Persistent acetylation of newly synthesized histones inhibits DNA replication origin activity

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ABSTRACT

Histone acetylation is a known regulator of DNA replication dynamics. In the yeast Saccharomyces cerevisiae, newly synthesized histone H3 deposited behind DNA replication forks are acetylated on lysine 56 (H3 K56ac) by the Rtt109 histone acetyltransferase. Two enzymes of the sirtuin family of deacetylases, Hst3 and Hst4, act redundantly to deacetylate this residue throughout chromosomes following S phase. Cells lacking Hst3 and Hst4 present constitutively acetylated H3 K56, which causes sensitivity to replication-blocking genotoxins and slow growth via mechanisms that remain poorly understood. Here, we present the results of a genome-wide screen aimed at identifying haploinsufficient genes that promote cell fitness in response to inhibition of sirtuins by nicotinamide (NAM). We find that heterozygous deletion alleles of genes involved in the regulation of DNA replication origins sensitize cells to NAM. Consistently, haploid cells harboring the hypomorph temperature sensitive allele cdc7-4 present striking NAM-induced S phase progression defects at their semi-permissive temperature. We further show i) that Rap1-interacting factor 1 (Rif1), an inhibitor of Cdc7-dependent activation of replication origins, causes DNA damage and replication defects in cells exposed to NAM and in hst3Δ hst4Δ mutants, and ii) that cdc7-4 hst3Δ hst4Δ triple mutant cells present strong synthetic temperature sensitivity associated with defective activation of replication origin and delayed S phase progression. Our data further demonstrate that such replication defects are not due to intra-S phase checkpoint activation leading to inhibition of origin activity. We finally show that Rtt109-dependent acetylation of histone H3 lysine 56 and its associated Rtt101-Mms1 ubiquitin ligase complex cause the DNA replication defects observed in cdc7-4 hst3Δ hst4Δ cells. Overall, these results argue that abnormal regulation of nascent chromatin structure negatively influences DNA replication initiation in cells presenting reduced Dbf4-dependent kinase activity including those experiencing replicative stress.
INTRODUCTION

DNA replication initiates at multiple origins throughout chromosomes during the S phase of the cell cycle [1]. During G1, Cdt1 and Cdc6 load the MCM helicase complex on DNA at origins of replication bound by the Origin Recognition Complex (ORC). At the beginning of S phase, cyclin-dependent (CDK) and Dbf4-dependent (DDK) kinase activities promote the recruitment of other factors including Cdc45 and the GINS complex to replication origins as well as the activation of the MCM helicase. Melting of origin DNA resulting from MCM helicase activity allows the formation of two replication forks (RF) that travel in opposite directions along chromosomal DNA. Depending on the timing of their activation in S phase, eukaryotic origins are classified as early, mid, or late. Such sequential activation of origins has been shown to result from the recycling of limiting replication initiation factors from early to mid, and then to late replicating genomic regions [2,3]. While the above-described temporal organization of DNA replication is evolutionarily conserved among eukaryotes, its functional relevance is incompletely understood.

RF progression can be halted upon encountering DNA lesions induced by any among a multitude of environmental or endogenous genotoxins [4]. This engenders a state of replicative stress which can forestall completion of chromosomal duplication, thereby causing genomic instability. Stalled RF cause the activation of Mec1 (ATR in humans), the apical kinase of the intra-S phase checkpoint response in yeast [4]. Mec1 then promotes activation of the kinase Rad53 via one of two pathways dependent upon either the RF component Mrc1 or the adaptor Rad9 [5]. Mec1 and Rad53 subsequently phosphorylate a multitude of substrates to i) promote the stability of stalled RF, and ii) to prevent further activation of replication origins [6]. In yeast, this latter effect depends on Rad53-dependent phosphorylation of the key replication factors Dbf4 and Sld3, which prevents the activation of the MCM helicase at origins that have not yet fired [7]. Such inhibition of origin activity is thought to prevent the accumulation and eventual collapse of stalled RF during periods of genotoxic stress [8].

Histone post-translational modifications are critical determinants of DNA replication dynamics and origin activity [9,10]. Of interest here, histone lysine acetylation has been shown to both promote and inhibit origin activity depending on the identity of the modified residue and/or chromosomal context [11]. The sirtuin family of histone deacetylases is well-conserved throughout evolution, and several of its members influence DNA replication and repair [12]. The yeast
*Saccharomyces cerevisiae* possesses 5 sirtuins; the founding member, Sir2, and Homologues of Sir Two 1 through 4 (Hst1-4) [12]. Sir2 targets histone H4 lysine 16 acetylation (H4 K16ac) thereby repressing the firing of origins at the rDNA locus and telomeres [13,14]. Hst1, which can also target H4 K16ac, forms a complex with Sum1 and Rfm1 and positively regulates a subset of origins genome-wide [15,16]. While the impact of Hst2 on DNA replication has not been directly assessed, at least some of the functions of this sirtuin are known to be partially redundant with those of Sir2, as overexpressed Hst2 rescues gene silencing defects caused by *sir2Δ* [17,18].

The only known histone substrate of the redundant sirtuins Hst3 and Hst4 is acetylated H3 lysine 56 (H3 K56ac) [19]. This modification is catalysed by the acetyltransferase Rtt109 on newly synthesized histones H3 before their deposition onto nascent DNA during S-phase [20,21]. After completion of DNA replication, Hst3 and Hst4 remove H3 K56ac chromosome-wide such that most nucleosomes do not harbor H3 K56ac at the start of the next S phase. While under normal circumstances the bulk of H3 K56ac is removed by Hst3 during G2, Hst4 can compensate for its absence. As such, the stoichiometry of H3 K56ac approaches 100% throughout the cell cycle in *hst3Δ hst4Δ* double mutants [19,22]. Such constitutive H3 K56ac has been shown to cause spontaneous DNA damage, thermosensitivity, and increased sensitivity to genotoxins that cause replicative stress [19,23,24]. However, the molecular mechanisms underlying the striking phenotypes of *hst3Δ hst4Δ* cells remain poorly understood.

Nicotinamide (NAM) is a non-competitive pan-inhibitor of sirtuins [25]. Our group previously performed genetic screens in *S. cerevisiae* with the goal of identifying genes whose homozygous deletion (i.e. complete loss-of-function) confers either fitness defects or advantages in response to NAM-induced sirtuin inhibition and consequent H3 K56 hyperacetylation [26]. These screens revealed that genes encoding regulators of the DNA replication stress response promote resistance to NAM-induced elevation in H3 K56ac caused by inhibition of Hst3 and Hst4 [26,27]. While the mechanisms are unclear, previous data indicate that cells lacking HST3 are defective in the maintenance of artificial chromosomes harboring a reduced number of DNA replication origins [28], further linking H3 K56ac with regulation of DNA replication dynamics. Here, we present the results of a genome-wide haploinsufficiency screen aimed at identifying genes modulating cell fitness in response to NAM-induced sirtuin inhibition. Overall, our results indicate that i) factors promoting DNA replication origin activation are critical for survival in the
absence of Hst3 and Hst4 activity, and ii) abnormal persistence of the acetylation of new histones deposited into chromatin during S phase compromises the activity of replication origins in yeast.

RESULTS

A genetic screen to identify genes modulating cellular fitness in response to NAM.

We performed a screen using the pooled strains of the heterozygote diploid yeast collection to identify haploinsufficient genes that affect cell fitness upon NAM-induced inhibition of sirtuins (Supplementary Table 1). Using a Z-score cut-off of +/- 2.58 (99% cumulative percentage), the screen identified 131 and 58 genes whose heterozygosity caused reduced or increased fitness, respectively, during propagation for 20 generations in YPD medium containing 41 mM NAM (Figure 1A). This list of genes presents only modest overlap with that obtained from our previously published screen using the homozygote deletion strain collection (Figure 1B), suggesting that most of the genes identified in the latter screen are not haploinsufficient with regards to NAM sensitivity. This lack of overlap between screens performed on the homozygous and heterozygous deletion collections is consistent with our previous observations [29]. Gene Ontology (GO) term analysis of genes whose heterozygosity sensitizes cells to NAM revealed an obvious enrichment in DNA replication and DNA damage response pathway, whereas terms reflecting proteasome-related and catabolic processes were associated with mutations promoting enhanced fitness upon NAM exposure (Figure 1C and Supplementary Tables 2-3).

We next sought to validate individual heterozygous mutations representing the main categories of “hits” identified in the screen. In these two-strains competitions, WT and heterozygote diploid mutant strains of interest were mixed in a 1:1 ratio and incubated for 20 generations in YPD +/- NAM. Appropriate dilutions of cells were then plated on YPD-agar +/- G418 and the ratio of the number of heterozygote mutant (G418-resistant) vs WT (G418-sensitive) colonies was calculated (Figure 1D). This competition analysis confirmed the expected impact of heterozygous mutation causing diminished cell fitness in NAM-containing medium, thereby validating our screening strategy. In contrast, significant improvement in growth was not observed for individual heterozygote mutants expected to elevate fitness (Supplementary Figure 1). We note that heterozygote mutations causing improved fitness in response to NAM displayed generally
lower absolute Z-scores than those reducing fitness (Figure 1A, Supplementary Table 1). This may reflect modest differences in growth that might be challenging to detect in 1:1 vs genome-wide competition. In view of the above considerations, we decided to focus on further characterization of genes whose heterozygosity reduced cell fitness in response to NAM.

**Deregulation of DNA replication origin activity sensitizes cells to NAM**

As mentioned previously, cells lacking Hst3 are defective in the maintenance of an artificial chromosome harboring reduced number of DNA replication origins, revealing a potential link between this sirtuin and origin activity whose mechanistic basis is unknown [28]. Interestingly, several essential DNA replication genes identified in the screen as promoting NAM resistance are involved in the assembly of the pre-replication complex (ORC3, ORC5, CDC6 and MCM4) or regulation of origins activation (CDC7, DBF4, SLD3 and GLC7). We chose to focus on the relationship between NAM sensitivity and the role of Cdc7-Dbf4 (DDK) in promoting origin activation. Consistent with results from the screen, haploid cells expressing hypomorphic temperature sensitive alleles of DBF4 (dbf4-1) and CDC7 (cdc7-4) are sensitized to NAM when incubated at the semi-permissive temperature of 30°C (Figure 2A). Our data also indicate that bob1-1 cdc7Δ cells, which harbor a mutation in MCM5 that bypasses DDK-dependent MCM phosphorylation for origin activation [30], are not NAM-sensitive (Figure 2B), arguing that the MCM complex is likely to be the relevant target of DDK in this context. Overall, these results suggest that impaired activation of DNA replication origins sensitizes cells to NAM.

Rap1-Interacting Factor 1 (Rif1) acts in concert with the phosphatase Glc7 to reverse DDK-dependent MCM phosphorylation, thereby inhibiting origin activation [31–34]. Moreover, a previous screen performed by our group identified RIF1 among the few yeast genes whose homozygous deletion improved cell fitness in NAM-containing medium [26]. Interestingly, we found that NAM-induced growth defects and accumulation of cells in S phase is rescued by deletion of RIF1 (Figure 2C and G). Moreover N-terminal truncation or mutations in the Glc7-interacting motif (rif1-RVxF/SILK) of Rif1, both of which were previously shown to partially suppress the temperature sensitivity of cdc7-4 mutants by eliminating Rif1 binding to Glc7 [33,35], also strongly suppressed the NAM sensitivity of cdc7-4 cells (Figure 2C). Overall, these data are
consistent with the notion that Rif1/Glc7-dependent dephosphorylation sensitizes cells with reduced DDK activity to NAM.

We and others previously showed that NAM treatment causes replicative stress and DNA damage in yeast [19,26,27]. Since lack of MCM phosphorylation by DDK causes sensitivity to replicative stress-inducing drugs [36,37], we tested the impact of RIF1 deletion on NAM-induced DNA damage. Compared to WT, rif1∆ cells presented reduced NAM-induced histone H2A S129 phosphorylation and Rad52-YFP foci formation (Figure 2D-E), two well-known markers of replicative stress-induced DNA damage [38,39]. Importantly, lack of Rif1 did not compromise the formation of ionizing radiation (IR)-induced Rad52 foci, which are not primarily caused by replication-associated DNA damage. We note that, in addition to its role in regulating DNA replication, Rif1 is known to limit telomere length by inhibiting telomerase activity [40]. Since we previously showed that cells with short telomeres are sensitive to NAM-induced sirtuin inhibition [27], we considered the possibility that abnormal telomere elongation in rif1∆ cells might favor NAM resistance. Contrary to this notion, deletion of RIF1 suppressed NAM-induced growth and S phase progression defects in telomerase-defective est2∆ cells (Figure 2F), indicating that the role of Rif1 in modulating NAM sensitivity is independent of its influence on telomere length.

**Lack of Hst3/Hst4 activity inhibits the initiation of DNA replication in cdc7-4 cells in a Rif1-dependent manner**

We previously showed that NAM-induced DNA damage is attributable to inhibition of H3 K56ac deacetylation by Hst3 and Hst4 [26]. We therefore tested the impact of Rif1 on the phenotypes caused by lack of HST3 and HST4. We found that deletion of RIF1 rescues the temperature sensitivity of hst3∆ hst4∆ cells as well as the synthetic lethality of hst3∆ hst4∆ sir2∆ without affecting the levels of H3 K56ac (Figure 3A-B). We note that for unknown reasons hst3∆ hst4∆ cells are only temperature sensitive in S288C-derived genetic backgrounds and not in W303 (our unpublished observations; e.g., compare Figure 3A and 3G). Because of this, while most of the experiments involving hst3∆ hst4∆ were done in W303-derived strains, certain experiments including the one presented in Figure 3A were done in the BY4741 background (see Supplementary Table 4 for a description of the yeast strains used in each figure of this study).
We previously demonstrated that transient exposure to methyl methane sulfonate, an alkylating agent that generates DNA replication blocking lesions such as 3-methyl adenine, prevents timely completion of S phase in hst3Δ hst4Δ cells [24]. DNA content flow cytometry analyses revealed that deletion of RIF1 noticeably rescued the S phase progression delay caused by transient MMS exposure in hst3Δ hst4Δ double mutants (Figure 3C), consistent with the notion that Rif1 compromises DNA replication completion in these cells. Nevertheless, deletion of RIF1 did not rescue the sensitivity of hst3Δ hst4Δ to MMS (Figure 3D), which is possibly due to the fact that Rif positively modulates the stability of stalled RF [41]. We also found that rif1Δ reduced spontaneous formation of Rad52 foci and histone H2A S129 phosphorylation in hst3Δ hst4Δ cells (Figure 3E-F), indicating that, in the absence of exogenous replicative stress-inducing genotoxins, Rif1 activity causes DNA damage in Hst3/Hst4-deficient cells. The above data suggest that Rif1/Glc7-mediated reversal of DDK-dependent phosphorylation, and consequent inhibition of origins of DNA replication, contribute to the phenotypes of cells lacking Hst3 and Hst4. Consistent with this idea, deletion of HST3 and HST4 considerably exacerbated the temperature sensitivity of cdc7-4 mutant cells in a Rif1-dependent manner (Figure 3G), and rif1Δ diminished H2A S129 phosphorylation in cdc7-4 hst3Δ hst4Δ mutants (Figure 3F). Deletion of either HST3 or HST4 alone did not increase the temperature sensitivity of cdc7-4 (Figure 3H), in accord with the known functional redundancy of Hst3/4 with respects to histone deacetylation [19,22].

We next evaluated DNA replication dynamics in cdc7-4 cells lacking Hst3/Hst4. cdc7-4 hst3Δ hst4Δ cells displayed strong Rif1-dependent S phase progression inhibition at the semi-permissive temperature of 30°C compared to either hst3Δ hst4Δ or cdc7-4 (Figure 4A). As expected, this phenotype was rescued by deletion of RIF1 (Figure 4A) or expression of plasmid-borne copy of HST3 (Figure 4B). The observed DNA replication defect does not appear to result from compromised release from alpha factor-mediated G1 arrest, since asynchronously growing cdc7-4 hst3Δ hst4Δ cells accumulated in early S when incubated at 30°C (Figure 4C). Moreover, the budding index of cdc7-4 hst3Δ hst4Δ cells released from alpha factor-mediated G1 block toward S phase at 30°C was comparable to that of cdc7-4 cells (~80%) even though the former cells present barely detectable S phase progression (Figure 4D-E). The above data indicate that cdc7-4 hst3Δ hst4Δ can enter S phase at the semi-permissive temperature but fail to synthesize DNA efficiently.
The very limited extent of DNA synthesis observed in \textit{cdc7-4 hst3}Δ \textit{hst4}Δ vs \textit{cdc7-4} cells raised the possibility that activation of early replication origins might be compromised in the absence of Hst3 and Hst4. Using quantitative PCR, we found that, compared to \textit{cdc7-4} mutants, duplication of DNA was inhibited at multiple early origins in \textit{cdc7-4 hst3}Δ \textit{hst4}Δ vs \textit{cdc7-4} cells 30 minutes after release from alpha factor arrest at 30°C (Figure 4F). This represents a strong delay in origin activation rather than permanent inhibition since \textit{cdc7-4 hst3}Δ \textit{hst4}Δ cells eventually initiate replication and complete S phase when incubated for longer periods at 30°C (240 minutes post-release from alpha factor arrest; Figure 4G-H). To independently confirm the above results, we used a previously described method based on alkaline gel electrophoresis and southern blotting to detect nascent DNA formation at the efficient early origin ARS305 [6]. Strikingly, the results indicate that minimal nascent DNA was formed at this origin within 1 h of release from alpha factor arrest at 30°C in \textit{cdc7-4 hst3}Δ \textit{hst4}Δ cells when compared to \textit{cdc7-4} mutants (Figure 4I). Taken together, the results reveal that lack of Hst3/4 activity compromises the activation of origins of replication in cells presenting reduced DDK activity.

\textbf{Inhibition of origin activity is not due to intra S phase checkpoint activation in \textit{cdc7-4 hst3}Δ \textit{hst4}Δ cells}

One of the key roles of intra S phase checkpoint signalling is to limit the activation of origins in response to replication stress [6,42], which occurs in part via negative regulation of DDK activity depending on the phosphorylation of Dbf4 by Rad53 [7]. Cells lacking Hst3/4 activity are well-known to present spontaneous DNA damage and constitutive activation of Rad53 [19,23,24,26,27]. We therefore sought to evaluate if the striking S phase progression delay observed in \textit{cdc7-4 hst3}Δ \textit{hst4}Δ cells is caused by elevated Rad53 activation at the semi-permissive temperature of \textit{cdc7-4} (30°C). While \textit{cdc7-4 hst3}Δ \textit{hst4}Δ cells constitutively present some Rad53 phosphorylation, no detectable elevation in Rad53 phosphorylation-induced electrophoretic mobility shift was observed upon release of cells from alpha factor arrest toward S phase at 30°C, either in the presence or absence of the replication-blocking genotoxin hydroxyurea (HU; Figure 5A-B). This result is consistent with the notion that few if any RF are progressing in these conditions (Figure 4F, I). This, in turn, is expected to reduce the number of stalled replication forks...
in either HU-treated or untreated conditions thereby compromising the activation of intra S phase checkpoint kinases.

Mrc1 and Rad9 are key mediators of intra S phase checkpoint activation [5]. We found that deletion of RAD9 led to modestly increased S phase progression, but only at later time points, in cdc7-4 hst3Δ hst4Δ cells (Figure 5C). This suggests that Rad9 might contribute to the maintenance rather than the establishment of the replication defects observed in cdc7-4 hst3Δ hst4Δ cells over longer time periods. In contrast, expression of a mutated allele of Mrc1 (Mrc1-AQ) which compromises its role in the intra S phase checkpoint [43] does not detectably improved S phase progression in cdc7-4 hst3Δ hst4Δ cells even at later time points (Figure 5D). We further found that inhibition of Mec using caffeine [44,45] completely abrogated Rad53 phosphorylation, as expected, but did not reverse the strong inhibition of DNA replication progression of cdc7-4 hst3Δ hst4Δ mutants (Figure 5E-F). Importantly, caffeine exposure did not prevent cdc7-4 cells from completing DNA replication at 30°C (Figure 5F). Expression of Dbf4 and Sld3 variants that cannot be phosphorylated by Rad53 was previously shown to abrogate intra S phase checkpoint-dependent inhibition of origin activity in yeast [7]. We found that introducing such mutated alleles of DBF4 and SLD3 in cdc7-4 hst3Δ hst4Δ cells does not alleviate their S phase progression defects at 30°C (Figure 5G). Taken together, the data argue that intra S phase checkpoint activity does not underlie the incapacity of cdc7-4 hst3Δ hst4Δ mutants to initiate DNA replication in a timely manner when incubated at the semi-permissive temperature for cdc7-4.

Constitutive histone H3 lysine 56 acetylation causes replication defects in cdc7-4 cells

Constitutive acetylation of histone H3 lysine 56 (H3 K56ac) was previously shown to cause most of the phenotypes associated with hst3Δ hst4Δ mutants, including their temperature and DNA damage sensitivity [19,22,23]. H3 K56ac strictly depends on the Rtt109 histone acetyltransferase [46]. We found that deletion of RTT109 rescued DNA replication progression and growth of cdc7-4 hst3Δ hst4Δ cells at semi-permissive temperatures for cdc7-4 (between 28°C and 30°C; Figure 6A-C). We next sought to directly implicate H3 K56ac in the phenotypes of cdc7-4 hst3Δ hst4Δ cells by expressing mutant histone H3 in which lysine 56 has been replaced by a non-acetylable arginine residue (H3 K56R). We tried to engineer appropriate histone mutant strains by deleting both copies of the genes encoding histone H3 (HHT1 and HHT2) and expressing one copy of the
HHT1 gene with or without a mutation resulting in expression of the H3 K56R variant. We failed to generate either an H3 WT- or H3 K56R-expressing cdc7-4 hst3Δ hst4Δ strain using the standard method described above, suggesting that abnormal histone gene copy number may be lethal in cdc7-4 hst3Δ hst4Δ cells. We therefore replaced only HHT1 by either WT or K56R alleles and left the endogenous copy of HHT2 intact to reduce H3 K56ac levels without changing histone gene dosage (Figure 6D-E). This strategy produced viable cdc7-4 hst3Δ hst4Δ strains in which H3 K56ac is either unchanged or noticeably reduced (Figure 6D-E). Strikingly, we observed a strong rescue of the temperature sensitivity of cdc7-4 hst3Δ hst4Δ upon expression of H3 K56R (compared to control cells expressing a WT version of H3; Figure 6D). Taken together, the above results indicate that constitutive Rtt109-dependent H3 K56ac inhibits growth and DNA replication in cdc7-4 hst3Δ hst4Δ cells.

Genetic and biochemical data indicate that Rtt109 and H3K56ac act at least in part by modulating the activity of a ubiquitin ligase complex composed of the Rtt101, Mms1 and Mms22 subunits [47–50]. Indeed, deletion of the genes encoding subunits of this complex is known to partially suppress the phenotypes of hst3Δ hst4Δ cells [47], although the precise mechanisms linking constitutive acetylation of nucleosomal H3 K56ac and the Rtt101/Mms1/Mms22 complex remain elusive. We found that deletion of either RTT101 or MMS1 suppresses the synthetic temperature sensitivity of cdc7-4 hst3Δ hst4Δ mutant cells. We were unable to generate cdc7-4 hst3Δ hst4Δ mms22Δ cells, suggesting that yet to be determined synthetic interactions between these loci prevent viability. Nevertheless, our results implicate Rtt101-Mms1-containing complexes in H3 K56ac-dependent modulation of DNA replication origin dynamics.

**DISCUSSION**

New histones are acetylated on several lysine residues which promotes efficient assembly of nucleosomes on replicated DNA [51–53]. In yeast, virtually all new histones H3 are acetylated on K56, leading to a genome-wide wave of histone acetylation at this residue during S phase [19,46]. H3 K56ac promotes timely formation of new nucleosomes by favoring the interaction of new histones with the chromatin assembly factors CAF1 and Rtt106 [51]. While this “pre-deposition” function of H3 K56ac in nucleosome assembly is well-established, several observations indicate that H3 K56ac plays other biological roles following its incorporation into chromatin. Indeed, lack
of nucleosomal H3 K56ac deacetylation in hst3Δ hst4Δ cells causes sensitivity to replication-blocking drugs and poor growth, suggesting that chromatin-associated H3 K56ac influences the cellular response to replicative stress [22,23]. Moreover, cells have evolved elaborate mechanisms to degrade Hst3 in response to genotoxin exposure during S phase [54,55], suggesting that the ensuing persistence of H3 K56ac may somehow be beneficial to the DNA damage response. Nevertheless, while several cellular pathways have been genetically linked to nucleosomal H3 K56ac [23,24,26,27,47], the molecular basis of the sensitivity of hst3Δ hst4Δ mutants to replicative stress, as well as the role of H3 K56ac persistence after DNA damage, remain poorly understood.

We previously showed that NAM elevates H3K56ac, thereby causing DNA damage and replicative stress similar to the situation observed for hst3Δ hst4Δ cells [26]. In accord with this, the screen presented here reveals that a large proportion of genes whose heterozygous inactivation confer NAM sensitivity participate in DNA replication. Our data further indicate that i) reduced activity of Cdc7 and Dbf4 causes growth defects in the presence of NAM, and ii) firing of early/efficient origins of DNA replication is strongly diminished in cdc7-4 hst3Δ hst4Δ cells at the semi-permissive temperature for cdc7-4. Deletion of Rif1 suppressed the inability of cdc7-4 hst3Δ hst4Δ to initiate replication and grow at the semi-permissive temperature of cdc7-4, in accord with the known role of Rif1 in promoting Glc7-dependent dephosphorylation of MCM complexes thereby inhibiting origin activation [33]. Moreover, lack of Rif1 also suppressed NAM-induced DNA damage, as well as the temperature sensitivity, spontaneous DNA damage, and elevated frequency of Rad52 foci in hst3Δ hst4Δ cells. Finally, we note that homozygous deletion of Rif1 was found to confer improved fitness in response to NAM in our previously published screen [26].

The above data are intriguing in light of the fact that Rif1 has been reported to promote the stability of stalled RF [41], which suggests that the contribution of limited origin activity to the phenotypes caused by elevated H3 K56ac overrides the negative impact of rif1Δ on replicative stress responses. We also note that published reports indicating that cells lacking Hst3 are unable to maintain a yeast chromosome harboring a reduced number of replication origins, which supports the notion that elevated H3 K56ac negatively influences the ability of cells to replicate their chromosomes under conditions where the number of active origins is limited [28,56].

It is now well-known that histone acetylation and chromatin structure influences DNA replication origin firing efficacy and timing of activation during S phase [10,57]. For example,
cells lacking the histone deacetylase Sir2 exhibit elevated origin activation at the ribosomal DNA locus and in subtelomeric regions [13,58]. Targeting the GCN5 acetyltransferase to an origin was also shown to favor its activation, while deletion of the Rpd3 deacetylase caused late origins to fire earlier in S phase [59]. While in the above-mentioned examples hyperacetylation was shown to promote origin activity, in contrast to hyperacetylation of H3 K56ac, elevated histone acetylation has also been reported to exert a negative influence on origin activity. For example, Sir2 and Rpd3 had opposite effects on the activation of origins located within the rDNA array [11], clearly indicating that histone acetylation can either promote or inhibit origin activity depending on chromosomal context.

RF stalling activates the kinases Mec1 and Rad53, which then phosphorylate a multitude of substrates to effect the various roles of the intra-S phase checkpoint [60]. Rad53 phosphorylates the replication proteins Dbf4 and Sld3 to inhibit the firing of replication origins which have not yet been activated [7]. In apparent contrast to our results, a previously published report revealed that hst3Δ hst4Δ cells present elevated activation of late origins in cells released from G1 toward S phase in medium containing the replication-blocking ribonucleotide reductase inhibitor HU [11]. However, this was not specific to hst3Δ hst4Δ cells; indeed, such effect was found to be an indirect consequence of elevated spontaneous DNA damage and constitutive Rad53 activity in various replicative stress response mutants, leading to Rad53-dependent elevation of dNTP pools and consequent HU-resistant DNA replication progression [61]. On the other hand, several observations presented here indicate that constitutive H3 K56ac can influence origin firing in a Rad53-independent manner. First, cdc7-4 hst3Δ hst4Δ cells do not display elevated Rad53 phosphorylation when released from G1 arrest toward S phase at the semi-permissive temperature for cdc7-4. Secondly, mutations or treatments that compromise Rad53 activation, or the ability of this kinase to phosphorylate Sld3 and Dbf4, do not rescue defective initiation of replication in cdc7-4 hst3Δ hst4Δ mutants. Taken together, the above data argue that the impact of constitutive H3 K56ac on origin activity does not require prior replication fork stalling and ensuing Rad53 activity, and that this histone modification can act before widespread initiation of replication origins in early S phase.

While our data argue that elevated H3 K56ac inhibits DNA replication initiation in a Rad53-independent manner, our results indicate that it does so only under conditions that reduce
DDK activity. Paradoxically, such conditions are met upon activation of the intra S phase checkpoint response; it is therefore expected that H3 K56ac-dependent inhibition of origins would contribute to S phase progression defects in \textit{hst3}\Delta \textit{hst4}\Delta cells only once RF emanating from early origins have been stalled, leading to Rad53 activation and consequent reduction of DDK activity. In other words, constitutive H3 K56ac and Rad53 activation are expected to act synergistically to inhibit late origin firing during genotoxic stress, thus interfering with the ability of cells to complete DNA replication. Consistent with this notion, we and others previously showed that i) \textit{hst3}\Delta \textit{hst4}\Delta cells cannot complete DNA replication in a timely manner after transient exposure to genotoxic drugs during S phase [24], ii) \textit{hst3}\Delta \textit{hst4}\Delta cells present strong and persistent activation of Rad53 upon DNA damage [23,24,26], iii) limiting Rad53 activation partially rescues the phenotypes of \textit{hst3}\Delta \textit{hst4}\Delta mutants [23,24,26,62], and iv) elevating the firing of late origins of replication by overexpression of Cdc45, Sld3 and Sld7 can rescue certain phenotypes caused by constitutive H3 K56ac [27,62]. Our data also show that deletion of \textit{RAD9} partially rescues defective S phase progression in \textit{cdc7-4 hst3}\Delta \textit{hst4}\Delta cells, but only at later time points. It is plausible that eventual initiation of a limited number of RF may cause spontaneous activation of Rad53 under these conditions, which in turn might maintain S phase progression defects caused by constitutive H3 K56ac over long periods of time. The combined negative effects of Rad53 activation and elevated H3 K56ac on origin activity is expected to force RF to travel unusually long distances before encountering a converging fork. Consequently, a substantial fraction of persistently stalled RF will not be “rescued” by converging forks, leading to under-replicated chromosomal regions, RF collapse, DNA damage, and eventual arrest in G2/M, which are all observed in \textit{hst3}\Delta \textit{hst4}\Delta cells [12,19,23,24].

The mechanism linking H3 K56ac to origin activity is currently unknown. As mentioned previously, Mec1 activation promotes degradation of Hst3, which causes H3 K56ac to persist during late S and G2 [54,55]. It is possible that such persistence might contribute to Mec1- and Rad53-dependent inhibition of replication origins. Interestingly, we demonstrated that deletion of \textit{RTT101} or \textit{MMS1}, which encode subunits of a ubiquitin ligase complex previously genetically linked to H3 K56ac [47,48], rescues the synthetic temperature sensitivity of \textit{hst3}\Delta \textit{hst4}\Delta \textit{cdc7-4}. Since deletion of either \textit{RTT101} or \textit{MMS1} does not influence H3 K56ac levels [47], it is likely that the effect of H3 K56ac is not strictly dependent on modulation of chromatin structure at origins. Rtt101 recruitment to chromatin upon DNA damage was shown to depend at least partly on the
H3 K56 acetyltransferase Rtt109 [50]. Moreover, Mms1 has been reported to interact directly with the Origin Recognition Complex subunit Orc5 [63], although the biological relevance of this interaction is unclear. The above considerations raise the possibility that DNA damage-induced persistence of H3 K56ac might modulate Rtt101/Mms1 activity, leading to regulation of Orc5-bound origins. Further experiments will be required to test the validity of such model, and to precisely ascertain the mechanistic basis of the impact of H3 K56ac on DNA replication dynamics.

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COMPETING INTERESTS

The authors declare no competing interests exist.

MATERIAL AND METHODS

Yeast strains and growth conditions. Yeast strains used in this study are listed in Table 1 and were generated and propagated using standard yeast genetics methods. Yeast strains used in Supplementary Figure 1 were taken from the heterozygote yeast deletion collection.
For nicotinamide (NAM) treatments, asynchronously growing cells were centrifuged and resuspended at 0.01-0.1 OD/mL in YPD or synthetic medium containing 20 mM NAM (Sigma-Aldrich). Cells were incubated on a shaker for indicated time. Cells synchronization in G1 was performed by incubating MATa yeasts in medium containing 2 µg/mL alpha-factor for 90 minutes followed by the addition of a second dose of 2 µg/mL of alpha-factor for another 75 minutes. Cells were then washed once in YPD or synthetic medium and released in S phase in medium supplemented with 5 µg/mL pronase (Protease from *Streptomyces griseus*, Sigma-Aldrich). For ionizing irradiation, exponentially growing cells were exposed to 40 Gy followed by a 60-minute incubation at 30°C prior to sample collection. For methyl methane sulfonate (MMS) treatment, cells were first synchronized in G1 using alpha factor, then incubated in YPD containing 0.01% MMS (Sigma-Aldrich) and 5 µg/mL pronase at a density of 1 OD₆₃₀/mL for 60 minutes. After treatment, cells were washed twice with YPD containing 2.5% sodium thiosulfate (Bioshop), followed by incubation in YPD. Caffeine (Sigma-Aldrich) was used at a concentration of 0.15%.

### TABLE 1. Yeast strains used in this study

<table>
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<tr>
<th>Strain name</th>
<th>Genotype</th>
<th>Figure</th>
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<td>6F</td>
<td>This study</td>
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**Genome-wide fitness screen.** The heterozygote diploid yeast fitness screen was realized as described [65–67]. Briefly, pools of the yeast heterozygote diploid deletion mutant collection (BY4743 background) were incubated at 30°C in YPD +/- 41 mM NAM. Cells were collected after 20 generations. PCR reactions were performed on extracted DNA to amplify sequence barcodes, and products were used to probe high-density oligonucleotide Affymetrix TAG4 DNA microarrays. Hybridization, washing, staining, scanning and intensity values calculation were performed as described [65–67]. For Z-score calculation, the intensity value of each mutant was divided by the standard deviation. Gene ontology (GO) Term Finder tool was used from the Saccharomyces Genome Database to identify cellular processes affected by NAM treatment [68,69]. Processes identified were considered significant if P-values ≤ 0.01. REVigo was used to summarized significant GO term identified by removing redundant ones [70]. Top 1% genes (Z-score > 2.58 or < -2.58) were compared to a previously published screen (performed on homozygote diploid mutants [26]) using Venn diagrams.

**Competitive growth assay**

0.0005 OD$_{630}$ of heterozygous deletion and WT diploid yeast cultures were mixed and incubated in YPD +/- 41 mM NAM at 25°C in a 96-well plate. Throughout the incubation, OD$_{630}$ were taken and cells were diluted appropriately to prevent saturation of the culture. After 20 generations, 0.01 OD$_{630}$ of cells was spread on YPD-agar +/- G418 plates. Plates were incubated at 30°C for 48 h and colonies were then counted. The following formula was used to describe growth +/- NAM:

\[
\frac{(\text{NAM}:\text{G418})(\text{NAM}:\text{YPD})}{((\text{YPD}:\text{G418})(\text{YPD}:\text{YPD}))}
\]

NAM:G418 is the number of colonies from cells that were grown in YPD + 41 mM NAM and then plated on YPD + 200 µg/mL G418. NAM:YPD is the number of colonies from cells grown on YPD + 41 mM NAM and then plated on YPD. YPD:G418 is the number of colonies from cells grown on YPD and then plated on YPD + 200 µg/mL G418. YPD:YPD is the number of colonies from cells grown on YPD and then plated on YPD.
Yeast growth assays. For growth in liquid medium, cells were grown to saturation in YPD in a 96-well plate. Cells were then diluted in fresh medium to 0.0005 OD_{630}/ml in 100 µL of YPD containing appropriate concentrations of NAM (Sigma-Aldrich). Cells were then incubated at the indicated temperature for 48-72 h. OD_{630} was then determined using a Biotek EL800 plate reader equipped with Gen5 version 1.05 software. Wells containing YPD were used as blanks. For spot growth assays on solid media, cells were grown in YPD in a 96-well plate to equivalent OD_{630}. Cells were serially diluted 1:5 and spotted on medium containing nicotinamide (Sigma-Aldrich), methyl methane sulfonate (Sigma-Aldrich), hydroxyurea (BioBasics), or 5-Fluoroorotic acid (Bioshop). Plates were incubated at the indicated temperature for 48-72 h.

Cell cycle analysis by flow cytometry. DNA content/cell cycle analysis by flow cytometry was performed as described previously [71]. Flow cytometry was performed using a BD Biosciences FACSCalibur instrument equipped with CellQuest software. Data were analyzed using FlowJo 10.8.1 (FlowJo, LLC).

Immunoblotting. 4 OD of cells were pelleted and frozen at -80°C prior whole-cell extraction. Cells were extracted using 0.1M NaOH for 5 minutes at room temperature as described before [72] or using standard tri-chloroacetic acid (TCA) and glass beads method [73]. Protein extracts were quantified using bicinechonic acid (BCA) protein assay kit according to the manufacturer’s protocol (Pierce). SDS-PAGE and transfer were performed using standard methods. Anti-H3 (ab1791) and anti-Rad53 (ab104232) were purchased from abcam. Anti-H3K56ac (AV105) and anti-H2A-S129-P (AV137) antibodies were generously provided by Dr. Alain Verreault (Université de Montréal, Canada). Goat anti-rabbit (BioRad; cat: 1705046), goat anti-mouse (Bio Rad; cat: 1705047) and goat anti-rat (Abcam; cat: ab97057) were used as secondary antibodies. Protein visualization was realized by chemiluminescence using Pierce ECL Western Blotting Substrate. Images were captured using an Azure c600 chemiluminescence Imaging System.

Fluorescence microscopy. Cells were fixed in 0.1M of potassium phosphate buffer pH 6.4 containing 3.7% formaldehyde (Sigma-Aldrich) and slides were prepared as described [48].
Images were taken by fluorescence microscopy using a 60X objective (numerical aperture [NA], 1.42) on DeltaVision instrument (GE Healthcare). Images analysis was performed using SoftWoRx 7 software and FIJI 1.53.

**Measurement of DNA content by quantitative PCR.** Genomic DNA from 1 OD$_{630}$ of cells was extracted and purified as described [74]. 3 ng of DNA was used per qPCR reaction (qPCR Master Mix, NEB). PCR was performed using an Applied Biosystems 7500 instrument (software version 2.3). PCR primers are listed in Table 1. Briefly, qPCR signal for a given origin was first normalized to the signal obtained from the NegV locus (ChrV: 532538-532516) [62]. This region is located $\approx 12$ Kb from ARS521, an origin which has not been detected to be active in several studies according to OriDB (http://cerevisiae.oridb.org/) and $\approx 18$ kb from ARS522, a subtelomeric origin of replication activated in late S. As such, the NegV locus is expected to be replicated in very late S, and therefore to generally remain unreplicated in a majority of $cde7-4$ and $cde7-4\ hst3\Delta \ hst4\Delta$ cells 30 minutes post-release from G1 arrest toward S phase. The NegV-normalized S phase signal was divided by the NegV-normalized signal obtained from alpha factor arrested (G1) cells. Complete replication of an origin is therefore expected to result in a ratio of S phase over G1 signal of 2.

**TABLE 2:** PCR primers used in this study.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
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<td>ARS305_qPCR_F</td>
<td>TACACGGGGGCTAAAAACGG</td>
</tr>
<tr>
<td>ARS305_qPCR_R</td>
<td>GCACTTTGATGAGGTCTCTAGC</td>
</tr>
<tr>
<td>ARS607_qPCR_F</td>
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</tr>
<tr>
<td>ARS607_qPCR_R</td>
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<td>ARS1211_qPCR_F</td>
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<tr>
<td>ARS315_qPCR_R</td>
<td>TTCTTGGCGCTACGATGTG</td>
</tr>
<tr>
<td>ARS300-F</td>
<td>TCACCCATCTCTCACCATCA</td>
</tr>
</tbody>
</table>
ARS300-R | GATGGGCATTATGCATGAAAT
NegV_qPCR_F | TAATTGCTGACGGTCATGTT
NegV_qPCR_R | GCCTCTACAGTACCGTGGGA
ARS305_probe_F | ATCGTGTAAGCTGGGGTGAC
ARS305_probe_R | AGTGGCGTTAGGTTCAATGC

Alkaline gel electrophoresis and Southern blotting. Samples were denatured by heating at 70°C in loading buffer (30 mM NaOH, 1 mM EDTA, 3% Ficoll 400, 0.01% bromocresol green). Denatured DNA was run in a 1% agarose gel in alkaline electrophoresis buffer (30 mM NaOH, 2 mM EDTA) at 3 V/cm. Southern blotting was performed using a digoxigenin (DIG)-labeled probe as described [75]. The ARS305 probe was generated by PCR using primers ARS305_probe_F and ARS305_probe_R (Table 1) and the PCR DIG Labeling Mix (Roche). Membranes were imaged using an Azure c600 chemiluminescence Imaging System.

Statistical analysis. Data are represented as mean ± standard error of the mean (SEM) unless otherwise specified. All analyses were performed using GraphPad Prism 8. Statistical tests used are described in figure legends.

FIGURE LEGENDS

FIGURE 1. A screen to identify haploinsufficient genes modulating cell fitness upon NAM exposure. (A) Z-score of individual heterozygote diploid yeast strains after 20 generations in medium containing 41mM NAM. GO-terms of genes for which the Z-scores is > 2.58 or < -2.58 were further analyzed in C. (B) Venn diagram comparing the heterozygote diploid screen presented in this article with a previously published screen using homozygote diploid deletion strains [26]. (C) GO-term associated with genes presenting Z-scores > 2.58 or < -2.58. (D) Growth competition assay for selected heterozygote mutant strains in 1:1 competition with a WT. WT and
mutant cells were mixed 1:1 and grown in YPD +/- 41 mM NAM for 20 generations. The fraction of mutant/WT cells in the culture was assessed by plating on selective medium followed by colony counting. Colors and numbers below strain names refer to GO-terms listed in C.

FIGURE 2. Compromised DDK-dependent activation of origins of DNA replication sensitizes cells to NAM. (A) Haploid dbf4-1 and cdc7-4 cells are sensitive to NAM at their semi-permissive temperature. Cells were incubated at 30°C in medium containing the indicated concentration of NAM. OD_{630} readings were taken at 72 h to evaluate cell proliferation (see Methods). (B) bob1-1 cdc7Δ cells are not sensitive to NAM. 5-fold serial dilutions of cells were spotted on YPD-agar and incubated at the indicated temperature. (C) The Glc7-interacting motif of Rif1 causes NAM sensitivity in cdc7-4 cells. Cells were treated as in B. (D) NAM-induced phosphorylation of H2A-S129 is reduced upon deletion of RIF1. WT and rif1Δ cells were exposed to 20 mM NAM for 8 h at 30°C before harvest for immunoblotting. (E) Deletion of RIF1 reduces the frequency of Rad52-YFP foci in response to NAM, but not upon IR treatment. Exponential growing cells were treated in medium containing 20 mM NAM for 8h at 30°C, or exposed to 40 Grey of ionizing radiations followed by incubation for 1 h at 30°C. Samples were then taken for fluorescence microscopy. Graph bars represent mean ± SEM of three independent experiments. (F) rif1Δ rescues the NAM sensitivity of est2Δ strains. OD_{630} readings of treated vs untreated cells after 48h of growth in 96-wells plates is presented. Graph bars represent mean ± SEM of three independent experiments each containing 4 technical replicates. **: p value < 0.01 and ***: p value < 0.001, Student’s t-test. (G) RIF1 deletion reduces NAM-induced S phase accumulation. Exponentially growing cells were incubated at 25°C in YPD containing 20 mM NAM for 8 h. Samples were taken for flow cytometry analysis of DNA content.
FIGURE 3. The activity of replication origins influences the phenotypes of hst3Δ hst4Δ cells.

(A) Deletion of RIF1 rescues the temperature sensitivity of hst3Δ hst4Δ cells (in the BY4741 genetic background) as well as the synthetic lethality of hst3Δ hst4Δ sir2Δ triple mutant. 5-fold serial dilutions of cells were spotted on the indicated medium and incubated at the indicated temperature. (B) Rif1 does not modulate H3 K56ac levels. Exponentially growing cells in YPD at 25°C were harvested and processed for immunoblotting. (C) RIF1 deletion improves S phase progression of hst3Δ hst4Δ cells after transient exposure to MMS. Exponentially growing cells at 25°C in YPD were arrested in G1 using alpha factor and released toward S in medium containing 0.01% of MMS for 90 minutes (MMS time point). After MMS inactivation using sodium thiosulfate, cells were released in fresh YPD. Samples were taken for flow cytometry analysis of DNA content at the indicated time points. As: asynchronous. (D) Deletion of RIF1 does not rescue the sensitivity of hst3Δ hst4Δ cells to MMS. 5-fold serial dilutions of cells were spotted on the indicated media and incubated at the indicated temperature. (E) RIF1 elevates the frequency of spontaneous Rad52 foci formation in hst3Δ hst4Δ cells. Rad52-GFP foci was assessed by fluorescence microscopy in exponentially growing cells at 25°C. Graph bars represent mean value ± SEM of 10 independent experiments. **: p value < 0.01 Student’s t-test. (F) Deletion of RIF1 reduces intra-S phase checkpoint activation in cdc7-4 hst3Δ hst4Δ strain. Exponentially growing cells in YPD at 25°C were harvested for immunoblotting. MMS: Cells were exposed to 0.03% MMS for 1 h prior to harvesting as control. (G) hst3Δ hst4Δ cdc7-4 cells present strong synthetic temperature sensitivity. 5-fold serial dilutions of cells were spotted on YPD-agar and incubated at the indicated temperature. (H) Individual deletion of HST3 or HST4 gene in cdc7-4 does not cause
synthetic temperature sensitivity. 5-fold serial dilutions of cells were spotted on YPD-agar and incubated at the indicated temperature.

**FIGURE 4.** Deletion of *HST3* and *HST4* inhibits the activation of origins of replication in *cdc7-4* cells. (A) S phase progression is impaired in *cdc7-4 hst3Δ hst4Δ* cells at the semi-permissive temperature for *cdc7-4*. Exponentially growing cells were arrested in G1 at 25°C and released toward S phase at 30°C. Samples were taken for DNA content analysis by flow cytometry at the indicated time points. As: Asynchronous exponentially growing cells. (B) Expression of Hst3 restores S phase progression of *cdc7-4 hst3Δ hst4Δ* cells at 30°C. Cells were treated as in A. (C) Incubation of asynchronous *cdc7-4 hst3Δ hst4Δ* cells at 30°C causes them to accumulate in early S phase. Asynchronously growing cells were transferred from 25°C to 30°C for the indicated time and harvested for DNA content determination by flow cytometry. (D-E) *cdc7-4 hst3Δ hst4Δ* cells accumulating with DNA content near 1N at 30°C are budded. (D) Cells were treated as in A and processed for DNA content analysis by flow cytometry. (E) Budding index of cells harvested in D. 200 cells were manually inspected per condition. (F) Activation of early origin of DNA replication is compromised in *cdc7-4 hst3Δ hst4Δ* cells released from G1 toward S phase at the semi-permissive temperature for *cdc7-4* (30°C). Cells were treated as in A and harvested 30 minutes post-release toward S phase at 30°C. DNA was extracted and processed for quantitative PCR analysis as described in Material and Methods. qPCR signal for a given origin was normalized to that obtained from the NegV locus (which is expected to be unreplicated in most cells 30 minutes post-release from G1 toward S). Normalized signals were then divided by the normalized signals obtained from alpha factor arrested (G1) cells. Graph bars represent mean ± SEM of five independent experiments. *: p value < 0.05 and **: p value < 0.01, Student’s t-test. (G) *cdc7-4*
*hst3Δ hst4Δ* eventually complete S phase with delayed kinetics. Cells were treated as in A. (H) Origin DNA is duplicated with delayed kinetics in *cdc7-4 hst3Δ hst4Δ* cells. As in F, except that cells were released toward S phase in presence of 200 mM HU for 120 minutes at 30°C prior harvest. Graph bars represent mean ± SEM of three independent experiments. (I) Formation of nascent DNA is inhibited at the early origin ARS305 origin in *cdc7-4 hst3Δ hst4Δ* cells. Cells were treated as in A. DNA samples were run on alkaline gels followed by Southern blotting to detect short ssDNA fragments generated at ARS305 upon origin activation.

**FIGURE 5. S phase progression defects of cdc7-4 hst3Δ hst4Δ is not due to the activation of the checkpoint.** (A-B) Rad53 activation is inhibited *cdc7-4 hst3Δ hst4Δ* upon release from G1 arrest toward S phase in the presence of HU. Cells were arrested in G1 using alpha factor at 25°C and released toward S phase at 30°C in presence or absence of 200 mM HU. (A) Cells were harvested for 60 minutes post-release toward S and processed for immunoblotting. (B) Samples were also taken for DNA content analysis by flow cytometry. As: Asynchronously growing cells. (C) *RAD9* deletion modestly improves S phase progression of *cdc7-4 hst3Δ hst4Δ* cells, but only at later time points. Exponentially growing cells were arrested in G1 using alpha factor at 25°C and released toward S at 30°C. Samples were taken for DNA content analysis by flow cytometry at the indicated time points. (D) Mutation of *MRC1* that inhibit its functions in the intra S phase checkpoint response (*mrc1-AQ*) does not improve S phase progression of *cdc7-4 hst3Δ hst4Δ* cells at 30°C. Cells were treated and collected as in C. (E-F) Caffeine does not improve S phase progression of *cdc7-4 hst3Δ hst4Δ* at 30°C. Exponentially growing cells were arrested in G1 using alpha factor at 25°C and released toward S phase at 30°C in YPD +/- 200 mM HU and +/- 0.15% caffeine. (E) After 60 minutes in S, cells were harvested for immunoblotting. (F) Samples were
taken for DNA content analysis by flow cytometry at the indicated time points. (G) The dbf4-4A sld3-38A mutations do not improve S phase progression of cdc7-4 hst3Δ hst4Δ cells at 30°C. Cells were treated as in C.

FIGURE 6. Constitutive H3K56 acetylation, Rtt101, and Mms1 cause S phase progression defects and synthetic temperature sensitivity in cdc7-4 hst3Δ hst4Δ cells. (A-B) Deletion of RTT109 rescues S phase progression in cdc7-4 hst3Δ hst4Δ cells at the semi-permissive temperature for cdc7-4 of 29°C. Exponentially growing cells were arrested in G1 at 25°C and released toward S for 60 minutes at 29°C. Samples were taken for DNA content analysis by flow cytometry. (B) Violin plot representation the Sytox Green value (DNA content) per cell from the 60 minutes time point in A. Red bars represent the median and quartiles. ****: p value < 0.0001 Mann-Whitney test. (C) RTT109 deletion rescue the synthetic temperature sensitivity of cdc7-4 hst3Δ hst4Δ cells. 5-fold serial dilutions of cells were spotted on YPD-agar and incubated at the indicated temperature. (D-E) Reducing H3 K56ac levels rescues mutant the synthetic temperature sensitivity of cdc7-4 hst3Δ hst4Δ cells. cdc7-4 hst3Δ hst4Δ HHT2 strains expressing either HHT1 or HHT1-K56R were generated. (D) 5-fold serial dilutions of cells were spotted on YPD-agar and incubated at the indicated temperature. (E) Exponentially growing cells at 25°C were processed for immunoblotting. (F) RTT101 or MMS1 deletion rescue the synthetic temperature sensitivity of cdc7-4 hst3Δ hst4Δ cells. Cells were treated as in C.
REFERENCES


**FIGURE 2**

A. Growth (% of untreated) vs. NAM (mM) for WT and dbf4-1.

B. Growth (% of untreated) vs. NAM (mM) for WT and cdc7-4.

C. Growth in YPD and NAM 25 mM for WT, cdc7-4, rif1Δ, cdc7-4 rif1Δ, cdc7-4 rif1-2-176, cdc7-4 rif1-RVxF/SILK.

D. Western blot analysis for Anti-H2A S129-P and Anti-H3.

E. Cells with Rad62-YFP foci at 0 mM, 20 mM, and 40 Gy NAM and IR.

F. Growth in 25 mM NAM (% of untreated) for WT, rif1Δ, est2Δ, rif1Δ est2Δ.

G. NAM peaks for WT, rif1Δ, est2Δ, rif1Δ est2Δ.
FIGURE 6