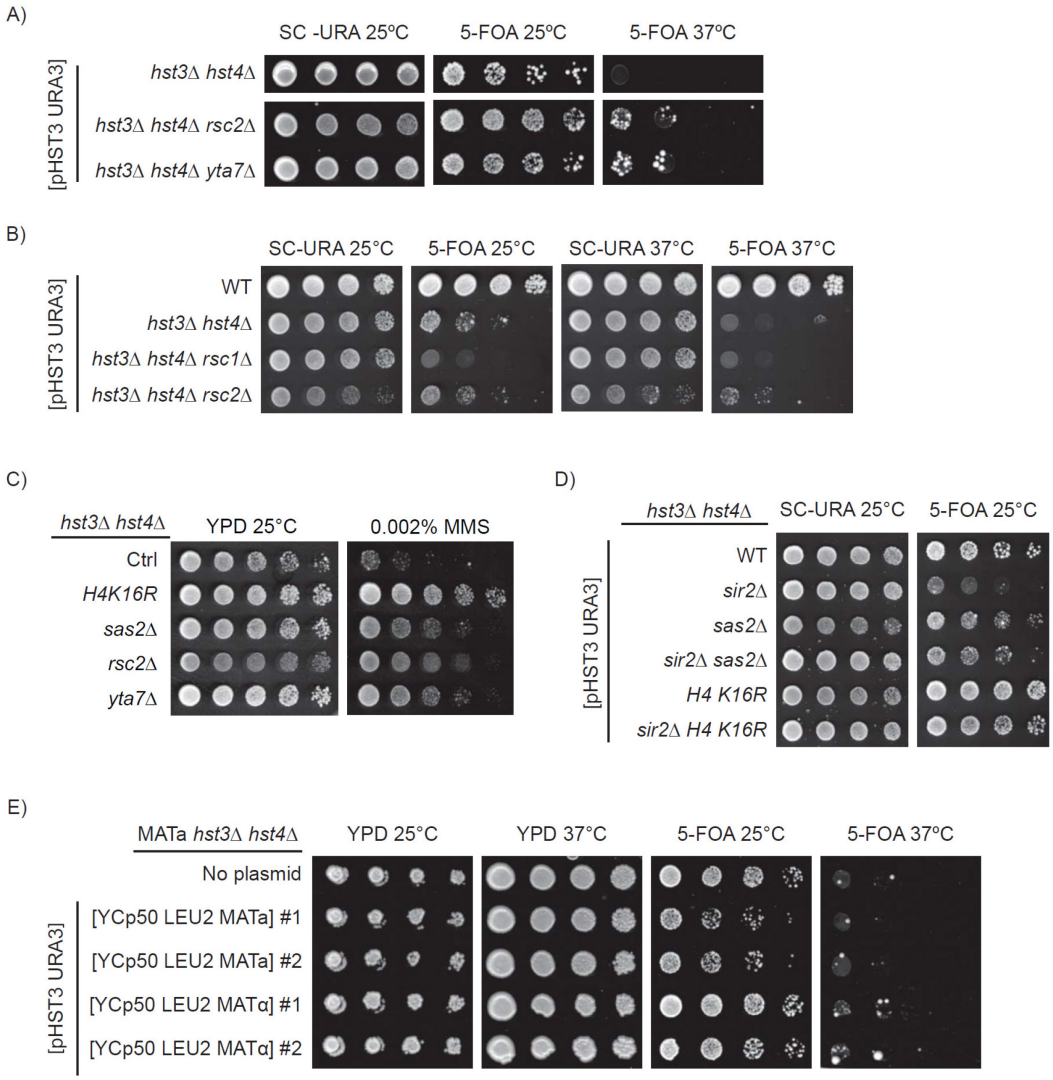

Figure 2.6. Mutation of genes involved in maintaining euchromatin-heterochromatin boundaries suppresses the phenotypes of *hst3*Δ *hst4*Δ mutants

A-B) Mutations of *YTA7* or *RSC2*, but not *RSC1*, partially suppress the temperature sensitivity of *hst3*Δ *hst4*Δ mutants. C) The sensitivity of *hst3*Δ *hst4*Δ mutants to MMS is partially rescued by *rsc2*Δ or *yta7*Δ mutations. Five-fold serial dilutions of cells were spotted onto YPD media lacking or containing MMS and incubated at 25°C. D) The suppressor effect of *sas2*Δ and *H4K16R* mutations on the temperature sensitivity of *hst3*Δ *hst4*Δ cells does not require *SIR2*. Cells were treated as in A. E) Constitutive expression of silent mating loci genes does not suppress the temperature sensitivity of *hst3*Δ *hst4*Δ cells. MATa *hst3*Δ *hst4*Δ cells harboring plasmids expressing either MATa or MATα genes and a centromeric plasmid expressing *URA3* and *HST3* were spotted on the indicated media and grown at either 25°C or 37°C.

Sas2, Rsc2 and Yta7 are involved in the maintenance of chromatin boundaries at the silent mating loci (83, 84, 89). Heterochromatin spreading in *sas2*Δ, *rsc2*Δ or *yta7*Δ mutants likely requires that limiting pools of Sir complexes spread beyond their normal domains of action (92, 93). Indeed, dilution of Sir2 over larger genomic domains has been proposed to reduce the efficacy of silencing in *sas2*Δ, *rsc2*Δ or *yta7*Δ mutants. Because of this, these mutants may abnormally express the *HMR***a** and/or *HML*α genes located at silent loci, thus generating pseudo-diploid cells (haploid cells that express genes from both mating types), which are more resistant than haploid *MAT***a** or *MAT*α cells to genotoxic agents such as MMS (47, 94). To test whether abnormal gene expression derived from *HML*α and *HMR***a** contributes to the suppression of *hst3*Δ *hst4*Δ mutant phenotypes, we transformed *hst3*Δ *hst4*Δ *MAT***a** cells with plasmids expressing either the *MAT***a** or *MAT*α mating cassettes (Figure 2.6E). These plasmids have been reported to suppress the MMS sensitivity of several DNA repair mutants of the opposite mating type (47). Our results revealed that ectopic expression of *MAT*α mating type genes did not rescue the temperature sensitivity of *hst3*Δ *hst4*Δ *MAT***a** cells (Figure 2.6E), which indicates that rendering *hst3*Δ *hst4*Δ mutants pseudo-diploid is not sufficient to suppress their phenotypes. In turn, these data suggest that suppression of *hst3*Δ *hst4*Δ mutant phenotypes by deletion of *SAS2*, *RSC2* or *YTA7* is unlikely to be explained by pseudo-diploidy. However, we cannot exclude the possibility that abnormal gene expression resulting from disruption of chromatin boundaries could contribute to the suppression of *hst3*Δ *hst4*Δ mutant phenotypes by deletion of *RSC2*, *YTA7* and mutations of H4K16 or H3K79.

2.4.4 DNA damage response kinases activity contributes to the severe phenotypes of *hst3*Δ *hst4*Δ cells.

Rad9 is an adaptor protein that permits Mec1-mediated phosphorylation and activation of Rad53 in response to DNA damage (71, 95). Rad9 was previously shown to be important for the constitutive activation of Rad53 observed in *hst3*Δ *hst4*Δ mutants (44). Rad9 binds methylated H3K79 via its Tudor domain, thereby promoting its recruitment to chromatin where it mediates Rad53 activation (96–99). We hypothesized that a decrease in H3K79Me would

impair Rad9 binding to chromatin and, consequently, reduce Rad53 activity to alleviate some of the phenotypes that result from H3K56 hyperacetylation. Consistent with this hypothesis, we found that deletion of *RAD9* partially suppressed the phenotypes of *hst3*Δ *hst4*Δ cells (Figure 2.7A), and that mutations of *DOT1, SAS2,* H4K16 or H3K79 noticeably decreased spontaneous and MMS-induced Rad53 activation (Figure 2.7B-C, Figure 2.8A, C-D). Interestingly, none of the suppressor mutations that we identified significantly modulated spontaneous Mec1/Tel1-mediated histone H2A phosphorylation, suggesting that these mutations may preferentially affect Rad53 activity (Figure 2.7B and Figure 2.8B).

Previously published data indicate that cells lacking Dot1 are more resistant to MMS-induced DNA damage than WT cells, and this increased resistance to MMS has been correlated with reduced levels of Rad53 activation (100–102). The sensitivity to MMS of certain yeast mutants (including *rad52*Δ and *rtt107*Δ*)* is also reduced in the absence of Dot1 or H3K79 tri-methylation (100–102). We sought to verify whether the sensitivity to MMS of mutants of the H3K56Ac pathway was also suppressed by *DOT1* mutations. We deleted *DOT1* in *rtt109*Δ and *ctf4*Δ strains, which are known to be extremely sensitive to MMS (19, 38, 44, 103). Importantly*, RTT109* and *CTF4* display extensive genetic and biochemical links to H3K56ac and *HST3/HST4* (19, 43, 44). In contrast to *hst3*Δ *hst4*Δ cells, deletion of *DOT1* in a *ctf4*Δ background caused synthetic sensitivity to MMS, whereas *dot1*Δ mutation did not appear to affect the MMS sensitivity of *rtt109*Δ mutants (Figure 2.7D). These data suggest that lack of H3K79 methylation does not increase cellular resistance to MMS in every mutant of the H3K56ac pathway.

We verified whether the identified suppressor mutations were able to alleviate the sensitivity of *hst3*Δ *hst4*Δ cells to replicative stress. Epitope tagging of replication enzymes such as Cdc45 causes severe growth defects in *hst3*Δ *hst4*Δ cells suggesting that these cells are exquisitely sensitive to subtle perturbations of the DNA replication machinery that have essentially no effect on the fitness of WT cells (44). Remarkably, we found that *sas2*Δ, *dot1*Δ, *H3K79A* or *H4K16R* mutations partially rescue the slow growth phenotype of *hst3*Δ *hst4*Δ cells that express Cdc45-HA (Figure 2.7E). We next sought to assess whether the *H4K16R* or *H3K79R* mutations improved the ability of *hst3*Δ *hst4*Δ mutants to complete DNA replication

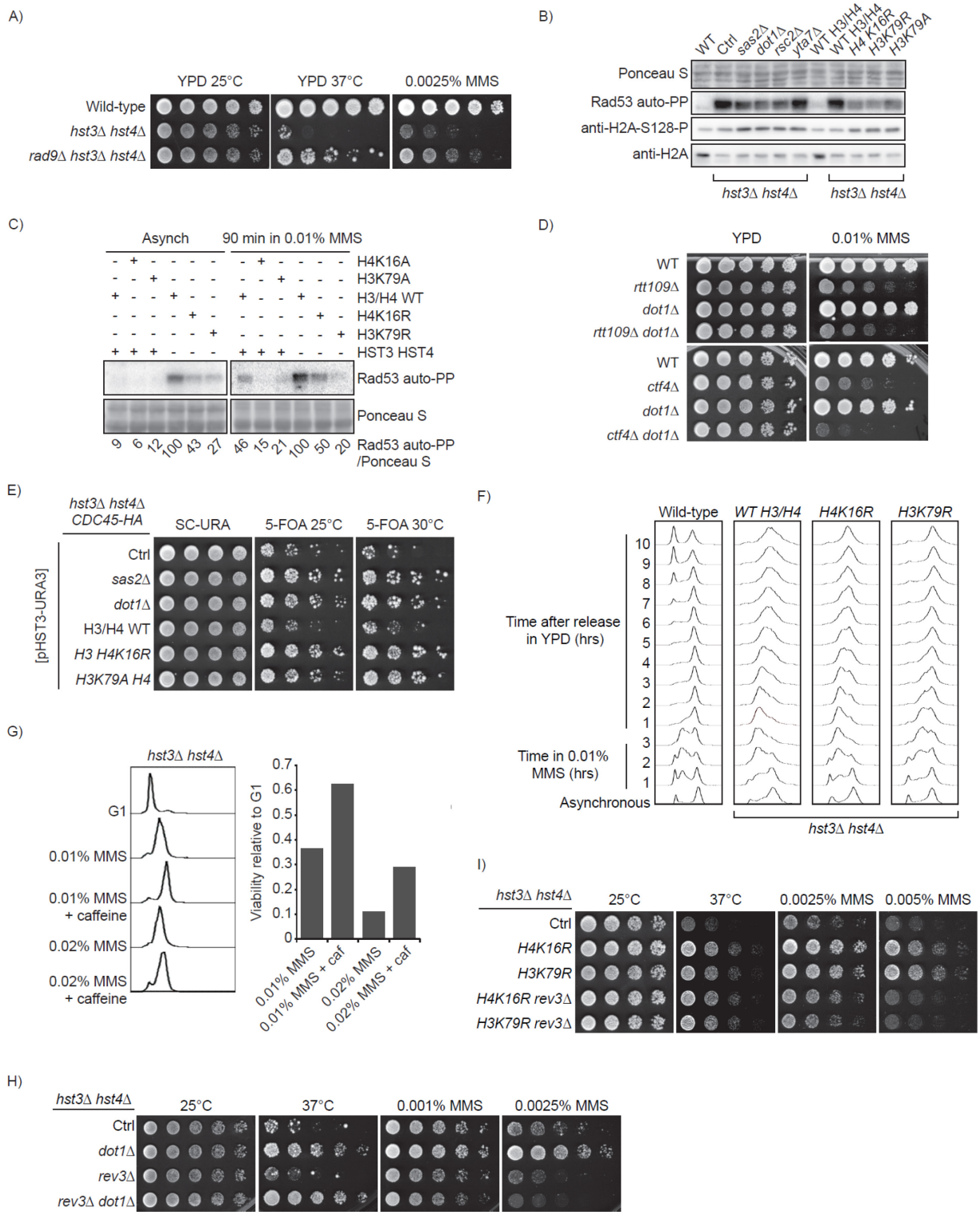

Figure 2.7. Mutations that decrease H4K16 acetylation or H3K79 methylation reduce the activity of the DNA damage response kinase Rad53 and suppress the phenotypes of *hst3Δ hst4Δ* cells.

Legend to figure 2.7. A) Deletion of *RAD9* partially rescues the phenotypes of cells lacking Hst3 and Hst4. Five-fold serial dilutions of cells were spotted onto YPD medium lacking or containing MMS, and incubated at 25°C or 30°C. B) Suppressor mutations reduce the spontaneous activity of Rad53 in *hst3*Δ *hst4*Δ mutants. Whole-cell lysates from cells growing exponentially at 25°C were prepared for immunoblotting and Rad53 autophosphorylation assays (see Materials and Methods). C) Suppressor mutations reduce Rad53 activation in *hst3*Δ *hst4*Δ mutants exposed to MMS. Exponentially growing cells of the indicated genotypes were exposed to 0.01% MMS for 90 minutes. Samples were then prepared for Rad53 autophosphorylation assays. Ponceaus S staining is used as loading control. D) Deletion of *DOT1* does not rescue the MMS sensitivity of *rtt109*Δ or *ctf4*Δ mutant cells. Cells were treated as in A except that they were incubated at 30°C. E) The sensitivity of *hst3*Δ *hst4*Δ mutants to replicative stress generated by epitope-tagging Cdc45 is rescued by mutations that reduce H3K79Me3 or H4K16Ac levels. Five-fold serial dilutions of cells were spotted onto SC-URA or 5-FOA plates and incubated at 25°C or at the semi-permissive temperature of 30°C. Ctrl: *hst3*Δ *hst4*Δ *cdc45-HA* strain without additional mutation. F) Exponentially growing cells were incubated in YPD containing 0.01% MMS for 180 minutes at 25°C. Cells were washed with YPD containing 2.5% sodium thiosulfate to inactivate MMS and then incubated in YPD. Samples were collected at the indicated times and processed to determine DNA content by FACS. G) Cells were arrested in G1 and released into the cell cycle in the presence of the indicated chemicals for 1.5h at 25°C (Right panel). Thes caffeine concentration was 0.1%. Viability was defined as the ratio of colonies that arose after MMS treatment to colonies formed by G1 cells that were not exposed to MMS. DNA content was analyzed by FACS for each sample (Left panel). H-I) Deletion of *REV3* compromises the effect of suppressor mutations on the MMS sensitivity of *hst3*Δ *hst4*Δ cells. Five-fold serial dilutions of cells were spotted onto YPD media lacking or containing MMS, and incubated at the indicated temperatures.

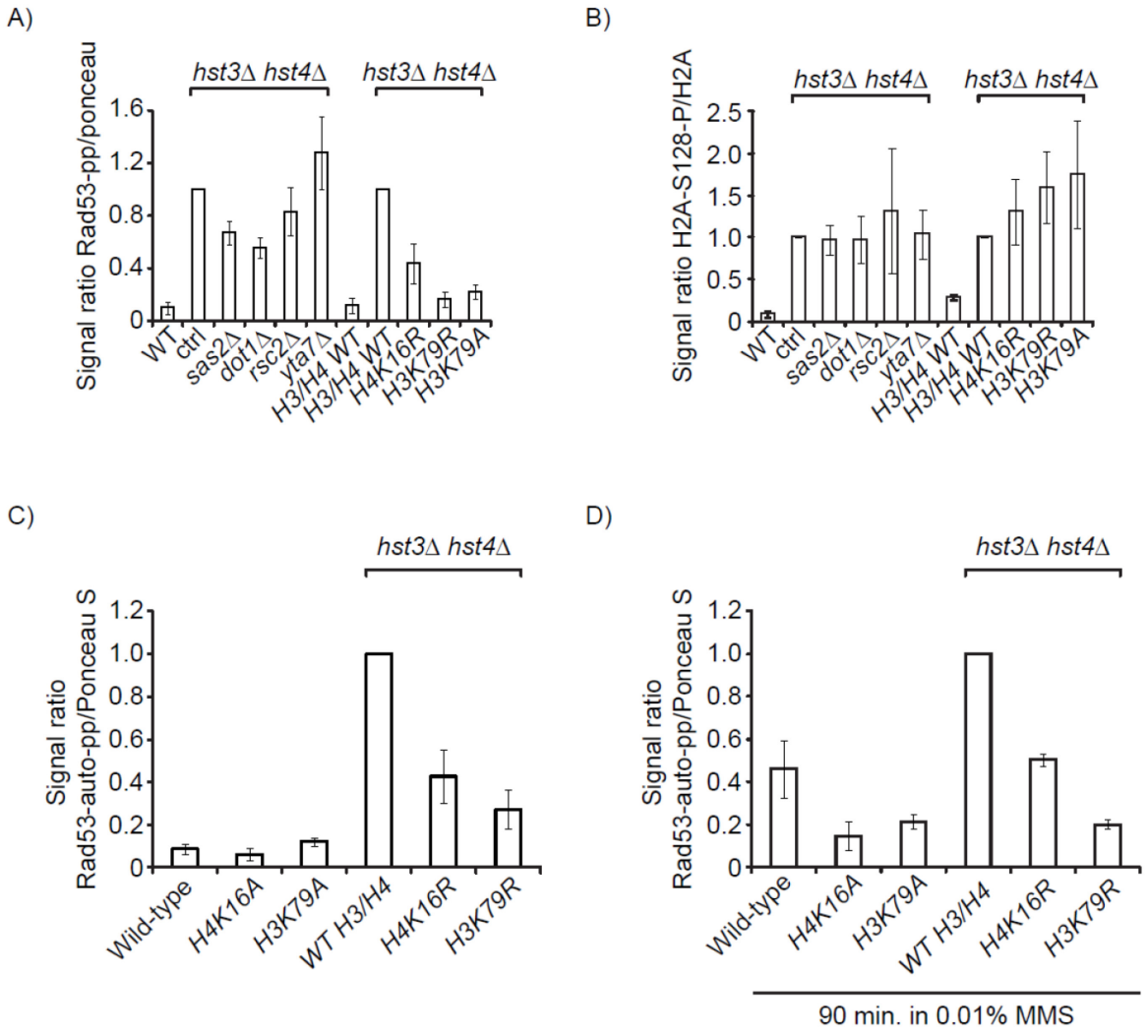

Figure 2.8. Densitometry analysis of immunoblots and Rad53 autophosphorylation assays shown in Figure 2.7B-C

A-B) Immunoblot or Rad53 autophosphorylation images from Figure 2.7B were analyzed by densitometry. Average signals relative to that observed in an isogenic *hst3*Δ *hst4*Δ strain are shown and error bars indicate the standard error of the mean (from at least 3 independent loadings of each sample).

after transient exposure to MMS (Figure 2.7F). DNA content analyses by FACS indicated that *hst3*Δ *hst4*Δ *H3K79R* mutant cells replicated a larger fraction of their genome after transient MMS exposure in comparison to *hst3*Δ *hst4*Δ cells. The effect of the *H4K16R* mutation was more subtle although, at late time points, G2/M peaks appeared sharper in *hst3*Δ *hst4*Δ *H4K16R* than in *hst3*Δ *hst4*Δ mutants. Taken together, these results indicate that mutations of H4K16 or H3K79, and gene mutations that cripple the acetylation or methylation of these residues, all enhance the ability of *hst3*Δ *hst4*Δ cells to survive conditions that induce replicative stress.

We investigated whether reduction of DNA damage response kinase activity would promote completion of DNA replication and survival of *hst3*Δ *hst4*Δ mutants exposed to MMS. *hst3*Δ *hst4*Δ cells were treated with MMS in the presence of caffeine, an inhibitor of the apical DNA damage response kinases Mec1 and Tel1 which are necessary for Rad53 activation (104). We found that this treatment significantly increased viability compared with the addition of MMS alone (Figure 2.7G). Moreover, FACS analysis demonstrated that caffeine treatment allowed *hst3*Δ *hst4*Δ cells to complete DNA replication more efficiently in the presence of MMS (Figure 2.7G). Importantly, the concentration of caffeine used had no effect on the survival of WT cells exposed to MMS (data not shown). These results are consistent with our hypothesis that partial reduction of DNA damage response kinases activity rescues the MMS sensitivity of cells lacking Hst3 and Hst4.

Published reports indicate that mutation of *DOT1,* and consequent reduction of DNA damage response kinase activity, promotes translesion DNA synthesis in response to MMS via molecular mechanisms that remain unclear (101, 102). Interestingly, we found that deletion of *REV3* (encoding the catalytic subunit of DNA polymerase Zeta involved in MMS-induced DNA lesion bypass) strongly reduced the suppressive effect of *dot1*Δ, *H4K16R* and *H3K79R* mutations on the MMS sensitivity of *hst3*Δ *hst4*Δ cells (Figure 2.7H-I). This suggest that the aforementioned suppressor mutations may act, at least in part, by promoting DNA damage tolerance via the translesion synthesis pathway in *hst3*Δ *hst4*Δ cells exposed to MMS. On the other hand, the *rev3*Δ mutation does not compromise the suppressive effect of *dot1*Δ, *H4K16R* and *H3K79R* on the temperature-sensitive (Ts-) phenotype of *hst3*Δ *hst4*Δ cells (Figure 2.7H-I), indicating that this effect is mediated via Rev3-independent pathways.

2.4.5 Links between the temperature and genotoxic agent sensitivity of *hst3*Δ *hst4*Δ mutants.

The basis of the Ts- phenotype, and its relationship to the genotoxic sensitivity of *hst3*Δ *hst4*Δ mutants is poorly understood. We isolated and characterized 12 independent spontaneous suppressors of the Ts- phenotype (see Materials and Methods). Aliquots from twelve cultures of *hst3*Δ *hst4*Δ cells carrying a *URA3 CEN* plasmid encoding WT *HST3* were plated on agar media containing 5-fluoro-orotic acid (5-FOA) at 37°C. This forces surviving cells to lose the plasmid encoding *HST3* and *URA3*, thus selecting for *hst3*Δ *hst4*Δ mutant cells that can form colonies at 37°C because they acquire a genetic/epigenetic change that suppresses the Ts- phenotype. We found that only 1 of the 12 thermoresistant (Tr) isolates was as sensitive to chronic MMS exposure as the parental *hst3*Δ *hst4*Δ strain, and that two of the Tr isolates were HU-sensitive (Figure 2.9A), demonstrating that the Ts- and genotoxic agent sensitivity of *hst3*Δ *hst4*Δ cells are generally linked.

As mentioned above, mutations that prevent H3K56 acetylation partially suppress the phenotypes of *hst3*Δ *hst4*Δ cells (17, 23, 44, 74). Nevertheless, a previous study reported that spontaneous *hst3*Δ *hst4*Δ suppressors rarely manifest reduced H3K56Ac levels (74). Here we found that H3K56Ac was below detection threshold in only one of the isolated suppressors of the Ts- phenotype (Figure 2.9B: Tr11). Rtt109 and Asf1 are both required for H3K56 acetylation (20, 21). In order to understand why H3K56Ac was undetectable in this Tr isolate, we epitope-tagged either Rtt109 or Asf1 in this strain. Tr11 showed no decrease in the abundance of Asf1, but the Rtt109-Flag protein was undetectable despite the fact that the *RTT109* gene was appropriately epitope-tagged at its endogenous locus (Figure 2.10F-G). Sequencing of the *RTT109* open reading frame in Tr11 revealed a cytosine to adenine mutation at position 597 that generates a premature stop codon. We conclude that spontaneous mutation of *RTT109* is a mechanism of phenotypic suppression in *hst3*Δ *hst4*Δ mutants.

The other Tr strains do not generally show strong decreases in H3K56Ac, H4K16 acetylation of H3K79 tri-methylation, as assessed by immunoblotting (Figure 2.9B, Figure 2.10A-C). In contrast, most of the Tr isolates that we generated displayed reduction of Rad53 activity and Mec1-mediated histone H2A serine 128 phosphorylation (Figure 2.9B, Figure

2.10D-E). Overall, the data indicate that phenotypic suppression of *hst3*Δ *hst4*Δ is frequently accompanied by a reduction in DNA damage response kinases activity.

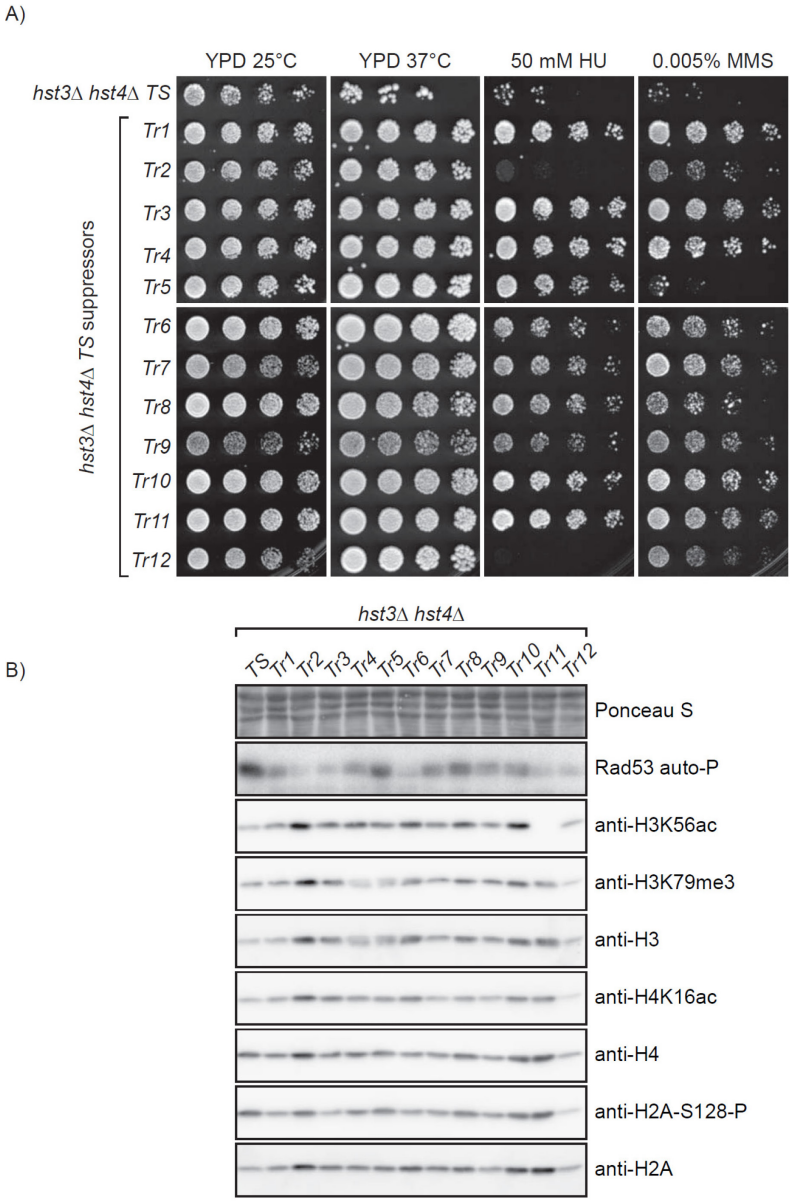

Figure 2.9. Spontaneous suppressors of *hst3*Δ *hst4*Δ mutant phenotypes exhibit reduced Rad53 activity.
A) Five-fold serial dilutions were spotted on YPD plates containing the indicated concentration of genotoxic agents and incubated at either 25°C or 37°C. TS is the starting hst3Δ hst4Δ mutant strain from which spontaneous thermo-resistant (Tr) suppressors were isolated. B) Immunoblotting were prepared from whole-cell lysates of exponentially growing cells. Ponceau S staining is shown as loading control.

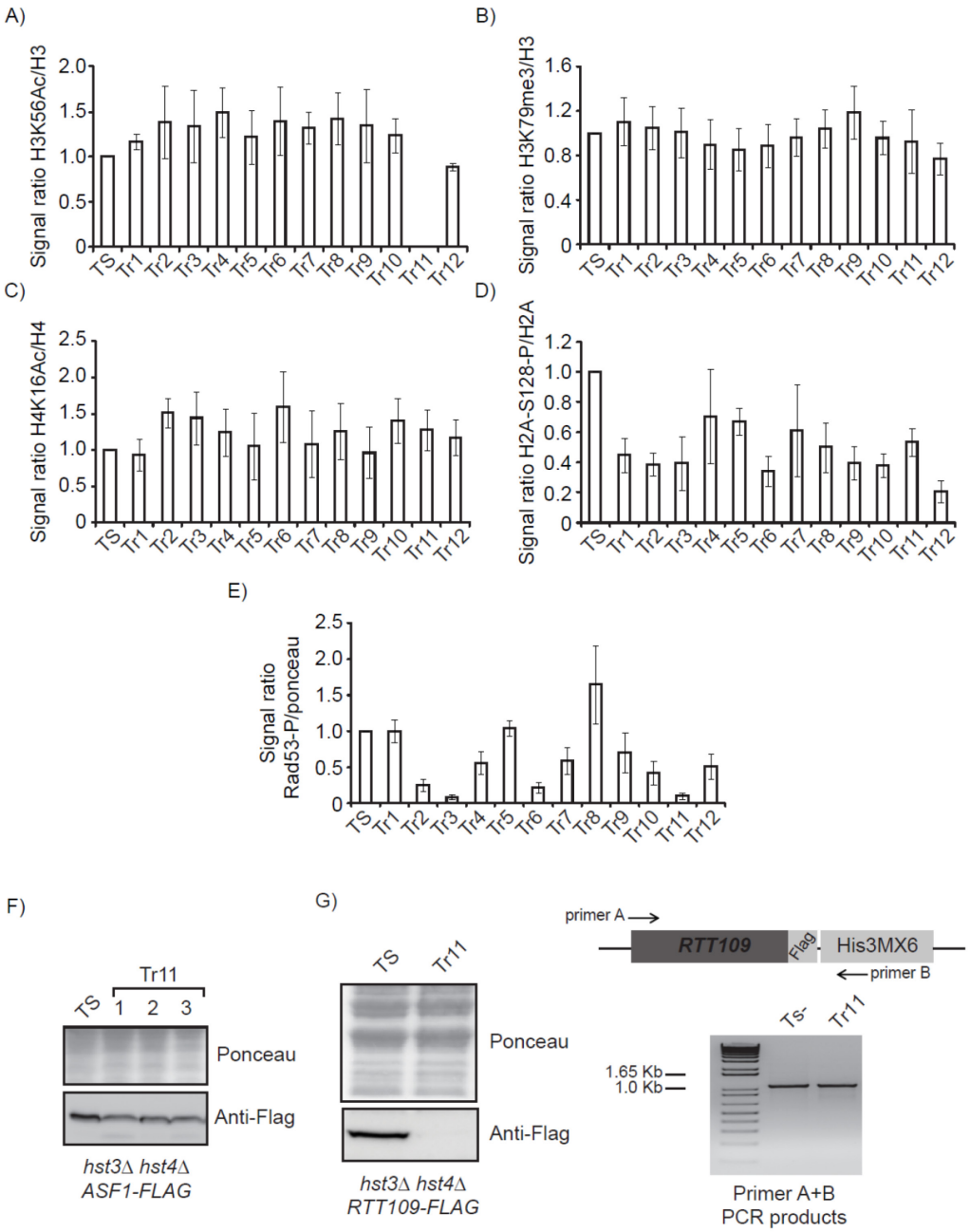

Figure 2.10. Densitometry analysis of immunoblots and Rad53 autophosphorylation assays shown in Figure 2.9B.

Legend to figure 2.10. A-E) Immunoblots and Rad53 autophosphorylation images from figure 2.9B were analyzed by densitometry. The y-axes represent ratio of the signals obtained in each thermo-resistant strain (Tr) relative to the signal observed in the parental *hst3*Δ *hst4*Δ TS strain. Error bars indicate the standard error of the mean (at least 3 independent loading of each sample). F) Asf1 was epitope-tagged in the *Tr11* thermo-resistant spontaneous suppressor derived from *hst3*Δ *hst4*Δ Ts- mutant cells. Three independent clones derived from tagging Asf1 in the Tr11 strain were selected. Immunoblots of whole-cell lysates were probed to detect Asf1-Flag. Ponceau S staining is shown as loading control. G) The Rtt109-Flag protein is not detectable in the *Tr11* spontaneous suppressor of *hst3Δ hst4Δ* that lack H3K56Ac. Rtt109-Flag was detected by immunoblotting in whole-cell lysates of exponentially growing cells probed with a Flag antibody. (Right panel) Location of PCR primers used to ensure that DNA integration correctly resulted in an *RTT109-Flag* gene and PCR results showing that the *RTT109-Flag* gene is present in each of the strains analyzed for Rtt109-Flag protein expression in the left panel.

2.5 Discussion

Previous genetic studies established that the temperature and genotoxic drug sensitivity of cells that are incapable of deacetylating H3K56 can be suppressed by secondary mutations (17, 27, 44) and that interfering with the DNA replication machinery was detrimental to *hst3*Δ *hst4*Δ mutants (44). Nevertheless, a detailed molecular analysis of the response to replicative stress in *hst3*Δ *hst4*Δ cells was lacking. Here, we showed that *hst3*Δ *hst4*Δ cells cannot complete chromosome duplication after transient exposure to MMS or HU during S-phase, leading to severe loss of cell viability and formation of persistent Rad52 foci. In general our results are consistent with studies that reported abnormally high frequencies of spontaneous Rad52 foci in *H3K56R*, *rtt109*Δ and *hst3*Δ *hst4*Δ mutants (32, 38, 105). Recently published data also indicate that replication-associated DNA double-strand breaks (DSBs) require proper levels of H3K56Ac for repair by HR-dependent sister chromatid exchange (105). Based on this, it is possible that persistent Rad52 foci in *hst3*Δ *hst4*Δ mutants transiently exposed to MMS represent abnormal sister chromatid exchange intermediates, which in turn could prevent replication restart (59). Even though our results reveal dysfunctional Rad52 activity in cells lacking Hst3 and Hst4, we previously showed that *hst3*Δ *hst4*Δ *rad52*Δ are not viable (44). In contrast, the Rad51, Rad54, Rad55 and Rad57 proteins are dispensable for viability of *hst3*Δ *hst4*Δ cells (44). We speculate that a subset of Rad52-dependent but Rad51-independent HR events may promote survival of *hst3*Δ *hst4*Δ mutants in response to DNA lesions that impede replication, and that other Rad52-dependent events (such as SCE) cannot be completed successfully in these mutants (105). Our observation of anaphase chromatin bridges in a large fraction of *hst3*Δ *hst4*Δ cells containing persistent Rad52 foci (Figure 2.2B) is consistent with this model. Indeed, such bridges are expected to form in cells that enter anaphase in the presence of incompletely replicated chromosomes and/or unresolved HR structures, and have been observed in response to MMS in several replicative stress-sensitive mutants (64).

Our results clearly show that H3K79 methylation and H4K16 acetylation contribute significantly to the phenotypes of cells presenting H3K56 hyperacetylation. Interestingly, our immunoblot and mass spectrometry assays indicate that the *H4K16R* mutation reduces global H3K79 tri-methylation levels, while increasing both mono- and di-methylation at this residue.

Potential links between H4K16 acetylation and Dot1-mediated H3K79 methylation have been investigated in previous studies using immunoblotting (81, 82, 106). Consistent with our mass spectrometry data, Evans *et al*. found that cells expressing *H4K16R* mutant histones presented elevated levels of mono- and di-methylated H3K79. However, no published study had yet reported decreased global H3K79 tri-methylation in *H4K16R* mutants. Dot1-mediated methylation of histone H3 depends on its interaction with a short basic patch of residues in the N-terminal tail of histone H4 (106). Current models propose that Dot1 and the Sir3 subunit of the SIR silencing complex compete for binding to this region of H4, and that H4K16 acetylation may promote Dot1-mediated H3K79 methylation by displacing Sir3. Indeed, overexpression of the Sas2 H4K16 acetyltransferase increased the levels of both H4K16ac and H3K79 trimethylation at subtelomeric regions, suggesting that these two modifications are functionally linked (106). Nevertheless, the extent to which reduction in H3K79 tri-methylation may contribute to the effect of *H4K16R* mutation on *hst3*Δ *hst4*Δ cells remains unclear. We also recognize that mutations of H3K79 and H4K16 to arginine residues may have consequences that go beyond reduction of their associated histone modifications, although the contribution of modification-independent effects in mediating the phenotypes of *hst3*Δ *hst4*Δ mutants is difficult to assess.

Based on mass spectrometry it was reported that approximately 85% of H4 molecules are K16-acetylated and 90% of H3 are K79-methylated in asynchronous WT yeast (75, 80). Although H4K16Ac and H3K79Me are very abundant in *S. cerevisiae*, they are absent from heterochromatic regions (77, 78, 80, 84). The boundaries between euchromatin and heterochromatin are characterized by a transition from nucleosomes that contain H4K16Ac/H3K79Me to nucleosomes lacking these modifications. Interestingly, we found that *rsc2*Δ and *yta7*Δ mutations partially suppress the temperature and MMS sensitivity of *hst3*Δ *hst4*Δ cells, albeit to a lesser extent than the *H4K16R* mutation (Figure 2.6A-C). Rsc2 and Yta7 have been implicated in preventing heterochromatin spreading and Yta7 can be detected near chromatin boundaries (83, 84, 89). Although Rsc2 and Yta7 have roles in other processes such as DSB repair (Rsc2) and gene transcription (Yta7) (88, 107, 108), it is tempting to speculate that mutations which reduce levels of either H3K79Me and H4K16Ac may suppress the phenotypes of *hst3*Δ *hst4*Δ cells in part by modulating the activity of Rsc2 and Yta7. The precise molecular mechanisms involved are currently unknown. The polypeptide subunits of the Rsc2

complex collectively contain five bromodomains (109), which are protein domains involved in binding acetylated lysine residues within specific structural contexts (110), and Yta7 also contains a bromodomain-like domain (83). It is possible that binding of RSC and/or Yta7 to chromatin containing both H4K16ac and abnormally elevated stoichiometries of H3K56Ac may interfere with the processing of DNA lesions that impede replication (*e.g.* MMS-induced 3-methyladenine). Alternatively, abnormal expression of specific genes due to crippled chromatin boundaries may partly account for the effect of *RSC2* or *YTA7* deletion on the phenotypes of *hst3*Δ *hst4*Δ cells. Further studies will be required to investigate the validity of these models.

Our data provide compelling evidence in support of a role for H4K16Ac and H3K79Me in promoting persistent activation of DNA damage response signalling in *hst3*Δ *hst4*Δ cells (Figure 2.7). H3K79 methylation is critical for chromatin binding and optimal activation of the Rad9 adaptor protein, which in turns permits full activation of the kinase Rad53 in response to DNA damage (96–99). Based on four main lines of evidence, we propose that this function of H3K79 methylation is deleterious to *hst3*Δ *hst4*Δ mutants. In *hst3*Δ *hst4*Δ cells: (i) reduction in H3K79Me, via mutations of the Dot1 methyltransferase or H3K79R, decreases spontaneous and MMS-induced Rad53 activation (Figure 2.7B-C); (ii) deletion of *RAD9* itself partially suppresses the temperature and genotoxic drug sensitivity (Figure 2.7A); (iii) caffeine treatment markedly alleviates the lethality induced by MMS (Figure 2.7G); and (iv) several spontaneous suppressors of the Ts- phenotype display reduced activation of the kinase Rad53 (Figure 2.9B). This interpretation is also consistent with our previously published observations indicating that a null mutation of *RAD24,* encoding the large subunits of a replication factor C-related complex responsible for loading the PCNA-like 9-1-1 complex, suppresses the Ts- phenotype of *hst3*Δ *hst4*Δ mutants (44). Similarly, mutation of the genes encoding the three 9-1-1 subunits (*MEC3*, *DDC1* and *RAD17*) also suppress the Ts- phenotype of *hst3*Δ *hst4*Δ mutants without decreasing H3K56Ac (44). Since loading of 9-1-1 clamps at sites of DNA damage promotes DNA damage checkpoint activation (111), these genetic data are consistent with the notion that persistent activation of DNA damage response kinases is detrimental to the survival *of hst3*Δ *hst4*Δ mutants.

It seems plausible that persistent activation of DNA damage response (DDR) kinases may contribute to the phenotypes of *hst3*Δ *hst4*Δ mutants through various mechanisms that are not mutually exclusive. For example, a well known function of Rad53 is to inhibit the firing of

late DNA replication origins (112, 113). Persistent activity of DDR kinases in *hst3*Δ *hst4*Δ cells transiently exposed to MMS could inhibit the firing of at least a subset of late DNA replication origins, thus preventing cells from completing replication where forks are permanently blocked by MMS lesion. Our results also argue that Rad53-mediated inhibition of translesion DNA synthesis (TLS) contributes to the MMS sensitivity of *hst3*Δ *hst4*Δ mutants (Figure 2.7H-I). This data is in line with published results showing that reduction of DDR kinase activity, and concomitant increase in TLS, partially suppresses the MMS sensitivity caused by deletion of genes such as *RAD52* or *RTT107*(100–102). Interestingly, deletion of the *REV3* gene encoding the catalytic subunit of translesion DNA polymerase Zeta does not influence suppression of the Ts- phenotype of *hst3*Δ *hst4*Δ cells by *dot1*Δ, *H3K79R* or *H4K16R* (Figure 2.7H-I). Further studies will be required to identify the mechanisms through which these mutations suppress the Ts- phenotype of *hst3*Δ *hst4*Δ mutants since its molecular basis remains poorly understood.

In fission yeast and human cells, the vast majority of histone H4 molecules are methylated at lysine 20 (H4K20Me) (114) (A. Verreault, unpublished results), a modification whose role in the DNA damage response is functionally related to that of *S. cerevisiae* H3K79Me. Indeed, orthologs of Rad9 in fission yeast (Crb2) and humans (53BP1) have been reported to interact with H4K20Me, which demonstrates an evolutionarily conserved link between histone methylation and DNA damage-induced signalling (115–117). Taken together, our results suggest that, in addition to abundant modifications such as H4K20Me in *S. pombe* and human cells or H3K79Me in *S. cerevisiae*, genome-wide deacetylation of newly synthesized histones (H3K56Ac in *S. cerevisiae* and *S. pombe* and, possibly, N-terminal acetylation sites of H3 and H4 in human cells) may be critical for appropriate regulation of DDR kinases and cell survival in response to DNA damage.

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Introduction au chapitre 3

Cet article a permis de révéler que la signalisation anormale en réponse aux dommages à l'ADN contribue aux phénotypes associés à l'acétylation constitutive d'H3K56. De plus, nous avons mieux caractérisé le lien entre H3K79me3 et H4K16ac et démontré que ces deux modifications influencent l'activation de la réponse aux dommages à l'ADN. Néanmoins, plusieurs questions demeurent, notamment pourquoi l'hyperacétylation d'H3K56 cause une hyperactivation de la réponse aux dommages à l'ADN, mais aussi, pourquoi cette hyperactivation est-t-elle toxique pour les cellules?

Pour tenter de répondre à ces questions nous avons effectué un second crible, celui-ci dans le but d'identifier des mutants qui influencent la croissance de cellules exposées au nicotinamide (NAM). Tel que mentionné plus haut, le NAM inhibe l'activité de toutes les sirtuines et cause ainsi l'accumulation d'H3K56ac et d'H4K16ac par la perte de fonctions d'Hst3, Hst4 et Sir2. Ceci nous a permis d'identifier une panoplie de gènes de la réponse aux dommages à l'ADN nécessaires à la croissance en présence d'hyperacétylation d'H3K56. Parmi ceux-ci, nous avons caractérisé plus en détails deux gènes, *SLX4* et *PPH3*, codant pour des facteurs qui antagonisent la signalisation en réponse aux dommages à l'ADN. Les résultats découlant de ces expériences ont été publiés dans la revue *Nucleic Acids Research* en 2016 (544).

J'ai activement participé à la conception du projet (50%) et j'ai effectué la plupart des expériences et analyses sous-jacentes aux figures de cet article (70%). J'ai monté toutes les figures (100%) et rédigé le premier brouillon du papier, qui a ensuite été corrigé et édité en collaboration avec Hugo Wurtele (50%).

Ricard É, Hammond-Martel I : 15% expériences et analyses
Weber S, Raymond M : 5% expériences et analyses
Wong LH, Sellam A, Giaever G, Nislow C : 10% expériences et analyses
Wurtele H : 50% rédaction 50% conceptualisation

Chapitre 3. Chromosome-wide histone deacetylation by sirtuins prevents hyperactivation of DNA damage-induced signalling upon replicative stress

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3.1 Abstract

The *Saccharomyces cerevisiae* genome encodes five sirtuins (Sir2 and Hst1-4), which constitute a conserved family of NAD-dependent histone deacetylases. Cells lacking any individual sirtuin display mild growth and gene silencing defects. However, *hst3*Δ *hst4*Δ double mutants are exquisitely sensitive to genotoxins, and *hst3*Δ *hst4*Δ *sir2*Δ mutants are inviable. Our published data also indicate that pharmacological inhibition of sirtuins prevents growth of several fungal pathogens, although the biological basis is unclear. Here, we present genome-wide fitness assays conducted with nicotinamide (NAM), a pan-sirtuin inhibitor. Our data indicate that NAM treatment causes yeast to solicit specific DNA damage response pathways for survival, and that NAM-induced growth defects are mainly attributable to inhibition of Hst3 and Hst4 and consequent elevation of histone H3 lysine 56 acetylation (H3K56ac). Our results further reveal that in the presence of constitutive H3K56ac, the Slx4 scaffolding protein and PP4 phosphatase complex play essential roles in preventing hyperactivation of the DNA damage-response kinase Rad53 in response to spontaneous DNA damage caused by reactive oxygen species. Overall, our data support the concept that chromosome-wide histone deacetylation by sirtuins is critical to mitigate growth defects caused by endogenous genotoxins.

3.2 Introduction

Post-translational modification of histones can directly influence chromatin structure, or serve as platforms for the recruitment of regulatory factors, thereby modulating DNA-associated processes (1). Acetylation of histone lysine residues is catalysed by histone acetyltransferases (HATs), and reversed by histone deacetylases (HDACs). Sirtuins are an evolutionarily conserved family of HDACs that deacetylate lysines in a reaction that consumes nicotinamide adenine dinucleotide (NAD^+) and releases nicotinamide and O-acetyl ADP ribose (2, 3). These enzymes are found in archaea, eubacteria, and eukaryotes (2) where they regulate key cellular pathways, e.g. metabolic processes, DNA replication and repair, telomere structure and function, gene expression, and replicative lifespan (4).

The *Saccharomyces cerevisiae* genome contains five sirtuin genes: *HST1-4* and *SIR2* (5, 6). Yeast Sir2 is the founding member of this family of enzymes, and was identified on the basis of its role in regulating gene silencing at the yeast mating loci (6), rDNA (7), and telomeres (8). These functions of Sir2 can be attributed in part to reversal of histone H4 lysine 16 acetylation (H4K16ac), an abundant and conserved modification of transcriptionally active chromatin (9, 10). Sir2 activity influences replicative life-span by limiting recombination in rDNA and consequent formation of age-associated extrachromosomal ribosomal DNA circles (ERCs) (11, 12). Hst1 (Homolog of Sir Two) shares sequence similarity with Sir2 but presents divergent functions (13, 14); this enzyme negatively regulates middle sporulation gene expression (15, 16), and controls intracellular NAD^+ levels and thiamine biosynthesis through transcriptional repression (17, 18). Although Hst2 contains a nuclear export signal that mediates its cytosolic localisation (19), it can deacetylate H4K16ac and influence cellular aging in the absence of Sir2 (20). Moreover, overexpression of Hst2 results in rDNA and telomeric silencing that can compensate for *sir2*Δ defects, indicating that its functions partially overlap with those of Sir2 in the nucleus (21).

Yeast mutants lacking any one of the five sirtuins display relatively mild growth phenotypes (5). In contrast, *hst3*Δ *hst4*Δ double mutants grow poorly, and combining these two mutations with *sir2*Δ causes synthetic lethality via poorly understood mechanisms (5). Hst3 and Hst4 present remarkable selectivity for acetylated H3K56 in several fungal species, and exert

partially redundant roles in deacetylating this residue (22–24). H3K56ac is catalyzed by the HAT Rtt109 and is found in virtually all newly-synthesized histone H3 deposited behind DNA replication forks in S phase (25–29). Hst3 and Hst4 are expressed in late S-G2/M and G1-G2/M, respectively, when they deacetylate nucleosomal H3K56ac genome-wide (23, 30). Cells lacking both Hst3 and Hst4 present constitutively acetylated H3K56 throughout the cell cycle, and exhibit thermosensitivity, spontaneous DNA damage, and extreme sensitivity to genotoxin-induced replicative stress (23, 31, 32). These severe phenotypes are partially suppressed by mutations that prevent H3K56ac, e.g. *H3K56R*, suggesting that they are caused in large part by defective regulation of H3K56ac (31).

DNA lesions that impede the progression of replication forks activate a signalling cascade which is regulated by the apical kinase Mec1 (33). In response to genotoxic stress, Hst3 is targeted for proteasomal degradation in a Mec1-dependent manner, causing chromatin-borne H3K56ac to persist in G2/M (27, 34). The fact that cells have evolved this capacity to preserve nucleosomal H3K56ac in response to replicative stress suggests that this modification may modulate certain aspects of the DNA damage response (DDR). Consistent with this, abnormal regulation of H3K56ac negatively influences homologous recombination-mediated sister chromatid exchange and break-induced replication (35–37). In addition, certain mutations in histone or DDR genes influence the severity of phenotypes caused by Hst3 and Hst4 deficiency (31, 32). For example, the temperature and genotoxin sensitivity of *hst3*Δ *hst4*Δ mutants is suppressed by mutations abolishing H3K79 methylation, a histone modification known to promote Rad9 chromatin binding and subsequent activation of the Rad53 DDR kinase (32). These data suggest that DNA damage-induced signaling may contribute to the phenotypes of cells presenting constitutive H3K56ac, although the mechanisms remain poorly understood at the molecular level.

Nicotinamide (NAM) is a non-competitive pan-inhibitor of several NAD-dependent enzymes, including HDACs of the sirtuin family (2, 38, 39). Our previously published results indicate that NAM-induced sirtuin inhibition prevents growth of the pathogenic fungus *Candida albicans* by causing constitutive H3K56ac (22). To further understand this phenomenon, we performed genome-wide fitness assays to identify genes that influence growth of *Saccharomyces cerevisiae* in the presence of NAM. The data reveal that sirtuin-mediated

deacetylation of H3K56ac promotes cell growth by preventing persistent activation of DNA damage-induced kinases in response to endogenous genotoxins.

3.3 Materials and methods

3.3.1 Yeast strains and growth conditions

Strains used in this study are listed in Table 3.I and were generated and propagated using standard yeast genetics methods. Nicotinamide and methyl methanesulfonate (MMS) were purchased from Sigma-Aldrich.

3.3.2 Growth assays in 96 well plates

Cells were grown overnight in YPD in a humid chamber at 30°C. Cells were then diluted to OD_{600} 0.0005 in 100 µl YPD containing nicotinamide in flat-bottomed 96 well plates. Plates were incubated for 48 hours at 30°C in a humid chamber and OD_{630} was measured using a Biotek EL800 plate reader equipped with Gen5 version 1.05 software (Biotek instruments). OD_{630} from blank wells (YPD) was subtracted from OD_{630} readings and growth was normalized to untreated controls for each strain. Experiments were performed at least in triplicate and error bars represent the standard error of the mean of normalized growth. To calculate population doubling time, cells were grown overnight in YPD at 30°C. Cells were then diluted to OD_{600} 0.01 in 100 µl YPD with or without 20 mM NAM in flat-bottomed 96 well plates. Cells were incubated for 48 hours at room temperature with shaking in a Biotek ELX808 and OD_{630} readings taken every 30 minutes. OD_{630} readings were plotted on a graph, and exponential regression was used to calculate doubling times.

Table 3.I. Strains used in this study

Strain	Genotype	Reference
BY4741	BY4741 *MATa ura3Δ0 leu2Δ0 his3Δ1*	(40)
BY4743	BY4743 *MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*	(40)
W303	W303 *MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* [*psi+*] *rad5-535*	(41)
W5094-1C	W303 *ADE2 RAD52-YFP RAD5*	(42)
HWY2493	W303 *ADE2 bar1Δ::LEU2 RFA1-8ala-YFP RAD5*	(32)
HWY297	BY4741 *rtt109Δ::KanMX*	This study
ASY3111	YBL574 *hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3* [*pCEN TRP1 HHT1-HHF1*]	(43)
ASY3113	YBL574 *hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3* [*pCEN TRP1 HHT-hhf1K16A*]	(43)
HWY2949	YBL574 *hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3* [*pCEN TRP1 HHT1-HHF1*] *rtt109Δ::URA3MX*	This study
ASY3180	BY4741 *dpb4Δ*::KanMx	This study
HWY2417	BY4741 *dun1Δ::KanMX*	This study
HWY634	BY4741 *srs2Δ::KanMX*	This study
ASY3188	BY4741 *tof1Δ::KanMX*	This study
ASY3193	BY4741 *sae2Δ::KanMX*	This study
HWY2477	BY4741 *mrc1Δ::KanMX*	This study
HWY2460	BY4741 *mrc1Δ::KanMX rtt109Δ::URA3MX*	This study
ASY1767	BY4741 *yku70Δ::KanMX*	This study
ASY3164	BY4741 *yku70Δ::KanMX rtt109Δ::HPHMX*	This study
EHY027	BY4741 *rad59Δ::KanMX*	This study
EHY029	BY4741 *rad59Δ::KanMX rtt109Δ::URA3MX*	This study
ASY2807	BY4741 *pol32Δ::KanMX*	This study
ASY3159	BY4741 *pol32Δ::KanMX rtt109Δ::HPHMX*	This study
HWY1608	BY4741 *slx4Δ::KanMX*	This study
ASY1875	BY4741 *slx4Δ*::HPHMX *rtt109Δ::URA3MX*	This study
HWY1610	BY4741 rad1Δ::kanMX 25C10	This study
HWY1609	BY4741 slx1Δ::kanMX 10E7	This study
ASY3147	BY4741 *mus81Δ::HPHMX*	This study
HWY3228	BY4741 *mms4Δ::KanMX*	This study
ASY2164	BY4741 *rtt101Δ::URA3MX*	This study
ASY2168	BY4741 *rtt101Δ::URA3MX slx4Δ::HPHMX*	This study
ASY2166	BY4741 *rtt107Δ::KanMX*	This study
ASY2163	BY4741 *rtt107Δ::KanMX slx4Δ::HPHMX*	This study
HWY525	BY4741 *rtt107Δ::KanMX*	This study
HWY530	BY4741 *rtt107Δ::KanMX rtt109Δ::URA3MX*	This study
ASY1763	BY4741 *psy2Δ::KanMX*	This study
ASY1764	BY4741 *psy4Δ::KanMX*	This study
ASY1765	BY4741 *pph3Δ::KanMX*	This study
ASY1840	BY4741 *pph3Δ::HPHMX rtt109Δ::URA3MX*	This study
EHY047	BY4741 *rad9Δ::KanMX*	This study
ASY2796	BY4741 *rad9Δ::KanMX pph3Δ::HPHMX*	This study
ASY3516	BY4741 *slx4Δ::KanMX rad9Δ::HPHMX*	This study
EHY071	BY4741 *dot1Δ::KanMX*	This study
ERY3386	BY4741 *slx4Δ::KanMX dot1Δ::URA3MX*	This study
ERY3389	BY4741 *pph3Δ::HPHMX dot1Δ::URA3MX*	This study
	FY406 *MATa hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* [*pCEN HIS3 HTB1-HTA1*]	(43)
ERY3394	FY406 *hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* [*pCEN HIS3 HTB1-HTA1*] *pph3Δ::HPHMX*	This study
ERY3396	FY406 *hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* [*pCEN HIS3 HTB1-hta1S128A*] *pph3Δ::HPHMX*	This study
HWY2878	FY406 *hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* [*pCEN HIS3 HTB1-HTA1*] *slx4Δ::KanMX*	This study
HWY2879	FY406 *hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* [*pCEN HIS3 HTB1-hta1S128A*] *slx4Δ::KanMX*	This study
HWY1936	FY406 *hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1* [*pCEN HIS3 HTB1-hta1S128A*]	This study
ASY2766	W303 *ADE2 RAD52-YFP RAD5 slx4Δ::HPHMX*	This study
ASY2764	W303 *ADE2 RAD52-YFP RAD5 pph3Δ::HPHMX*	This study
Y2573	W303 *dbf4Δ::TRP1 his3::PDBF4-dbf4-4A::HIS3 sld3-38A-10his-13MYC::KanMX4*	(44)
ERY3414	W303 *dbf4Δ::TRP1 his3::PDBF4-dbf4-4A::HIS3 sld3-38A-10his-13MYC::KanMX4 slx4Δ::HPHMX*	This study

Table 3.I (continued)

Strain	Genotype	Reference
ERY3415	W303 *dbf4Δ::TRP1 his3::PDBF4-dbf4-4A::HIS3 sld3-38A-10his-13MYC::KanMX4 pph3Δ::HPHMx*	This study
ASY2798	W303 *pph3Δ::HPHMX*	This study
HWY2882	W303 slx4Δ::*HPHMX*	This study
HWY2942	BY4741 *slx4Δ::KanMX dot1Δ::URA3MX rev3Δ::HIS3MX*	This study
ASY3534	BY4741 *pph3Δ::HPHMX dot1Δ::URA3MX rev3Δ::KanMX*	This study
ASY3667	BY4741 *pol30Δ::KANMX trp1Δ::KANMX slx4Δ::HPHMX rad9Δ::HIS3MX* [*pCEN-POL30-TRP1*]	This study
ASY3668	BY4741 *pol30Δ::KANMX trp1Δ::KANMX slx4Δ::HPHMX rad9Δ::HIS3MX* [*pCEN-pol30-K164R-TRP1*]	This study
ASY3669	BY4741 *pol30Δ::KANMX trp1Δ::KANMX pph3Δ::HPHMX rad9Δ::HIS3MX* [*pCEN-POL30-TRP1*]	This study
ASY3670	BY4741 *pol30Δ::KANMX trp1Δ::KANMX pph3Δ::HPHMX rad9Δ::HIS3MX* [*pCEN-pol30-K164R-TRP1*]	This study
HWY630	BY4741 *rad18Δ*::KanMX	This study
HWY636	BY4741 *mms2Δ*::KanMX	This study
ASY3522	BY4741 *slx4Δ::KanMX dot1Δ::URA3 rad18Δ::HIS3MX*	This study
HWY2939	BY4741 slx4Δ::kanMX dot1Δ::URA3MX *mms2Δ::HIS3MX*	This study
ASY3651	BY4741 *pph3Δ::KanMX rad18Δ::HIS3MX rad9Δ::URA3MX*	This study
ASY3654	BY4741 *pph3Δ::KanMX mms2Δ::HPHMX rad9Δ::URA3MX*	This study
ASY3519	BY4741 *pph3Δ::HPHMX rev3Δ::KanMX*	This study
HWY2940	BY4741 *slx4Δ::KANMX rev3Δ::HIS3MX*	This study
ICY703	FY833 *hst3Δ::HIS3 hst4Δ::TRP1* [p*CEN URA3 HST3*]	(23)
ASY3537	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 pph3Δ*::HPHMX [p*CEN URA3 HST3*]	This study
ASY3657	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 rtt107Δ*::HPHMX [p*CEN URA3 HST3*]	This study
ASY2156	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 slx4Δ*::KanMX [p*CEN URA3 HST3*]	This study
ASY3675	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 slx4Δ*::KanMX *rtt107Δ*::HPHMX [p*CEN URA3 HST3*]	This study
ASY3678	BY4741 *srl4Δ::KanMX*	This study
ASY3679	BY4741 *him1Δ::KanMX*	This study
ASY3680	BY4741 *hug1Δ::KanMX*	This study
ICY1164	*MATa his3D200 leu2Δ1 lys2Δ202 trp1Δ63 ura3-52 bar1Δ::hygMX hst4Δ::TRP1 hst3::td-HST3- 13MYC::KanMX4*	(23)
ASY3139	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 sml1Δ::KanMX* [p*CEN URA3 HST3*]	This study
ASY3143	FY833 *hst3Δ::HIS3 hst4Δ::TRP1 sml1Δ::KanMX rad53-3HA::HPHMX* [p*CEN URA3 HST3*]	This study
ASY3682	BY4741 *sml1Δ::KanMX slx4Δ::HIS3MX*	This study
ASY3684	BY4741 *sml1Δ::KanMX pph3Δ::HIS3MX*	This study
ASY3718	BY4741 *sml1Δ::KanMX slx4Δ::HIS3MX rad53-3HA::HPHMX*	This study
ASY3720	BY4741 *sml1Δ::KanMX pph3Δ::HIS3MX rad53-3HA::HPHMX*	This study
ASY4003	BY4741 *rev3Δ::KanMX dot1Δ::URA3MX*	This study
ASY4014	BY4741 *rad9Δ::KanMX rad18Δ::HPHMX*	This study
ASY4020	BY4741 *dot1Δ::KanMX rad18Δ::HPHMX*	This study
ASY4023	W303 *RAD5 pph3Δ::HPHMX*	This study
ASY4024	W303 *rad5-535*	This study
ASY4025	W303 *RAD5*	This study
ASY4026	W303 *rad5-535 pph3Δ::HPHMX*	This study
ASY4027	W303 *RAD5 slx4Δ::HPHMX*	This study
ASY4029	W303 *rad5-535 slx4Δ::HPHMX*	This study

3.3.3 Cell synchronization and treatment with MMS

Cells were grown overnight in YPD medium at 25°C and arrested in G1 at 30°C in YPD containing 5 μg/ml α-factor for 90 minutes, followed by addition of a second dose of 5 μg/ml α-factor for 75 minutes. Cells were released into the cell cycle by washing them once with YPD and resuspending them in medium containing 50 μg/ml pronase (Sigma-Aldrich, P6911-1G) and methyl methanesulfonate (MMS).

3.3.4 Measurement of DNA content by flow cytometry

Cells were fixed with 70% ethanol prior to flow cytometry analysis, and DNA content was determined using Sytox Green (Invitrogen) as described (45). Flow cytometry was performed on a FACS Calibur instrument using the Cell Quest software. Graphs were generated using FlowJo 7.6.5 (FlowJo, LLC).

3.3.5 Immunoblots

Whole-cell lysates were prepared for SDS–polyacrylamide gel electrophoresis using an alkaline cell lysis (46) or standard glass beads/trichloroacetic acid precipitation methods. SDS-PAGE and protein transfers were performed using standard molecular biology protocols. Monoclonal anti-myc antibody (9E10) was purchased from Sigma-Aldrich. The antibody against histone H2A was purchased from Active Motif (Cat. No 39236). Anti-H3 (AV100), anti-H3K56ac (AV105) and anti-phosphorylated H2A (AV137) antibodies (27) were kindly provided by Dr. Alain Verreault (Université de Montréal).

3.3.6 Rad53 autophosphorylation assays

Protein samples were prepared by the glass beads/tricholoroacetic acid precipitation method, resolved by SDS-PAGE and transferred to PVDF membranes using standard Towbin buffer (25mM Tris and 192mM glycine) without methanol or SDS at 0.8 mA/cm^2 for 2 h in a Bio-Rad SD semi-dry transfer apparatus. Membranes were then processed as previously described (47).

3.3.7 Fluorescence microscopy

Cell samples were fixed using formaldehyde as described (42) and examined using a Zeiss Z2 Imager fluorescence microscope equipped with the AxioVision software or a DeltaVision fluorescence microscope equipped with SoftWorx (GE Healthcare). Images were analyzed with Image J 1.46E.

3.3.8 Determination of intracellular ROS by dihydrorhodamine 123 Staining

Intracellular Reactive Oxygen Species (ROS) levels were monitored as described (48). Briefly, cells were washed with water, resuspended in PBS + 15 µg/mL dihydrorhodamine 123 and incubated for 90 min at 30°C with shaking. Cell pellets were washed twice with Phosphate Buffered Saline (PBS) and fluorescence was measured in 10 000 cells using a FACS Calibur Flow cytometer equipped with the Cell Quest software. FlowJo 7.6.5 (FlowJo, LLC) was used for statistical analysis of the fluorescence distribution in the queried samples.

3.3.9 Fitness assays

Genome-wide fitness assays were performed as described (49). Briefly, duplicate pools of strains from the "barcoded" BY4743 homozygous diploid mutant collection were incubated in YPD with or without 20 mM NAM at 30°C. Cells were collected at 0 generation (population doubling) to assess initial strain representation, and after 5 and 20 generations of exponential growth. Genomic DNA was extracted using the Zymo Research YeaStar kit as described (50). For each sample, two PCR reactions were performed (to amplify uptag and downtag sequences). Amplified DNAs were combined, and used to probe high-density oligonucleotide Affymetrix TAG4 DNA microarrays (51). These arrays contain at least 5 replicate features for each up- and downtags that are dispersed across the array so that outlier features can be discarded before calculating average intensity values for each tag. Hybridization, washing, staining and scanning were performed as described (50). After removal of outliers, intensity values for each tag were calculated by averaging unmasked replicates. Methods for outlier masking, correction for saturation, normalization and calculating sensitivity scores are described in detail elsewhere (49, 50). Z-scores for each deletion mutant were calculated after 5 and 20 generations using the equation (X-mean/standard deviation). Gene ontology (GO) analysis was performed using the GO Term Finder tool of the Saccharomyces Genome Database (52, 53). P-values ≤ 0.01 were considered significant. Redundant GO terms were removed using the REViGO server (54).

3.3.12 Gene set enrichment analysis (GSEA)

Fitness assay and RNA profiling data were analyzed as previously described (55).

3.3.10 Venn diagrams

Venn diagrams were generated using genes whose mutation was associated with an absolute z-score of over 2.58 (p-value < 0.01) from our data sets and those of Hillenmeyer *et al*.

(56). *p*-values associated with the overlap in datasets were calculated using a hypergeometric function, taking into account that 4767 mutants were included in our analysis.

3.3.11 RNA profiling assay, microarray hybridization and data analysis

BY4743 cells were grown as described for fitness assays, in the absence or presence of 20 mM NAM for 1, 5 and 20 generations. Three independent biological replicates were processed, and 50 OD_{600nm} units of cells were harvested at each time point. Cells were pelleted and washed with diethylpyrocarbonate (DEPC)-treated water. Cells were centrifuged at 3,000 rpm for 3 min at room temperature, and cell pellets were snap-frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using a "hot phenol" protocol (57) and purified using a QIAGEN RNeasy mini kit. Total RNA was quantified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc.) and its integrity assessed using a 2100 Bioanalyzer (Agilent Technologies). Three independent RNA preparations were prepared for each time point (1, 5 or 20 generation) and NAM concentration (0 or 20 mM). Double stranded cDNA was synthesized from 100 ng of total RNA and *in vitro* transcription was performed to produce biotin-labeled cRNA using the Affymetrix Gene Chip 3' IVT Express reagent kit (Affymetrix). After fragmentation, 5 µg of cRNA was hybridized on Yeast Genome 2.0 arrays (Affymetrix) and incubated at 45°C in a Genechip® Hybridization oven 640 (Affymetrix) for 16 hours at 60 rpm. GeneChips were then washed in a GeneChips® Fluidics Station 450 (Affymetrix) using Affymetrix Hybridization Wash and Stain kit. Microarrays were scanned on a GeneChip® scanner 3000 (Affymetrix), and data was analyzed using the Partek Genomic Suite. Gene ontology (GO) analysis was performed using the GO Term Finder tool of the Saccharomyces Genome Database (52, 53). P-values ≤ 0.005 were considered significant. Redundant GO terms were removed using the REViGO web server (54).

3.4 Results

3.4.1 Nicotinamide is genotoxic in *Saccharomyces cerevisiae*

We investigated the consequences of NAM-induced inhibition of sirtuins in fungi using *S. cerevisiae* as a model. Addition of 20, 50, or 100 mM NAM inhibited cell proliferation in a dose-dependent manner (Figure 3.1A). As reported, NAM exposure elevated H3K56ac levels, which is consistent with inhibition of Hst3 and Hst4 (Figure 3.1B) (23). As assessed by *in situ* autophosphorylation assay (Figure 3.1B), NAM also caused activation of Rad53, a critical DDR kinase acting downstream of Mec1 (47). In contrast, we did not detect NAM-induced modulation of H4K16ac, a well-known target of Sir2 and Hst2 in yeast (9, 10, 20). Exposure to 20 mM NAM also caused formation of Rad52-YFP and Rfa1-YFP foci, which are hallmarks of DNA damage and repair in yeast (Figure 3.1C) (58). We conclude that NAM either causes DNA lesions in yeast, or prevents the repair of endogenous DNA damage, leading to elevated DDR marks. Intriguingly, we found that 100 mM NAM did not induce Rad53 activation as strongly as 20 or 50 mM (Figure 3.1B), suggesting that growth inhibition caused by high NAM concentrations may not result solely from DNA damage.

Published data clearly indicates that most, if not all, phenotypes observed in *hst3*Δ *hst4*Δ cells are due to increased H3K56ac (22, 23, 31). Consistent with a central role for this modification in causing NAM-induced DNA damage, lack of the H3K56 acetyltransferase Rtt109 abrogated growth inhibition and Rad53 activation caused by 20 mM NAM (Figure 3.1D-E). Although we did not observe any obvious increase in H4K16 acetylation levels by immunoblot assays (Figure 3.1B), it remains possible that subtle or localized increase in levels of this modification may contribute to NAM-induced DNA damage induction and growth inhibition. Consistently, we found that mutation of H4K16 to non-acetylatable arginine or alanine residues (*H4K16R/A*) improved growth in NAM-containing medium (Figure 3.1D) and reduced Rad53 activation (Figure 3.1E). We conclude that H3K56 hyperacetylation plays a dominant role in NAM-induced genotoxicity, but that other factors including H4K16ac may also contribute.

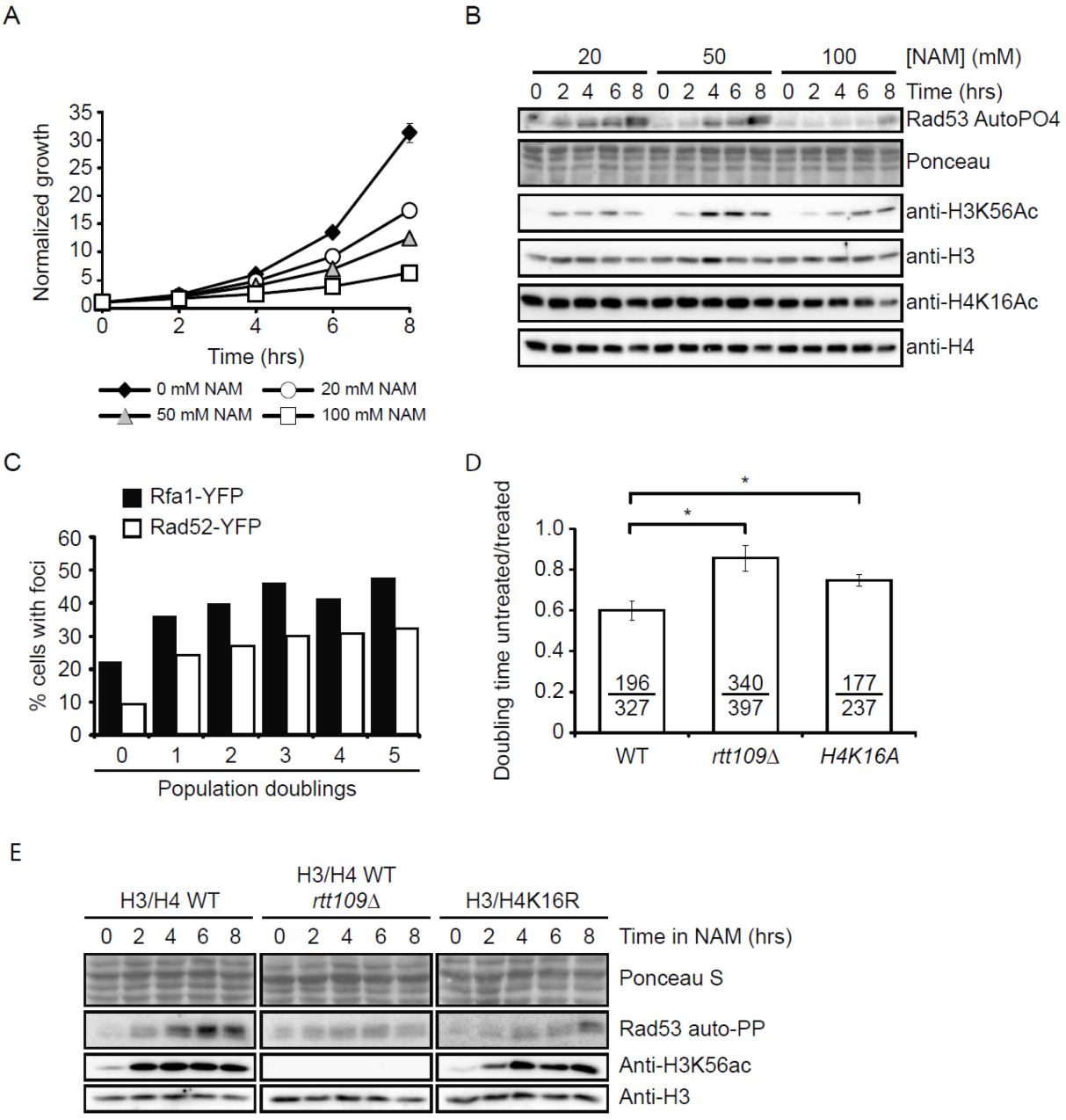

Figure 3.1. Exposure to NAM causes DNA damage in yeast.

Legend to figure 3.1. A) NAM inhibits cell proliferation in a dose-dependent manner. Cells were grown in YPD containing the indicated concentrations of NAM. Cell growth was monitored by OD_{630} measurements. B) NAM activates the DNA damage response kinase Rad53 and causes H3K56 hyperacetylation. Exponentially growing yeast cells were treated with NAM and samples were collected at the indicated time for immunoblotting and Rad53 autophosphorylation assays. C) NAM causes the formation of Rad52-YFP and Rfa1-YFP foci. Exponentially growing yeasts were treated with 20 mM NAM and samples were examined by microscopy at the indicated time points. D) NAM-induced growth defects result from H3K56 and H4K16 acetylation. Doubling times for strains of indicated genotypes were measured in YPD or YPD + 20 mM NAM, and values are represented as a ratio of the doubling time in NAM vs YPD. Error bars: standard error of the mean (3 to 6 experiments). Doubling time with/without NAM are indicated (in minutes; untreated/treated). E) Lack of H3K56ac or H4K16ac attenuates NAM-induced activation of Rad53. Exponentially growing yeasts were treated with 20 mM NAM and processed as in C). NAM: nicotinamide, *: p-value < 0.05 as calculated by unpaired one-tailed student's T-Test.

3.4.2 A genome-wide screen for *Saccharomyces cerevisiae* genes that modulate fitness in the presence of nicotinamide.

To investigate the basis of NAM-induced inhibition of cell proliferation, we used two genome-wide approaches: a fitness assay to identify genes whose deletion results in sensitivity or resistance to NAM, and mRNA profiling to identify genes that are up- or down-regulated in response to NAM. Fitness assays using "barcoded" yeast deletion collections have been used to study the genetic networks that respond to various stimuli (56, 59). Briefly, strains of the "barcoded" yeast homozygote diploid non-essential gene deletion collection were pooled and grown in the absence or presence of 20 mM NAM for 5 and 20 population doublings. Genomic DNA from treated and untreated cells was extracted, and DNA barcode sequences were amplified by PCR and hybridized to microarrays to assess their relative abundance (see Materials and Methods). Z-scores were derived from the comparison between treated and non-treated samples, and scores above or below ± 2.58 (99% cumulative percentage) were further considered for analysis. Using these criteria, we found that more genes conferred fitness defects than advantage after 5 (84 vs 12, respectively) or 20 (467 vs 342, respectively) generations in the presence of NAM (Figure 3.2A-B). Gene Ontology (GO)-term analysis of genes whose mutation caused significant fitness defects in NAM at 5 generations revealed an obvious enrichment for processes related to the DDR and replicative stress (Table 3.II). This was also the case at 20 generations, although other cellular processes were also identified that may be linked to reduced cell proliferation associated with long term growth in NAM (e.g. catabolic processes). Interestingly, the set of genes whose deletion conferred growth disadvantage in NAM significantly overlaps with published fitness analyses of pools of deletion strains treated with either of two genotoxic drugs: camptothecin (CPT) or methyl methanesulfonate (MMS) (Figure 3.2C-D) (56). We also applied Gene Set Enrichment Analysis (55) to correlate our fitness assays with available *S. cerevisiae* genome annotations including GO terms, protein-protein complexes (60) and genetic interactions (61). This analysis indicated a strong overlap between our dataset and others describing physical or genetic interactions with genes involved in DNA repair, DNA damage signalling, and DNA replication (Figure 3.2F). These observations confirm that growth of *S. cerevisiae* in the presence of NAM solicits cellular DDR pathways.

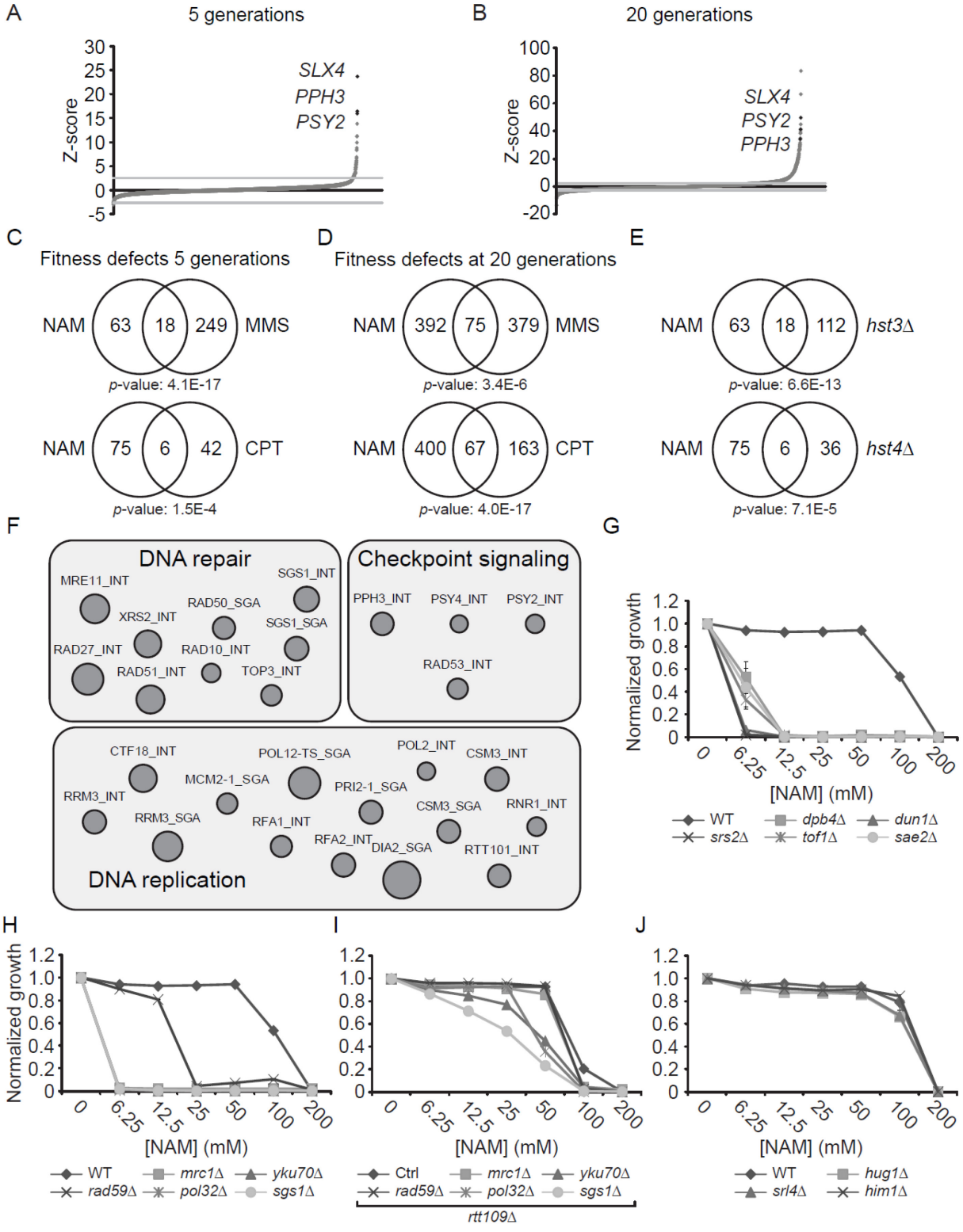

Figure 3.2. Genome-wide response to NAM-induced sirtuin inhibition

Legend to figure 3.2. A-B) Graphical representation of results from NAM fitness assays at 5 (A) and 20 (B) generations. Mutants were plotted according to their Z-score from lowest to highest. C-D) Growth in NAM, methyl methane sulfonate (MMS) and camptothecin (CPT) share similar genetic requirements. Fitness assays datasets were compared and Venn diagrams were generated as described in Materials and Methods. Statistically significant results from the NAM fitness test were compared to published fitness assays in which cells were treated with either CPT or MMS for 5 (C) or 20 (D) generations. E) Genes whose mutation reduces fitness in NAM overlap with those presenting negative genetic interaction with *HST3* and *HST4*. F) Gene set enrichment analysis was performed on statistically significant positive hits from the NAM fitness assay (see text for details). G-I) Validation of fitness assays results using haploid deletion strains. Cells were grown in 96 well plates and OD_{630} readings were acquired as described in Materials and Methods. J) Mutation of DNA damage response genes that are overexpressed in response to NAM do not influence growth in NAM-containing medium.

Table 3.II. GO term analysis of fitness test data

GO-Term ID	Description	Cluster Frequency	Background Frequency	p-value	Generation	Fitness
0006974	cellular response to DNA damage stimulus	23/81	299/7163	3.29E-11	5	-
0006259	DNA metabolic process	26/81	448/7163	4.13E-10	5	-
0006950	response to stress	28/81	655/7163	7.02E-09	5	-
0050896	response to stimulus	35/81	996/7163	1.30E-08	5	-
0006310	DNA recombination	14/81	181/7163	4.70E-06	5	-
0051052	regulation of DNA metabolic process	11/81	100/7163	5.42E-06	5	-
0006260	DNA replication	12/81	153/7163	5.16E-05	5	-
0051053	negative regulation of DNA metabolic process	7/81	36/7163	5.46E-05	5	-
0065007	biological regulation	38/81	1536/7163	9.81E-05	5	-
0090304	nucleic acid metabolic process	38/81	1573/7163	1.70E-04	5	-
0019222	regulation of metabolic process	28/81	958/7163	3.50E-04	5	-
0006261	DNA-dependent DNA replication	10/81	128/7163	6.80E-04	5	-
0043170	macromolecule metabolic process	54/81	2946/7163	8.20E-04	5	-
0044260	cellular macromolecule metabolic process	53/81	2870/7163	9.60E-04	5	-
0007530	sex determination	6/81	35/7163	1.03E-03	5	-
0022616	DNA strand elongation	6/81	37/7163	1.45E-03	5	-
0007049	cell cycle	21/81	637/7163	2.71E-03	5	-
0044237	cellular metabolic process	61/81	3724/7163	3.42E-03	5	-
0006139	nucleobase-containing compound metabolic process	38/81	1773/7163	4.01E-03	5	-
0071897	DNA biosynthetic process	5/81	27/7163	5.22E-03	5	-
0044238	primary metabolic process	58/81	3499/7163	7.11E-03	5	-
0071704	organic substance metabolic process	60/81	3708/7163	8.45E-03	5	-
0065007	biological regulation	163/467	1536/7163	1.22E-09	20	-
0022402	cell cycle process	79/467	594/7163	2.28E-06	20	-
0007049	cell cycle	80/467	637/7163	6.80E-06	20	-
0051276	chromosome organization	64/467	495/7163	5.15E-05	20	-
0050896	response to stimulus	103/467	996/7163	6.29E-05	20	-
0006974	cellular response to DNA damage stimulus	45/467	299/7163	8.15E-05	20	-
0048285	organelle fission	52/467	390/7163	8.16E-05	20	-
0006259	DNA metabolic process	59/467	448/7163	9.67E-05	20	-
0006950	response to stress	73/467	655/7163	2.20E-04	20	-
0044699	single-organism process	286/467	3588/7163	2.70E-04	20	-
0009057	macromolecule catabolic process	53/467	392/7163	4.30E-04	20	-
0009056	catabolic process	79/467	710/7163	8.20E-04	20	-
0044248	cellular catabolic process	74/467	657/7163	1.24E-03	20	-
2000113	negative regulation of cellular macromolecule biosynthetic process	42/467	302/7163	2.04E-03	20	-
0044265	cellular macromolecule catabolic process	49/467	370/7163	2.36E-03	20	-
0044763	single-organism cellular process	254/467	3165/7163	4.14E-03	20	-
0044767	single-organism developmental process	38/467	268/7163	4.48E-03	20	-
0032502	developmental process	38/467	271/7163	5.87E-03	20	-
0007533	mating type switching	10/467	28/7163	6.95E-03	20	-
0010526	negative regulation of transposition, RNA-mediated	3/12	8/7163	2.06E-05	5	+
0031297	replication fork processing	2/12	4/7163	1.59E-03	5	+
0007005	mitochondrion organization	40/342	390/7163	2.35E-03	20	+
0017182	peptidyl-diphthamide metabolic process	5/342	7/7163	4.25E-03	20	+

To assess the contribution of individual sirtuins to NAM-induced fitness defects, we compared our results with available genetic interaction data (53) and found that our dataset significantly overlaps with negative genetic interactions involving *HST3* and *HST4*, but not *SIR2*, *HST1* or *HST2* (Figure 3.2E and data not shown). Our experiments also identified a limited number of genes whose mutation improved fitness in response to NAM (12 and 342 genes at 5 and 20 generations respectively). In particular, deletion of genes that are genetically and biochemically linked to the H3K56ac cellular pathway (*RTT101, RTT107* and *MMS1*) (42, 62) improved fitness in the presence of NAM. These three genes are negative regulators of retrotransposition (63), and promote the response to damaged DNA replication forks, explaining their associated GO terms in Table 3.II (64, 65).

To validate these results, we tested the influence of NAM on the growth of individual mutant strains presenting high Z-scores in our fitness assays. This was done by evaluating the optical density (OD_{630}) of cultures of the corresponding haploid deletion mutants after 48 hours of growth in NAM-containing medium (Figure 3.2G-I). Results from these experiments are in line with our fitness assays, as mutations in DDR genes caused NAM sensitivity. Importantly, deletion of the gene encoding the H3K56 acetyltransferase Rtt109 rescued the NAM sensitivity of several DDR mutants, e.g., *pol32*Δ, *yku70*Δ, *mrc1*Δ, *rad59*Δ, *slx4*Δ and *pph3*Δ (Figure 3.2I, 3.3A, 3.4B). These data confirm that various DDR pathways respond to H3K56ac-dependent DNA damage induction caused by NAM.

As a complementary approach to our fitness assays, mRNA profiling was performed to document transcriptional changes caused by NAM. To permit comparison between transcriptional and phenotypic responses, cells were grown under the same conditions as for the fitness assays, i.e. using the same *S. cerevisiae* diploid strain (BY4743), NAM concentration (20 mM), and time points (5 and 20 generations). We also included a short time point (1 hour) to allow detection of early changes in mRNA expression patterns. We identified 213, 430, and 306 genes that were differentially expressed in cells exposed to NAM for 1 hour, and for 5 and 20 generations, respectively (absolute fold change ≥ 2.0). A majority of the identified genes (91-95%) were influenced by NAM at every time point analysed, with a core set of 133 induced genes. Results from GO term analysis are only presented for genes whose expression is significantly modulated after 5 generations, since analyses performed with genes modulated at other time points yielded similar results (data not shown). Many significantly enriched GO terms

were clustered into subsets of processes known to be regulated by Hst1 (and to a lesser extent Sir2) at the transcriptional level, and are expected to respond to NAM-induced inhibition of these sirtuins, e.g. sexual reproduction (sporulation) (66, 67) and metabolism ('*de novo*' NAD biosynthetic process, sulfur amino acid, carboxylic acid, energy reserve metabolic processes) (17, 18) (Table 3.III). Gene Set Enrichment Analysis (GSEA) also revealed that transcripts repressed in NAM-treated cells were significantly enriched in ribosome biogenesis, translation, and tRNA modification (data not shown). Genes involved in general transcription regulation including mediators and RNA elongation complexes were also repressed. Transcripts upregulated by NAM were significantly correlated with those found to be activated by NAM treatment in a previous study, thereby validating our methodology (18). Consistent with the GO term analysis presented in Table 3.III, GSEA indicated that genes involved in sporulation were activated by NAM. Finally, we found that genes bound by the transcription factor Sum1, which acts together with Hst1 to repress middle sporulation-specific genes (16), were significantly upregulated by NAM.

Table 3.III. GO term analysis of genes whose expression is modulated by NAM

GO-Term ID	Description	Cluster Frequency	Background Frequency	*p*-value
0048646	anatomical structure formation involved in morphogenesis	53/430	142/7164	1.85E-26
0043935	sexual sporulation resulting in formation of a cellular spore	49/430	120/7164	1.97E-26
0070726	cell wall assembly	34/430	54/7164	6.59E-26
0048856	anatomical structure development	56/430	165/7164	1.33E-25
0043934	sporulation	51/430	138/7164	3.95E-25
0048869	cellular developmental process	58/430	199/7164	1.13E-22
0051704	multi-organism process	62/430	260/7164	2.96E-19
0022414	reproductive process	63/430	276/7164	1.60E-18
0044767	single-organism developmental process	60/430	270/7164	6.74E-17
0032502	developmental process	60/430	273/7164	1.21E-16
0045229	external encapsulating structure organization	39/430	154/7164	2.87E-12
0000003	reproduction	73/430	464/7164	3.70E-12
0071554	cell wall organization or biogenesis	39/430	201/7164	2.56E-08
0006112	energy reserve metabolic process	12/430	34/7164	2.30E-04
0034627	'de novo' NAD biosynthetic process	5/430	5/7164	5.80E-04
0019752	carboxylic acid metabolic process	44/430	349/7164	1.25E-03
0044281	small molecule metabolic process	71/430	684/7164	1.48E-03
0006082	organic acid metabolic process	44/430	363/7164	3.59E-03
0000096	sulfur amino acid metabolic process	11/430	36/7164	3.72E-03

The above data suggest that transcriptional changes are unlikely to significantly contribute to DNA damage-induced inhibition of cell proliferation caused by NAM, which

depends largely on H3K56 hyperacetylation (Figure 3.1D). No GO term associated with the DDR was identified in our analyses of mRNA profiling data. Nevertheless, keyword searches identified three DDR genes in our lists of NAM-modulated genes: *HUG1* encoding a protein involved in the Mec1p-mediated checkpoint pathway (68); *SRL4* of unknown function but whose deletion suppresses the lethality of *rad53* mutations (69); and *HIM1* encoding a poorly characterized protein involved in DNA repair (70). Northern blot analysis of these three genes confirmed their induction in response to NAM (data not shown). However, mutant cells lacking these genes are not sensitive to NAM (Figure 3.2J), suggesting that their NAM-induced expression likely reflects non-specific responses to DNA damage.

3.4.3 Slx4 is essential for growth in the presence of NAM-induced sirtuin inhibition

Slx4 acts as a scaffold by forming complexes with several important DDR proteins (71). *slx4*Δ homozygote diploid cells displayed strong NAM-induced fitness defects in our assays, and the corresponding haploid deletion strain grew poorly in medium containing NAM (Figure 3.3A). Deletion of *RTT109* restored growth of *slx4*Δ cells in NAM, suggesting that Slx4 promotes cell survival in response to H3K56 hyperacetylation (Figure 3.3A). Consistently, we confirmed that deletion of *SLX4* in *hst3*Δ *hst4*Δ mutants was synthetically lethal (Figure 3.3D), as previously reported (31).

We sought to exploit the NAM sensitivity of *slx4*Δ mutants as a means of probing the function of this gene in the H3K56ac pathway. Slx4 interacts with several DDR proteins including Rtt107 (72–74), Dpb11 (71), Mus81-Mms4 (75), Rad1-Rad10 (76) and Slx1 (77). In agreement with fitness data, *rtt107*Δ cells presented improved cell growth compared to wild-type in NAM (Figure 3.3B) and mutation of *RTT107* abrogated NAM-induced growth defects in *slx4*Δ (Figure 3.3B). One model to explain this intriguing observation would be that Rtt107 contributes to induction of DNA lesions in an H3K56ac-dependent manner, which would require Slx4 for their resolution. In such a case, the absence of Rtt107-dependent NAM-induced DNA lesions would circumvent any eventual requirement for the Slx4-Rtt107 complex.

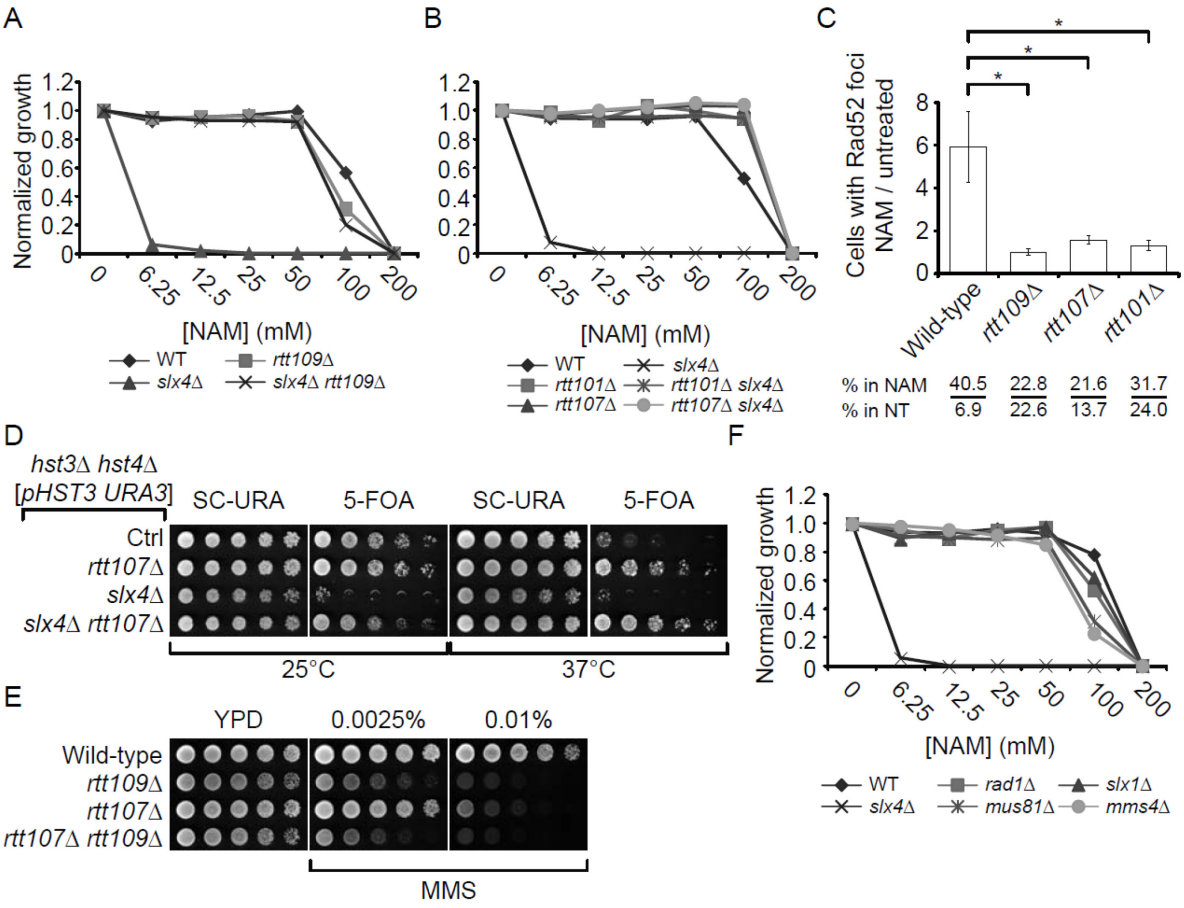

Figure 3.3. The NAM sensitivity of *slx4*Δ cells requires H3K56ac, Rtt107 and Rtt101. A) *slx4*Δ mutants are sensitive to NAM-induced H3K56 constitutive acetylation. Cells were grown in 96 well plates and OD readings were acquired as described in Materials and Methods. B-C) Mutation of Rtt101 and Rtt107 cause growth inhibition and DNA damage in NAM. B) Cells were treated as in A. C) Exponentially growing yeast cultures were exposed for 8 hours to 20 mM NAM and samples were collected for microscopy analysis of Rad52-YFP foci. Results are represented as the ratio of cells with Rad52-YFP foci after and before NAM treatment. The numbers below the graph indicate the fraction of cells containing foci in NAM-treated and untreated cells. At least 300 cells were examined per time point, and the experiment was performed in triplicate. D-E) *RTT107* is part of the H3K56ac genetic pathway. Cells were serially diluted, spotted on the indicated medium and incubated at the indicated temperature (D) or 30°C (E). F) Evaluation of the NAM sensitivity of mutants of genes encoding nucleases interacting with Slx4. Cells were treated as in A. MMS: Methyl methanesulfonate, NT: Non-treated, *: p-value < 0.05 as calculated with an unpaired one-tailed student's T-test. SC-URA: synthetic medium lacking uracil. 5-FOA: 5-Fluoroorotic Acid-containing medium.

Consistent with this, the fraction of cells presenting Rad52-YFP foci was increased after 8 hours in NAM to a significantly greater extent in wild-type cells (Figure 3.3C, ~5-fold) as compared to *rtt107*Δ and *rtt109*Δ mutants (Figure 3.3C, ~1 to 1.5-fold). Rtt107 physically interacts with the Rtt101-Mms1-Mms22 complex, which is genetically linked to H3K56ac (42, 62, 78, 79). Interestingly, deletion of *RTT101* also diminished the frequency of NAM-induced Rad52-YFP foci as compared to WT cells (Figure 3.3C), suggesting that Rtt107 and Rtt101 promote the formation of DNA lesions in conjunction with NAM-induced H3K56ac.

The genetic relationship between *RTT107* and H3K56ac is controversial as recently published data suggest, in direct contradiction with our results, that deletion of *RTT107* does not suppress the phenotypes of *hst3*Δ *hst4*Δ cells (80), and that *slx4*Δ *hst3*Δ *hst4*Δ cells are viable. We sought to address these discrepancies by further examining the genetic relationships between H3K56ac, *RTT109, HST3, HST4,* and *RTT107*. We found that *rtt109*Δ is epistatic to *rtt107*Δ in response to MMS, a genotoxic drug causing replication-blocking DNA lesions, and that mutation of *RTT107* clearly rescues the temperature sensitivity of *hst3*Δ *hst4*Δ mutants (Figure 3.3D-E). Furthermore, our results indicate that deletion of *RTT107* abolishes the synthetic lethality caused by *hst3*Δ *hst4*Δ *slx4*Δ mutations (Figure 3.3D). Taken together, our results suggest that cells with constitutive H3K56ac require Slx4 to process Rtt107-induced DNA lesions.

3.4.4 Dampening DDR activity promotes growth in response to NAM-induced H3K56 hyperacetylation.

We next sought to identify functions of Slx4 that are important for the processing or tolerance of H3K56ac-dependent DNA damage. Mus81/Mms4, Rad1/Rad10 and Slx1 form distinct structure-specific endonuclease complexes that physically interact with Slx4 and are involved in resolving branched DNA structures (77, 81, 82). We found that the haploid deletion strains of each of these genes displayed only modest NAM sensitivity in comparison to *slx4*Δ (Figure 3.3F). This suggests that loss of function of any among these Slx4-containing complexes cannot explain the NAM sensitivity of *slx4*Δ mutants, although we cannot exclude that

simultaneously compromising the activity of all these complexes may sensitize *slx4*Δ cells to NAM.

Alternatively, we hypothesized that other functions of Slx4 could account for the extreme NAM sensitivity of *slx4*Δ mutants. Slx4 acts with Rtt107 to dampen DDR signaling by competing with Rad9 for binding to phosphorylated H2A, thus limiting Rad53 activation in response to DNA damage (73). Consistently, NAM engendered strong Rad53 activation in *slx4*Δ mutants compared to WT cells (Figure 3.4A). Interestingly, 3 genes encoding subunits of the yeast PP4 phosphatase complex presented high Z-scores in our fitness assays: *PPH3*, *PSY2* and *PSY4* (83, 84) (Figure 3.2A-B,). This complex dephosphorylates Rad53 and H2A S128, well-known targets of Mec1 kinase, thereby counteracting DNA damage-induced signaling (85, 86). Consistent with a model in which constitutive H3K56ac causes lethality in the absence of Pph3, haploid *psy2*Δ and *pph3*Δ mutants, but not *psy4*Δ, displayed *RTT109*-dependent growth defects in NAM-containing medium, while deletion of *PPH3* in *hst3*Δ *hst4*Δ cells caused synthetic lethality (Figure 3.4B-C). Psy4 is required for dephosphorylation of H2A S128, but not Rad53 (85, 86), indicating that activated Rad53 is likely to be a critical PP4 substrate in the context of NAM exposure. Consistently, Rad53 was strongly activated in NAM-treated *pph3*Δ cells (Figure 3.4A). NAM did not cause a higher frequency of Rad52-YFP foci in *slx4*Δ or *pph3*Δ cells as compared to WT (Figure 3.4D), arguing against the notion that DNA lesions are induced at higher frequency in response to NAM in these mutants.

We and others showed that spontaneous activation of Rad53 in *hst3*Δ *hst4*Δ cells depends in large part on the Rad9 adaptor protein (31, 32). Strikingly, mutation of *RAD9* abrogated NAM-induced Rad53 activation in *slx4*Δ and *pph3*Δ mutants (Figure 3.4E) and rescued growth of these mutants in NAM-containing medium (Figure 3.4F). Rad9 recruitment to damaged chromatin depends on its interaction with trimethylated histone H3 lysine 79 (H3K79me3) and with phosphorylated serine 128 of histone H2A (H2A-P) via its tudor and BRCT domains, respectively (87, 88). Dot1 is the methyltransferase responsible for H3K79me3 in yeast (89). We found that introducing a histone mutation abolishing H2A-P (H2A S128A) or deleting *DOT1* in *slx4*Δ and *pph3*Δ mutants improved their growth in NAM (Figure 3.4G-H). However, for unknown reasons suppression of NAM sensitivity in *pph3*Δ by *dot1*Δ was not as striking as in *slx4*Δ mutants, although the sensitivity of both mutants was similarly suppressed by

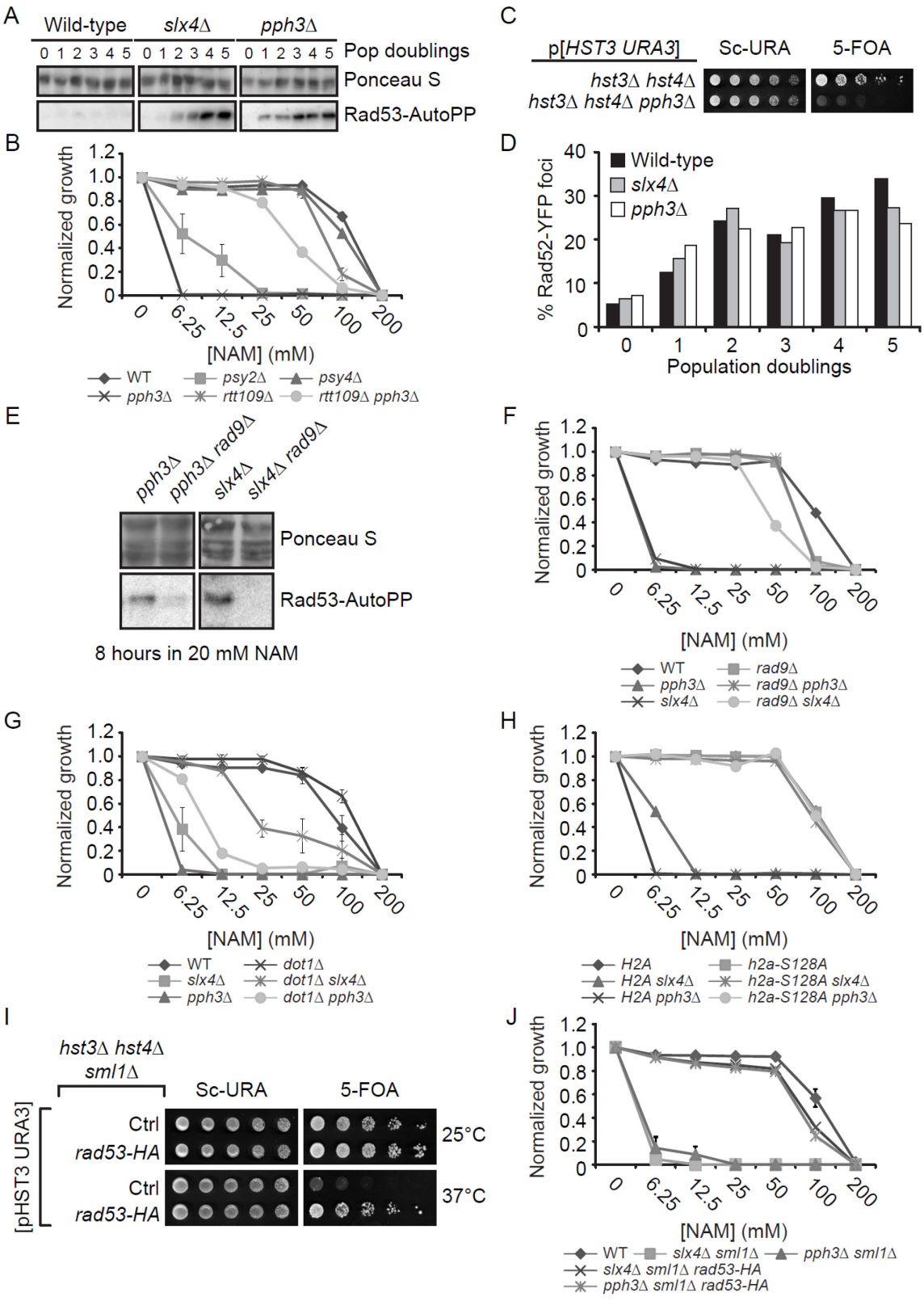

Figure 3.4. DNA damage-induced signaling inhibits cell growth in NAM

Legend to figure 3.4. A) *slx4*Δ and *pph3*Δ mutants strongly activate Rad53 in response to NAM. Exponentially growing cells were incubated in YPD with 20 mM NAM and samples were taken for Rad53 *in situ* autophosphorylation assays. A population doubling is defined as the doubling time of wild-type cells in NAM. The "0 population doubling" sample was taken immediately prior NAM exposure and therefore represents an untreated control. B) NAM inhibits growth of *pph3*Δ and *psy2*Δ but not *psy4*Δ mutants in an H3K56ac-dependent manner. Cells were grown in 96 well plates and OD readings were acquired as described in Materials and Methods. C) Deletion of *PPH3* in *hst3*Δ *hst4*Δ cells causes synthetic lethality. Cells were serially diluted, spotted on the indicated medium and incubated at 30°C. D) *slx4*Δ and *pph3*Δ mutations do not increase the frequency of NAM-induced Rad52-YFP foci. Samples were taken at indicated population doublings and processed for fluorescence microscopy analysis. Population doublings are defined as in A. E-F) *RAD9* deletion inhibits NAM-induced Rad53 activation and growth defects in *slx4*Δ and *pph3*Δ mutants. Cells were incubated for 8 hours in YPD + 20 mM NAM at 30°C, and samples were processed for Rad53 autophosphorylation assays. G-H) Abolishing H3K79me and H2A-S128 phosphorylation suppress NAM-induced growth defects in *slx4*Δ and *pph3*Δ mutants. Cells were treated as in B. I) The *rad53-3HA* hypomorphic allele rescues the thermosensitivity of *hst3*Δ *hst4*Δ mutants. Cells were serially diluted, spotted on the indicated medium and incubated at the indicated temperature. J) Reducing Rad53 activity rescues the NAM-induced growth defects of *slx4*Δ and *pph3*Δ mutants. Cells were treated as in B.

H2A S128A. Expression of a hypomorphic *rad53-HA* allele (90) also rescued both thermosensitivity of *hst3*Δ *hst4*Δ mutants and growth defects of *pph3*Δ and *slx4*Δ mutants in NAM (Figure 3.4I-J). Overall, these results indicate that Rad9-mediated Rad53 activation inhibits cell growth in response to NAM-induced H3K56 hyperacetylation.

3.4.5 Hyperactive DDR signaling compromises completion of DNA replication in response to NAM

Rad53 delays S phase completion by inhibiting activation of late origins of DNA replication in response to DNA damage (91). Using flow cytometry-based DNA content analysis, we found that compared to WT cells *slx4*Δ and *pph3*Δ mutants accumulated in S phase during NAM exposure (Figure 3.5A). This phenotype was abolished by *rtt109*Δ or *rad9*Δ mutations, indicating that defects in S phase progression were caused by H3K56 hyperacetylation and Rad9-dependent DNA damage signaling (Figure 3.5A). Rad53-mediated phosphorylation of several residues in Dbf4 and Sld3 prevents late origin firing in response to DNA damage (44). Expression of non-phosphorylatable *dbf4-4A* and *sld3-A* alleles in yeast does not influence Rad53 activation (44), but bypasses the inhibition of late replication origins in response to DNA lesions. Interestingly, we found that the *dbf4-4A sld3-A* mutations partially rescue the growth defects of *slx4*Δ and *pph3*Δ cells in NAM (Figure 3.5B), and that *dbf4-4A sld3-A pph3*Δ and *dbf4-4A sld3-A slx4*Δ mutants display reduced accumulation in S phase in response to NAM in comparison to *slx4*Δ and *pph3*Δ cells (Figure 3.5C). We note that cells of the W303 genetic background accumulate in late S phase in NAM, in contrast to BY4741 cells which accumulate in mid S (compare Figure 3.5C and 3.5A). Although the reasons for this remain unclear, we excluded the possibility that the *rad5-G535R* mutation present in certain W303 backgrounds explains these differences, as cells harboring this mutation respond to NAM in a manner indistinguishable from W303 *RAD5* cells (data not shown). Overall, these data are consistent with a model in which Rad53-mediated phosphorylation of Dbf4 and Sld3 contributes to inhibition of cell cycle progression in cells with constitutive H3K56ac.

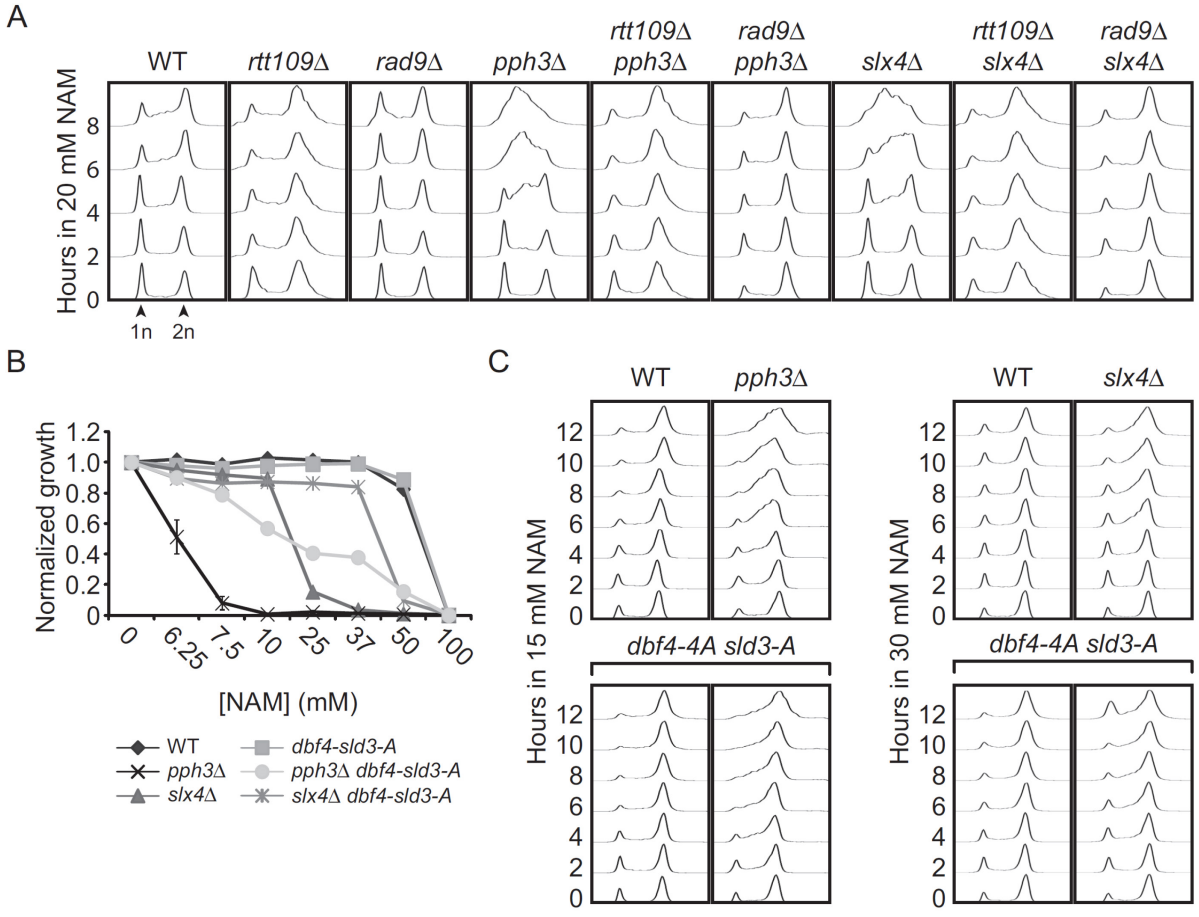

Figure 3.5. NAM inhibits the completion of DNA replication in *slx4*Δ and *pph3*Δ mutants

A) NAM-induced S-phase arrest in *slx4*Δ and *pph3*Δ mutants depends on Rad9 and Rtt109. Exponentially growing cells were incubated in YPD with 20 mM NAM and samples were taken at the indicated time points for DNA content analysis by flow cytometry. B-C) Rad53-dependent inhibition of the activation of late DNA replication origins contributes to growth defects of *slx4*Δ and *pph3*Δ in NAM. B) Cells were grown in 96 well plates and OD readings were acquired as described in Materials and Methods. C) Cells were treated as in A. 1n, 2n: DNA content.

Rad53 activity has been shown to inhibit the mutagenic translesion synthesis (TLS) pathway of DNA damage tolerance in response to MMS (90, 92). Because of this, mutations that compromise Rad9-dependent Rad53 activation, i.e. *dot1*Δ or *H3K79R*, increase cellular resistance to MMS in a TLS-dependent manner (90, 92). Our previous data suggested that the MMS sensitivity of *hst3*Δ *hst4*Δ cells may result in part from Rad53-dependent inhibition of Pol Zeta, a TLS polymerase involved in the bypass of MMS-damaged DNA bases such as 3-methyl adenine (32, 93, 94). We sought to verify whether Rad53-mediated inhibition of TLS contributes to cell cycle progression defects of NAM-treated *slx4*Δ and *pph3*Δ mutants. *REV3* encodes the catalytic subunit of Pol Zeta, and has been shown to mediate TLS-induced spontaneous mutagenesis in cells lacking Slx4 (76). We found that deletion of *REV3* had no effect on NAM sensitivity of *slx4*Δ *dot1*Δ or *pph3*Δ *dot1*Δ mutants (Figure 3.6A and data not shown). This suggests either that DNA lesions caused by NAM are not bypassed by Pol Zeta, or that relief of Rad53-mediated TLS inhibition does not contribute to the suppressive effect of *dot1*Δ on NAM-associated phenotypes in *slx4*Δ and *pph3*Δ mutants. To distinguish these possibilities, we tested whether ubiquitination (ub) of the DNA replication processivity clamp PCNA was involved in the suppressive effect of *dot1*Δ and *rad9*Δ on NAM-induced phenotypes. PCNA mono-ub on lysine 164 by the Rad6/Rad18 ub ligase complex favors TLS in response to damaged DNA bases, whereas subsequent poly-ub of this residue by Mms2-Rad5-Ubc13 promotes error-free homologous recombination-mediated template switching (95). Interestingly, mutation of PCNA lysine 164 to a non-ubiquitylable arginine residue diminished the extent of *rad9*Δ-dependent rescue of the NAM sensitivity of *slx4*Δ and *pph3*Δ mutants (Figure 3.6B). Moreover, deletion of *RAD18* abrogated the rescue of NAM sensitivity conferred to *slx4*Δ and *pph3*Δ mutants by the *dot1*Δ and *rad9*Δ mutations, respectively (Figure 3.6C-D). This effect depends on TLS-promoting PCNA mono-ub rather than subsequent Mms2-Rad5-Ubc13-mediated poly-ub since deletion of *MMS2* had no impact on the NAM sensitivity of *slx4*Δ *dot1*Δ and *pph3*Δ *rad9*Δ mutants (Figure 3.6C-D). However, we note that even though our data suggest that relief of Rad53-mediated TLS inhibition may contribute to the rescue of the phenotypes of *slx4*Δ and *pph3*Δ mutants in the context of NAM exposure, the identity of eventual TLS polymerase (s) involved in the bypass of NAM-induced DNA lesions remain unknown.

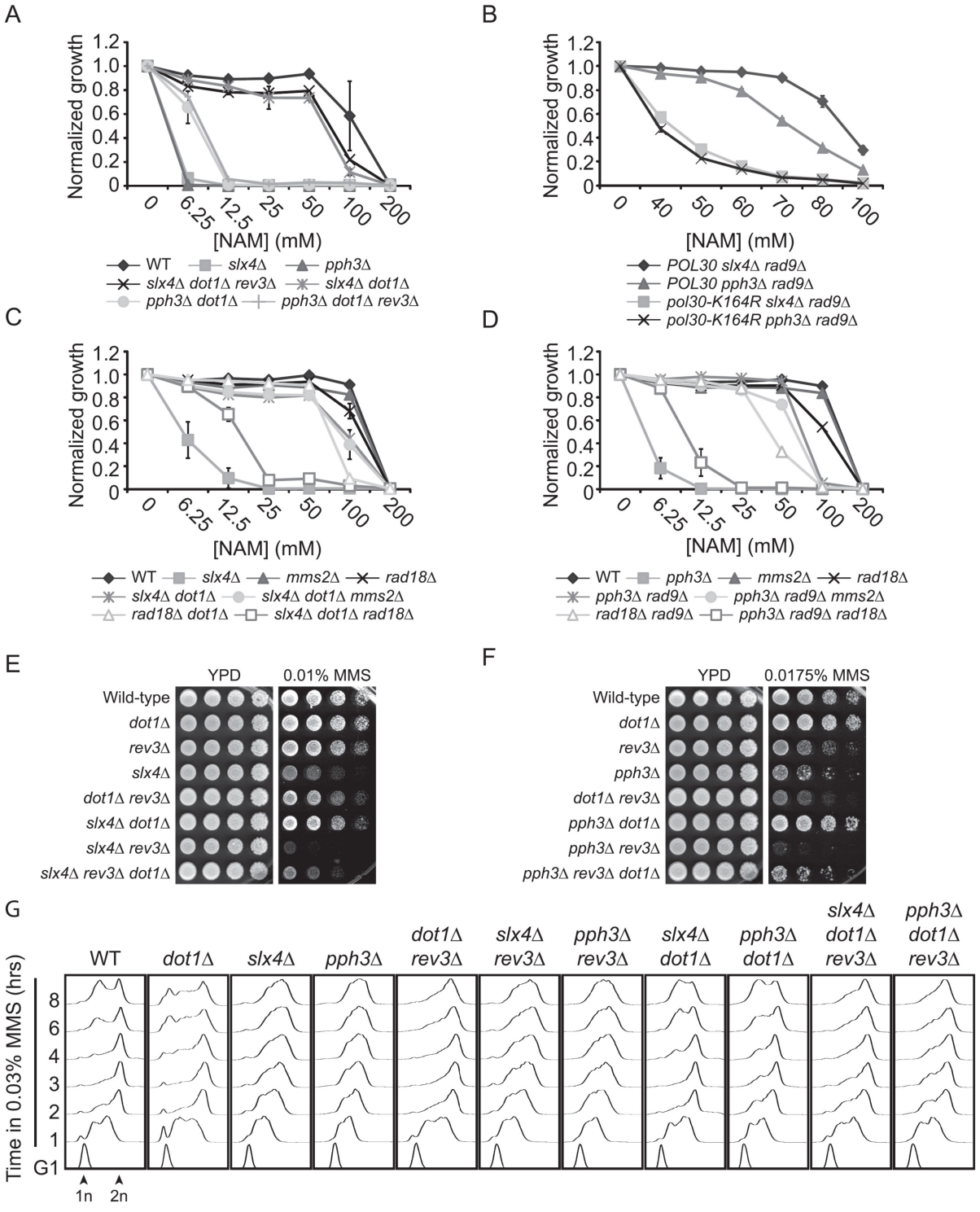

Figure 3.6. The translesion synthesis pathway of DNA damage tolerance promotes growth in response to NAM and MMS in *slx4*Δ and *pph3*Δ mutants

Legend to figure 3.6. A) *dot1*Δ-mediated suppression of the NAM sensitivity of *slx4*Δ and *pph3*Δ mutants does not depend on the Rev3 subunit of translesion DNA polymerase Zeta. Cells were grown in 96 well plates and OD reading were assessed as described in Materials and Methods. B) *rad9*Δ-mediated suppression of the NAM sensitivity of *slx4*Δ and *pph3*Δ mutants depends on PCNA K164. Cells were treated as in A. C-D) *dot1*Δ- and *rad9*Δ-mediated rescue of the NAM sensitivity of *slx4*Δ and *pph3*Δ mutants require *RAD18* but not *MMS2*. Cells were treated as in A. E-F) *dot1*Δ-mediated rescue of the MMS sensitivity of *slx4*Δ and *pph3*Δ depends on the Rev3 subunit of translesion DNA polymerase Zeta. Cells were serially diluted, spotted on the indicated medium and incubated at 30°C. G) Translesion synthesis promotes completion of the cell cycle, but not S phase progression, in *dot1*Δ, *dot1*Δ *slx4*Δ and *dot1*Δ *pph3*Δ mutants treated with MMS. Cells were synchronized in G1 with alpha factor and released in YPD containing 0.03% MMS. Samples were taken at indicated time and processed for DNA content analysis by flow cytometry. MMS: methyl methane sulfonate. 1n, 2n: DNA content.

To further investigate the role of TLS in *slx4*Δ and *pph3*Δ cells, we decided to use MMS as a model mutagen, which produces 3-methyl adenines that are bypassed in large part by Pol Zeta. As was the case with NAM, *DOT1* deletion partially suppressed MMS sensitivity of both *slx4*Δ and *pph3*Δ mutants (Figure 3.6E-F). This suppression was reversed in large part by *REV3* deletion, consistent with the notion that TLS could be involved in this phenomenon (Figure 3.6E-F). However, we found that *DOT1 slx4*Δ *rev3*Δ and *pph3*Δ *rev3*Δ cells are also extremelysensitive to MMS, and that *dot1*Δ slightly suppressed the severe MMS sensitivity of these mutants. We conclude that even though *dot1*Δ-mediated suppression of the phenotypes of *slx4*Δ and *pph3*Δ mutants requires Rev3 in large part, other cellular pathways also appear to contribute.

Deletion of *SLX4* or *PPH3* causes delays in S phase completion in response to MMS (76, 96). To investigate the impact of Rad53-dependent inhibition of TLS on this phenomenon, we synchronized cells in G1 using alpha factor and released them toward S-phase in MMS-containing medium. Cell cycle progression was monitored by flow cytometry at regular intervals. As expected, *slx4*Δ and *pph3*Δ mutants exhibited delays in passage through S in MMS compared to WT cells (Figure 3.6G). In contrast, deletion of *DOT1* in these mutants permitted completion of DNA replication with kinetics similar to WT, and furthermore allowed cells to progress toward the next G1 and S-phase. The *rev3*Δ mutation did not enhance the replication defects observed in *pph3*Δ and *slx4*Δ single mutants, and also did not dramatically compromise S phase progression in *dot1*Δ, *dot1*Δ *slx4*Δ, and *dot1*Δ *pph3*Δ. Instead, these latter strains were found to accumulate in late S-G2/M in the absence of Rev3. The above data (i) are in agreement with the notion that cells lacking Dot1 rely on Rev3-mediated TLS for growth in the presence of MMS (Figure 3.6E-F) (92), and (ii) further suggest that Rev3 acts to bypass MMS-induced DNA lesions mostly in late S-G2/M under these conditions, thus operating in a manner that is uncoupled from the bulk of DNA replication, as described (97). Our overall results therefore suggest that Rad53-mediated inhibition of TLS contributes at least in part to the phenotypes of *slx4*Δ and *pph3*Δ mutants in response to DNA damage, but does not explain the striking S-phase progression defects of these mutants when challenged with MMS. These data also highlight the fact that the genetic requirements for *dot1*Δ-mediated suppression of *slx4*Δ and *pph3*Δ differ in cells treated with NAM vs MMS.

3.4.6 Reactive oxygen species generate DNA damage in NAM-treated cells

We next sought to identify the source of DNA lesions observed in NAM-treated cells (Figure 3.1C, 3.3D). Reactive oxygen species (ROS) are generated as by-products of ATP production during oxidative phosphorylation. ROS induce highly-mutagenic replication-blocking DNA lesions that can be bypassed by TLS polymerases (98, 99). Interestingly, data from our NAM fitness assays significantly overlaps with that of similar assays performed in the presence of paraquat, a chemical known to induce ROS (56, 100) (Figure 3.7A-B). Moreover, addition of the antioxidants N-acetylcysteine (NAC) or glutathione (GSH) improved growth of *slx4*Δ and *pph3*Δ mutants in NAM (Figure 3.7C-D), and NAC reduced both the frequency of NAM-induced Rad52-YFP foci in WT cells and activation of Rad53 in *slx4*Δ and *pph3*Δ mutants following NAM exposure (Figure 3.7E-F). Overall, these data are consistent with the notion that cellular ROS may contribute at least in part to NAM-induced DDR and growth defects.

As initial explanation for the above-described observations, we hypothesized that NAM exposure could lead to increased ROS production, thereby causing growth defects and DDR induction. This model predicts that mutations causing ROS sensitivity should also sensitize cells to NAM, and vice-versa. Contrary to this, growth of *slx4*Δ and *pph3*Δ mutants was only mildly hampered by H_2O_2 (Figure 3.8A). In addition, deletion of *RTT109* in *slx4*Δ and *pph3*Δ cells caused synthetic growth defects in response to H_2O_2, but rescued their NAM sensitivity. To more directly assess whether intracellular ROS levels were influenced by NAM, we stained control and NAM-treated cells with dihydrorhodamine 123 (DHR123), a reagent that fluoresces by reacting with ROS, and analysed cell fluorescence by flow cytometry (Figure 3.8B). We also assessed DHR123 fluorescence in cells exposed to 2.5 mM H_2O_2, a concentration that inhibits cell proliferation to a similar extent as 20 mM NAM (data not shown). NAM exposure did not increase DHR123 fluorescence compared to untreated control cells, whereas H_2O_2 caused strong increase in fluorescence signal, as expected (Figure 3.8B). Overall, our data do not support the concept that NAM exposure *per se* directly causes elevated intracellular ROS.

As an alternative explanation, we reasoned that NAM-induced H3K56 hyperacetylation could sensitize cells to DNA damage caused by normal ROS levels, thereby compromising cell

proliferation. Consistent with this, we found that combined exposure to NAM and ROS-inducing agents led to strong synergistic growth defects (Figure 3.8C), and that *hst3*Δ *hst4*Δ mutants are exquisitely sensitive to H_2O_2 (Figure 3.8D). These results strongly suggest that H3K56 hyperacetylation sensitizes cells to ROS. To further validate this idea, we exploited an *hst4*Δ strain expressing a temperature-inducible degron allele of *HST3* (td-Hst3) under the control of a methionine-repressible promoter. This strain exhibits strong temperature sensitivity in YPD, but not in synthetic medium lacking methionine (SC-MET; Figure 3.8E). Our results show that cells expressing td-Hst3 arrest in S phase when grown in YPD at the restrictive temperature of 37°C and accumulate elevated H3K56ac and H2A-P, a marker of DNA damage (Figure 3.8F). We found that addition of NAC to the growth medium noticeably improved growth at 37°C, alleviated S phase accumulation, and reduced H2A-P in cells expressing td-Hst3 grown at 37°C (Figure 3.8F-H). Overall, these data support a model in which endogenously-produced ROS contribute to growth defects caused by NAM-induced constitutive H3K56ac in yeast.

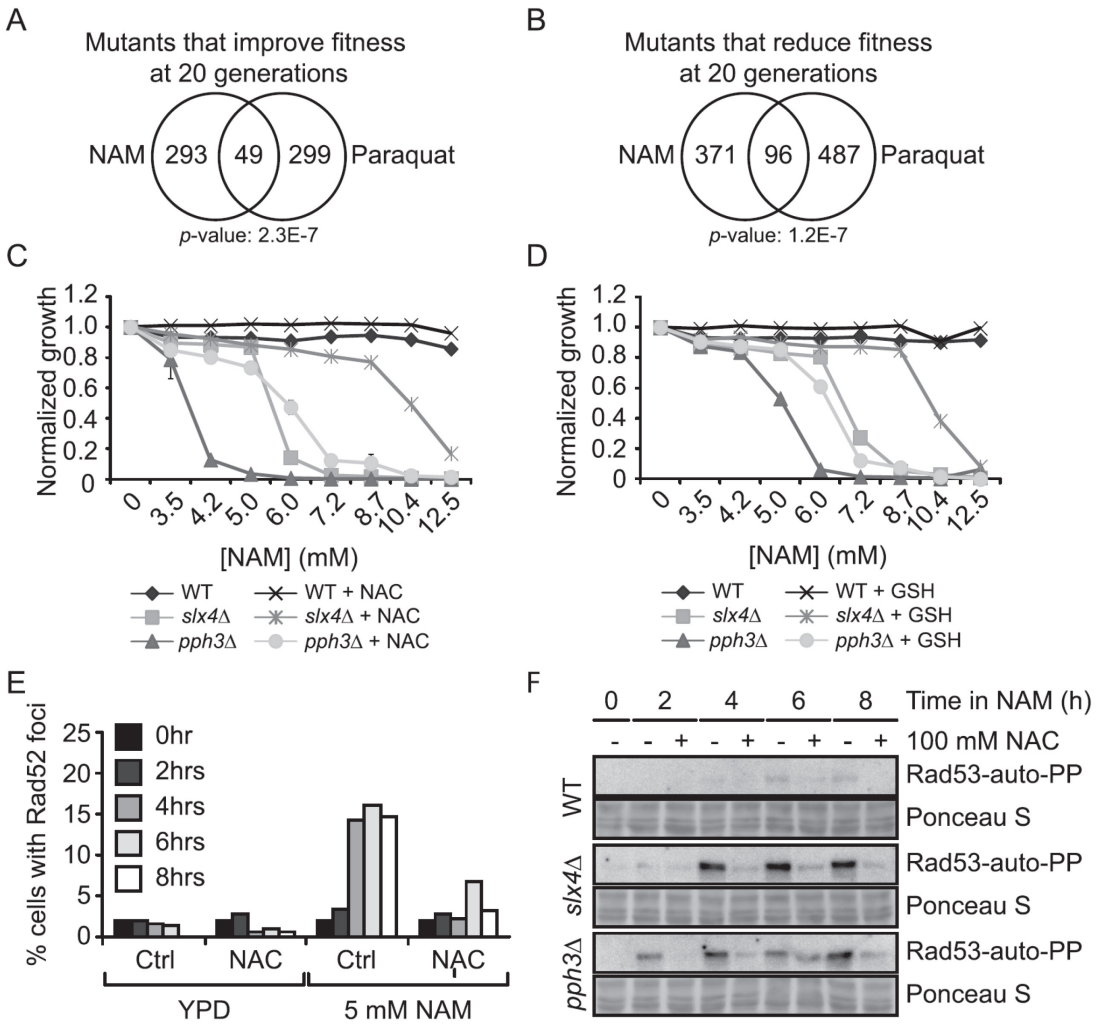

Figure 3.7. Reactive oxygen species generate DNA damage in NAM-treated cells.
A-B) Growth in NAM and paraquat share similar genetic requirements. Fitness assays datasets were compared and Venn diagrams were generated as described in Materials and Methods. C-D) NAC and GSH partially suppress NAM-induced growth defects in *slx4*Δ and *pph3*Δ mutants. OD_{630} was measured after 48 h in YPD containing NAM at 30°C in 96 well plates, with or without 100 mM NAC or GSH. E) NAC suppresses NAM-induced formation of Rad52-YFP foci. Exponentially growing cells were incubated in YPD at 30°C with or without 5 mM NAM and/or 100 mM NAC. Samples were taken at indicated times and processed for fluorescence microscopy. F) NAC suppresses the NAM-induced Rad53 activation in *slx4*Δ and *pph3*Δ mutants. Cells were treated as in E) and samples were taken at indicated time points for Rad53 *in situ* autophosphorylation assays. NAC: N-acetylcysteine, GSH: Glutathione

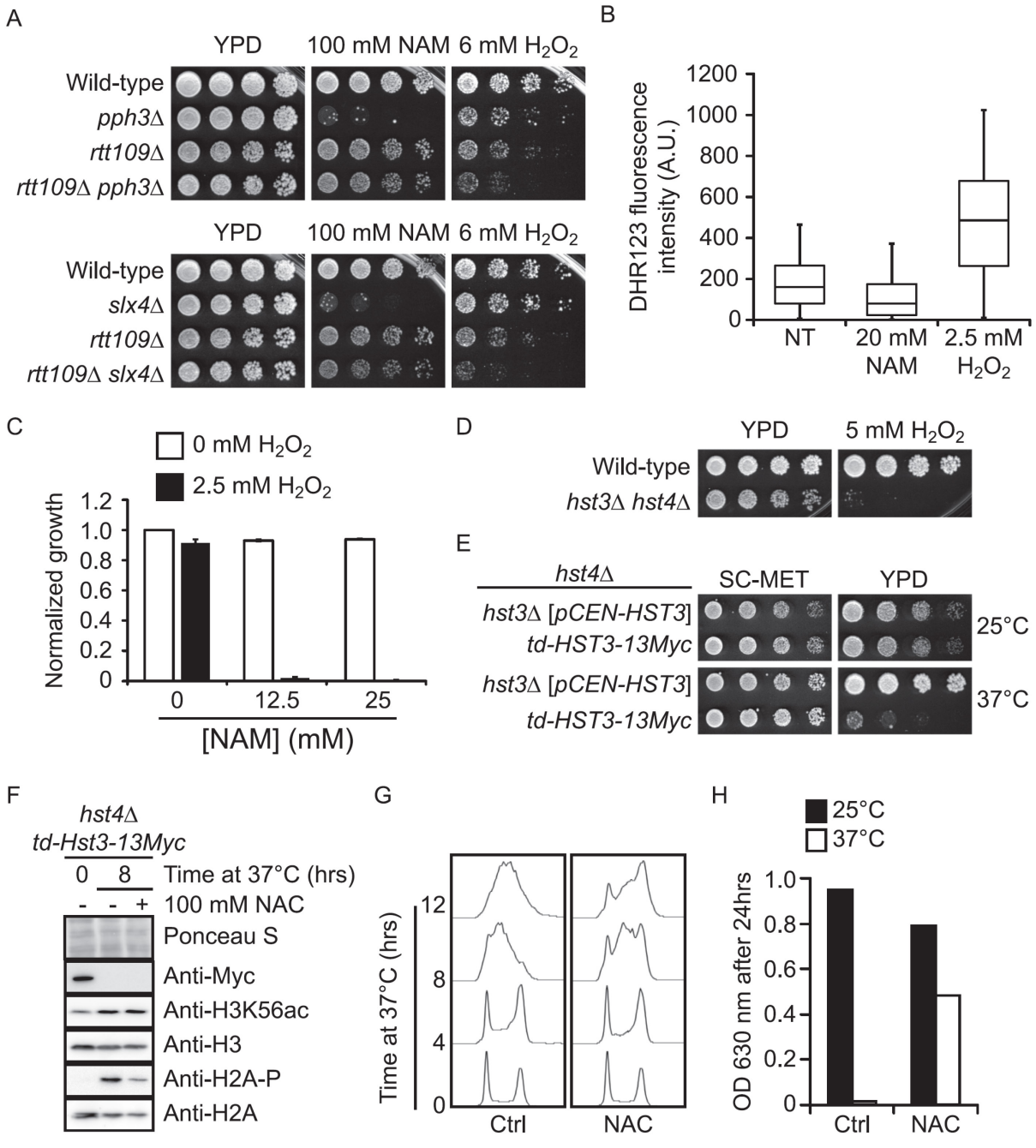

Figure 3.8. NAM-induced H3K56 hyperacetylation sensitizes cells to reactive oxygen species.

Legend to figure 3.8. A) Growth in NAM and H2O2 require different genetic pathways. Cells were serially diluted, spotted on the indicated media, and incubated at 30°C. B) NAM does not elevate intracellular ROS. Exponentially growing cells were incubated in synthetic medium at 25°C with or without 20 mM NAM or 2.5 mM H2O2 for 8 h. Cells were stained with dihydrorhodamine 123 and cellular fluorescence was analyzed by flow cytometry. The distribution of fluorescence signals is represented in a box and whiskers plot in which the whiskers show the 5th and 95th percentiles. C) NAM and H2O2 cause synergistic growth defects. Cells were grown in YPD at 30°C with indicated concentrations of NAM or H2O2. OD630 measurements were taken after 48 h and values were normalized relative to the untreated control. D) hst3Δ hst4Δ cells are hypersensitive to H2O2. Cells were treated as in A, but were incubated at 25°C. E) hst4Δ cells expressing a temperature sensitive-degron of Hst3 under the control of a methionine-repressible promoter grow poorly at 37°C. td: temperature-sensitive degron, including methionine-repressible promoter. SC-MET: synthetic medium lacking methionine. Cells were treated as in A, and incubated at the indicated temperature. F) NAC reduces H2A serine 128 phosphorylation upon growth of td-HST3 at 37°C. Cells were grown to the exponential phase in synthetic medium lacking methionine. The medium was then changed to YPD and cells were incubated at 37°C. Samples were taken after 8 h and analyzed by immunoblotting. G) NAC promotes cell cycle progression in cells lacking Hst3 and Hst4. Cells were treated as in B, and DNA content was analyzed by flow cytometry at the indicated time after changing the medium to YPD from SC-MET. H) NAC improves the growth of cells lacking Hst3 and Hst4 activity at the restrictive temperature. td-Hst3-13Myc cells were grown overnight in SC-MET. An identical number of cells were then diluted in YPD containing either 0 or 100 mM NAC. Cells were then incubated at 25°C or 37°C, and OD readings at 630 nm were taken after 24 hours. NT: non-treated, A.U.: Arbitrary units.

3.5 Discussion

In the present study, we probed the role of the sirtuin family of histone deacetylases in promoting cell proliferation in *Saccharomyces cerevisiae*. Our results show that NAM-induced sirtuin inhibition promotes the induction of DNA lesions that impede cell growth, and that such DNA damage results largely from inhibition of Hst3 and Hst4 and consequent constitutive H3K56ac. This is consistent with known phenotypes of *hst3*Δ *hst4*Δ cells, which grow poorly and present spontaneous HR foci and activation of DNA damage-induced kinases (31, 32). Mutations in several genes known to cause synthetic lethality when combined with *hst3*Δ *hst4*Δ, e.g. *SRS2, SLX4* and *DUN1,* reduced cell fitness in NAM, thereby supporting our interpretation of the data (31). Our screens provide a comprehensive assessment of genetics networks responding to misregulated H3K56ac, and as such represent an important resource toward elucidation of the biological functions of this histone modification. Even though H3K56ac plays a dominant role, we note that compromising H4K16ac (via a *H4K16A* mutation) also partially suppressed NAM-induced growth inhibition. This is in agreement with our previous results indicating that lack of H4K16ac partially suppresses the temperature and genotoxin-sensitivity of *hst3*Δ *hst4*Δ cells, although the molecular mechanisms involved in such suppression remain unclear (32). We speculate that localized increase in H4K16ac in specific regions of the genome in response to NAM, e.g. at subtelomeric regions or other silent loci known to be deacetylated by the SIR complex, may cause DNA damage via unknown mechanisms. Further experiments will be required to understand the basis of such putative H4K16ac-induced DNA lesions, and to verify whether DNA damage specifically occurs at silent genomic loci in response to NAM.

Our data support a model in which cells with constitutive H3K56ac require Slx4- and Pph3-containing complexes to counteract Rad9-dependent activation of Rad53 in response to spontaneous DNA damage. Such a model is consistent with our published results indicating that histone mutations known to cripple Rad9 binding to chromatin, e.g. *H3K79R,* partially suppress the phenotypes of *hst3*Δ *hst4*Δ mutants (32). Interestingly, while formation of the Rtt107-Slx4 complex is necessary to limit Rad53 activity in response to replicative stress (73), deletion of either gene caused opposite effects in NAM. We propose that Slx4-Rtt107 complexes permit cells to tolerate NAM-induced DNA lesions, whereas complexes containing Rtt101 and/or

Rtt107 generate DNA damage in an H3K56ac-dependent manner. We note that the biological significance of the Rtt101-Rtt107 complex and its links to H3K56ac is poorly characterized. Rtt101 is part of a ubiquitin ligase complex which promotes chromatin assembly behind DNA replication forks by ubiquitinating newly synthesized histones before their deposition into chromatin, thereby promoting the flow of new histones from the chaperone Asf1 to the CAF1 and Rtt106 chromatin assembly factors (101). However, Rtt107 is not required for such ubiquitination events to occur (101), suggesting that abnormal chromatin assembly is unlikely to explain Rtt107-dependent DNA damage in NAM-treated cells. On the other hand, Rtt109 and Rtt101 have been shown to promote Rtt107 recruitment to chromatin in response to replicative stress induced by MMS (79), and recently published data indicate that the Slx4-Rtt107 complex is recruited specifically behind stalled DNA replication forks during genotoxic stress (73, 102). Since H3K56ac is normally only present behind DNA replication forks, we hypothesize that this histone modification may serve to restrict recruitment of the Slx4-Rtt107 or Rtt107-Rtt101 complexes behind stalled replisomes. We further speculate that constitutive H3K56ac may cause inappropriate localization of these complexes during DNA replication, leading to the formation of DNA lesions. Additional studies will be required to investigate the validity of such models.

NAM-induced activation of Rad53 was found to impede S phase progression, and lack of *PPH3* and *SLX4* strongly enhanced this effect. Our genetic data suggest that these phenotypes are attributable in part to Rad53-mediated inhibition of late replication origin firing. Our data also indicate that while Rad53-mediated inhibition of TLS (90, 92) does not contribute significantly to S phase progression defects in *slx4*Δ and *pph3*Δ cells, such inhibition does prevent these mutants from initiating subsequent cell cycles after MMS exposure. This is consistent with the known ability of the TLS DNA damage tolerance pathway to act in G2 (97), and clarify the molecular mechanisms that impede cell cycle progression in *slx4*Δ and *pph3*Δ mutants. Our results and those of others (76) also show that deletion of *REV3* in *pph3*Δ and *slx4*Δ cells cause synergistic sensitivity to MMS, raising the possibility that alternative pathways of DNA damage tolerance, e.g., homologous recombination, may be compromised in these mutants. In agreement with this, recently published data indicate that Slx4- and Pph3-mediated dampening of Rad53 activity promotes the functions of the Mus81-Mms4 structure-specific endonuclease, thereby permitting resolution of homologous recombination structures in

response to MMS (103). Our finding that reduction of Rad9-dependent Rad53 activation via *DOT1* deletion partially rescues the MMS sensitivity of *slx4*Δ *rev3*Δ and *pph3*Δ *rev3*Δ mutants also supports this notion.

Several observations suggest that NAM-induced constitutive H3K56ac promotes the induction of DNA lesions that differ from those bypassed by Pol Zeta, a TLS polymerase known to act on methylated DNA bases resulting from MMS exposure: i) *hst3*Δ *hst4*Δ mutations cause increased rates of spontaneous *CAN1* mutation in a Pol Zeta-independent manner (104), ii) Rev3 is required for Dot1-mediated rescue of the MMS, but not temperature sensitivity of *hst3*Δ *hst4*Δ mutants (32), and iii) suppression of NAM sensitivity in *slx4*Δ and *pph3*Δ cells by *dot1*Δ or *rad9*Δ is unaffected by *REV3* deletion. Nevertheless, our results indicate that TLS-promoting PCNA mono-ub, but not PCNA poly-ub (which favours template switching), mitigates NAM-induced growth defects in *slx4*Δ *dot1*Δ and *pph3*Δ *rad9*Δ mutants. This in turn suggest that Rad53-mediated inhibition of TLS DNA polymerases other than Pol Zeta may contribute to growth defects caused by constitutive H3K56ac. Although the precise nature of the DNA lesions causing the phenotypes of cells with constitutive H3K56ac remains elusive, our data suggest that such lesions result at least in part from endogenous ROS. ROS-induced DNA adducts can be bypassed by mutagenic TLS polymerases (105), and can cause severe replicative stress (106). Indeed, we found that H3K56 hyperacetylation strongly sensitizes cells to H_2O_2, and that treatment with antioxidants mitigates DDR induction in both NAM-treated WT cells and in *hst3*Δ *hst4*Δ mutants. Overall, our results suggest a model in which sirtuin-mediated deacetylation of marks associated with newly synthesized histones is critical towards preventing growth inhibition due to endogenous genotoxins.

Sirtuins are the focus of intense investigation because of their conserved roles in modulating aging in several model systems (107). In addition, misregulated sirtuin expression is observed in several tumor types, and pharmacological inhibition of these enzymes may hold promise for cancer treatment (108). Our published results also demonstrate that pan-inhibition of sirtuins by nicotinamide prevents growth in several species of pathogenic fungi (22). Further understanding the biology of sirtuins therefore harbours important clinical ramifications. However the therapeutically-relevant targets of these enzymes, and their molecular mechanism of action, are far from being completely characterized. Towards enhancing our knowledge in this regard, results presented herein provide novel insight into the cellular and genetic networks

responding to sirtuin inhibition and outline their role in modulating important aspects of the DNA damage response.

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Introduction chapitre 4

Dans le denier chapitre, nous avons décelé que l'incapacité à réparer les dommages à l'ADN issus du stress oxydatif intrinsèque au métabolisme cellulaire est responsable, du moins en partie, des défauts de croissances des mutants *hst3*Δ *hst4*Δ. Nos résultats suggèrent que cet effet est causé par l'hyperactivation de la réponse aux dommages à l'ADN résultant de l'acétylation constitutive d'H3K56. Ceci a notamment pour conséquence d'empêcher l'activation des origines de réplication tardives en plus d'inhiber la voie de TLS, résultant en difficultés à traverser la phase S. Les travaux présentés dans cette publication aident donc à mieux comprendre pourquoi l'activation de la réponse aux dommages à l'ADN est nocive en présence d'hyperacétylation d'H3K56.

Cependant, la question à savoir pourquoi l'amplitude de la réponse aux dommages à l'ADN est aussi élevée demeure sans réponse. De plus, bien que cette étude fournit des indices supplémentaires sur les fonctions d'H3K56ac, celles-ci demeurent nébuleuse. Nous n'avions évidemment pas terminé d'étudier et de caractériser les gènes qui affectent la croissance des cellules en présence de NAM. À cet effet, deux des gènes les plus sensibles au NAM identifiés dans le crible, *YKU70* et *YKU80*, ont d'ailleurs suscité notre curiosité. Yku70 et Yku80 forment un hétérodimère impliqué dans la réparation des DSB par NHEJ et dans le maintien de l'intégrité des télomères. La sensibilité des mutants *yku70*Δ et *yku80*Δ au NAM sous-entends qu'H3K56ac, ou une autre cible des sirtuines, est impliqué dans l'une ou l'autre de ces voies qui n'ont à priori pas de lien direct avec la réponse au stress réplicatif. Ainsi, nous avons tenté d'élucider les mécanismes sous-jacents à la sensibilité de ces mutants au NAM et les travaux à ce sujet sont présentement en révision dans la revue *PLoS Genetics*.

J'ai joué un rôle crucial dans la conceptualisation de ce projet (60%) et j'ai effectué presque toutes les expériences et analyses qui sont présentées dans l'article (95%). J'ai monté toutes les figures (100%) et j'ai rédigé la première version du manuscrit qui a été corrigé et édité en collaboration avec Hugo Wurtele (50%).

Ricard É : 5% expériences et analyses
Wurtele H : 50% rédaction, 40% conceptualisation

Chapitre 4. An interplay between multiple sirtuins promotes completion of DNA replication in cells with short telomeres

Authors: Antoine Simoneau, Étienne Ricard and Hugo Wurtele

4.1 Abstract

Sirtuins are an evolutionarily conserved family of histone deacetylases (HDAC) that regulate a multitude of DNA-associated processes. A recent genome-wide screen conducted in the yeast *Saccharomyces cerevisiae* identified Yku70/80, which regulate both nonhomologous end-joining and telomere structure, as being essential for cell survival in the presence of the pan-sirtuin inhibitor nicotinamide (NAM). Here, we show that sirtuin-dependent deacetylation of histone H3 lysine 56 and H4 lysine 16 is necessary for the growth of cells with short telomeres, e.g. *yku70/80*Δ or telomerase (*est1/2*Δ) mutants. Our results further indicate that early activation of DNA replication origins at short telomeres is the root cause of the sensitivity of such mutants to NAM-induced sirtuin inhibition. We present evidence suggesting that misregulation in the timing of telomeric replication origin activation titrates replication initiation factors away from other origins located at internal chromosomal locations, thereby compromising their activity and sensitizing cells harboring short telomeres to replicative stress. Overall, our results reveal the existence of a novel interplay between sirtuins and telomere-regulating factors in promoting timely completion of DNA replication in eukaryotes.

4.2 Introduction

Histone post-translational modifications influence chromatin structure and serve as recruitment platforms for diverse protein complexes (1). Histone acetylation is catalysed by histone acetyltransferases (HAT) and can be reversed by histone deacetylases (HDAC). Four HDAC classes have been defined based on sequence identity and catalytic mechanism (2). Class III HDACs are referred to as sirtuins because of their sequence homology to yeast Sir2. These enzymes deacetylate lysine residues in both histone and non-histone proteins in a reaction that requires nicotinamide adenine dinucleotide (NAD^+) and releases nicotinamide and O-acetyl ADP ribose (3, 4). Sirtuins are evolutionarily conserved, and regulate several DNA-associated processes including gene silencing, DNA replication, and DNA repair (5).

The genome of the budding yeast *S. cerevisiae* encodes 5 sirtuins: Sir2 and Homolog of Sir Two (Hst) 1-4 (6, 7). Sir2 controls gene silencing at the yeast mating and ribosomal DNA (rDNA) loci (7, 8) and at telomeres (9), and has been shown to modulate replicative lifespan (10, 11). Hst1 regulates sporulation gene expression (12, 13), and also controls thiamine biosynthesis and intracellular NAD^+ levels at the transcriptional level (14, 15). Hst2 appears to be partially redundant with Sir2 at the functional level; indeed, Hst2 influences cellular aging in the absence of Sir2 (16), and its overexpression can rescue rDNA and telomeric silencing defects in *sir2*Δ mutants (17). Hst3 and Hst4 reverse histone H3 lysine 56 acetylation (H3K56ac) (18), a modification catalyzed by the HAT Rtt109 on virtually all newly synthesized histones (19, 20). H3K56ac-harboring nucleosomes are assembled behind DNA replication forks to maintain normal nucleosomal density on daughter chromatids following parental histone segregation, and are then deacetylated chromosome-wide by Hst3/4 during the G2/M phase. Cells lacking both Hst3 and Hst4 present constitutive H3K56ac throughout the cell cycle, which causes severe phenotypes including chromosomal instability, spontaneous DNA damage, and extreme sensitivity to drugs that impede the progression of DNA replication (18, 21, 22). *hst3*Δ single mutants were also recently shown to be defective in the maintenance of artificial chromosomes engineered to contain reduced number of DNA replication origins, which are sites on chromosomes where DNA replication initiates during S phase, suggesting that H3K56ac deacetylation facilitates replication of long stretches of chromosomal DNA (23). Nevertheless,

the mechanisms through which Hst3/4-mediated H3K56ac deacetylation promote DNA replication and the resistance to replicative stress remain poorly understood.

In *S. cerevisiae,* DNA replication is initiated in a temporally ordered manner, with specific origins being activated in early, mid, or late S phase (24). Genomic context influences the timing of origin activation (or "firing"): origins located near telomeres and within rDNA repeats are activated late during S phase, while those next to centromeres fire earlier (25–28). Of relevance here, control of replication origin activation at telomeres appears to depend on an interplay between chromatin structure and the activity of telomere-associated proteins. Indeed, the silent information regulator (SIR) HDAC complex, comprising the Sir2-Sir3-Sir4 subunits, mediates telomere silencing and is critical to prevent early firing of telomeric origins (29). The Rif1 telomeric chromatin component also enforces late firing of telomeric/subtelomeric origins by recruiting the PP1 phosphatase Glc7, which counteracts Dbf4-dependent kinase (DDK)-dependent phosphorylation and activation of MCM replicative helicase complexes (30–34). Yeast strains presenting short telomeres, including mutants lacking components of the evolutionarily conserved Yku70/80 complex, initiate DNA replication at telomeric and subtelomeric regions abnormally early during S phase (35–38). This appears to result from increased recruitment of the Tel1 kinase to short telomeres, which counteracts Rif1-dependent repression of origins (39, 40). While the mechanistic basis of the above-described temporal regulation of telomeric DNA replication is increasingly well characterized, its functional significance is still unclear.

DNA replication initiation factors are limited in abundance, and current models propose that during an unperturbed S phase, sequential activation of replication origins requires recycling of these factors from early to mid, and eventually, to late origins (36, 41). Because of this, origins located in various genomic regions compete for limiting pools of initiation factors throughout S phase. For example, an increased proportion of active rDNA repeats caused by *SIR2* deletion reduces the activity of origins at other loci (42, 43). Conversely, de-repression of 100 Rpd3-regulated chromosomal origins is associated with a proportionate decrease in rDNA origin activity in the *rpd3*Δ mutant (42). These observations suggest that titration of replication factors caused by abnormal timing of origin activation at specific loci may influence DNA replication dynamics at other genomic regions. Importantly, it remains unclear whether

abnormal timing of replication origin firing caused by short telomeres influences the availability of initiation factors elsewhere in the genome.

A recent genome-wide screen conducted by our group in *Saccharomyces cerevisiae* identified *yku70*Δ and *yku80*Δ mutants as sensitive to pharmacological inhibition of sirtuin HDACs by nicotinamide (NAM) (44). Since NAM exposure influences DNA replication and the response to replicative stress (44), we originally postulated that the Yku70/80 complex may promote DNA replication progression in the absence of sirtuin activity. Here, we show that an interplay between multiple sirtuins is essential for completion of DNA replication in *yku70Δ* and *yku80Δ* mutants, and that telomere shortening is the root cause of their sensitivity to NAM-induced sirtuin inhibition. Our data further suggest that proper regulation of replication origin firing at telomeres promotes resistance to both NAM- and genotoxin-induced replicative stress by preventing titration of DNA replication initiation factors from origins throughout the genome.

4.3 Materials and methods

4.3.1 Yeast strains and growth conditions

Experiments were performed using standard yeast growth conditions. Yeast strains used in this study are listed in Table 4.I. To avoid the frequent emergence of spontaneous suppressor mutations in cells presenting constitutive H3K56 hyperacetylation, *hst3*Δ *hst4*Δ mutants used in this study are propagated with a *URA3*-harboring centromeric plasmid encoding Hst3. To evaluate the phenotypes caused by *hst3*Δ *hst4*Δ, cells are plated on 5-Fluoroorotic Acid (5-FOA)-containing medium immediately before experiments to select cells that spontaneously lost the plasmid, or during the experiment (spot assays on 5-FOA-containing plates). For experiments involving telomerase mutants (*est1*Δ or *est2*Δ), fresh haploid clones were obtained from tetrad dissection of heterozygous diploids to ensure that cells were not undergoing senescence during experiments. For spot assays, cells were grown to saturation in YEP with 2% glucose or 2% raffinose in a 96-well plate. Five-fold serial dilutions of these cultures with

identical OD were then plated on indicated medium and allowed to grow for 2 to 5 days. Growth assays in NAM were done as previously described (44). Cells were diluted to OD_{600} 0.0005 in 100 µL of YPD with increasing NAM concentrations in a 96-well plate. OD_{630} were acquired using a BioTek EL800 plate reader, and growth of each strain was normalized relative to an untreated control well. For doubling time assessments, cells were diluted to OD_{600} 0.01 in 100 µL of YPD in a 96-well plate and incubated at 30°C in a BioTek EL808 plate reader for 48h. Every 30 minutes, plates were shaken for 30 seconds and OD_{630} readings were acquired. Doubling times were derived from exponential regression of the resulting growth curve.

Table 4.I. Strains used in this study

Strain	Genotype	Reference
BY4741	BY4741 *MATa ura3Δ0 leu2Δ0 his3Δ1*	(45)
BY4743	BY4743 *MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*	(45)
ASY4249	BY4741 *MATa hst3Δ::HPHMX hst4Δ::NATMX [pHST3 URA3]*	(44)
ASY5043	BY4741 *MATa hst3Δ::HPHMX hst4Δ::NATMX yku70Δ::KanMX [pHST3 URA3]*	This study
ASY1767	BY4741 *MATa yku70Δ::KanMX*	(44)
ASY4526	BY4741 *MATa sas2Δ::KanMX*	This study
HWY4447	BY4741 *MATa sas2Δ::KanMX yku70Δ::HPHMX*	This study
ASY3111	YBL574 *hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3* [*pCEN TRP1 HHT1-HHF1*]	(46)
ASY3113	YBL574 *hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3* [*pCEN TRP1 HHT-hhf1K16A*]	(46)
ERY3398	YBL574 *hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3* [*pCEN TRP1 HHT1-HHF1*] *yku70Δ::KanMX*	This study
ERY3400	YBL574 *hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3* [*pCEN TRP1 HHT-hhf1K16A*] *yku70Δ::KanMX*	This study
ASY4460	BY4741 *MATa hst3Δ::HPHMX hst4Δ::NATMX sir3Δ::KanMX [pHST3 URA3]*	This study
ASY4282	BY4741 *MATa hst3Δ::HPHMX hst4Δ::NATMX sir4Δ::KanMX [pHST3 URA3]*	This study
ASY4528	BY4741 *MATa hst3Δ::HPHMX hst4Δ::NATMX sir3Δ::HIS3MX yku70Δ::KanMX [pHST3 URA3]*	This study
ASY4516	BY4741 *MATa hst3Δ::HPHMX hst4Δ::NATMX sir4Δ::HIS3MX yku70Δ::KanMX [pHST3 URA3]*	This study
ASY4868	BY4741 *MATa hst3Δ::HPHMX hst4Δ::NATMX sir3Δ::HIS3MX sir4Δ::KanMX [pHST3 URA3]*	This study
ASY5108	BY4741 *MATa hst3Δ::HPHMX hst4Δ::NATMX hst1Δ::HIS3MX [pHST3 URA3]*	This study
ASY5110	BY4741 *MATa hst3Δ::HPHMX hst4Δ::NATMX yku70Δ::KanMX hst1Δ::HIS3MX [pHST3 URA3]*	This study
ASY5118	BY4741 *MATa hst3Δ::HPHMX hst4Δ::NATMX sum1Δ::HIS3MX [pHST3 URA3]*	This study
ASY5121	BY4741 *MATa hst3Δ::HPHMX hst4Δ::NATMX yku70Δ::KanMX sum1Δ::HIS3MX [pHST3 URA3]*	This study
ASY4038	BY4741 *MATa hst1Δ::KanMX*	This study
ASY4040	BY4741 *MATa sir2Δ::KanMX*	This study
ASY3975	BY4741 *MATa hst1Δ::KanMX yku70Δ::HPHMX*	This study
ASY3727	BY4741 *MATa sir2Δ::KanMX yku70Δ::HPHMX*	This study
ASY5130	BY4741 *MATa sir2Δ::KanMX hst1Δ::HIS3MX*	This study
ASY5132	BY4741 *MATa sir2Δ::KanMX hst1Δ::HIS3MX yku70Δ::HPHMX*	This study
ASY5113	BY4741 *MATa sum1Δ::HIS3MX*	This study
ASY5116	BY4741 *MATa sum1Δ::HIS3MX yku70Δ::KanMX*	This study
ASY5147	BY4741 *MATa sum1Δ::URA3MX yku70Δ::HPHMX sir2Δ::KanMX hst1Δ::HIS3MX*	This study
ASY2229	BY4741 *MATa dnl4Δ::KanMX*	This study
ASY2230	BY4741 *MATa nej1Δ::KanMX*	This study
ASY2231	BY4741 *MATa lif1Δ::KanMX*	This study

Table 4.I. (continued)

Strain	Genotype	Reference
ASY1762	BY4741 *MATa yku80Δ::KanMX*	This study
ASY4105	BY4743 *MATa/α est2Δ::KanMX/EST2*	This study
HWY2678	BY4741 *TIR1-Myc::URA3MX*	This study
ASY4083	BY4741 *TIR1-Myc::URA3MX YKU70-6FLAG-AID::HPHMX*	This study
ASY3687	BY4741 *MATa rif1Δ::KanMX*	This study
ASY3688	BY4741 *MATa rif2Δ::KanMX*	This study
ASY3709	BY4741 *MATa rif1Δ::KanMX yku70Δ::HPHMX*	This study
ASY3712	BY4741 *MATa rif2Δ::KanMX yku70Δ::HPHMX*	This study
ASY4234	BY4741 *TIR1-Myc::URA3MX RIF1-6FLAG-AID::HPHMX yku70Δ::KanMX*	This study
ASY3689	BY4741 *MATa tel1Δ::KanMX*	This study
ASY3715	BY4741 *MATa tel1Δ::KanMX yku70Δ::HPHMX*	This study
ASY4476	BY4741 *MATa tel1Δ::KanMX yku70Δ::HPHMX rif1Δ::URA3MX*	This study
HWY1608	BY4741 *MATa slx4Δ::KanMX*	(44)
ASY1835	BY4741 *MATa pph3Δ::HPHMX*	(44)
ASY4063	BY4741 *MATa slx4Δ::KanMX rif1Δ::HPHMX*	This study
ASY4066	BY4741 *MATa pph3Δ::HPHMX rif1Δ::KanMX*	This study
W5094-1C	W303 *ADE2 RAD52-YFP RAD5*	(47)
HWY2841	W303 *ADE2 RAD52-YFP RAD5 yku70Δ::KanMX*	This study
ASY5104	BY4741 *MATa pRS316*	This study
ASY5105	BY4741 *MATa pRS316*	This study
ASY5106	BY4741 *MATa yku70Δ::KanMX pRS316*	This study
ASY5107	BY4741 *MATa yku70Δ::KanMX pRS316*	This study
1962	W303 *MATa his3::GAL-MHT*	(41, 42)
1963	W303 *MATa his3::GAL-SLD3/SLD7/CDC45* (1 copy)	(41, 42)
1964	W303 *MATa his3::GAL-SLD3/SLD7/CDC45* (2 copies)	(41, 42)
ASY4876	W303 *MATa his3::GAL-MHT yku70Δ::KanMX*	This study
ASY4879	W303 *MATa his3::GAL-SLD3/SLD7/CDC45* (1 copy) *yku70Δ::KanMX*	This study
ASY4882	W303 *MATa his3::GAL-SLD3/SLD7/CDC45* (2 copies) *yku70Δ::KanMX*	This study
ASY5175	W303 *MATa his3::GAL-MHT sum1Δ::HPHMX*	This study
ASY5179	W303 *MATa his3::GAL-SLD3/SLD7/CDC45* (2 copies) *sum1Δ::HPHMX*	This study
ASY5169	W303 *MATa his3::GAL-MHT sum1Δ::HPHMX yku70Δ::KanMX*	This study
ASY5173	W303 *MATa his3::GAL-SLD3/SLD7/CDC45* (2 copies) *sum1Δ::HPHMX yku70Δ::KanMX*	This study
ASY5181	W303 *MATa his3::GAL-MHT rad52Δ::HPHMX*	This study
ASY5185	W303 *MATa his3::GAL-SLD3/SLD7/CDC45* (2 copies) *rad52Δ::HPHMX*	This study
ASY5268	W303 *MATa his3::GAL-MHT rad52Δ::HPHMX yku70Δ::KanMX*	This study
ASY5272	W303 *MATa his3::GAL-SLD3/SLD7/CDC45* (2 copies) *rad52Δ::HPHMX yku70Δ::KanMX*	This study
HWY289	BY4741 *MATa fob1Δ::KanMX*	This study
ERY4186	BY4741 *MATa fob1Δ::KanMX yku70Δ::HPHMX*	This study

4.3.2 Telomere southern blot

Monitoring of telomere length by southern blotting was performed as described (48). Briefly, genomic DNA was digested with XhoI (New England Biolabs) and run on a 1.2% agarose gel for 17 hrs in 1x TBE buffer. Telomeric repeats were detected with a TG_{1-3} probe kindly provided by Dr Raymund Wellinger (Université de Sherbrooke).

4.3.3 Immunoblotting

Proteins were extracted from samples by alkaline cell lysis (49) and run on 10% or 6% acrylamide gels to resolve Yku70 and Rif1 respectively. Flag epitopes were detected using an anti-Flag-M2 antibody (Sigma).

4.3.4 DNA content analysis by flow cytometry

Cells were fixed in ethanol 70%, sonicated, treated with 0.4 ug/mL RNAse A in 50mM Tris-HCl pH 7.5 for 3 hours at 42°C followed by treatment with 1mg/mL Proteinase K in 50mM Tris-HCl pH 7.5 for 30 minutes at 50°C. DNA content was assessed by Sytox Green (Invitrogen) staining as previously described (50). DNA content analysis was performed on a FACS Calibur flow cytometer equipped with Cell Quest software. Graphs were produced using FlowJo 7.6.5 (FlowJo, LLC).

4.3.5 Fluorescence microscopy

Cells expressing either RAD52-YFP or RFA1-YFP were fixed with formaldehyde as previously described (47, 51) and stained with DAPI. Fluorescence was examined with a DeltaVision microscope equipped with SoftWorx version 6.2.0 software (GE Healthcare). Rad52-YFP images were analysed with ImageJ version 1.51n. Rfa1-YFP images were examined using a custom MATLAB script (version R2017a; MathWorks) to extract the number of cells with Rfa1-YFP foci and their average fluorescence. Briefly, a mask was created based on DAPI signals to identify cell nuclei and count the number of cells within an image. A second mask was created with the YFP channel to mark foci by finding spots with elevated YFP fluorescence compared to surrounding regions. Nuclei with at least one focus were listed as cells with Rfa1-YFP foci. The average intensity of every Rfa1-YFP focus located within a nucleus was calculated and the results from 250 or more foci are represented in a box plot.

4.3.6 *in situ* Rad53 autophosphorylation assays

Protein samples were prepared by trichloroacetic acid/glass beads lysis, run on 10% acrylamide gels and transferred to a PVDF membrane. Autophosphorylation assays were then carried out as previously described (52).

4.3.7 Auxin-induced degradation

Cells were maintained in logarithmic phase for indicated number of days by dilution in fresh YPD ± 2 mM Auxin (3-indoleacetic acid, Sigma). To release from Auxin, cells were spun, washed once with YPD, and then resuspended into fresh YPD. For each time point, a growth assay was performed in YPD with increasing concentrations of NAM ± 2 mM Auxin. Growth was normalized to the untreated control.

4.3.8 Plasmid loss assay

Cells were grown to saturation in SC-URA to maintain the pRS316 plasmid. Cells were then diluted in YPD or YPD + 5 mM NAM, and incubated overnight at 30°C. Cell dilutions were plated on either YPD or 5-FOA. Plasmid loss frequency was calculated as the number of colonies growing on 5-FOA divided by the number of colonies growing on YPD plates. 6 independent colonies were picked and used for each condition.

4.4 Results

4.4.1 An interplay between the activities of multiple sirtuins is necessary for growth of cells lacking Yku70/80.

We previously found that *S. cerevisiae yku70*Δ and *yku80*Δ mutants are extremely sensitive to NAM (44), a pan-sirtuin inhibitor (3, 53). In order to identify which of the five yeast sirtuins (Sir2, Hst1-4) were responsible for this phenomenon, single deletions of each sirtuin gene were combined with *yku70*Δ by mating, and double mutants isolated via tetrad dissection (Figure 4.1A). None of the double mutants displayed noticeable growth defects, suggesting that NAM-induced growth inhibition in *yku70*Δ mutants is likely due to concurrent inhibition of multiple sirtuins. We previously showed that deletion of the H3K56ac acetyltransferase *RTT109* strongly rescues the sensitivity of *yku70/80*Δ mutants to NAM ((44) and Figure 4.1B). We therefore tested whether this reflects NAM-induced inhibition of the H3K56ac-deacetylases Hst3 and Hst4 (18), and indeed found that *yku70*Δ *hst3*Δ *hst4*Δ cells display significant, albeit moderate, decrease in growth rate and doubling time compared to *hst3*Δ *hst4*Δ cells (Figure 4.2A-B). While these results indicate that H3K56 hyperacetylation is an essential component of NAM-induced growth inhibition of *yku70*Δ mutants, the limited growth defects of *yku70*Δ *hst3*Δ *hst4*Δ cells suggest that other sirtuins probably contribute to this phenomenon.

We demonstrated previously that mutations inhibiting H4K16 acetylation (H4K16ac), whose levels are regulated by Sir2 and Hst1 *in vivo* (54, 55), partially rescue several phenotypes of *hst3*Δ *hst4*Δ cells (22). Interestingly, deletion of *SAS2*, a gene encoding the catalytic subunit of the H4K16 acetyltransferase complex SAS-I (54, 56, 57), or mutation of H4K16 to alanine (H4K16A), rescued growth of *yku70*Δ cells in NAM (Figure 4.2C-D). We could not directly test whether reduced Sir2 activity exacerbates the growth defects of *yku70*Δ *hst3*Δ *hst4*Δ mutants since *sir2*Δ causes synthetic lethality when combined with *hst3*Δ *hst4*Δ (22, 58). Sir2 is recruited to rDNA repeats and *HMR*/*HML*/telomeres as part of either the RENT

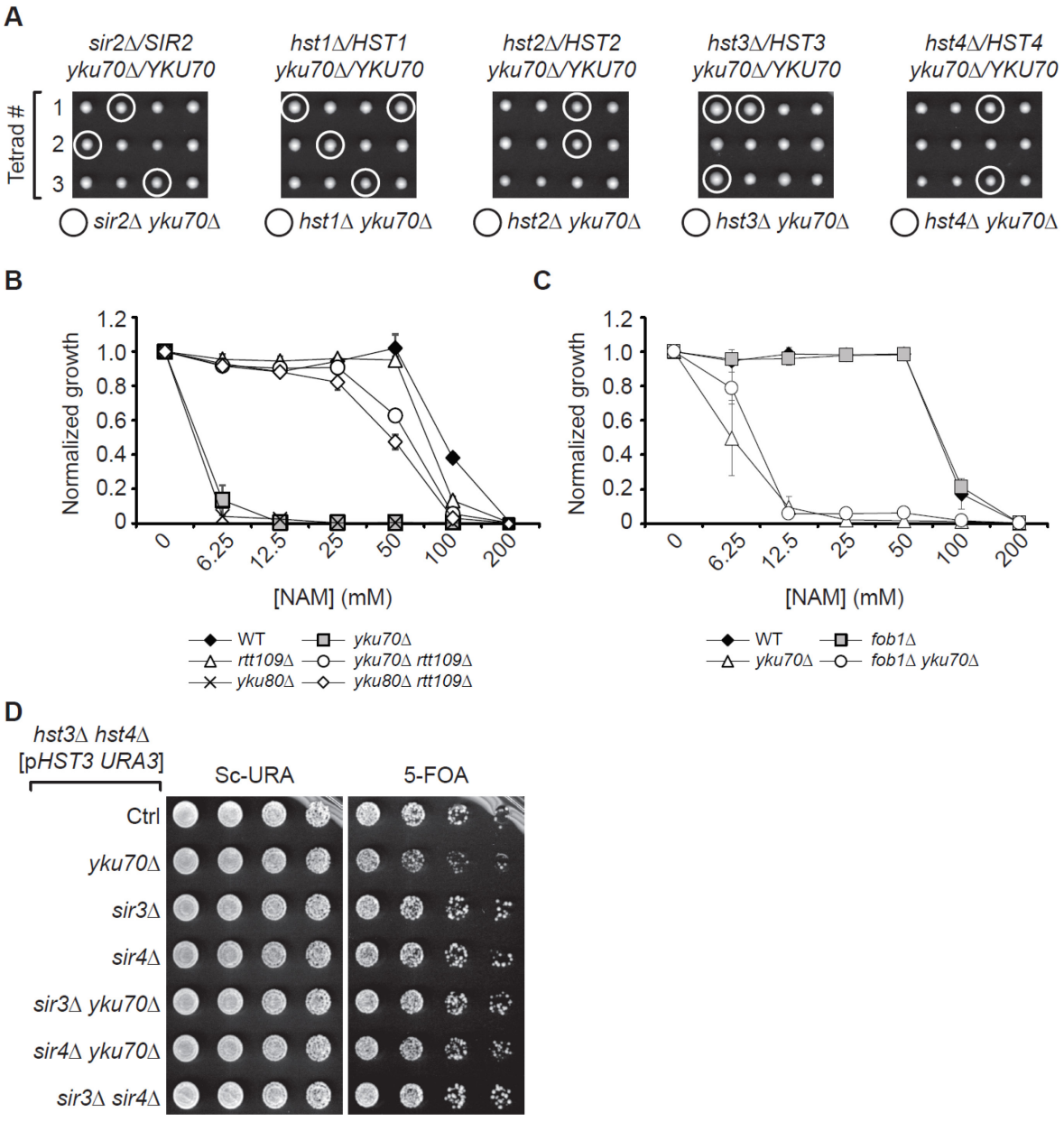

Figure 4.1. Analysis of genes involved in the sensitivity of *yku70*Δ/*80*Δ mutants to NAM.

(A) *yku70*Δ does not display synthetic growth defects with single sirtuin mutants. (B) Preventing H3K56 acetylation rescues the growth of *yku70*Δ and *yku80*Δ mutants in NAM. (C) *fob1*Δ does not rescue the growth defects of *yku70*Δ in NAM (D) Inhibition of the Sir2-Sir3-Sir4 complex is not responsible for the sensitivity of *yku70*Δ mutants to NAM

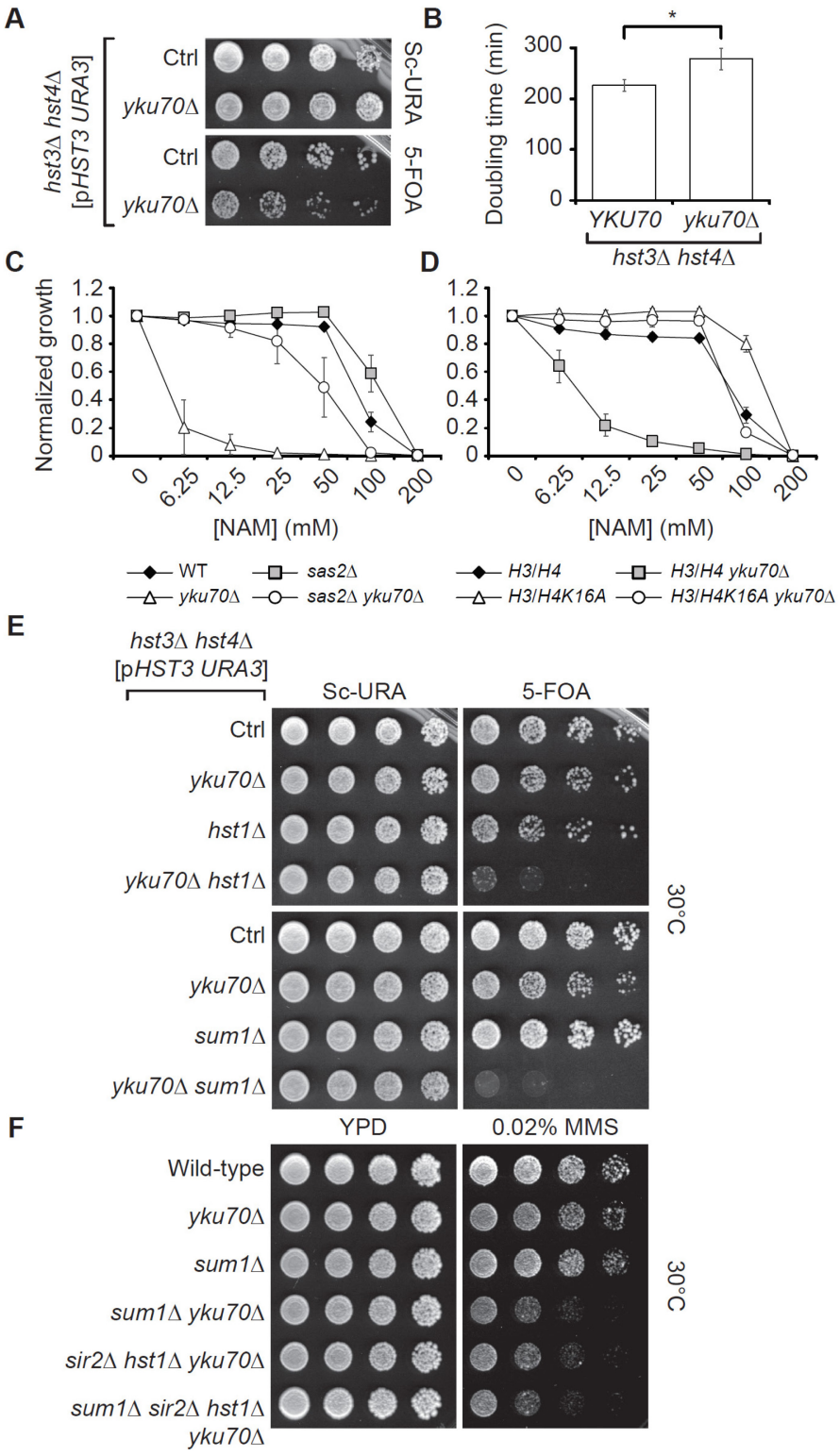

Figure 4.2. Multiple sirtuins permit growth of cells lacking Yku70/80

Legend to figure 4.2. (A-B) *yku70*Δ causes synthetic growth defects when combined with *hst3*Δ *hst4*Δ. Five-fold serial dilution of cells were spotted on the indicated solid media and incubated at 25°C. (B) Doubling time for strains in A incubated in YPD at 30°C (see material and methods). Error bars: standard deviation, p-value=7.42×10^{-7} (two-sided student's T-test). (C-D) Preventing H4K16 acetylation rescues the growth of *yku70*Δ mutants in NAM. Yeast cells were incubated in a 96-well plate containing increasing concentrations of NAM. OD_{630} readings were acquired after 48 h at 30°C and results were normalized to untreated controls. Error bars: standard deviation. (E) Deletion of genes encoding subunits of the Hst1-Sum1-Rfm1 complex cause synthetic lethality when combined with *hst3*Δ *hst4*Δ *yku70*Δ. (F) Lack of Sum1 causes synthetic sensitivity to MMS-induced replicative stress when combined *yku70*Δ.

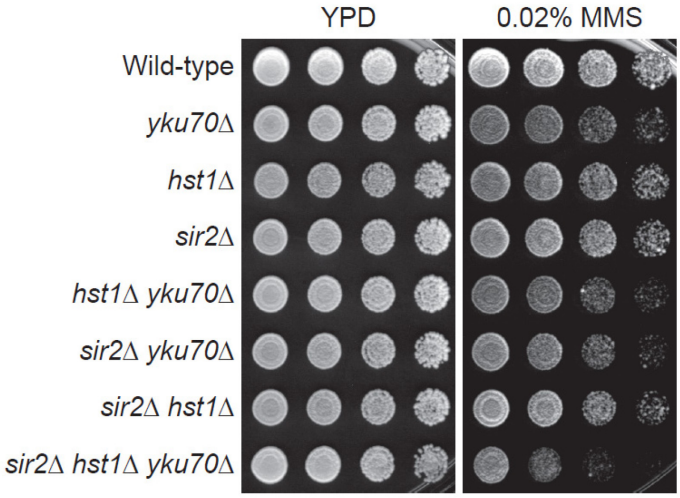

Figure 4.3. Deletion of both *SIR2* and *HST1* exacerbates the growth defects of *yku70*Δ cells in MMS.
Five-fold serial dilution of cells were spotted on the indicated solid media and incubated at 30°C.

(Sir2/Cdc14/Net1) or SIR (Sir2/Sir3/Sir4) complexes, respectively (59–61). While deletion of the RENT subunit-encoding *CDC14* or *NET1* genes causes cell lethality, rDNA silencing defects arising from lack of Sir2 activity are rescued by deletion of *FOB1*, which encodes a component of the rDNA replication fork barrier (10, 62, 63). Deletion of *FOB1* did not rescue the sensitivity of *yku70*Δ cells to NAM (Figure 4.1C); moreover, combining *hst3*Δ *hst4*Δ *yku70*Δ with either *sir3*Δ or *sir4*Δ also did not cause synthetic growth defects (Figure 4.1D). We conclude that growth of *yku70*Δ cells depends on the activity of Hst3/4 and on either i) sirtuins other than Sir2, or ii) Sir2-dependent processes that are not associated with the RENT or SIR complexes.

The Hst1-Sum1-Rfm1 complex promotes H4K16ac deacetylation *in vivo* (55). Interestingly, we found that deletion of either *HST1* or *SUM1* provoked synthetic growth defects when combined with *hst3*Δ *hst4*Δ *yku70*Δ (Figure 4.2E). Since constitutive hyperacetylation of H3K56 in *hst3*Δ *hst4*Δ mutants causes spontaneous DNA damage (18), we reasoned that elevated sensitivity to genotoxic stress in cells lacking both Hst1-Sum1-Rfm1 and Yku70/80 might explain the observed synthetic lethality. Consistently, we found that deletion of *SUM1* sensitized *yku70*Δ mutants to the DNA alkylating agent methylmethane sulfonate (MMS; Figure 4.2F). However, this was not the case for *hst1*Δ, implying that Hst1-independent Sum1functions might influence growth of *yku70*Δ cells in MMS (Figure 4.3). Published data indicate that Sir2 interacts with Sum1 and that this complex can promote transcriptional silencing in the absence of Hst1 (64). In accordance with this, while deletion of *SIR2* did not confer increased MMS sensitivity in *yku70*Δ mutants, the *sir2*Δ *hst1*Δ *yku70*Δ triple mutant was strongly sensitized to MMS compared to control double mutants (Figure 4.2F, Figure 4.3). Moreover, the MMS sensitivity of *sir2*Δ *hst1*Δ *yku70*Δ was similar to that of *sum1*Δ *yku70*Δ, and was not further increased in *sir2*Δ *hst1*Δ *sum1*Δ *yku70*Δ cells (Figure 4.2F). Overall, these data are consistent with a model in which the inability of *yku70*Δ mutants to grow in the presence of NAM is due to lack of Sum1-Hst1/Sir2 activity, which in turn sensitizes cells to replicative stress caused by constitutive H3K56ac.

4.4.2 Short telomeres sensitize cells to NAM-induced sirtuin inhibition.

The Yku70/80 complex is required for both DNA repair by non-homologous end joining (NHEJ) and telomere length maintenance (65, 66). Indeed, as for cells lacking the telomerase-encoding genes *EST1* and *EST2*, *yku70*Δ and *yku80*Δ mutants present short telomeres which, in contrast to the situation for *est1*Δ and *est2*Δ mutants, remain stable across cell generations (66, 67). While none of the NHEJ-abolishing mutations (*lif1*Δ, *nej1*Δ and *dnl4*Δ) that we tested caused notable growth defects in NAM (Figure 4.4A), *est1*Δ and *est2*Δ pre-senescent haploid mutants phenocopied *yku70*Δ (Figure 4.4B). This suggests that Yku70/80-dependent telomere homeostasis may be critical for growth in the absence of sirtuin activity. To test this, we performed a time course experiment with a strain expressing an auxin-inducible degron (AID)-tagged *YKU70* allele (68, 69). Auxin addition to the growth media provoked rapid (within one hour) degradation of Yku70 and progressive telomere shortening over several days of cell growth, while auxin removal from the growth medium allowed rapid Yku70 re-expression and progressive telomere length recovery (Figure 4.4C-D, Figure 4.5).

We reasoned that if Yku70 activity/presence within the cell was important for NAM resistance, then sensitivity to this agent should dramatically increase within hours of auxin treatment. On the other hand, if telomere homeostasis is critical for NAM resistance, progressive decrease in telomere length caused by Yku70 depletion should lead to concomitant increase in NAM sensitivity over several days of cell growth. At every time point analyzed, we tested the capacity of cells to grow in NAM with or without auxin, *i.e.* with or without Yku70 re-expression during NAM exposure (Figure 4.4E). Strikingly, NAM sensitivity correlated well with overall telomere length of the cell population, and re-expression of Yku70 during the growth assay (by omitting auxin in the NAM-containing medium) did not significantly reverse this trend. Together, these results indicate that i) continued presence of Yku70 is not essential for survival in the presence of NAM-induced sirtuin inhibition, and ii) telomere length is a critical determinant of NAM sensitivity in *yku70*Δ mutants.

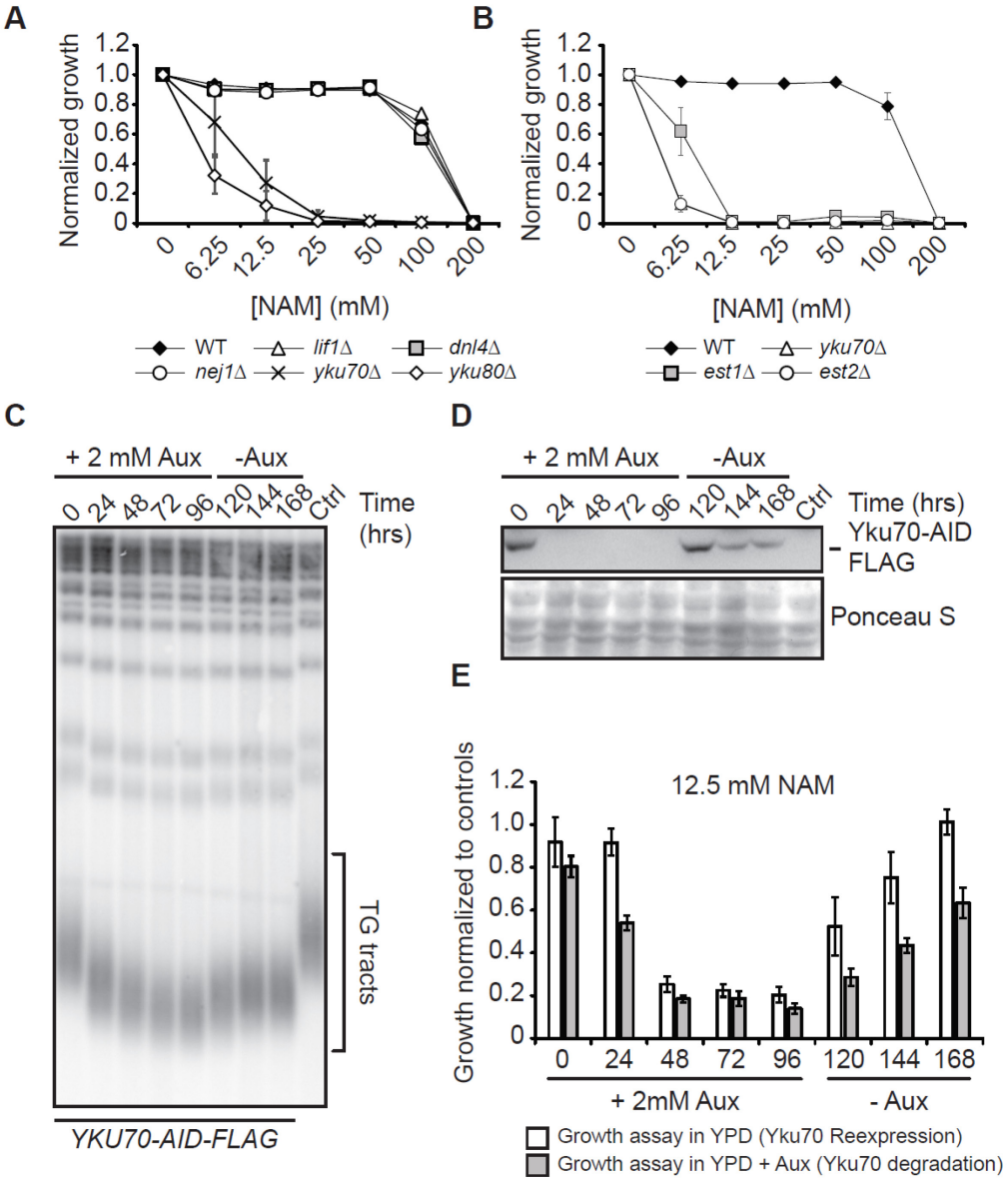

Figure 4.4. Short telomeres sensitize cells to NAM-induced sirtuin inhibition.
(A) Lack of NHEJ does not cause growth defects in NAM. (B) Telomerase mutants display severe growth defects in NAM. (A-B) Growth assay in 96-well plates (see materials and methods). Error bars: standard deviation. (C-E) Reduction of telomere length associated with Yku70 depletion causes NAM-induced growth defects. Yku70-AID-Flag-expressing yeasts were incubated in YPD at 30°C in the presence of auxin for 4 days to degrade Yku70. Cells were then transferred to YPD media without auxin to allow Yku70 re-expression. (C) Southern blot analysis of telomere length. (D) Yku70 degradation and re-expression was monitored by immunoblotting. (E) Samples were taken at every time point to evaluate cell growth in 12.5 mM NAM with or without auxin for 24h. Error bars: Standard deviation.

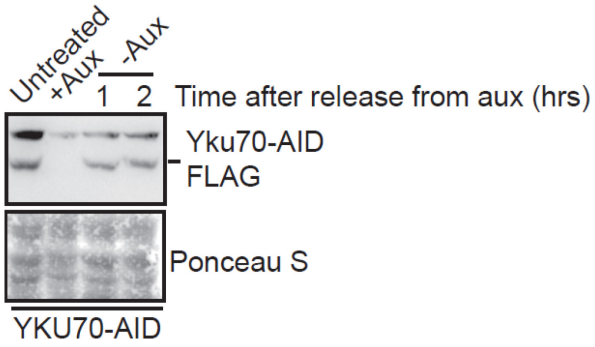

Figure 4.5. Yku70 is re-expressed within an hour after auxin removal
Yeast cells from the 96h time point from fig. 2C-D were resuspended in YPD medium without auxin. Yku70-AID-Flag re-expression was monitored by immunoblotting.

4.4.3 *RIF1* is deleterious to the growth of *yku70*Δ mutants in the presence of NAM.

Rif1 and Rif2 bind to telomeric TG_{1-3} repeats and limit telomere length (70) and, as such, cells lacking either protein present elongated telomeres (71–73). Since short telomeres appear to increase NAM sensitivity, we reasoned that mutating *RIF1* or *RIF2* might revert this effect and rescue the growth of *yku70*Δ in NAM. We found that telomeres are noticeably longer in *yku70*Δ *rif1*Δ than in WT cells (Figure 4.6A). In contrast, they remain shorter than WT in *rif2Δ yku70*Δ cells, even though they are modestly elongated when compared to those of *yku70Δ* single mutants (Figure 4.6A). Interestingly, we found that *rif1*Δ completely rescued the growth of *yku70*Δ in NAM whereas *rif2*Δ had no effect (Figure 4.6B). We note that NAM exposure did not significantly alter telomere length in any of these mutants (Figure 4.6A). *RIF1* deletion also rescued NAM resistance in *est1*Δ and *est2*Δ telomerase mutants (Figure 4.6C), suggesting that Rif1 negatively influence NAM resistance in cells with short telomeres of various genetic backgrounds. Importantly, *rif1*Δ is not a general suppressor of NAM-induced growth defects as *RIF1* deletion had no effect on the extreme NAM sensitivity of *slx4*Δ and *pph3*Δ mutants, which act via molecular mechanisms that are not directly related to telomere homeostasis (Figure 4.7) (44).

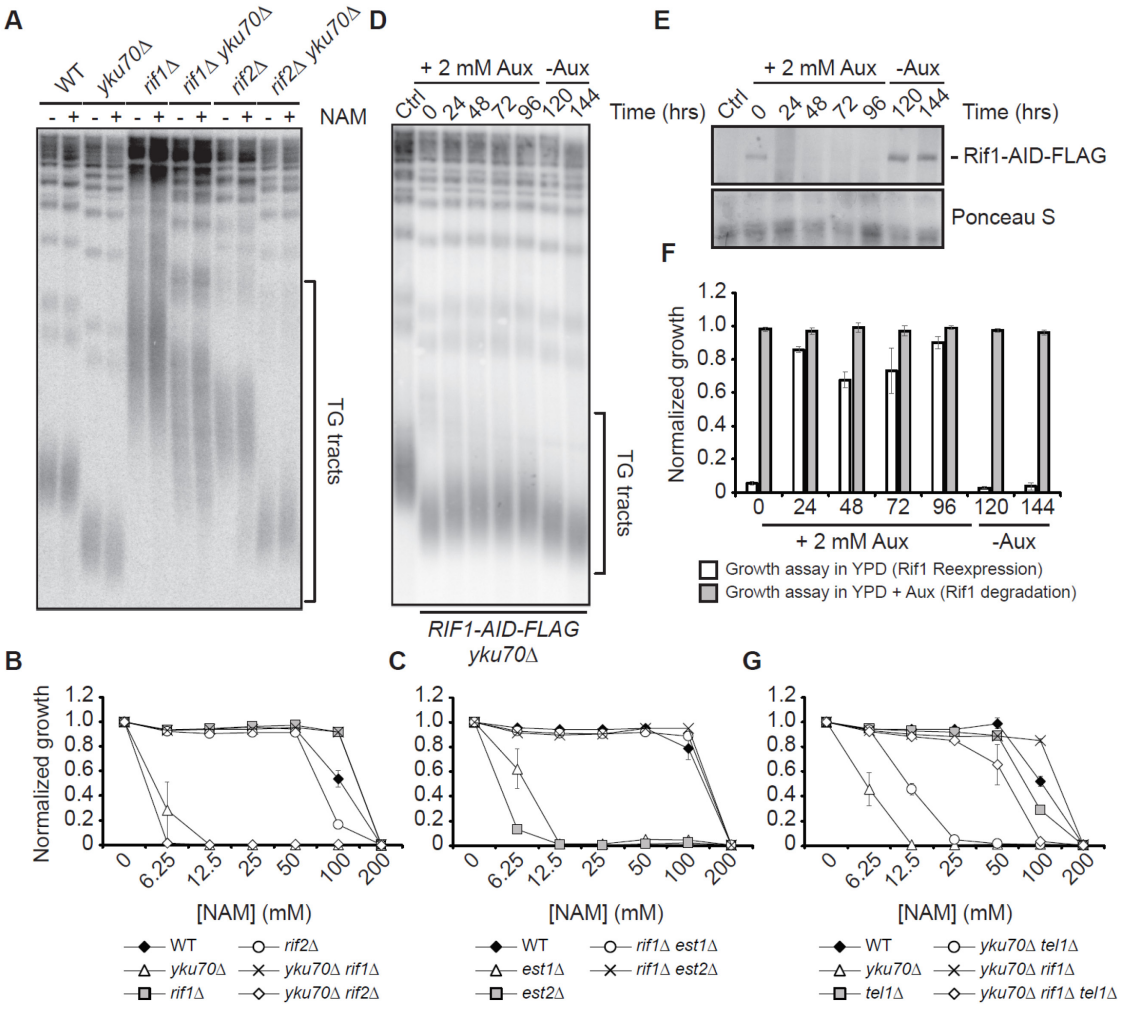

Figure 4.6. Rif1 is detrimental to the growth of cells with short telomeres in the presence of NAM.

(A) Southern blot analysis of telomere length of cells of the indicated genotype exposed or not to 20 mM NAM for 8 hours. (B) *rif1*Δ, but not *rif2*Δ, rescues growth of *yku70*Δ mutants in NAM. (C) *rif1*Δ rescues growth of pre-senescent telomerase mutants in NAM. (B-C) Growth assay as described in materials and methods. Error bars: standard deviation. (D-F) Depletion of Rif1 permits growth of *yku70*Δ mutants in NAM. *yku70*Δ *RIF1-AID-6Flag* strain was incubated with 2 mM auxin for 4 days in YPD at 30°C. Cells were then transferred to YPD media without auxin and allowed to grow for 2 days at 30°C. (D-E) Telomere length and Rif1 expression were analysed by southern and immuno blotting at every time point. (F) Samples were taken at every time point to evaluate cell growth in 6.25 mM NAM with or without 2 mM auxin for 48 h. (G) *rif1*Δ rescues the growth of *tel1*Δ *yku70*Δ in NAM. Growth assay were performed as described in materials and methods. Error bars: standard deviation.

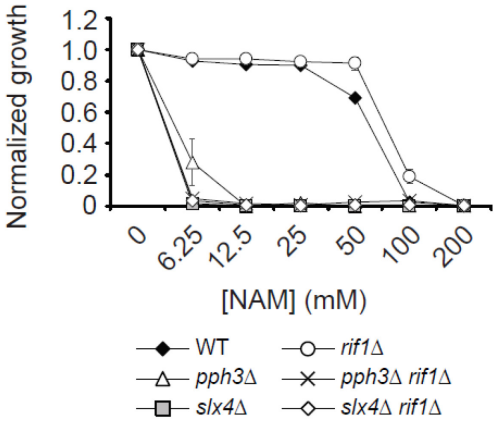

Figure 4.7. *rif1*Δ does not rescue the NAM sensitivity of *slx4*Δ and *pph3*Δ cells. Growth assay in 96-well plates (see materials and methods). Error bars: standard deviation.

To further evaluate the contribution of telomere lengthening in *rif1*Δ-dependent rescue of NAM sensitivity in *yku70*Δ cells, we performed an experiment similar to the one described in Figure 4.4C-E, except that we used a *RIF1-AID yku70*Δ strain. Auxin-induced Rif1-AID degradation provoked a moderate increase in telomeric length (in comparison to *RIF1* deletion, Figure 4.6A) over time in *yku70*Δ cells (Figure 4.6D-E). The ability of *yku70*Δ to grow in the presence of NAM after Rif1 re-expression (no auxin in the NAM-containing medium) correlated with increased telomere length, suggesting that the rescue of *yku70*Δ by *rif1*Δ may partly depend on increased telomere length (Figure 4.6F). However, in contrast to results presented in Figure 4.4 we noted that Rif1-AID degradation during the growth assay in NAM-containing medium allowed cells to grow as well as control strains from the very beginning of our experiment, when telomere length has not yet been modified (Figure 4.6F grey bars). Since telomere length significantly increased within 24 hrs of continuous Rif1 degradation in our experiments, it is possible that rapid auxin-induced telomere extension occurs in the *RIF1-AID yku70*Δ strain before the onset of irreversible NAM-induced cytotoxicity. Alternatively, the fact that the NAM sensitivity of cells with short telomeres is rapidly reversed upon Rif1 depletion may suggest that another function of this protein undermines cell growth under these conditions. We reasoned that if the latter was true, preventing telomere elongation should not inhibit the growth of *rif1*Δ *yku70*Δ cells in NAM. *rif1*Δ *tel1*Δ mutants present constitutively short telomeres that are similar

to those of *tel1*Δ or *yku70*Δ cells (39, 74). Interestingly, we found that deletion of *RIF1* strongly rescued the NAM sensitivity of *yku70*Δ *tel1*Δ mutants despite their short telomeres (Figure 4.6G), which is consistent with the notion that Rif1 functions other than telomere length regulation may contribute to NAM-induced growth defects in *yku70*Δ cells.

4.4.4 Cells with short telomeres present DNA replication defects upon NAM-induced inhibition of sirtuins.

Intriguingly, *tel1Δ* single mutants have short telomeres (75), but are not sensitive to NAM (Figure 4.6G). In fact, *TEL1* deletion partially rescues the sensitivity of *yku70*Δ cells to this chemical (Figure 4.6G). Even though *yku70*Δ and *tel1Δ* cells exhibit similar telomere length, one key difference between these mutants is that telomeric/subtelomeric replication origins are activated early in S phase in *yku70*Δ, but not in *tel1Δ* or *yku70*Δ *tel1Δ* strains (39). As mentioned above, aberrant timing of origin activation at a given locus, e.g., rDNA repeats in *sir2*Δ mutants, can influence DNA replication dynamics at unlinked loci by titrating replication initiation factors (42, 43). Based on this, we hypothesized that early activation of telomeric origins in cells with short telomeres could sequester limiting replication initiation factors away from internal loci, thereby reducing the overall density of active origins along chromosomes. This would force replication forks to process extended chromosomal regions before encountering a converging fork, and reduce the overall progression of DNA replication. Stalled replication forks under such conditions also cannot be "rescued" by a converging fork, which may increase sensitivity to replication-blocking genotoxins. The fact that Rif1 inhibits early firing of origins along chromosomes in addition to its effect on telomeric origins timing (32) is concordant with the above-described model, i.e., deletion of *RIF1* could compensate for the deleterious effects of early telomeric origin activation in *yku70*Δ mutants by increasing the activity of origins along chromosomes. Importantly, this effect of *rif1*Δ is not expected to depend on increased telomere length, which is consistent with the data presented in Figure 4.6G. Conversely, the Sum1 complex positively regulates the activity of a subset of internally located replication origins (76, 77). As such, the elevated sensitivity to MMS and synthetic lethality

with *hst3*Δ *hst4*Δ of the *yku70*Δ *sum1*Δ mutant (Figure 4.2E-F) may result from further decrease in internal origin activity, as compared to either *yku70*Δ or *sum1*Δ single mutants.

One prediction of our working model is that cells with short telomeres may fail to complete DNA replication in a timely manner in response to NAM. Consistently, we observed striking accumulation of *yku70*Δ and telomerase mutants (*est1*Δ and *est2*Δ) in early-mid S phase upon NAM exposure compared to WT (Figure 4.8A). We also note that this phenotype becomes more pronounced with increasing generations in *est1*Δ and *est2*Δ cells (Figure 4.8A), which is expected to be correlated with progressive telomeres shortening in these mutants. We previously showed that NAM promotes the formation of Rad52 and Rfa1 foci, which is associated with induction of replicative stress (44). We did not detect any increases in the proportion of *yku70*Δ cells presenting Rad52-YFP and Rfa1-YFP foci in response to NAM, suggesting that replication defects in cells with short telomeres are not due to more frequent DNA lesions (Figure 4.8B-C). We did however observe increased Rfa1-YFP foci fluorescence intensity and activation of the intra-S phase checkpoint kinase Rad53 (Figure 4.8D, Figure 4.9), concordant with the fact that a larger proportion of cells are experiencing replicative stress in response to NAM in *yku70*Δ mutants compared to WT cells (Figure 4.8A). Both *tel1*Δ and *rif1*Δ mutations were found to prevent S phase accumulation of *yku70*Δ cells (Figure 4.8E), although neither mutation completely abolished Rad53 activation (Figure 4.9). This suggests that while *tel1*Δ and *rif1*Δ do not resolve the source of NAM-induced replicative stress (constitutive H3K56ac), they favour cell survival under these conditions by allowing completion of DNA replication.

One possible consequence of the observed DNA replication defects in NAM-treated *yku70*Δ mutants is that chromosomes may remain incompletely replicated at the end of S phase. If so, one would expect chromosome loss to occur frequently in *yku70*Δ mutants upon NAM exposure. Consistently, we found that cells lacking Yku70 lose a non-essential ARS209-containing pRS316 plasmid at an increased frequency compared to WT, and that exposure to non-lethal doses of NAM amplifies this effect in *yku70*Δ but not WT cells (Figure 4.8F). Overall, our results indicate that *yku70*Δ mutants present DNA replication defects in response to NAM-induced sirtuin inhibition, which probably explains their inability to grow in the presence of this agent.

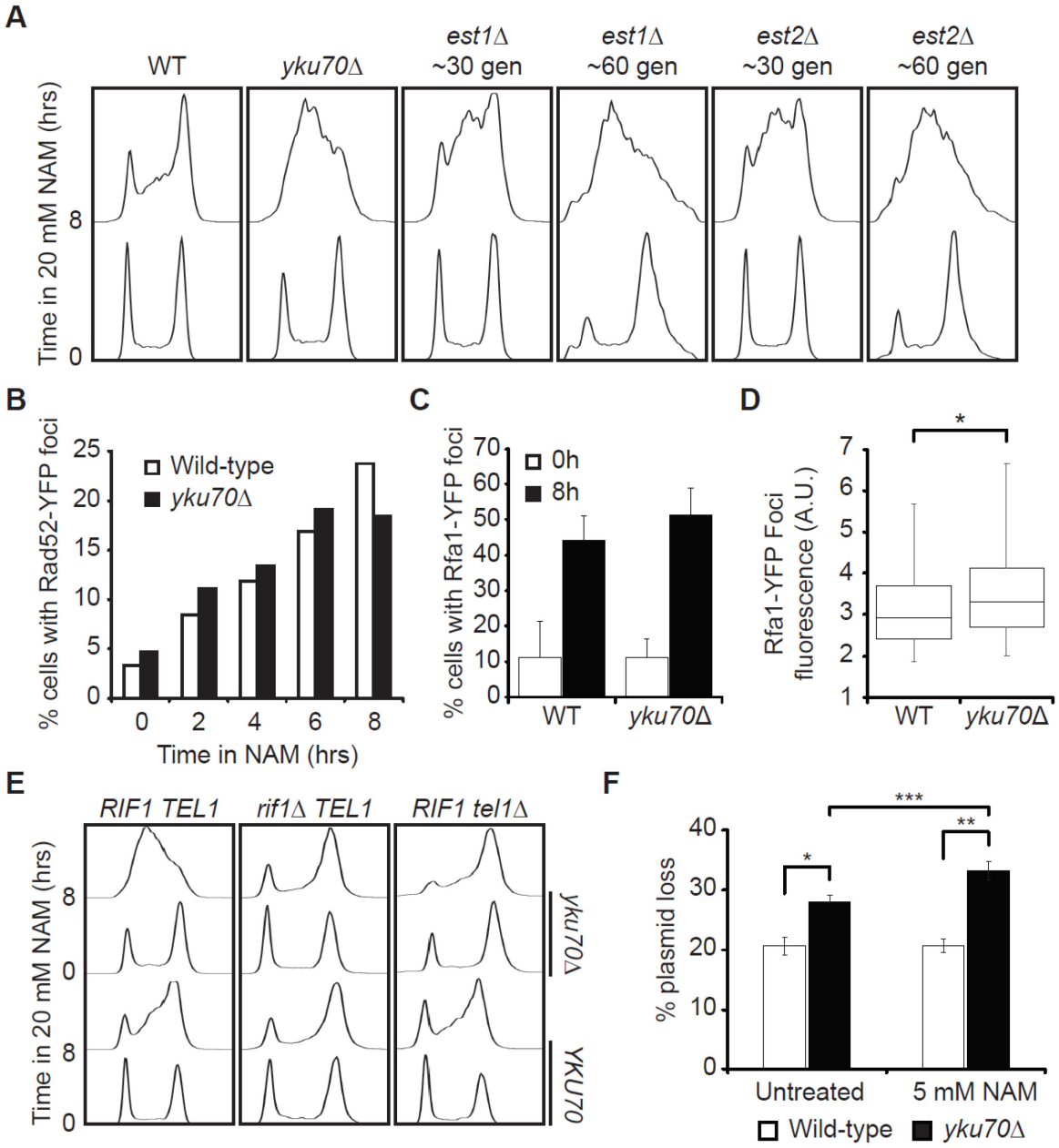

Figure 4.8. Cells with short telomeres present defects in completing DNA replication upon NAM exposure

Legend to figure 4.8. (A) Asynchronous cells were incubated in YPD for 8 hours at 30°C in the presence of 20 mM NAM. Samples were taken at indicated time for flow cytometry-based DNA content analysis. (B) Asynchronous cells were incubated for 8 hours in synthetic complete media at 30°C in the presence of 50 mM NAM. Samples were taken at indicated time for fluorescence microscopy (Rad52-YFP foci). (C-D) Upon NAM exposure, *YKU70* deletion does not increase the proportion of cells with Rfa1-YFP foci, but intensifies their fluorescence. Asynchronous cells were exposed to 20 mM NAM for 8 hours in synthetic complete medium at 30°C. Samples were taken at indicated time and Rfa1-YFP foci were analysed by fluorescence microscopy. (E) *rif1*Δ and *tel1*Δ rescue the S phase progression defects of *yku70*Δ mutants in NAM. Cells of indicated genotypes were treated as in (A) and DNA content was analysed by FACS. (F) *yku70*Δ mutants exposed to NAM frequently lose a ARS209-containing pRS316 plasmid. Error bars: standard error of the mean, *: p-value=0.004, **: p-value=0.002, ***: p-value=5.9E-5.

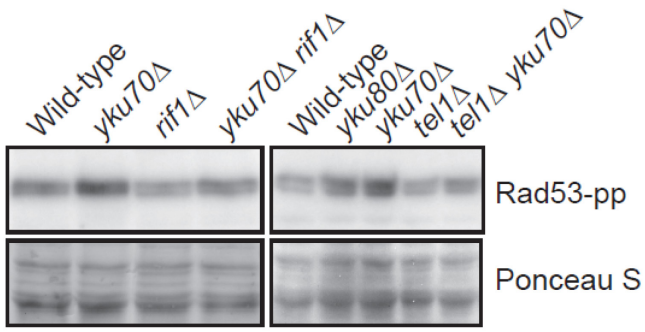

Figure 4.9. *rif1*Δ and *tel1*Δ do not significantly alter checkpoint activation of cells exposed to NAM
Rad53 activation in cells exposed to 20 mM NAM for 8h was examined by in situ auto-phosphorylation assay (see materials and methods).

Another prediction from our working model, is that increasing the number of active origins in a *yku70*Δ mutant, similar to what occurs in the absence of Rif1, should alleviate its sensitivity to replicative stress. Published results indicate that Cdc45, Sld3 and Sld7 (45/3/7) represent the minimal set of initiation factors required to trigger activation of licensed replication origins, and that their overexpression increases the number of active origins during S phase (41, 42). Interestingly, 45/3/7 overexpression rescued the growth of *yku70*Δ mutants in response to either NAM or MMS (Figure 4.10A-B). Moreover, we found that the MMS sensitivity of *yku70*Δ *sum1*Δ cells is also rescued by 45/3/7 overexpression (Figure 4.10C), which suggests that the elevated sensitivity to MMS of the *sum1*Δ *yku70*Δ mutant results from further reduction in the number of active internal origins.

Published data indicate that *yku70*Δ and *rad52*Δ mutants present synthetic sensitivity to MMS, which is assumed to result from the loss of two important DSB repair pathways, NHEJ and homologous recombination (HR) (78). However, the fact that MMS does not produce detectable DSBs in yeast (79) raises the possibility that cellular functions of Yku70 that are unrelated to NHEJ-dependent DSB repair may contribute to such synthetic sensitivity. Indeed, our results show that 45/3/7 overexpression partially rescues the synthetic sensitivity to MMS of *rad52*Δ *yku70*Δ mutants (Figure 4.10D), suggesting that reduced availability of DNA replication initiation factors may contributes to the sensitivity of these cells to replicative stress. Collectively, our results suggest that dysregulation of the timing of DNA replication origins caused by short telomeres influences the sensitivity of cells to replicative stress, and that the activity of multiple sirtuin-family HDACs is essential to permit completion of DNA replication under these conditions.

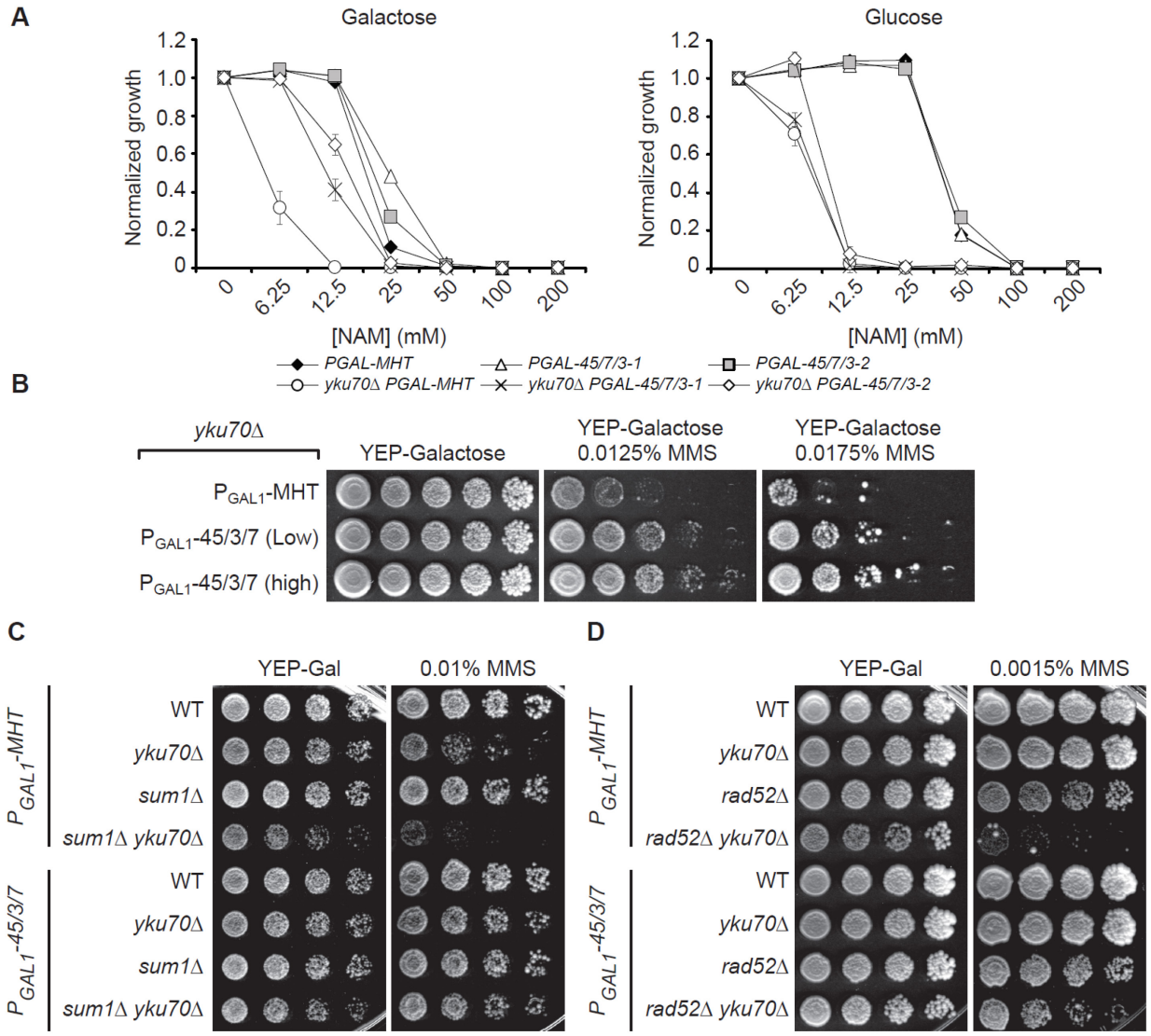

Figure 4.10. Overexpression of replication initiation factors rescues the sensitivity of cells lacking Yku70 and Sum1 complexes to replicative stress caused by MMS or NAM

(A-B) Galactose-induced overexpression of *CDC45*, *SLD3* and *SLD7* (45/3/7) improves the growth of *yku70*Δ mutants in NAM (A) and MMS (B) in a dose-dependent manner. (C-D) Overexpression of *CDC45*, *SLD3* and *SLD7* (45/3/7) rescues the synthetic growth defects of *yku70*Δ *sum1*Δ and *yku70*Δ *rad52* mutants exposed to MMS.

4.5 Discussion

Results herein presented reveal novel mechanisms by which short telomeres may influence the ability of cells to respond to replicative stress. We suggest the following model: abnormal activation of telomeric/subtelomeric origins in early S phase in cells with short telomeres, *e.g.* in *yku70*Δ mutants, restricts the availability of limiting DNA replication initiation factors such that the density of active replication forks is reduced in a pan chromosomal manner. Under these conditions individual replication forks would be forced to replicate extended chromosomal regions before encountering a converging fork, which is expected to compromise: i) timely S phase completion, and ii) rescue of stalled replication forks by converging forks emanating from adjacent origins. Conceptually, this model is in line with previously published evidence indicating that reducing the number of origins on a chromosome extends the duration of S phase (80), and that abnormal sequestration of origin firing factors at specific loci dysregulates global patterns of replication origin timing (31, 42, 43). Haploid yeast cells possess 32 telomeres which normally repress origins at both telomeric and subtelomeric regions (35), rendering plausible the notion that abnormal timing of telomeric/subtelomeric origin activation may cause sufficient sequestration of limiting replication initiation factors to cause replicative stress-associated phenotypes.

Sudden telomere shortening occurs naturally *in vivo* in WT yeast and is thought to result from replication fork collapse in telomeric tracts (37). Early firing of origins proximal to shortened telomeres promotes recruitment of telomerase and subsequent telomere elongation (38), suggesting that regulation of replication initiation at telomeres may favor genomic integrity by allowing prompt elongation of critically shortened telomeres. Since such telomere shortening events are relatively infrequent and are expected to involve only one telomere in any given cell, the impact on the availability of replication factors, and hence on global replication timing patterns, is expected to be minor. This is in contrast with senescence caused by lack of telomerase activity, which causes uniform reduction in length of all telomeres. Interestingly, such conditions have been shown to induce several classical markers of the DNA damage response in both yeast and human cells (81, 82). In light of our results, we propose that misregulation of the timing of telomeric and subtelomeric origins in cells with uniformly

shortened telomeres (e.g. telomerase mutants) may contribute to the induction of DNA damage response markers by sequestering limiting replication initiation factors.

We note that DNA replication progresses slowly at telomeres (83–85). It is therefore conceivable that increasing the number of active replication forks at short telomeres may generate replicative stress specifically in these regions, thereby elevating NAM sensitivity. While our results do not allow us to exclude this possibility, several lines of evidence suggest that disruption of genome-wide origin firing is the main cause of DNA replication defects in NAM-treated cells with short telomeres. First, telomeric $TG_{1\text{-}3}$ tracts are devoid of histones, and some subtelomeric regions present low nucleosome occupancy (85). Such paucity of H3K56ac-harboring nucleosomes at telomeres suggest that NAM-induced replicative stress probably stems from defective replication of intrachromosomal regions rather than telomeres. Second, *45*/*3*/*7* overexpression and *RIF1* deletion both rescued the growth defects of *yku70*Δ mutants in NAM, even though they are expected to promote early firing of telomeric origins. Finally, we did not detect strongly increased DNA damage response markers upon NAM exposure in *yku70*Δ mutants as compared to WT, *e.g*. Rad53 autophosphorylation or Rad52/Rfa1 foci, which appears inconsistent with frequent induction of replication fork stalling/collapse at short telomeres. Thus, further experiments will be required to assess the contribution of replicative stress arising specifically at short telomeres to NAM-induced growth defects.

Our results clearly indicate that constitutive H3K56ac interferes with DNA replication in cells with short telomeres. While the mechanisms through which genome-wide hyperacetylation of H3K56 negatively influence DNA replication are poorly understood, lack of Hst3/4 activity has recently been shown to prevent extensive DNA synthesis in the context of DNA double-strand break repair by break-induced replication (86). Deletion of *HST3* also causes, in a H3K56ac-dependent manner, frequent loss of a disomic chromosome III in which several efficient ARS have been removed (23). Finally, deletion of *SIR2*, which causes sequestration of DNA replication initiation factors at the rDNA locus (42), leads to synthetic lethality in *hst3*Δ *hst4*Δ cells. The results presented herein are generally consistent with these observations, and reinforce the notion that reduced origin activity is deleterious in cells that misregulate H3K56ac levels.

Previous genome-wide screens found that simultaneous deletion of *SUM1* and *YKU70* confers synthetic fitness defects (87). Moreover, *SUM1* deletion provokes growth defects when

combined with various mutations that perturb replication initiation, e.g., *orc2-1*, *orc5-1*, *cdc6-1*, *cdc7-1* and *cdc45-1* (76, 88). Our data are generally consistent with these observations, and suggest that these genetic interactions may result from synergistic perturbation of cellular replication patterns. Indeed, Sum1, in conjunction with the sirtuin Hst1, promotes replication initiation at several ARS (76, 77). Consistent with previous reports indicating that Sir2 can associate with Sum1 (64), our results also suggest that the effects of the Sum1 complex on origin firing may result from its interaction with either Sir2 or Hst1. Intriguingly, we found that the ability of *yku70*Δ mutants to grow in the presence of NAM can be rescued by reducing H4K16ac levels. Since both Hst1 and Sir2 influence H4K16ac levels *in vivo* (54, 55), our results raise the possibility that regulation of H4K16ac by Sum1-containing complexes may influence DNA replication dynamics.

The model we propose herein may have implications for cancer biology. Human somatic cells generally do not express telomerase, leading to progressive telomere shortening over cell generations. When telomeres become critically short, cells activate DNA damage checkpoints, stop dividing, and undergo replicative senescence (81). A large fraction of human tumours escape this arrest by expressing telomerase (89), which confers the ability to proliferate indefinitely. This has led to the development and clinical trials of telomerase inhibitors for cancer treatment (90, 91). In addition, several human sirtuins are deregulated in various cancers, and pharmacological inhibition of sirtuins is considered a promising avenue for anti-cancer therapy (92). While the impact of short telomeres on global patterns of DNA replication in human cells has not yet been characterized, our results suggest that combinatorial approaches using sirtuin and telomerase inhibitors could potentiate the cytotoxicity of replication-blocking genotoxic chemotherapy drugs in cancer cells.

4.6 Acknowledgements

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Chapitre 5. Discussion

Dans le cadre des trois articles présentés dans cette thèse, nous nous sommes intéressés aux rôles d'H3K56ac en utilisant des systèmes dans lesquels H3K56 est constitutivement acétylé. Nos résultats indiquent que prévenir la désacétylation d'H3K56ac anéantit la capacité des cellules à répondre au stress réplicatif et que l'une des composantes majeures des phénotypes extrêmes engendrés provient de l'hyperactivation de la signalisation en réponse aux dommages à l'ADN. De plus, les résultats du troisième article suggèrent que l'activité des sirtuines est nécessaire pour la survie de cellules qui présentent des télomères courts. Nos données soutiennent un modèle selon lequel la longueur des télomères affecte la distribution d'activation des origines, ce qui en contrepartie sensibilise les cellules au stress réplicatif. Bien que les fonctions d'H3K56ac expliquant ces phénotypes sont encore mal comprises, les travaux présentés ici fournissent plusieurs pistes pour la compréhension future du rôle de cette modification.

5.1 L'acétylation d'H3K56 et la réponse aux dommages à l'ADN

5.1.1 H3K56ac favorise l'activation de la signalisation en réponse au stress réplicatif

Un concept récurrent dans les deux premiers articles présentés est que l'hyperactivation de la réponse aux dommages à l'ADN issue de sources endogènes est particulièrement nocive pour les cellules comportant l'acétylation constitutive d'H3K56. La plupart des défauts observés chez un mutant *hst3*Δ *hst4*Δ ou dans des cellules traitées au NAM peuvent être au moins partiellement éliminés par l'inhibition de l'activation de la réponse aux dommages à l'ADN. À l'inverse, la mutation de gènes impliqués dans l'inactivation ou la réduction de l'activation de Rad53 exacerbe les phénotypes reliés à l'hyperacétylation d'H3K56ac (543, 544). Une

explication possible pour réconcilier ces phénomènes serait qu'H3K56ac joue un rôle dans l'activation de la signalisation en réponse aux dommages à l'ADN.

H3K56ac affaiblit le contact nucléosome-ADN et rend l'ADN accessible à la nucléase micrococale (528). Il est ainsi concevable qu'H3K56ac facilite l'association à l'ADN ou la chromatine de facteurs de réponse aux dommages à l'ADN lorsque H3K56 est acétylé (528). En ce sens, puisque la mutation de *RAD9* supprime les défauts de croissance du mutant *hst3*Δ *hst4*Δ et ceux de *slx4*Δ et *pph3*Δ en NAM, il est possible que Rad9 puisse interagir plus fortement avec la chromatine en présence d'H3K56ac. Toutefois, comment H3K56ac pourrait agir de la sorte demeure nébuleux. Une possibilité est qu'H3K56ac provoque des changements dans la structure de la chromatine qui favorise la présentation et l'exposition d'H3K79me et H2A-S128P, permettant ainsi à Rad9 de s'y lier sous sa forme de dimère. Autrement, la relaxation de la chromatine par H3K56ac pourrait éliminer un encombrement stérique qui préviendrait normalement l'association de Rad9 (Figure 5.1A).

Alternativement, l'interaction affaiblie ADN-nucléosome en présence d'H3K56ac pourrait faciliter l'éviction ou le glissement des histones sur l'ADN, favorisant alors l'association directe de facteurs avec l'ADN. La suppression des défauts de croissance et d'activation de Rad53 du mutant *hst3*Δ *hst4*Δ par la délétion de *RSC2* est en accord avec cette hypothèse (Figure 2.6, (543)). Rsc2 fait partie du complexe de remodelage de la chromatine RSC qui dégage les nucléosomes des promoteurs de certains gènes et est impliqué dans la réponse aux dommages à l'ADN (325, 545–547). En son absence, il est donc possible que les nucléosomes bloquent le recrutement de facteurs de réponse aux dommages à l'ADN aux fourches de réplication arrêtées, réduisant alors l'amplitude de l'activation de Rad53. Ce concept n'est pas sans précédent et il a été démontré que le remodelage de la chromatine joue un rôle dans la réponse aux dommages à l'ADN. En particulier, RSC, Ino80 et Fun30 sont nécessaires pour l'étape de résection dans la réparation des DSBs et Rad54 délocalise les nucléosomes de la matrice d'ADN lors de l'invasion (324, 325, 327, 328, 331, 332). Donc, l'éviction des nucléosomes pourrait être plus substantielle aux fourches arrêtées en présence d'hyperacétylation d'H3K56 comparativement à une situation normale où les niveaux d'H3K56ac atteignent 50%. Dans ce contexte, le complexe 9-1-1 est un candidat potentiel qui pourrait s'associer plus fortement ou à plus haute fréquence à l'ADN dénudé (Figure 5.1B). En effet, ce complexe interagit directement avec les jonctions dsDNA et ssDNA au niveau des

lésions et la délétion des gènes codant pour chacun de ses membres améliore la croissance du mutant *hst3*Δ *hst4*Δ (253, 531). Enfin, il sera intéressant de tester si l'association à la chromatine ou à l'ADN de Rad9 et la clampe 9-1-1 peut être influencée par la présence d'H3K56ac pour déterminer si cette modification joue un rôle direct dans la signalisation.

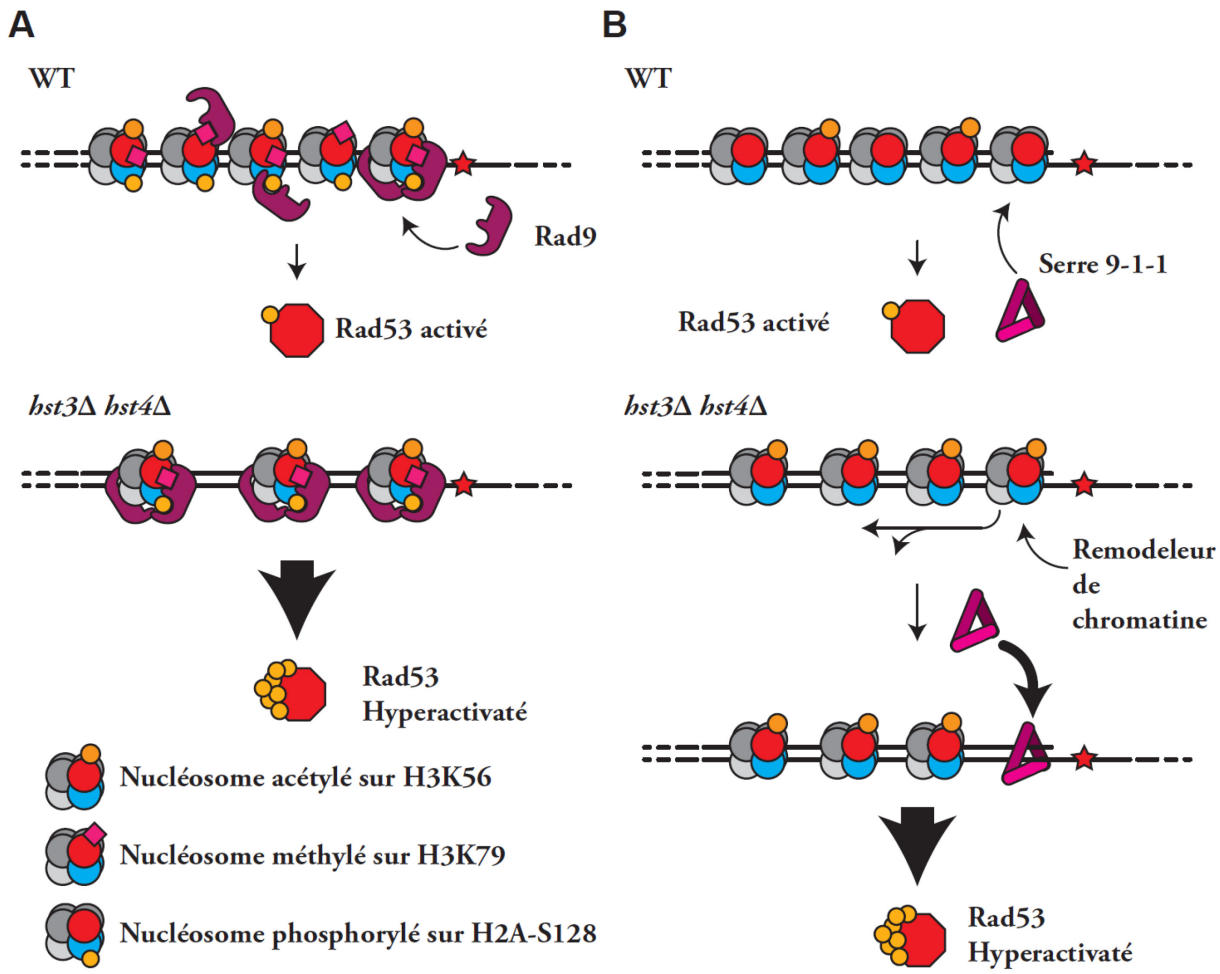

Figure 5.1. Modèles selon lesquels H3K56ac pourrait influencer l'activation de la signalisation en réponse aux dommages à l'ADN

5.1.2 Les causes de l'hypersensibilité à l'activation de la signalisation en réponse au stress réplicatif

Nos résultats suggèrent que l'hyperactivité de Rad53 est toxique en présence d'acétylation constitutive d'H3K56 en conséquence d'au moins deux mécanismes: l'inhibition de la voie de TLS et l'inhibition de l'activation des origines tardives. Le TLS est considéré comme une voie propice aux erreurs, ou mutagénique, et il est intriguant que celle-ci soit inhibée par l'activité de Rad53 (441, 442). En effet, il paraîtrait plus intuitif que cette voie soit plutôt utilisée en dernier recours, lorsqu'il y a présence d'une quantité substantielle de dommages à l'ADN et que Rad53 est hyperactivé. Une explication alternative est que Rad53 canalise la réparation vers une voie qui devient inefficace, e.g. la recombinaison homologue et le TS (permutation de matrice, template switching), en présence aberrante d'H3K56ac ou de certains agents induisant du stress réplicatif. Nonobstant, dans les deux cas, la diminution de l'activité de Rad53 permet de recanaliser le PRR vers le TLS, assurant la tolérance des dommages à l'ADN chez le mutant *hst3*Δ *hst4*Δ. Il sera nécessaire d'identifier les cibles de Rad53 dans ce contexte pour comprendre comment les cellules régulent la voie de TLS et de déceler plus précisément dans quelles situation cette voie est souhaitable pour le maintien de la stabilité génomique.

La dérégulation du complexe Mus81-Mms4, en conséquence de l'activation de Rad53, pourrait également contribuer aux défauts de croissance du mutant *hst3*Δ *hst4*Δ. En effet, l'activité de Mus81-Mms4 dans la résolution des structures de recombinaison homologue est réduite lorsque Rad53 est hyperactif (421). De plus, la mutation de Mus81 provoque l'apparition de foyers spontanés de Rad52 (535). Il est donc probable que l'apparition des foyers spontanés de Rad52 chez le mutant *hst3*Δ *hst4*Δ découle au moins en partie de défauts à résoudre certaines structures de recombinaison homologue suite à l'inhibition de Mus81-Mms4, ce qui pourrait avoir pour conséquence de réduire la viabilité de ces cellules. À ce titre, il sera intéressant de tester si la réduction de l'activation de la signalisation en réponse au stress réplicatif, notamment par la mutation de *RAD9, SAS2 ou DOT1*, pourrait réduire la fréquence d'apparition de foyers de réparation.

5.1.3 H3K56ac dans la recombinaison homologue

Le fait que des mutants *rtt109*Δ et H3K56R, dont les nucléosomes ne comportent pas d'H3K56ac, présente lui aussi une hyperactivation constitutive de Rad53 (529) n'est pas entièrement cohérent avec une fonction unique d'H3K56ac dans la signalisation en réponse aux dommages à l'ADN et suggère que cette modification régule des mécanismes supplémentaires. La présence cyclique d'H3K56ac fait en sorte que cette marque est normalement présente sur les chromatides sœurs, et donc à une position idéale pour définir quelles régions peuvent servir de matrice pour la recombinaison homologue. De plus, cette modification persiste sur la chromatine jusqu'à ce que les dommages à l'ADN soient réparés ce qui suggère qu'elle joue un rôle dans la réparation des dommages (528). Un modèle attrayant, qui a d'ailleurs déjà été proposé (541), serait qu'H3K56ac soit nécessaire pour diriger l'étape d'invasion de la recombinaison homologue vers la chromatide sœur. Ainsi, en l'absence d'H3K56ac, les filaments Rad51 seraient incapables d'envahir la chromatide sœur et à l'inverse, la présence aberrante d'H3K56ac devant les fourches de réplications provoqueraient des invasions toxiques à des régions non répliquées (Figure 5.2). Perturber le cycle d'acétylation d'H3K56 dans un sens ou l'autre aurait donc un impact négatif sur la recombinaison homologue et causerait l'hyperactivation de Rad53 en conséquence de la persistance de dommages non-réparés.

Plusieurs observations sont en accord avec ce modèle; la présence élevée de foyers de Rad51 et Rad52, la diminution de la fréquence de recombinaison et la sensibilité au stress réplicatif des mutants *rtt109*Δ et *hst3*Δ *hst4*Δ (213, 216, 520, 529, 531, 541, 543). La toxicité des événements de recombinaison en présence d'H3K56 constitutivement acétylé prédit que la formation de filaments Rad51 est néfaste pour la réponse au stress réplicatif. En ce sens, la mutation des gènes impliqués dans la formation et stabilisation des filaments de Rad51 (*RAD51*, *RAD54*, *RAD55* et *RAD57*) ne confère pas de défauts de croissance au mutant *hst3*Δ *hst4*Δ (531). À l'inverse, la mutation de *SRS2*, qui entraîne la formation aberrante de filaments Rad51 non-productifs (338, 339), figure parmi les mutations les plus sensible au NAM (544) et cause une létalité synthétique avec *hst3*Δ *hst4*Δ (531). De plus, la mutation d'*HST3* et *HST4* augmente fortement la fréquence de délétion complète du gène *CAN1* (548), ce qui est compatible avec la

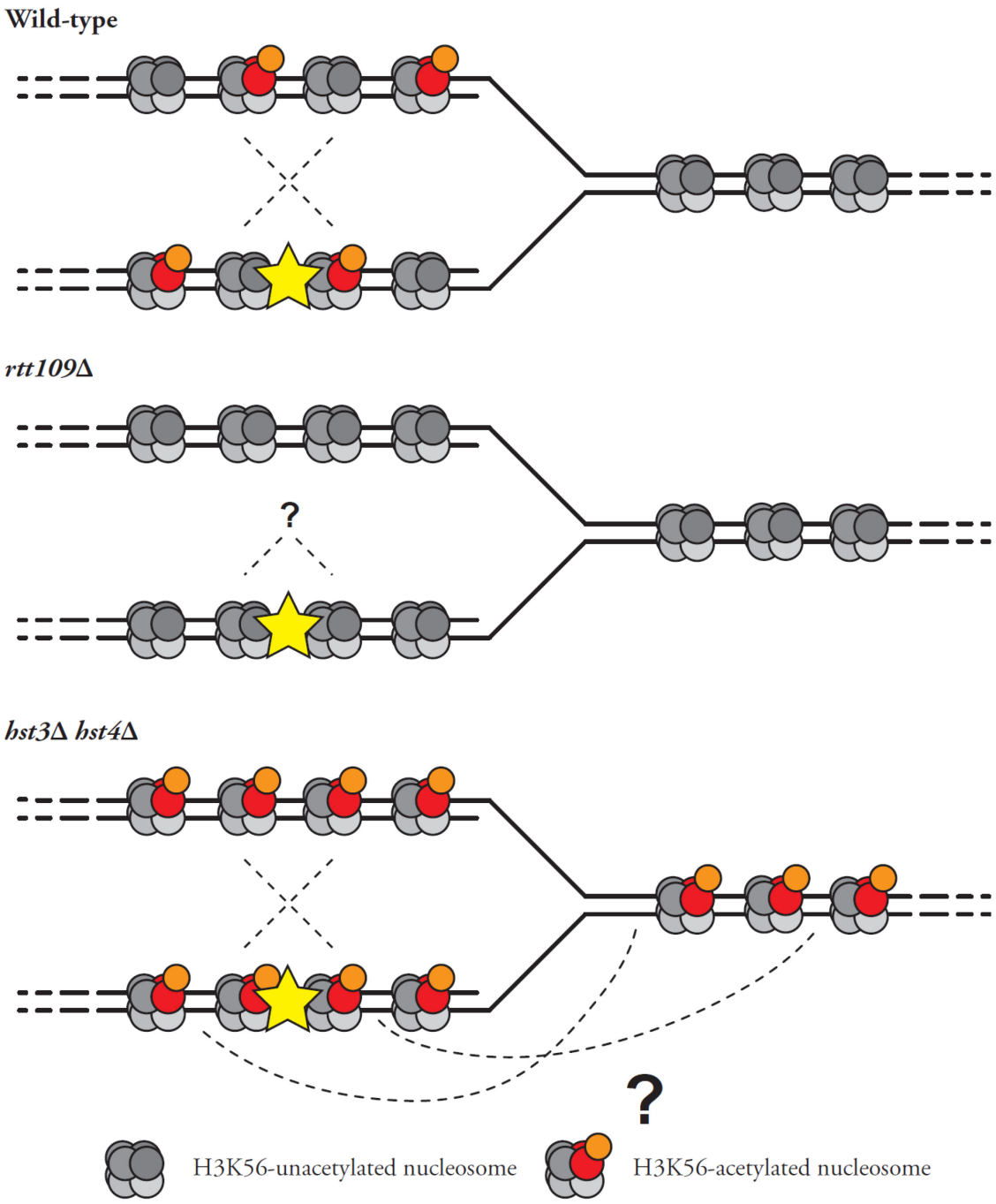

Figure 5.2. Les conséquences perturbation du cycle d'H3K56ac sur la recombinaison homologue

notion que des événements de recombinaison entre l'avant et l'arrière des fourches de réplication provoquent la perte d'information génétique.

Ce modèle pourrait être validé à l'aide d'un dérivé de l'expérience de recombinaison par échange inégal du gène *HIS3* sur les chromatides sœurs, qui repose sur la reconstitution du marqueur d'auxotrophie *HIS3* par recombinaison entre deux fragments du gène placés à une courte distance l'un de l'autre (549). Deux de ces constructions seraient placés à une grande distance l'une de l'autre sur le même chromosome. La première des constructions *HIS3* (celle en 5') serait flaquée d'une ARS (du côté 5'), tandis que l'autre serait éloignée de toute ARS. Une séquence RFB (identique à celle présente dans le rDNA), puis le gène *ADE2* seraient insérés dans la région entre les deux séquences *HIS3* (Figure 5.3). Au cours de la phase S, la fourche de réplication provenant de l'ARS s'arrêtera nécessairement à la RFB, et pendant la majeure partie réplication, seule l'une des deux constructions *HIS3* serait répliquée et présente sur les deux chromatides sœurs (celle proche de l'ARS). Dans cette situation, la recombinaison devrait avoir lieu strictement entre les marqueurs *HIS3* répliqués, ce qui permettrait la reconstitution du gène *HIS3* ainsi que la croissance des levures sur un milieu dépourvu d'histidine (Figure 5.3, 1). Or, un événement de recombinaison entre une des séquences sur la chromatide sœur et celle non-répliquée reconstituerait aussi le marqueur *HIS3*, mais entraînerait également la disparition du gène *ADE2* (Figure 5.3, 2). L'absence de ce dernier provoque l'accumulation d'un pigment rouge et ainsi des cellules ayant accompli la recombinaison selon le mode 2 pourraient croître sur un milieu dépourvu d'histidine, mais acquerraient une couleur rouge. Ainsi, cet essai permettrait de distinguer entre des événements de recombinaison entre chromatides sœurs ou entre régions répliquées et non répliquées. Selon le modèle décrit plus haut, un mutant *hst3*Δ *hst4*Δ devrait accomplir la recombinaison de type 2 à une beaucoup plus haute fréquence que des cellules de type sauvage. À l'inverse, le mutant *rtt109*Δ devrait être beaucoup moins efficace dans les deux types de recombinaison. Il serait donc possible de confirmer, ou d'infirmer, l'hypothèse selon laquelle H3K56ac dirige l'invasion des filaments Rad51 vers les régions répliquées.

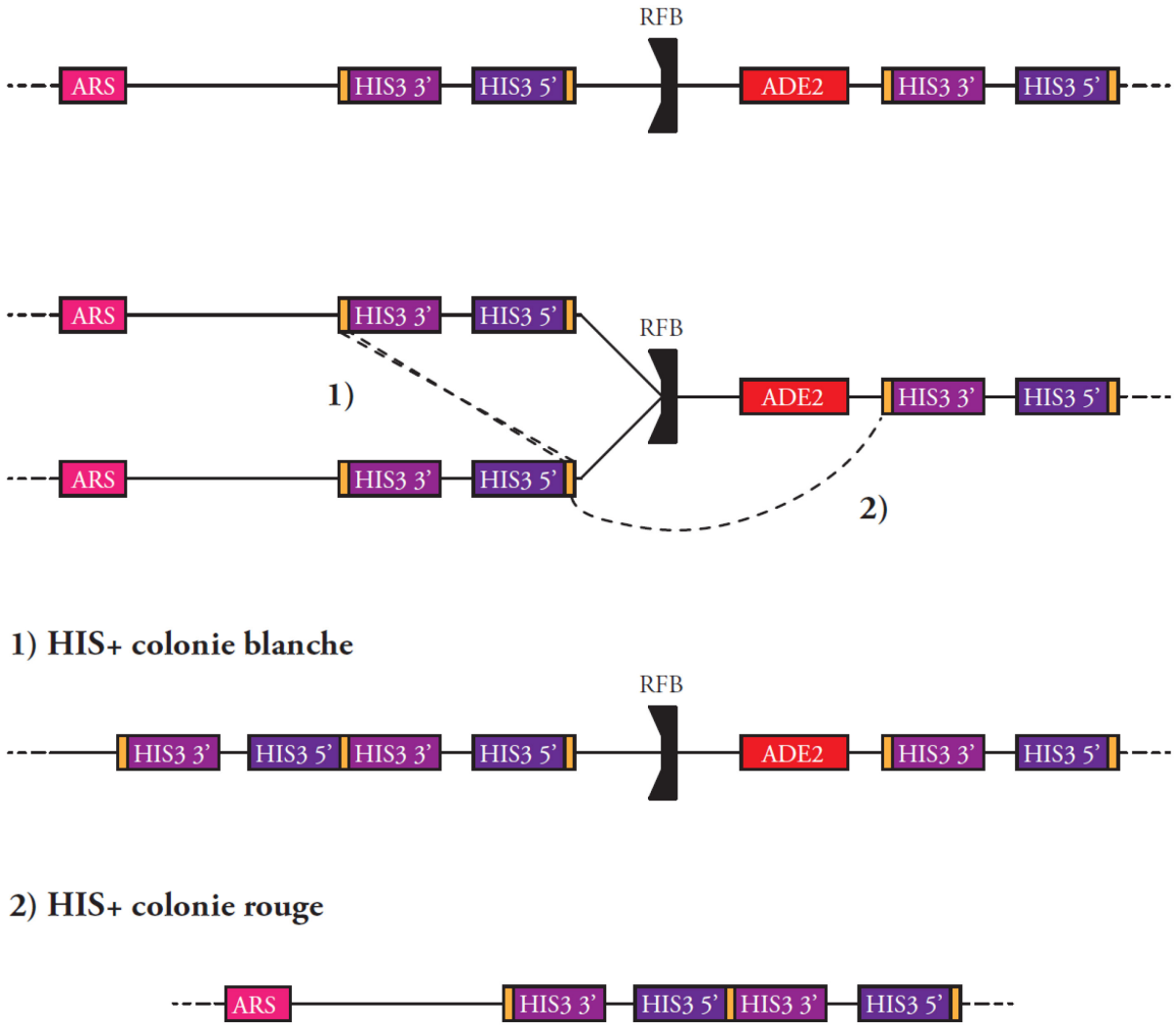

Figure 5.3. Essai de pour tester la sélectivité de la recombinaison homologue

Comment H3K56ac peut-elle réguler la sélectivité de l'invasion des filaments Rad51 dans recombinaison homologue? Tel que mentionné plus haut, il est plausible qu'H3K56ac agisse indirectement en relaxant la structure de la chromatine. Dans le cadre de la recombinaison homologue, il n'est donc pas inconcevable qu'H3K56ac, en affaiblissant l'interaction nucleosome-ADN, facilite le glissement des nucléosomes sur les chromatides sœurs et la dislocation de l'ADN par la recombinase Rad54 pendant l'étape de recherche d'homologie. Lorsque le cycle d'acétylation suit son cours normal, la recherche d'homologie ne pourrait ainsi

n'avoir lieu que sur l'ADN répliqué et comportant H3K56ac, i.e. là où les nucléosomes sont facilement déplaçables. Chez un mutant *hst3*Δ *hst4*Δ, virtuellement 100%, plutôt que 50%, des histones H3 sont acétylées sur la lysine 56, et ce, à l'avant et l'arrière des fourches de réplication. La présence constitutive d'H3K56 favoriserait substantiellement l'invasion aberrante de filaments Rad51 partout à travers le génome et expliquerait les dépendances génétiques d'*hst3*Δ *hst4*Δ décrites plus haut. Ceci n'est pour l'instant que spéculatif, mais il sera très intéressant de vérifier si les niveaux d'H3K56ac influencent le glissement des nucléosomes par Rad54.

Nos résultats indiquent que Rtt107 et le complexe Rtt101-Mms1-Mms22 sont nécessaires pour l'induction de foyers Rad52 lorsque Hst3 et Hst4 sont inhibés par l'exposition au NAM (Figure 4.3C, (544)). Ces facteurs semblent impliqués dans la recombinaison homologue en conjonction avec Rtt109 et H3K56ac et leur délétion supprime les phénotypes du mutants *hst3*Δ *hst4*Δ, suggérant qu'ils sont responsables au moins en partie des défauts de recombinaison en présence d'acétylation constitutive d'H3K56 (212, 529, 540, 541). Or, le mécanisme selon lequel ces protéines influencent la recombinaison homologue est encore mal compris. Rtt101-Mms1-Mms22 forment un complexe ubiquitine ligase qui peut interagir avec Rtt107 (222, 223) et il est plausible que Rtt101-Mms1-Mms22, en conjonction avec Rtt107, régule par leur ubiquitination certains facteurs de la recombinaison homologue encore non-identifiés. Alternativement, le complexe Rtt101-Mms1-Mms22 a récemment été impliqué dans l'établissement de la cohésion derrière les fourches de réplication selon un mécanisme qui dépend d'H3K56ac (550). La cohésion des chromatides sœurs est importante pour le processus de recombinaison homologue (551) et l'apparition aberrante de foyers Rad52 dans les mutants *hst3*Δ *hst4*Δ et *rtt101*Δ/*mms1*Δ/*mms22*Δ pourrait ainsi être la conséquence de la dérégulation de la cohésion. En support avec cette notion, l'acétylation constitutive d'H3K56 augmente substantiellement la fréquence de perte de chromosome (520), un phénotype associé avec des défauts dans l'établissement la cohésion. Néanmoins, des travaux supplémentaires seront nécessaires pour déterminer les fonctions d'H3K56ac, Rtt101-Mms1-Mms22 et Rtt107 dans le processus de cohésion.

5.2 Patron d'activité des origines de réplication

Les résultats présentés dans le troisième article révèlent une fonction peu explorée du complexe Yku70/80 dans la préservation du génome en réponse au stress réplicatif qui n'a aucun lien avec son rôle classique dans la réparation des DSBs par NHEJ. Nos données suggèrent plutôt que l'activation précoce des origines aux télomères courts durant la phase S, tel qu'observé en l'absence de Yku70/80, sensibilise les cellules au stress réplicatif en affectant l'activité des origines à travers le génome. Ceci soulève un concept intéressant : le patron d'activation des origines de réplication influence la capacité des cellules à répondre au stress réplicatif. Nos résultats suggèrent en effet que la séquestration des facteurs d'initiation de la réplication aux télomères courts réduit la résistance des cellules au stress réplicatif en limitant le nombre d'origines internes actives. Une fourche effondrée suite à la rencontre avec une lésion non réparable ne peut être sauvée que par une fourche de réplication convergente. Or, l'initiation à partir des origines télomériques est nécessairement moins efficace pour la synthèse d'ADN qu'une origine interne, car une des fourches de réplication émergeante atteindra promptement la fin d'un chromosome. Ceci implique que la distance inter-origines actives augmente lorsque les facteurs d'initiations sont séquestrés aux télomères. Il est ainsi concevable que la probabilité qu'une fourche de réplication converge vers un réplisome endommagé diminue, ce qui augmente en contrepartie la fréquence de régions non répliquées (figure 5.4). Ce modèle est d'ailleurs compatible avec la corrélation négative entre la fragilité des chromosomes et le nombre d'origines actives (552, 553). Bien que nos résultats supportent cette interprétation, il est important de noter que nous n'avons pas de preuve directe de celui-ci. Il sera donc important de vérifier si l'activation des origines internes est en effet réduite lorsque les télomères sont raccourcis.

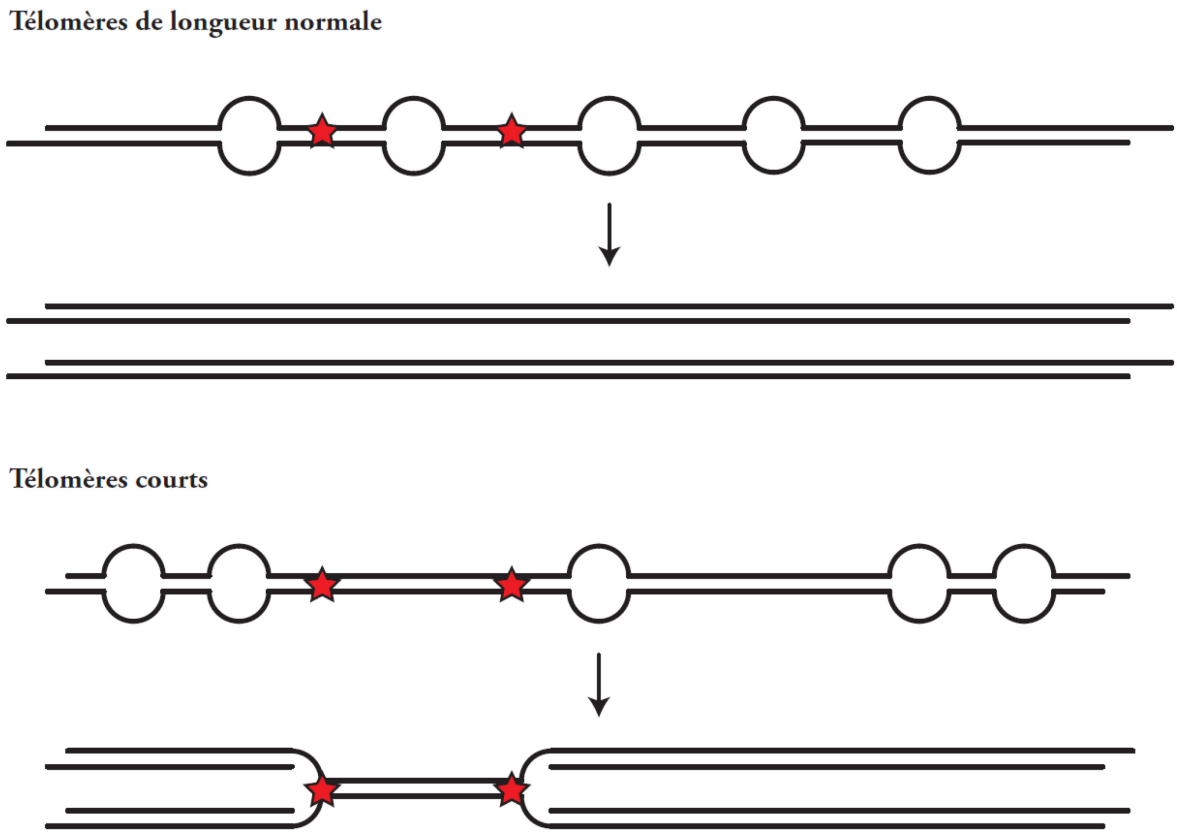

Figure 5.4. Modèle selon lequel la longueur des télomères pourrait influencer la résistance au stress réplicatif

L'hyperacétylation d'H3K56 est nécessaire, mais non suffisante, pour causer les défauts de croissance observés chez le mutant *yku70*Δ en présence de NAM et l'inhibition de deux autres sirtuines, Hst1 et Sir2, est additionnellement requise. Nos résultats suggèrent que cette interaction génétique découle du stress réplicatif induit par l'inhibition d'Hst3 et Hst4, de la séquestration de facteurs de réplication aux télomères et de la réduction de l'activité certaines origines à travers le génome par l'inactivation d'Hst1 et Sir2. Comment le complexe Sum1-Rfm1-Hst1/Sir2 accomplit ses fonctions au niveau de l'activation des origines demeure incompris, mais le fait que la diminution des niveaux H4K16ac supprime la sensibilité de *yku70*Δ au NAM indique que le mécanisme implique probablement l'ablation de cette modification par Hst1 ou Sir2 au niveau des ARS. Il n'est pas impossible que des cibles supplémentaires, autres que des histones, soient impliquées dans ce processus. D'ailleurs, chez l'humain, il a été démontré que SIRT1^{Sir2} régule l'activité des origines par la désacétylation de

MCM10 et TOPBP1^{Dpb11}, suggérant en outre que la régulation de l'activité des origines par la famille des sirtuines est conservée dans l'évolution (554–556).

La répression des origines télomériques au début de la réplication chez *S. cerevisiae* est un phénomène intéressant. Il implique que l'évolution a sélectionné pour leur suppression en temps normal, mais que des mécanismes ont émergé pour assurer leur activation plus tôt durant la réplication lorsque les télomères sont courts. Deux fonctions peuvent expliquer ce mécanisme : i) l'utilisation de ces origines comme dernier recours pour terminer la réplication du télomère lorsqu'une fourche convergente s'effondre avant d'atteindre l'extrémité du chromosome (557) et ii) promouvoir les fonctions de la télomérase en allongeant la fenêtre d'action de celle-ci lorsque les télomères sont raccourcis (186). Nos travaux présentent une troisième fonction qui implique la distribution d'activité optimale des origines à travers le génome pour faire face au stress réplicatif. Curieusement, la répression des origines télomériques n'est pas conservée chez les eucaryotes supérieurs (558). Leurs chromosomes comportent aussi des origines télomériques et sous-télomériques, mais celles-ci ne sont pas soumises à un contrôle par la longueur des télomères, au moins en partie parce que RIF1 n'y est pas associé (559). Les origines télomériques peuvent donc être activées à tout moment durant la phase S et leur activité dépend plutôt du chromosome sur lesquels elles sont localisées (560, 561). Les raisons derrières cette divergence évolutive sont nébuleuses, mais une explication pourrait résider dans la dépendance des différentes espèces envers la télomérase. Les fourches de réplication s'effondrent fréquemment aux télomères des levures (562). Celles-ci remédient à la situation en activant les origines sous-télomériques, favorisant de ce fait l'action de la télomérase (186). Or, plusieurs espèces de mammifères, dont les humains, n'expriment pas la télomérase de manière constitutive dans leurs cellules somatiques. Leurs télomères ont plutôt une structure (boucle T) et des facteurs additionnels, notamment Shelterin (composé de TRF1, TRF2, hRAP1, POT1, TIN2 et TPP1) (563) ainsi qu'un plus grand nombre d'origines pouvant être activées, même à l'intérieur des séquences répétées (564). Peut-être qu'ainsi les télomères des eucaryotes supérieurs favoriseraient la protection des télomères plutôt que la dépendance sur la télomérase.

5.3 La sensibilité du mutant *hst3*Δ *hst4*Δ aux perturbations de la réplication de l'ADN

Il est intéressant de noter que le mutant *hst3*Δ *hst4*Δ est particulièrement sensible à tout phénomène qui perturbe la réplication de l'ADN, de la déstabilisation du réplisome par l'ajout d'épitope sur les facteurs de réplication à la diminution du nombre d'origines internes actives par la mutation de *YKU70* et *SUM1*. Tel que décrit dans les sections précédentes, ceci pourrait être le résultat d'une réponse au stress réplicatif défective dans le mutant *hst3*Δ *hst4*Δ. L'inhabilité des cellules comportant l'acétylation constitutive d'H3K56 à gérer les fourches de réplication arrêtées ferait en sorte qu'elles dépendent plus fortement sur la stabilité du réplisome et le nombre d'origines actives pour leur survie en présence de stress réplicatif.

Il est néanmoins difficile d'expliquer la sensibilité au stress réplicatif et l'apparition aberrante de foyers Rad52 chez le simple mutant *hst3*Δ (535). Celui-ci n'affecte pas la stœchiométrie d'H3K56ac et ne fait que prolonger son temps de résidence sur la chromatine en G2/M (520). Une récente étude démontre que la simple mutation d'*HST3* induit la perte d'un chromosome disomique comportant une faible densité d'origines de réplication (536). Ceci implique que la présence prolongée d'Hst3 pendant un cycle cellulaire influence la réplication de l'ADN dans un cycle cellulaire subséquent. Une interprétation attrayante de ces phénomènes serait qu'H3K56ac régule négativement l'autorisation des origines. Les modèles actuels suggèrent que l'inhibition de l'autorisation des origines dépend entièrement de l'activité CDK (38), mais celle-ci est inhibée par la signalisation en réponse aux dommages à l'ADN (565, 566). En présence de dommages à l'ADN, il n'y aurait donc aucun mécanisme prévenant l'autorisation des origines en phase S ou G2, ce qui pourrait en conséquence provoquer la re-réplication de certaines parties du génome. H3K56ac, en étant localisé sur la chromatine et en persistant jusqu'à la réparation des dommages à l'ADN, est à un endroit idéal pour combler ce vide et limiter l'autorisation des origines en présence de lésions (Figure 5.5A). Donc, selon cette prémisse, les phénotypes des mutants *rtt109*Δ, *hst3*Δ et *hst3*Δ *hst4*Δ pourraient, au moins en partie, résulter de défaillances dans l'autorisation d'origines. La persistance d'H3K56ac en l'absence de dommages chez les mutants *hst3*Δ et *hst3*Δ *hst4*Δ diminuerait la période

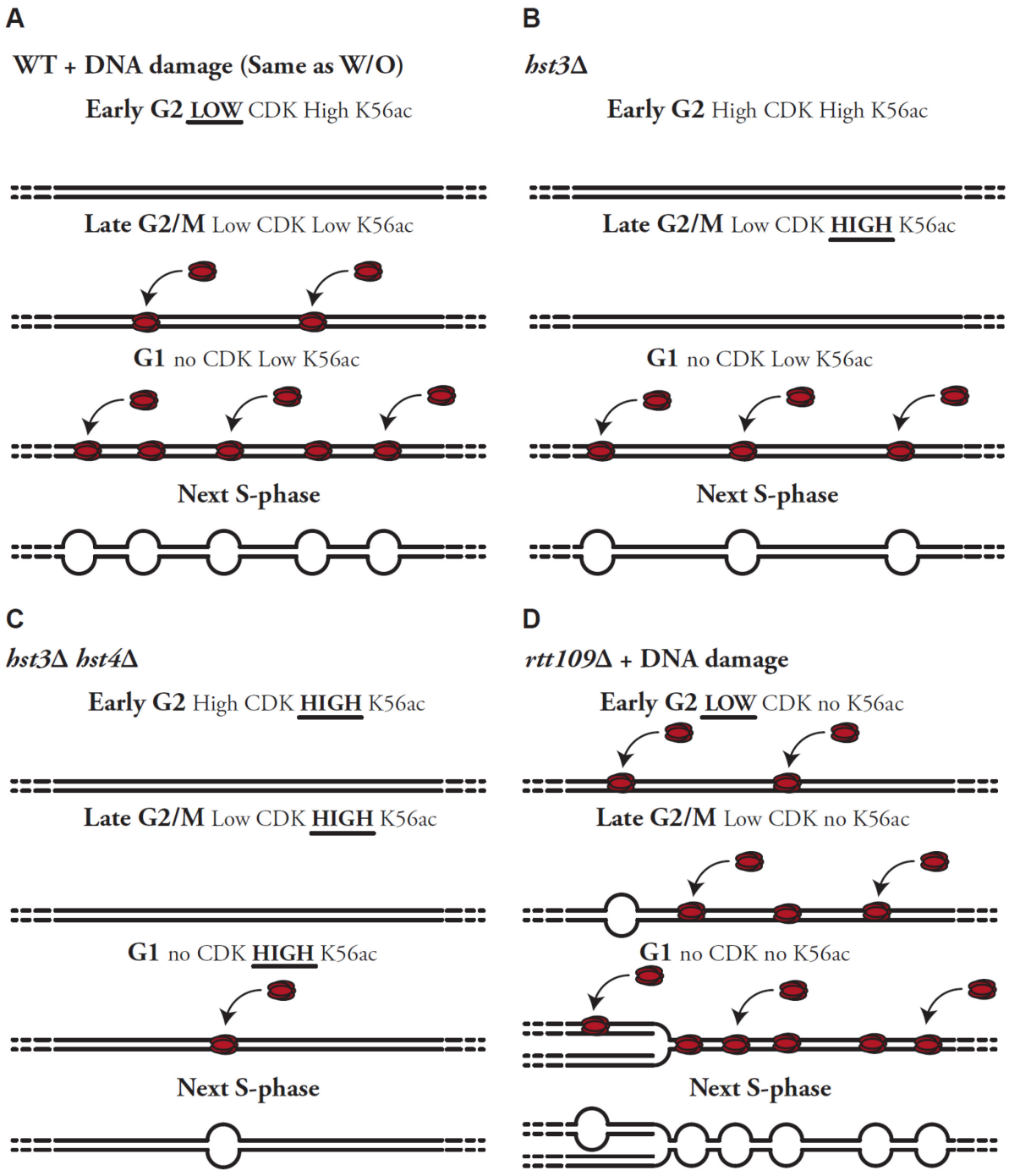

Figure 5.5. Modèle selon lequel H3K56ac pourrait influencer l'autorisation des origines

pendant laquelle les origines peuvent être autorisées (Figure 5.5B-C). À l'inverse, l'absence d'H3K56ac en présence de dommages résulterait en autorisation aberrante d'origines pendant la phase G2 (Figure 5.5D).

Plusieurs prédictions de ce modèle sont facilement vérifiables. Premièrement, l'absence d'H3K56ac devrait entraîner une re-réplication au moins partielle en G2/M lorsque les cellules sont exposées au stress réplicatif. On observe d'ailleurs en général un contenu en ADN légèrement plus élevé chez le mutant *rtt109*Δ dans cette condition (529). Deuxièmement, un mutant *hst3*Δ, et encore plus drastiquement *hst3*Δ *hst4*Δ, devrait avoir moins d'origines autorisées à l'initiation de la réplication. Finalement, le mutant *hst3*Δ devrait être sensibilisé à toute perturbation de l'autorisation des origines. En ce sens, il a déjà été démontré qu'*hst3*Δ entraîne une létalité synthétique avec la mutation de *SIC1*, un inhibiteur de CDK (567).

Puisqu'ils semblent agir dans la même voie en réponse aux dommages à l'ADN, il est probable que l'inhibition de l'autorisation des origines par H3K56ac nécessite les fonctions de l'ubiquitine ligase Rtt101-Mms1-Mms22. En ce sens, il a d'ailleurs été démontré que Mms22 interagit avec Orc5, suggérant que Rtt101-Mms1-Mms22 peut réguler les fonctions d'autorisation des ORC ou d'autres facteurs qui s'y associent (223). Par exemple, l'ubiquitination de Mcm7 par Dia2 entraîne le désassemblage du réplisome et l'exclusion de celui-ci de l'ADN par Cdc48 (568). Il est donc possible que Rtt101-Mms1-Mms22 puisse ubiquitiner les MCM et causer leur éviction de la chromatine selon un processus similaire.

5.4 Conservation du cycle d'acétylation d'H3K56 dans l'évolution

Le cycle d'acétylation d'H3K56 est vraisemblablement conservé à travers les mycètes et cette modification a notamment été retrouvée chez *Candida albicans* et *Schizosaccharomyces pombe* (521, 522, 569). Chez ces espèces, il existe un homologue d'Hst3 ou d'Hst4 qui désacétyle H3K56ac et occupe des fonctions conservées au sein de la réponse aux dommages à l'ADN. Plusieurs études proposent qu'H3K56 est aussi acétylé chez les eucaryotes supérieurs par p300/CBP et que cette modification joue un rôle similaire dans la réponse au stress réplicatif (570–573). Cependant, la stœchiométrie d'H3K56ac est de loin plus modeste que celle retrouvée chez *S. cerevisiae*. De plus, la spécificité des anticorps commerciaux contre H3K56ac utilisés

pour arriver à ces conclusions a été sérieusement remise en question, suggérant que la détection d'H3K56ac chez les eucaryotes supérieurs est en partie issu d'artéfacts d'immunodétection (574, 575).

Le rôle d'H3K56ac chez les mammifères semble plutôt être repris par la forme non-méthylée de la lysine 20 de l'histone H4 (H4K20me0). Celle-ci n'est présente que sur les nucléosomes nouvellement synthétisés et déposés sur l'ADN naissant durant la réplication, puis H4K20 est rapidement méthylé en phase G2 par SET8, de façon (inversement) similaire à H3K56ac (528, 576, 577). La déposition des dimères H3-H4 nouvellement synthétisés suit également un processus analogue et dépend de l'interaction entre TONSL-MMS22L (MMS22L est un homologue de Mms22), ASF1, MCM2 et H3-H4K20me0 (577, 578). De plus, tout comme Hst3, SET8 est dégradé en réponse aux dommages à l'ADN, ce qui résulte en persistance d'H4K20me0 (579–581). Il est intéressant de noter que, de façon similaire à Rtt101-Mms22-Mms1, MMS22L-TONSL est impliqué dans la recombinaison homologue et stabilise les filaments RAD51 (582). De plus, la méthylation de H4K20 par SET8 induit le recrutement à la chromatine de 53BP1, un homologue de Rad9 se liant également à H3K79me et nécessaire à l'activation de la cascade de signalisation en réponse aux dommages à l'ADN (583–585). Ceci soutient l'idée qu'H3K56ac puisse influencer la liaison de Rad9 à la chromatine et suggère que la relation que nous avons identifiée entre Rad9, H3K79me, la recombinaison homologue et la voie d'H3K56ac est au moins en partie conservée dans l'évolution.

5.5 Le cycle d'acétylation d'H3K56ac, une cible pour le traitement des infections fongiques?

Candia albicans est un pathogène fongique qui peut causer de sérieuses infections nosocomiales, en particulier chez les patients immunosupprimés (586–588). Les traitements actuels reposent sur l'utilisation d'azoles et d'échinocandins qui ciblent des enzymes qui influencent la stabilité de la membrane cellulaire et de la paroi cellulaire, respectivement (589, 590). Cependant, bien que ces drogues soient initialement efficaces, l'apparition de souches résistantes limite leur usage. Il a récemment été démontré que *C. albicans* est extrêmement

sensible à l'inhibition d'Hst3 et de la désacétylation d'H3K56ac par le NAM (522), et ici, nous avons découvert que l'induction de stress oxydatif synergise avec le NAM (544). Or, un des effets secondaires de l'exposition aux échinocandins est la génération d'espèces oxygénées réactives (ROS) (591), ce qui suggère qu'il pourrait y avoir une synergie entre le NAM et ces drogues, Ainsi, il sera intéressant de déterminer si l'élimination des cellules de *C. albicans* par les échinocandins est potentialisée par la combinaison avec avec le NAM, ce qui pourrait révéler une nouvelle approche thérapeutique plus efficace que celles utilisées actuellement.

5.6 Conclusion

Enfin, les travaux présentés dans le cadre de cette thèse aident à mieux comprendre comment une modification cyclique, dont le concept est conservé de la levure à l'humain, agit dans la réponse au stress réplicatif et le maintien de l'intégrité génomique. Il en reste beaucoup à découvrir sur ce type de modification, mais j'ose croire que mes labeurs ont permis de révéler des pistes sur desquelles d'autres pourront se baser pour déceler les fonctions d'H3K56ac.

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