Gcn5 and NuA4 lysine acetyltransferases cooperatively promote DNA replication

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Abstract

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Eukaryotic cells must replicate DNA within the confines of chromatin. The basic unit of chromatin is the nucleosome, which consists of approximately 147 base pairs of DNA wrapped around an octamer of histone proteins. Because chromatin is generally inhibitory to mechanisms requiring a DNA template, enzymes that modify nucleosomes have evolved. Post-translational histone modifications can influence all DNA-dependent processes, including replication, which is initiated at sites called origins. Acetylation of histone lysine tails is catalyzed by lysine acetyltransferases, or KATs. The work of this thesis presents evidence that the cooperative action of

two KATs, Gcn5 and NuA4, combine in the budding yeast *Saccharomyces cerevisiae* to promote replication origin firing. Inducible knock-down of Gcn5 results in reduced levels of BrdU incorporation at replication origins during S-phase, and BrdU levels are further reduced with the addition of *eaf5Δ,* a deletion allele of a subunit of the NuA4 KAT complex. Gcn5 and NuA4 are also required for efficient growth of *7oriΔ,* a yeast strain with an origin-deficient chromosome. Although replication origins have been deleted in the *7oriΔ* strain, *de novo* origin firing can still occur at these sites. These results show that the combined action of Gcn5 and NuA4 KAT enzymes play critical roles in replication, and that origin identity in *S. cerevisiae* possesses surprising plasticity.

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Introduction

Complete, accurate, and timely duplication of the genome is essential for cell propagation. Failures in DNA replication can be catastrophic as they can lead to chromosome instability, fragile sites, and cancer (Letessier et al., 2011; Ozeri-Galai et al., 2011). As a result, precise regulation to control each step in the replication process has evolved. The work of this dissertation describes how two well-conserved lysine acetyltransferase enzymes promote DNA replication, and how they contribute to replication when the cell is faced with massive origin failure.

Replication

In eukaryotic cells, replication initiates at multiple sites along the chromosome called origins. In the budding yeast *Saccharomyces cerevisiae*, origins are defined by discrete sequences called autonomously replicating sequences (ARSs), named for their ability to support plasmid maintenance (Stinchcomb et al., 1979). Because origins are well defined in *S. cerevisiae*, replication mechanisms have been studied intensely in this organism. Although origin identification and selection mechanisms are more complex in higher eukaryotes, the core protein machinery is conserved.

In order to achieve activation, each origin must be bound by an ordered assembly of replication proteins (Sclafani and Holzen, 2007). In *S. cerevisiae*, each origin is bound by the six-subunit origin recognition complex (ORC) throughout the

cell cycle (Figure 1). A process termed origin licensing occurs in early G1 phase of the cell cycle when Cdc6, Cdt1, and the Mcm2-7 complex are recruited to a subset of ORC-bound origins to form the pre-replicative complex (pre-RC) (Bell and Dutta, 2002). Pre-RC formation can only occur when cyclin-dependent kinase (CDK) activity is low, but the pre-RCs are then activated by the S-phase kinases DDK (Cdc7) and CDK (Cdc28), ensuring that origins are licensed only once during the cell cycle to prevent re-replication (Heller et al., 2011). CDK also phosphorylates the Sld2 and Sld3 proteins. This phosphorylation results in recruitment of Dpb11 and is required for the pre-initiation complex (pre-IC) to assemble during S phase when Cdc45 and the GINS complex (Labib, 2010) are loaded to origins. Once these steps have been executed, origins are considered activated when fire, or initiate replication.

Although all active origins are subjected to the same initiation process, origin firing does not occur synchronously during S phase (Aparicio, 2013). Individual origins vary both in the likelihood that they will initiate replication in any given cell cycle, as well as in the firing time within S phase. Efficient origins fire in most cell cycles, whereas inefficient origins fire in few cell cycles and are usually passively replicated by forks originating from neighboring origins. Early firing origins reproducibly tend to fire early in S phase, whereas late firing origins fire in the later portion of S phase. Some factors have been shown to influence origin firing and timing, for instance, the timing of Cdc45 assembly at origins reflects origin firing times (Aparicio et al., 1999). However, the factors that set up and enforce these

qualities are still not completely understood. The complex characteristics of origin firing make identification of functional origins difficult.

At the core of each replication origin is the ARS consensus sequence (ACS), a conserved 17 base pair sequence that binds the ORC complex (Theis and Newlon, 1997). The ACS is partially degenerate, and only 11 base pairs are somewhat conserved. In addition, not all conserved positions in the ACS must be present in order to function. This level of degeneracy results in thousands of ACSs with the potential to support replication origin firing. Additional cis elements surrounding the ACS have been identified that are necessary but not sufficient to create a replication origin, but they are not well conserved and differ between individual ARSs. Clearly, other factors are required to define an active replication origin and attempts to identify functional origins cannot depend on sequence alone.

The development of microarray technology provided a major breakthrough to map and characterize active replication origins genome-wide. The results from the first set of origin mapping experiments showed that the ~12Mb *S. cerevisiae* genome has approximately 400 origins (Raghuraman et al., 2001). When replication fork rate and cell cycle length are taken into consideration, it is clear that there are more origins than required in the genome.

Origin mapping and replication profiling studies also revealed general replication patterns. On each chromosome, centromeric DNA typically replicates early in S phase, while telomeres are replicated late. As technologies such as tiling

arrays, BrdU immunoprecipitation in yeast, and next generation sequencing advance, more accurate origin maps continue to develop and become more resolved.

When replication is inhibited, either due to lesions in the DNA strand or from the presence of replication inhibitors such as hydroxyurea (HU), the cell responds by activating the intra-S phase checkpoint (Paulovich and Hartwell, 1995). This checkpoint signal cascade is critical to stabilize stalled replication forks, which are detected by the ssDNA-binding protein RPA, to which the Mec1/Ddc2 kinase binds (Branzei and Foiani, 2009). Mec1 then phospho-activates Mrc1, which acts with Tof1 to slow replication forks, and also activates Rad9, a mediator kinase in the checkpoint pathway. Rad9, and possibly Mrc1, then phosphorylate the Rad53 effector kinase, which amplifies the checkpoint signal via autophosphorylation, and whose downstream targets act to halt the cell cycle, further stabilize replication forks, and prevent unused origins from firing (Lopes et al., 2001; Santocanale and Diffley, 1998; Shirahige et al., 1998).

Chromatin

Like all DNA-dependent processes, eukaryotic DNA replication is strongly influenced by the chromatin status around origins. Chromatin refers to the combination of DNA and proteins that packages DNA into the nucleus. The basic unit of chromatin is the nucleosome, which consists of approximately 147 base pairs of DNA wrapped around an octamer of histone proteins (Luger et al., 1997). The core histone octamer is comprised of four dimers of the histones H3, H4, H2A, and

H2B, and each histone has flexile peptide tails that extend from the core. Each histone makes multiple contacts with the DNA double helix, and as a result is generally inhibitory to processes occurring on a DNA template, including transcription, replication, and repair (Luger et al., 1997). However, histones can be reversibly post-translationally modified with a variety of covalent chemical groups, including phosphorylation, acetylation, methylation, and ubiquitination. These modifications typically occur on histone tails and can change accessibility to DNA.

The addition of a negatively charged acetyl group to a histone lysine residue likely neutralizes the positive charge of the amino acid and can weaken histone/DNA interactions (Anderson et al., 2001; Workman and Kingston, 1998). There is evidence that histone tail acetylation can disrupt the higher order structure of chromatin packaging (Luger et al., 1997) and can act as a signal for targeting trans-acting factors (Strahl and Allis, 2000).

Lysine acetyltransferase (KAT) enzymes (formerly referred to as histone acetylases, or HATs) (Allis et al., 2007) catalyze the transfer of an acetyl group from acetyl Co-A to N-terminal histone tail lysine residues. KAT are divided into families based on sequence homology and functional roles. Gcn5 was the first nuclear KAT to be identified (Brownell et al., 1996), and is the founding member of the Gcn5-related *N*-acetyltransferase (GNAT) family (Carrozza et al., 2003). It is the catalytic subunit of multiple complexes and subcomplexes, including SAGA (Spt-Ada-Gcn5-acetyltransferase), ADA, SLIK/SALSA (SAGA-like/SAGA-altered, Spt8 absent), and HAT-A2 (Lee and Workman, 2007). The residue specificity of Gcn5 depends on its

complex composition and whether it is acting on free histones or chromatin templates, but is thought to acetylate histone H3 at lysines 9, 14, 18, and 23, and residues on histone H2B to a lesser extent (Grant et al., 1997). The Sas3-dependent NuA3 complex acetylates similar residues and has overlapping functions with Gcn5, but hasn't been characterized to the same extent (Howe et al., 2001; Lafon et al., 2007).

Esa1 is the essential catalytic subunit (Clarke et al., 1999) of the MOZ, Ybf2/Sas3, Sas2, Tip60 (MYST) family KAT NuA4. NuA4 is composed of many protein subunits (Table 1), and acetylates histone H4 lysines 5, 8, 12, and 16, as well as residues on histone H2A (Allard et al., 1999). The NuA4 complex can be dissociated into multiple sub-complexes, and their assembly into the full NuA4 complex is dependent on the Eaf1 subunit (Auger et al., 2008; Mitchell et al., 2008). One of the sub-complexes, piccolo NuA4 (picNuA4) is thought to be responsible for global, non-specific histone acetylation while the larger complex (NuA4) is recruited to promoters and represents targeted activity (Boudreault et al., 2003).

Chromatin and KATs have roles in all DNA-dependent processes (Kurdistani and Grunstein, 2003), but most studies have focused on transcription. However, a picture for how histone acetylation affects replication is gradually emerging.

Replication in the context of chromatin

Replication is highly influenced by chromatin. At the basic level, the ACS sites of active ARSs are positioned asymmetrically in a nucleosome-depleted region,

and are flanked on either side by well-positioned nucleosomes (Eaton et al., 2010) that are dependent on ORC binding (Lipford and Bell, 2001). DNA accessibility around early-firing origins is greater than the accessibility at dormant origins (Rodriguez and Tsukiyama, 2013), which suggests that chromatin is a major determinant of origin timing and efficiency characteristics.

Histone modifications have been shown to influence replication origin function. Loss of phosphorylation at histone H3 threonine 45 results in phenotypes consistent with replication defects (Baker et al., 2010). The Set2 methyltransferase has been shown to recruit Cdc45 to replication origins through histone H3 lysine 36 mono-methylation (Pryde et al., 2009). Enzymes required for histone H3K4 di-methylation, Set1 and Bre1, have also been implicated in promoting replication origin function (Rizzardi et al., 2012).

Work with histone deacetylase mutants has shown that histone acetylation can advance origin firing timing and efficiency (Aparicio et al., 2004; Vogelauer et al., 2002). Loss of function of the *SIR2* histone deacetylase (HDAC) rescues the pre-RC assembly mutant *cdc6-4* (Pappas et al., 2004). Interestingly, artificial targeting of the GCN5 KAT to a late-replicating origin in *S. cerevisiae* causes it to fire early (Vogelauer et al., 2002). Histone acetylation is also associated with origin activation in metazoans (Aggarwal and Calvi, 2004; Danis et al., 2004; Hartl et al., 2007; Schwaiger et al., 2009). For instance, targeting the Drosophila *Chameau* gene, a human KAT homolog, to a replication origin promotes firing (Aggarwal and Calvi, 2004).

Since histone acetylation influences DNA replication, then histone acetyltransferase enzyme mutants should also reflect replication defect phenotypes. Indeed, the Gcn5 and Sas3 KATs acetylate histone H3 lysines 9 and 14, and have been shown to function in cell cycle progression (Howe et al., 2001). Loss of function of Esa1, the catalytic subunit of NuA4, is lethal, but mutations of non-essential subunits suggest a role in replication, as they are sensitive to replication inhibitors and show cell cycle delays (Choy and Kron, 2002). In human cells, the HBO1 (histone acetyltransferase bound to Orc) complex is required for replication origin licensing (Iizuka et al., 2006; Miotto and Struhl, 2010). Interestingly, the catalytic subunit of HBO1 is homologous to Esa1, the catalytic subunit of the *S. cerevisiae* histone H4 KAT NuA4 (Doyon et al., 2004).

These studies point to histone acetylation as an important mechanism in DNA replication. In this thesis, we hypothesize that the histone H3 and H4 KATs Gcn5 and NuA4 cooperate to promote replication, and that acetylation by these enzymes are required for replication of an origin deficient chromosome.

Histone Acetylation Accelerates Replication Origin Firing

Introduction

Like all DNA dependent processes, replication is regulated through its chromatin status. Previous studies from our lab aimed to identify and understand histone modifications that contribute to replication origin firing (Unnikrishnan et al., 2010). The findings in Unnikrishnan et al. identified many new histone modifications that fluctuate during the cell cycle. Importantly, a quantitative analysis of histone acetylation around an origin of replication showed that acetylation levels on histone H3 and H4 tails increase from S phase through G2/M, before decreasing dramatically in G1. Interestingly, the analysis also showed that it is not individual lysine acetylation levels that change during the cell cycle, but the number of acetylated lysine residues on the same histone peptides. Furthermore, these modifications were found to be required for replication origin firing.

Analysis of histone deacetylase (HDAC) mutants has indeed shown that histone acetylation promotes origin firing timing and efficiency (Aparicio et al., 2004; Knott et al., 2009; Pasero et al., 2002; Stevenson and Gottschling, 1999; Vogelauer et al., 2002). However, less is known about the specific histone lysine acetyltransferases (KATs) that should positively regulate replication. The histone H3 KAT Gcn5 has emerged as a likely candidate, as tethering Gcn5 to a late-firing origin causes it to fire early (Vogelauer et al., 2002), and deletion of *GCN5* results in

increased mini-chromosome loss. (Espinosa et al., 2010). However, results from our lab show that acetylation of histone H4 is also important in DNA replication, suggesting that Gcn5 is likely acting in concert with a histone H4 lysine acetyltransferase.

In this study, we aimed to understand the mechanism of histone acetylation in promoting replication by investigating how the cooperative action of KATs contributes to replication origin firing. We used an inducible knock-down allele of *GCN5* to show that diminishing levels of Gcn5 results in replication defects, which are increased when combined with the *eaf5Δ* mutation, a deletion of a non-essential subunit of the histone H4 KAT NuA4. We show evidence that Gcn5 and NuA4 cooperate to promote replication origin firing, and that the relevant substrate for H4 KAT activity is indeed the N-terminal tail of histone H4.

Methods

Strains, plasmids, and genetic methods

Strains used in this study were created using standard yeast manipulations (Adams, 1998) and are listed in Table 1. Cells were grown in standard YPD medium or synthetic selective medium as appropriate. All strains were derived from W303-1a but contain a corrected weak *rad5* mutation (Thomas and Rothstein, 1989; Zhao et al., 1998).

Histone mutants were generated in plasmid-shuffle strains where both endogenous copies of histones H3 and H4 had been deleted, and single copies of wild-type histones were expressed from a plasmid containing the *URA3* marker. Plasmids expressing mutated histones were constructed using site-directed mutagenesis (QuikChange, Stratagene) and integrated into the genome. Wild-type histone plasmids were then eliminated by counter-selection on medium containing 5-fluoroorotic acid. Single integration of plasmids and histone genotype was confirmed by PCR.

Degron-gcn5 strains were constructed by cloning the first 500 bp of Gcn5 into the PstI and NotI sites of the pSB229 plasmid. A sense mutation was introduced into the Gcn5 plasmid fragment by site-directed mutagenesis (QuikChange, Stratagene) to create a BstBI restriction site for plasmid linearization and integration via homologous recombination at the endogenous Gcn5 locus that had been previously tagged with the 3XFlag epitope. Single integration was confirmed by PCR. Integration of the pSB229-Gcn5 plasmid creates a UBR degron-Gcn5-3XFlag gene fusion under the control of the Tet operator.

Plasmid loss assay

Plasmids were transformed into the required strains, and three independent transformants were chosen for analysis. Cells were grown under selective conditions at 30˚C until the culture reached saturation. Samples from this initial culture were plated on YPD and the percentage of colonies that had lost the ADE

plasmid was determined by counting the proportion of pink versus white colonies. Cells were then diluted 1:1000 in non-selective medium and allowed to reach saturation (approximately 10 generations). Samples were plated and loss rates were determined according to the formula: plasmid loss rate (L) = $1\text{-}10^{x}$ with x = $[\log(F_i) - \log(F_f)]$/number of doubling times. (F_i) and (F_f) = the fraction of cells containing the plasmid before and after incubation in full medium, respectively.

Cell viability assay

Asynchronous mid-log phase cells were stained using the LIVE/DEAD FungaLight yeast viability kit (Molecular Probes, Invitrogen). Samples were washed and suspended in 50 mM Tris buffer (pH7.5) with 1 µl each of SYTO9 dye and propidium iodide. Cells were incubated at 37˚C for 15 min then fluorescence was measured by flow cytometry on a FACSCanto cell analyzer (BD Biosciences). Viability was measured as the percentage of cells that were able to exclude propidium iodide and therefore fluoresce within the range of SYTO9. Dead cells (heat killed) were used as a control and to guide gating parameters. Cell Quest software (BD Biosciences) was used for data collection and analysis.

BrdU IP-Chip

DNA was harvested from cells harboring a single integrated BrdU incorporation vector (Viggiani and Aparicio, 2006) and replicated DNA was immunoprecipitated as described (Viggiani et al., 2010) with the following

modifications: Lysis buffer contained 100mM Tris-HCl pH8.0 with 20% glycerol, cell lysate was sonicated using a Bioruptor sonicator bath (Diagenode), and BrdU immunoprecipitation was performed using Dynabeads Protein G (Novex). Input (G1) and immunoprecipitated DNA was amplified using whole genome amplification (GenomePlex, Sigma-Aldrich), and then UDG fragmented and terminally labeled as previously described (Yadon et al., 2010). Samples were competitively hybridized to custom tiling arrays (Roche NimbleGen) as previously described (Yadon et al., 2010). Arrays contain 50-mer probes overlapping by an average of 42 bp, covering both strands of chromosomes III, VI, and XII and representing 14% of the yeast genome. Array usage and data manipulation were performed as described (Rodriguez and Tsukiyama, 2013), where data was smoothed with a 50 base-pair pseudomedian sliding window. Each data set was then normalized to the average signal of un-replicated regions in 200mM HU. The sequences of microarray probes are available upon request.

Results

KAT mutation alleles suggest replication roles for Gcn5 and NuA4

Previous work from our lab revealed that multiple N-terminal lysine residues on histones H3 and H4 located around active replication origins are acetylated during S-phase in *S. cerevisiae,* and that multiple acetylation is required for efficient origin firing (Unnikrishnan et al., 2010). To investigate how histone acetylation

facilitates origin function, we hypothesized that because lysine point mutations on histone H3 and H4 result in synthetic sickness when grown in the presence of HU, mutations of the corresponding lysine acetyltransferases (KATs) that act cooperatively in replication origin firing would produce the same phenotype. To this end, we first generated mutations in the catalytic subunits of KAT complexes that can acetylate histone H3 N-terminal tails: Gcn5 and Sas3. In histone H4 tail KAT complexes, activities are catalyzed by the Esa1, Hat1, and Sas2 subunits (Roth et al., 2001). Because Esa1, the catalytic subunit of the histone H4 KAT NuA4, is essential for viability, we deleted non-essential subunits of the NuA4 complex to elucidate its function.

We generated a series of seven double mutations of H3 and H4 KAT subunits and tested their growth on nutrient rich media (YPD) as well as on YPD supplemented with the replication inhibitor hydroxyurea (HU). These analyses revealed that deletion of *GCN5* or *SAS3* combined with deletion of various NuA4 subunits leads to synthetic sensitivity to HU (Figure 2), suggesting parallel functions of H3 and H4 KATs in replication. The double mutants *gcn5Δ eaf1Δ* and *gcn5Δ yng2Δ* result in cell inviability (data not shown) whereas *sas3Δ eaf1Δ* and *sas3Δ yng2Δ* are viable (Figure 2b). Therefore, we concluded that the *gcn5Δ* mutation generally exhibits stronger genetic interactions with NuA4 mutations than the *sas3Δ* mutation, and therefore focused on *gcn5Δ* in the following studies. To study NuA4, we decided to use the *eaf5Δ* deletion mutation, as Eaf5 is a rare subunit that is not shared with other chromatin modification complexes (Table 1) (Carrozza et al.,

2003). It should be noted that the *eaf5Δ* mutation has only a small effect on NuA4 function, as it does not cause detectable growth defects by itself (Figure 2a).

Although the constitutive *gcn5Δ eaf5Δ* mutant strain exhibited striking HU sensitivity, the strain had severe growth defects, and suppressors frequently arose. To circumvent this problem, we constructed a conditional mutant, *degron-gcn5*, where a ubiquitin degron was fused to the N-terminus of Gcn5, and expression was controlled by the Tet promoter (Cormack and Struhl, 1992) (Figure 3a). Addition of doxycycline to the growth medium shuts off expression and the presence of the degron targets Gcn5 for ubiquitin-mediated proteasome degradation. Gcn5 protein level in the *deg-gcn5* strain is lower than endogenous levels, even under permissive conditions (no dox). However, this level of Gcn5 is sufficient to support wild-type levels of cell growth even in the presence of the *eaf5Δ* mutation (Figure 3b,c). When doxycycline is added to the growth medium, Gcn5 levels are greatly diminished (Figure 3b). As expected, although the *eaf5Δ* single mutation does not cause detectable sensitivity to HU, combining the *deg-gcn5* allele with *eaf5Δ* results in greater sensitivity to HU than either KAT mutation alone (Figure 3c).

KAT mutant phenotypes are not due to cell death alone

Growth defects and sensitivity to HU can be attributed to either slower cell growth or cell death. To determine the contribution of each possibility, we first calculated the doubling time for each strain in mid-log phase liquid cultures (Figure 4). Interestingly, mutating either *GCN5* or the NuA4 subunit *EAF5* caused a slight

increase in doubling time, even though there is no detectable change in growth between wild-type and *eaf5Δ* cells when grown on solid media (Figure 4, 3c). As expected, combining both the *deg-gcn5* and *eaf5Δ* alleles caused a 19.2% increase in doubling time versus wild type (Table 3). When Gcn5 degradation was induced by growing cells in medium containing doxycycline, doubling time increased to 55.4% longer than wild-type in *deg-gcn5* cells, and more than doubled in the *deg-gcn5 eaf5Δ* mutant (Figure 4 and Table 3).

To measure how much of these differences were due to cell death, we performed a FungaLight viability assay (Molecular Probes, Invitrogen) on the same cultures, which quantifies the fraction of cells with the ability to maintain intact membranes and exclude propidium iodide. The results of this assay are shown in Figure 5. All strains have a proportion of cells that are measured as dead, including wild-type. To determine how cell death contributes to doubling time, we constructed a set of hypothetical growth curves with different percentages of cells "dying" and unable to contribute to the next generation. Doubling times for each hypothetical culture were calculated, and the difference in doubling time compared to wild-type was calculated and presented in Table 3. By comparing the expected increase in doubling time between strains to the doubling times actually observed, we can conclude that the growth defects are not due to cell death alone (Table 3). This indicates that KAT mutations cause the cell cycle to slow down.

Gcn5 and NuA4 KATs cooperate to promote replication

Cells with defective replication mechanisms have difficulty supporting the maintenance of mini-chromosomes, and show increased plasmid loss rates compared to wild-type cells (Tye, 1999). To test if the loss of both H3 and H4 KAT activity affects replication to a greater degree than the individual mutants, we performed a plasmid loss assay in wild-type and KAT mutant cells. Plasmid loss rates for *eaf5Δ* and *deg-gcn5* single KAT mutation cells were similar to wild-type rates (Figure 6). In contrast, when KAT mutations were combined, the *deg-gcn5 eaf5Δ* strain showed an increased plasmid loss rate. This result suggests that replication is dependent upon the concerted effort of both H3 and H4 KATs.

To examine the roles of histone H3 and H4 KATs on DNA replication on a global scale, we measured BrdU incorporation to generate replication profiles for each strain (Viggiani et al., 2010). Wild-type and KAT mutant cells were grown in media containing doxycycline, arrested them in G1 with alpha factor, then released into media containing BrdU, doxycycline, and 200mM HU to localize BrdU incorporation in the vicinity of early origins. Replicated DNA was immunoprecipitated using an anti BrdU antibody, amplified, and hybridized to custom-made high-density tiling arrays of chromosomes III, VI, and XII (Figure 7) (Yadon et al., 2010). It has been demonstrated that BrdU signals reflect the level of origin activity in 200mM HU (Knott et al., 2009; Poli et al., 2012). BrdU profiles at the chromosome level revealed that strains with strains with *deg-gcn5* alleles have lower levels of BrdU incorporation compared to wild-type, whereas *eaf5Δ* cells no

detectable defects (Figure 7). The double KAT mutant *deg-gcn5 eaf5Δ* exhibited a replication profile similar to that of *deg-gcn5*.

To closely examine the differences in BrdU replication pro between wild-type and mutant cells, we averaged data for eight early-firing efficient origins (*ARS305, 306, 307, 606, 607, 1209, 1211*, and *1213*) represented on our tiling arrays and aligned their BrdU signals at the origin midpoints (Rodriguez and Tsukiyama, 2013). Comparison of BrdU signal between wild-type and KAT mutant strains showed that BrdU incorporation is greatly diminished in *deg-gcn5* cells (Figure 8a). Importantly, although the single *eaf5Δ* mutation did not affect the replication profiles, the combination of both *deg-gcn5* and *eaf5Δ* resulted in slightly lower BrdU signal at all origins compared to the *deg-gcn5* allele alone. The cooperative effect of the *deg-gcn5* and *eaf5Δ* mutations was also apparent at early-firing origins with lower efficiency and at late-firing origins (data not shown).

To test if this cooperative effect was detectable without constitutive Gcn5 knockdown, we repeated the experiment described above, but only added doxycycline to arrested cells for 30 minutes before releasing into media containing BrdU, doxycycline, and 200mM HU. BrdU signal at origins was similar for wild-type, *eaf5Δ* and *deg-gcn5* cells, and slightly diminished BrdU signal was only seen in the double KAT mutant *deg-gcn5 eaf5Δ* strain (Figure 8b).

Histone acetylation mimic rescues *deg-gcn5 eaf5Δ* phenotype

A known major function of Gcn5 and NuA4 is to acetylate histones, but they have also been shown to modify other proteins (Charles et al., 2011; Paolinelli et al., 2009). To test whether histones are important substrates of KATs in facilitating replication, we constructed strains in which histone lysine residues (K) that are acetylated by Gcn5 and Nua4 were changed to glutamine (Q), which mimics lysine acetylation (Hecht et al., 1995). The histone H4 acetyl mimic mutations strongly rescued the HU sensitivity of *deg-gcn5 eaf5Δ* cells (Figure 9). In contrast, H3 acetyl mimic mutations did not cause detectable rescue of the HU sensitivity of the KAT mutants. This may reflect a more prominent role for histone H4 in origin firing, which is consistent with previous results from our lab (Unnikrishnan et al., 2010). Our results strongly support our model that NuA4-dependent acetylation of histone H4 tails plays critical roles in promoting DNA replication.

Subunits in the Set3 HDAC complex rescue the *deg-gcn5 eaf5Δ* phenotype

Histone acetylation by KATs is counteracted by histone deacetylases (HDACs). To screen for potential HDACs that balance replication-specific acetylation caused by cooperation between Gcn5 and NuA4, we tested HDAC deletion mutations for rescue of the HU sensitivity seen in *deg-gcn5 eaf5Δ* cells. We hypothesized that because KAT mutants have abnormally low levels of histone acetylation, deletion of the counteracting HDAC(s) should restore acetylation levels and rescue the growth phenotype in the presence of replication inhibitors. We found that mutations in *HOS2*, *HOS4*, and *HST1* rescue the growth phenotype on HU

(Table 4). Interestingly, these subunits are all found in the Set3 HDAC complex, suggesting that it is the enzyme responsible for counteracting the effects of Gcn5 and NuA4 in replication.

Discussion

These results provide evidence that the histone H3 KAT Gcn5 and the histone H4 KAT NuA4 act cooperatively to promote DNA replication, as shown with BrdU incorporation profiles. Gcn5 has been reported to affect DNA replication (Aparicio et al., 2004; Knott et al., 2009; Vogelauer et al., 2002), but its effect on DNA replication on a genome-wide scale has not been determined. In addition, we found that replication defects of *gcn5Δ* mutants are enhanced when the *eaf5Δ* allele is added. To confirm that these phenotypes were not specific to the Eaf5 subunit of NuA4, we also tested *deg-gcn5 eaf1Δ* and *deg-gcn5 esa1-1851^{ts}* mutants, and found that they had additive sensitivity to HU (data not shown). However, their extremely strong growth defects prevented their use in other assays.

Because KATs play an important role in transcription, it is difficult to exclude the possibility that Gcn5 and Nua4 affect replication indirectly through transcription. However, we believe they at least partly function directly in DNA replication because Gcn5 has been shown to associate with origins (Espinosa et al., 2010; Robert et al., 2004). Additionally, transcriptional analysis of *gcn5Δ* cells does not show altered levels of replication factors (Huisinga and Pugh, 2004). Gcn5 has also

been suggested to play a role in histone deposition, mostly because of its genetic interactions with histone chaperones (Burgess et al., 2010). While this may contribute to Gcn5 mutant phenotypes, we believe Gcn5 plays a role in promoting origin firing, as more direct evidence such as 2D gels and BrdU profiles suggest (Vogelauer et al., 2002).

HBO1 (human acetylase bound to ORC1) is a histone H4-specific acetyltransferase that is required for replication licensing (Doyon et al., 2006; Iizuka et al., 2006). Interestingly, Esa1, the catalytic subunit of Nua4, is homologous to Tip60, the catalytic subunit of HBO1, and the HBO1 complex is suggested to be orthologous to yeast piccolo NuA4 based on subunit interactions and specificity (Avvakumov and Côté, 2007; Doyon et al., 2004). If this is the case, it means that roles of H4 KATs in DNA replication may be evolutionarily conserved.

Although histone lysine acetylation is important in replication origin firing, KATs also acetylate other substrates. Indeed, a recent study in human cells identified numerous non-histone proteins with increased acetylation when treated with HDAC inhibitors (Choudhary et al., 2009). However, rescue of HU sensitivity phenotypes of KAT mutants by acetyl mimic mutations on histone H4 tails strongly suggests that histone H4 is the relevant KAT substrate in replication.

It is difficult to study KAT association specifically with replication origins because the yeast genome is so compact, and ARSs frequently overlap with gene promoters and coding regions where KATs are actively recruited. Indeed, attempts to measure ChIP signals of Gcn5 and multiply acetylated histones at chromosomal

origins showed Gcn5 localization and robust histone acetylation at many origins, yet these signals were typical of promoter loci or reflective of the particular gene structure in which the origin was located (data not shown). Nevertheless, a recent study of KAT activity on episomes removed ARS elements away from other KAT binding sites and found Gcn5 weakly associated with origins (Espinosa et al., 2010).

The results presented in this section support a model in which Gcn5 and NuA4 KATs cooperate to promote DNA replication. We suggest that both enzymes work directly at origins, with NuA4 acetylating histone lysines to promote origin firing, and Gcn5 either acetylating histones to a lesser degree than NuA4, or acetylating a non-histone substrate. A non-histone substrate for is supported by a study in human cells showing that Gcn5 acetylates Cdc6 (Paolinelli et al., 2009), and our results that histone H3 acetyl mimics do not rescue HU sensitivity of KAT mutants (Figure 9). If this is the case, Gcn5 could play a role in replication origin licensing. The mechanism of histone H4 acetylation in promoting replication remains unknown, but previous studies from our lab show that it is not the specific residues on histone tails that change during the cell cycle, but the number of acetylated residues per peptide (Unnikrishnan et al., 2010). This suggests a mechanism in which acetylation keeps chromatin in an open state to promote replication factor access to DNA, instead of specific residue marks recruiting trans-acting factors.

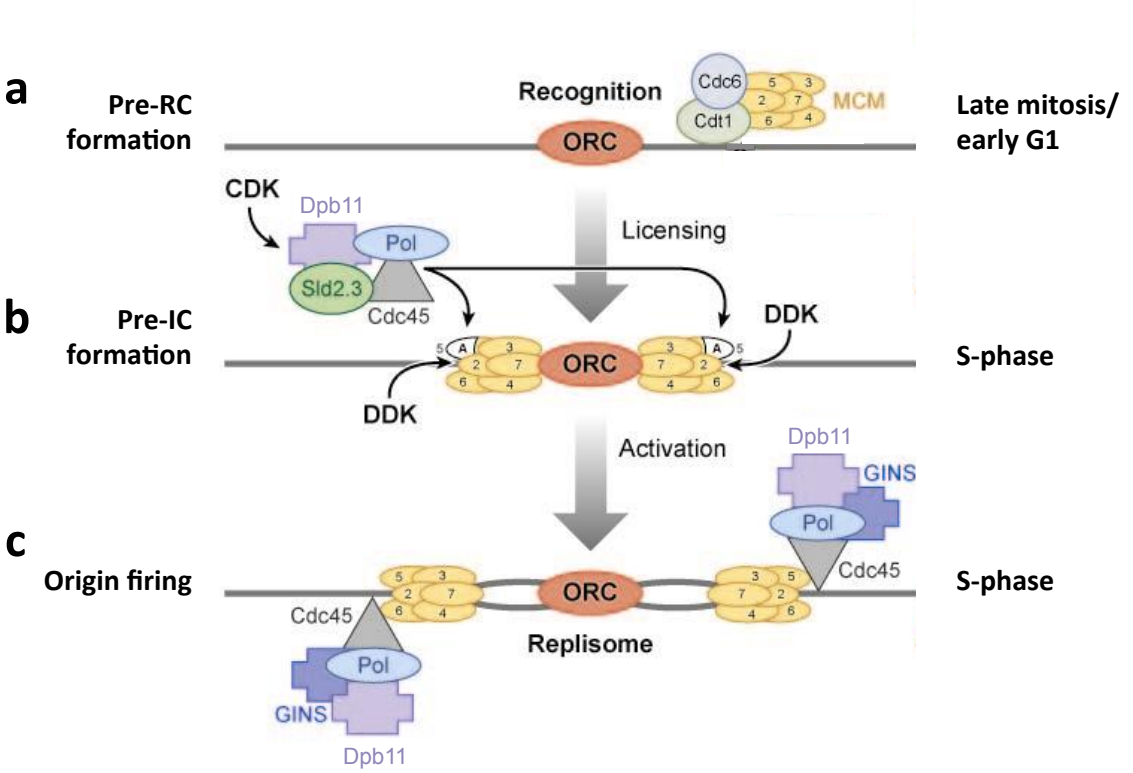

Figure 1: Eukaryotic DNA replication. (a) pre-RC formation occurs when origins are recognized by the ORC complex, then Cdc6 and Cdt1 proteins load the MCM helicase to form licenced origins. (b) CDK and DDK become active in late G1 phase to activate the MCM helicase and (c) load the polymerase-containing replisome. Modified from Sclafani & Holzen 2007.

Table 1: NuA4 lysine acetyltransferase subunits and complexes.

Subunit	Notes	Mammalian homolog
Esa1	Catalytic subunit, knockout lethal in S. cerevisiae, part of piccolo NuA4	Tip60
Epl1	Knockout lethal in S cerevisiae, part of piccolo NuA4	Enhancer of Polycomb
Yng2	Part of piccolo NuA4	Ing1
Arp4	also found in several chromatin remodelers	-
Tra1	ATM-family cofactor, also found in SAGA, knockout lethal in S. cerevisiae	-
Eaf3	-	-
Eaf1	platform for Nua4 assembly	-
Eaf6	also found in SAS3/NuA3 acetyltransferase	-
Eaf7	-	-
Yaf9	also found in SWR1	-
Eaf5	-	-
Swc4	also found in SWR1	-

Table 2: Yeast strains used to analyze KAT contribution to replication

Strain	Genotype	Reference
W1588-4C	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5+*	Zhao et al. 1998
YTT4032	*MATa gcn5Δ::KanMX*	This study
YTT3175	*MATa eaf5Δ::KanMX*	This study
YTT4952	*MATa gcn5Δ::HYG eaf5Δ::NAT*	This study
YTT3182	*MATa eaf6Δ::HYG*	This study
YTT6008	*MATa gcn5Δ::HYG eaf6Δ::NAT*	This study
YTT2462	*MATa eaf3Δ::KanMX*	This study
YTT4034	*MATa sas3Δ::KanMX*	This study
YTT2329	*MATa eaf1Δ::NAT*	This study
YTT6010	*MATa sas3Δ::HYG eaf1Δ::NAT*	This study
YTT5317	*MATa yng2Δ::KanMX*	This study
YTT6011	*MATa sas3Δ::HYG yng2Δ::NAT*	This study
YTT5266[a]	*MATa 3XTet:UBRdeg-Gcn5-2L3XFlag-klTRP1-HYG*	This study
YTT5267[a]	*MATa 3XTet:UBRdeg-Gcn5-2L3XFlag-klTRP1-HYG eaf5Δ::NAT*	This study
YTT3846	*MATa hht2-hhf2::NAT hht1-hhf1::HYG TRP::hht2K9,14R-hhf2K5,8,12R*	Unnikrishnan et al. 2010
YTT5521[b]	*MATa URA3::pRS306-BrdUinc*	This study
YTT5903[b]	*MATa URA3::pRS306-BrdUinc eaf5Δ::NAT*	This study
YTT5905[a,b]	*MATa URA3::pRS306-BrdUinc 3XTet:UBRdeg-Gcn5-2L3XFlag-klTRP1-HYG*	This study
YTT5658[a,b]	*MATa URA3::pRS306-BrdUinc 3XTet:UBRdeg-Gcn5-2L3XFlag-klTRP1-HYG eaf5Δ::NAT*	This study
YTT5923	*MATa hht2-hhf2::NAT hht1-hhf1::HYG LEU::hht2K9,14Q-HHF2*	This study

Continued on next page

Table 2: Yeast strains used to analyze KAT contribution to replication (cont.)

Strain	Genotype	Reference
YTT5925	*MATa hht2-hhf2::NAT hht1-hhf1::HYG LEU::HHT2-hhf2K5,8Q*	This study
YTT5926	*MATa hht2-hhf2::NAT hht1-hhf1::HYG LEU::hht2K9,14Q-hhf2K5,8Q*	This study
YTT5927[a]	*MATa hht2-hhf2::NAT hht1-hhf1::HYG LEU::hht2K9,14Q-HHF2 3XTet:UBRdeg-Gcn5-2L3XFlag-klTRP1-HYG eaf5Δ::NAT*	This study
YTT5929[a]	*MATa hht2-hhf2::NAT hht1-hhf1::HYG LEU::HHT2-hhf2K5,8Q 3XTet:UBRdeg-Gcn5-2L3XFlag-klTRP1-HYG eaf5Δ::NAT*	This study
YTT5931[a]	*MATa hht2-hhf2::NAT hht1-hhf1::HYG LEU::hht2K9,14Q-hhf2K5,8Q 3XTet:UBRdeg-Gcn5-2L3XFlag-klTRP1-HYG eaf5Δ::NAT*	This study

All strains are isogenic to W1588-4C, a RAD5+ strain of W303. Only alleles that differ from W303 are listed.

a BrdU incorporation plasmid provided by O. Aparicio (Viggiani et al. 2006).

b Degron plasmid provided by S. Biggins (Cormack & Struhl 1992).

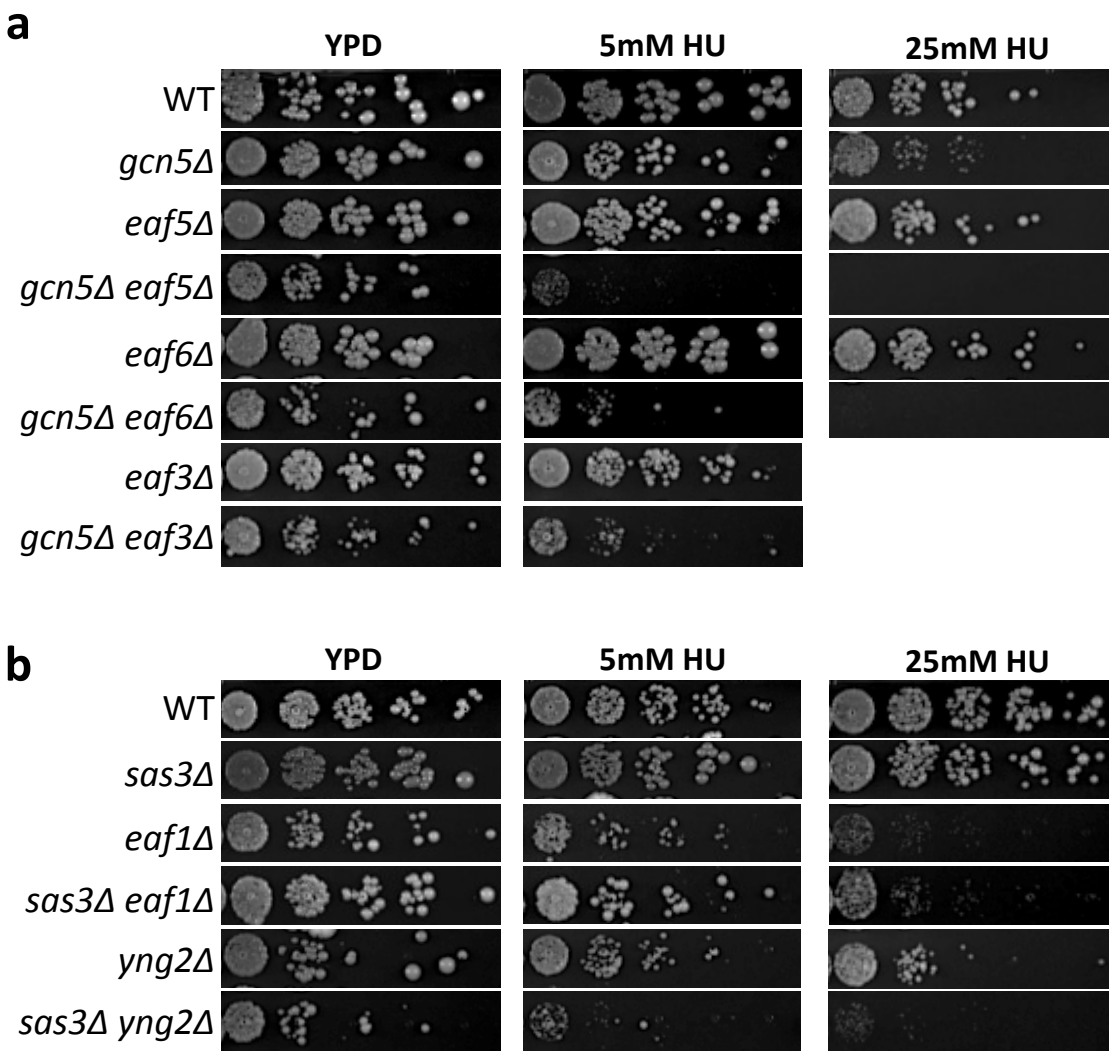

Figure 2: Histone H3 and H4 KATs act cooperatively to promote growth during replication stress. (a) Fivefold serial dilutions of *gcn5Δ* combined with indicated Nua4 subunit deletions. (b) Fivefold serial dilutions of *sas3Δ* combined with indicated Nua4 subunit deletions. Cells were spotted onto indicated media and grown for 2 days at 30˚C either with or without the addition of the replication inhibitor hydroxyurea (HU).

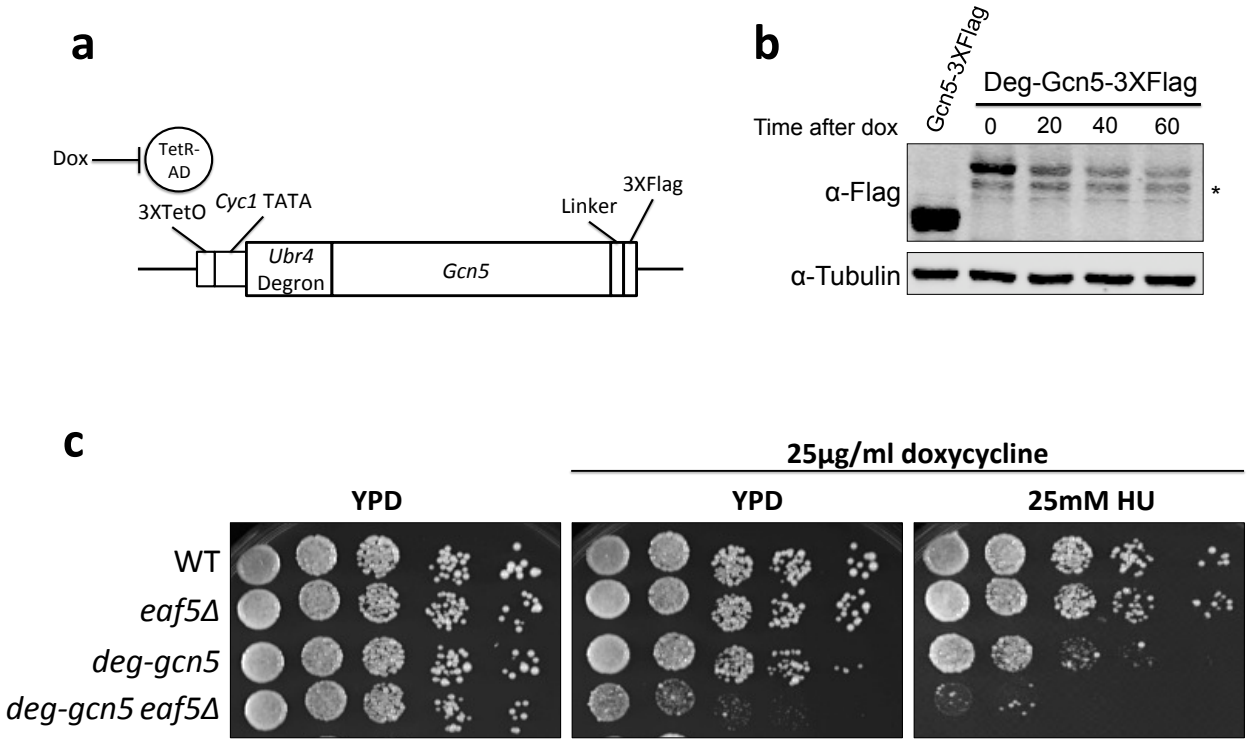

Figure 3: The *degron*-gcn5 allele is sensitive to hydroxyurea and works in parallel with NuA4. (a) Schematic of *degron-gcn5* construct. The activation domain of the tet repressor is constitutively expressed, binding to the three copies of the tet operator and triggering expression of the degron-gcn5 fusion protein. Doxycycline inhibits binding of the TetR to TetO. (b) Western blot showing Gcn5 depletion after addition of doxycycline. Gcn5-3XFlag cells were arrested in G1 and a sample was taken for protein extraction. Deg-gcn5-3XFlag cells were arrested in G1, doxycyline was added and protein samples were taken at the indicated times. Tubulin is shown as a loading control. Asterisk indicates a non-specific band. (c) Five-fold serial dilution assays showing growth and hydroxyurea (HU) sensitivity of the indicated strains.

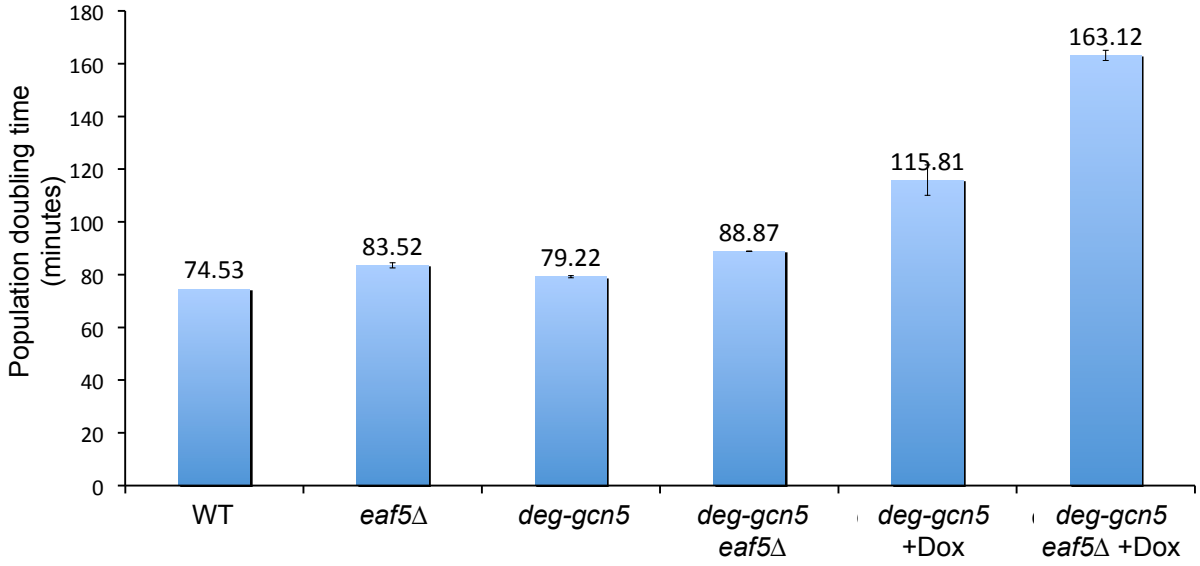

Figure 4: Abrogating KAT activity increases population doubling time. Doubling times of indicated strains at 30˚C. Cell density was counted at two time points in mid-log phase using a hemocytometer. Doubling time was calculated using the formula $\ln(2)/(\ln(A/A_o)/t)$, where A_o is cell density at an early time point and A is cell density at a later time point, and t is the amount of time that passed between the two. Error bars represent standard error.

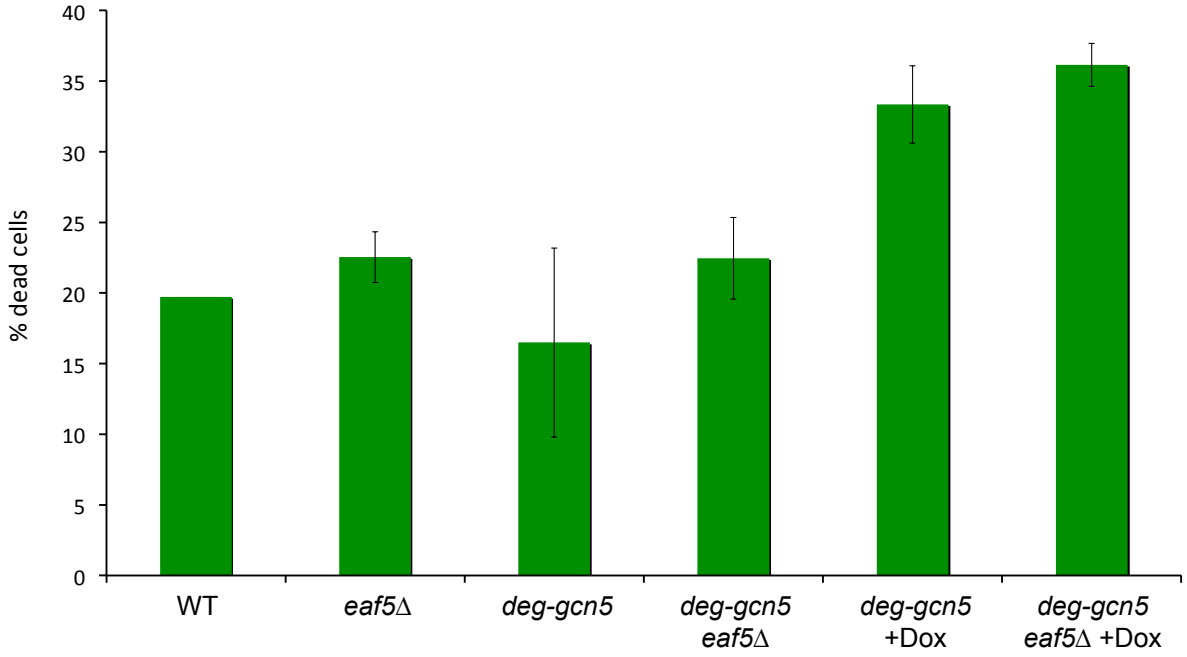

Figure 5: Abrogating KAT activity increases cell death. Comparison of viability in wild-type and KAT mutant cells with and without doxycycline. Cell death was determined by using the FungaLight Yeast Viability Kit to determine the percentage of dead cells in an asynchronous population. Error bars represent standard deviation.

Table 3: KAT mutant growth defects are not due to cell death alone.

Strain	% Dead cells in log phase	Modeled increase in doubling time versus wild-type due to cell death	Observed increase in doubling time versus wild-type
Wild-type	19.7	-	-
eaf5Δ	22.5 +/- 1.8	3.9%	12.1%
deg-gcn5	16.5 +/- 6.7	-2.47%	6.3%
deg-gcn5 eaf5Δ	22.5 +/- 2.9	3.9%	19.2%
deg-gcn5 + dox	33.3 +/- 2.7	16.2%	55.4%
deg-gcn5 eaf5Δ + dox	36.1 +/- 1.5	19.7%	118.9%

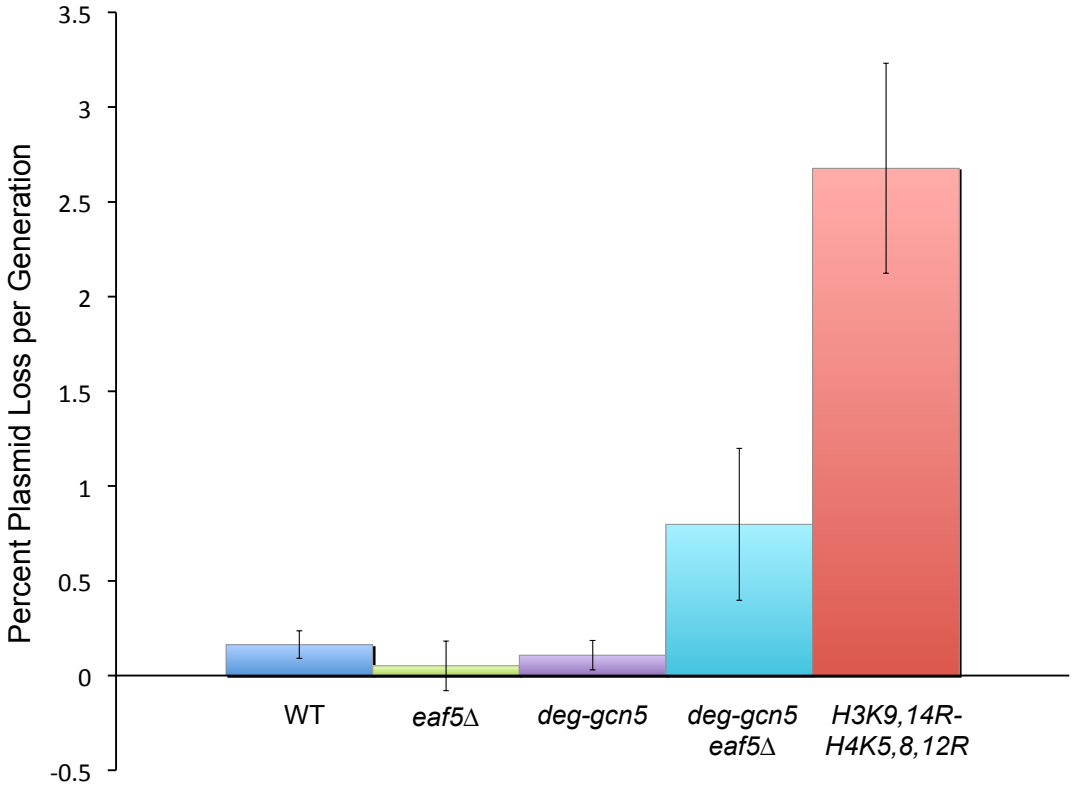

Figure 6: Plasmid instability increases when H3 and H4 HAT mutants are combined. A plasmid containing the ADE metabolic marker and the early efficient ARS1 was transformed into each strain. Loss rates were measured by the presence of pink versus white colonies in the presence of doxycycline as described in methods. Bars represent standard error.

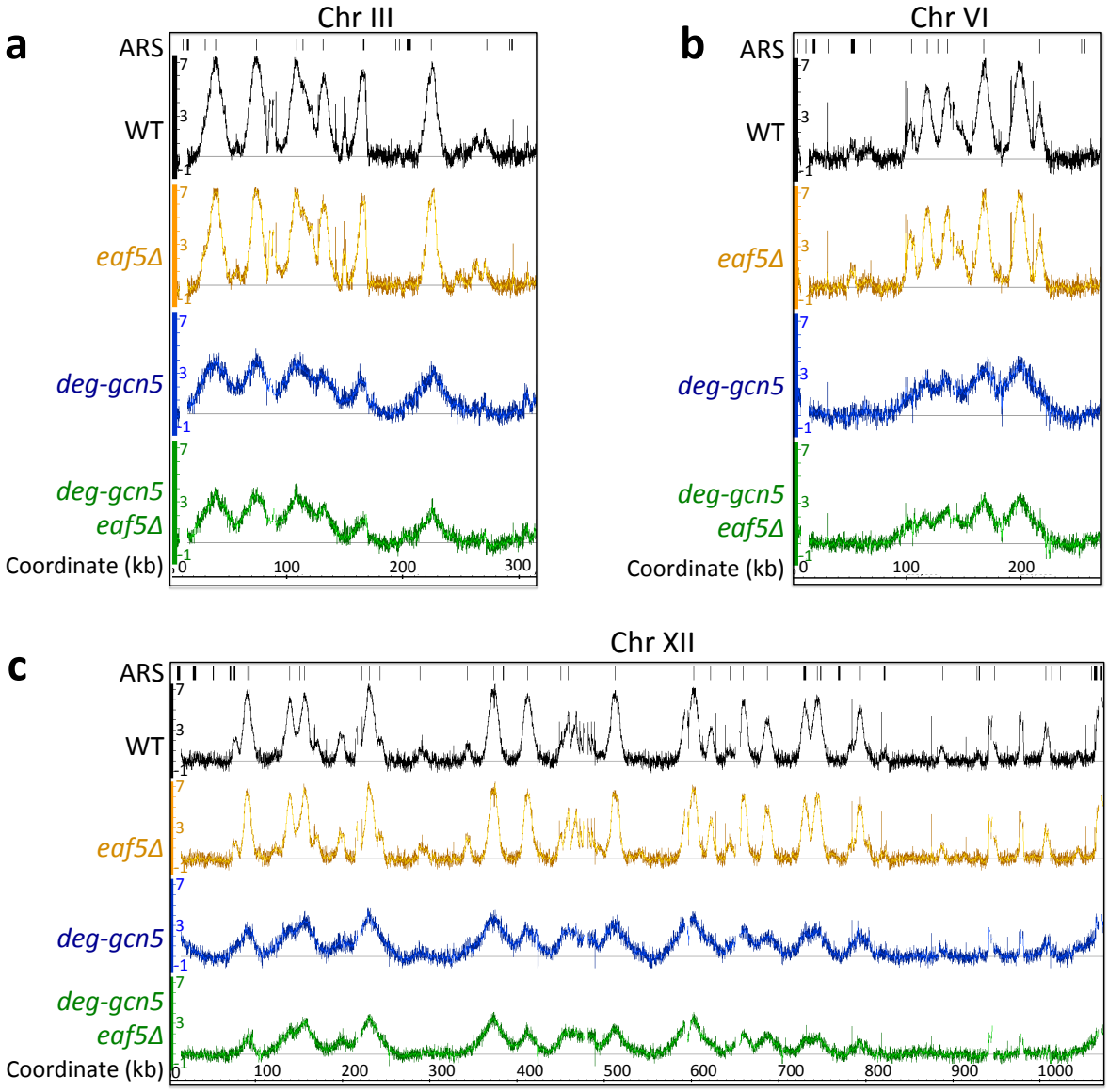

Figure 7: Gcn5 KAT is required for robust replication origin firing. BrdU IP-ChIP replication profile analysis of chromosomes (a) III (b) VI and (c) XII. Wild-type *eaf5Δ, deg-gcn5,* and *deg-gcn5 eaf5Δ* cells were grown to early log phase in the presence of doxycycline, arrested in G,1 and released into S phase in doxycycline, BrdU, and 200mM HU for 60 min. The y axes show BrdU signal ratio in log2 scale using an unreplicated sample (G1) as a reference, and the x axes show chromosomal coordinates in kilobases. Positions of ARS sequences are labeled with a black bar.

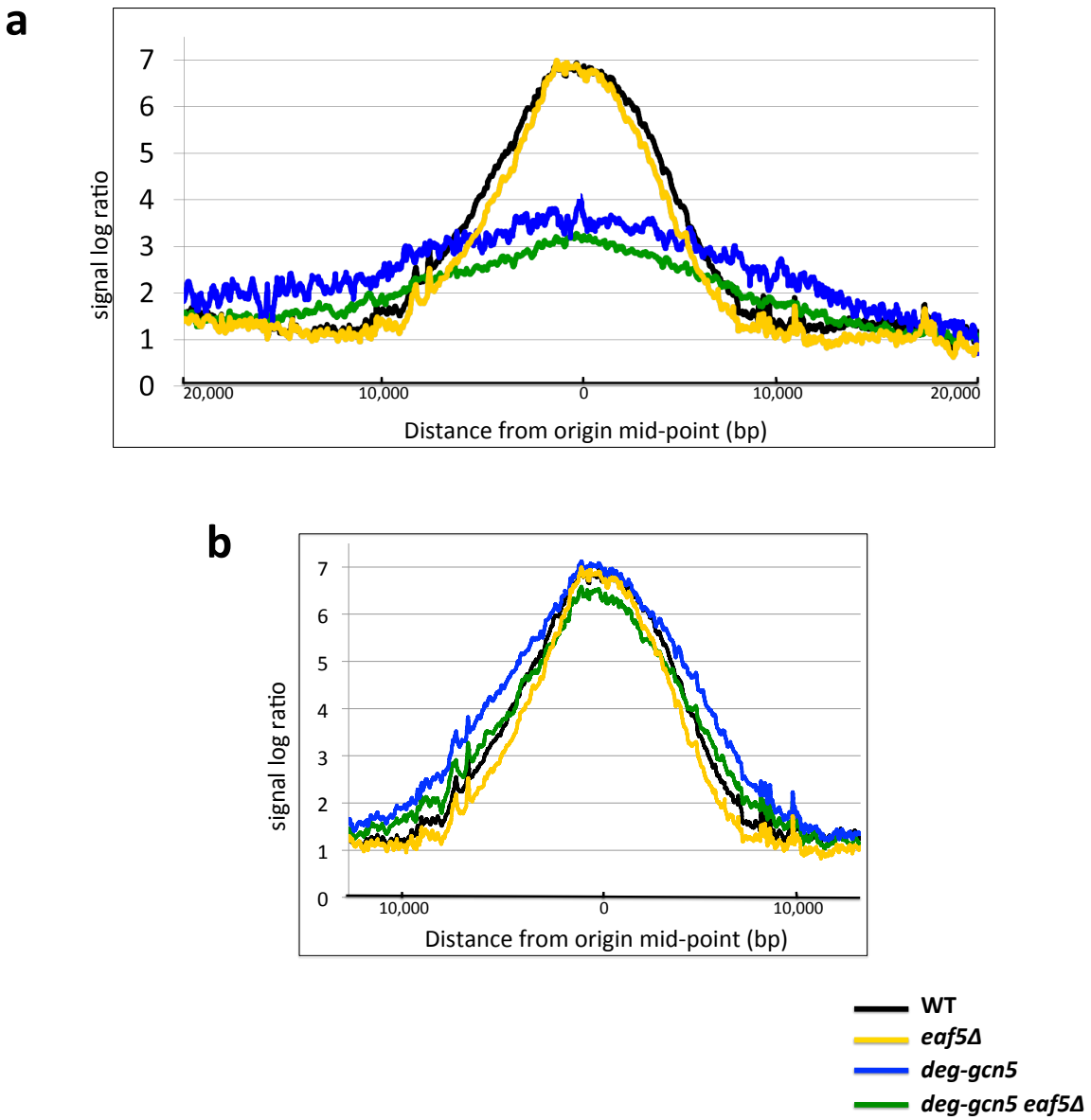

Figure 8: Gcn5 and NuA4 KATs cooperate to promote replication origin firing. BrdU signals averaged from eight early efficient origns. Wild-type, *eaf5Δ, deg-gcn5,* and *deg-gcn5 eaf5Δ* cells were grown to early log phase, arrested in G1, and released into S phase in doxycycline, BrdU, and 200mM HU for 1 hour min. (a) cells were treated with doxycycline overnight and (b) cells were treated with doxycycline fr 30 minutes before release into BrdU. Wild-type signal is black, *eaf5Δ* is yellow, *deg-gcn5* is blue, and *deg-gcn5 eaf5Δ* is shown in green. Data sets were aligned at the mid point of each origin (0 on the x-axis). The y axes show average BrdU signal ratio in log2 scale using an unreplicated sample (G1) as a reference, and the x axes show distance from origin midpoint in base pairs.

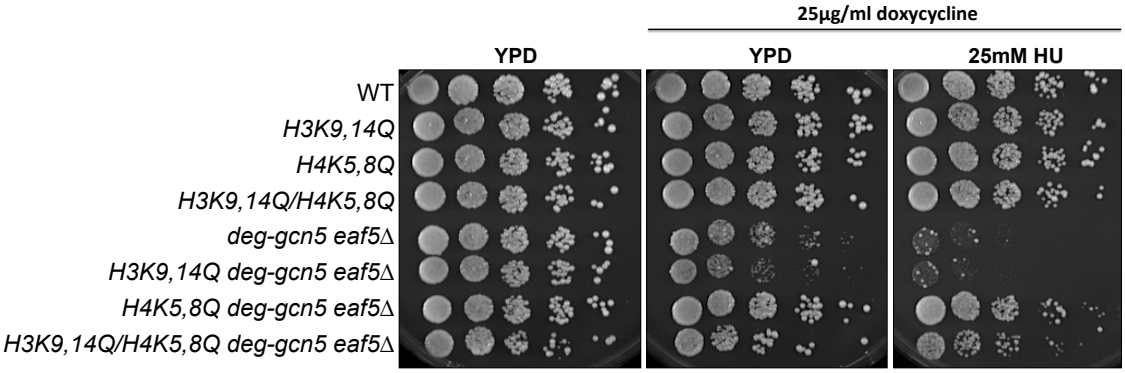

Figure 9: KAT mutant growth defects are rescued by histone lysine acetylation mimics. Fivefold serial dilutions of indicated strains. Cells were spotted onto indicated media and grown for 2 days at 30˚C either with or without the addition of doxycycline and the replication inhibitor hydroxyurea (HU).

Table 4: HDAC mutant interactions with *deg-gcn5 eaf5*Δ in 5mM HU.

HDAC Deletion	Interaction with deg-*gcn5 eaf5Δ* on 5mM HU
hos1Δ	--
hos2Δ	+
hos3Δ	none
hos4Δ	+
hst1Δ	+
hst2Δ	none
hst3Δ	--
hst4Δ	none
hda1Δ	+
sir2Δ	none
rpd3Δ	not tested
hos2Δ hos4Δ	+
hos2Δ hst1Δ	+
hda1Δ rpd3Δ	--

\+ growth defect of *deg-gcn5 eaf5*Δ is partially rescued
\- growth defect is worse when mutations are combined

Note: Experiments for this table were performed by Tanya Cunningham

Replication Dynamics of an Origin-Deficient Chromosome

Introduction

Eukaryotic DNA replication initiates at multiple sites on each chromosome called origins. In the budding yeast *Saccharomyces cerevisiae*, origins are specified by defined sequences, where an ordered assembly of protein complexes binds to activate replication. Each origin fires at a distinct time during S-phase, and have different usage efficiencies. It is generally believed that these properties, along with proper origin spacing, are essential for chromosome stability, as late-firing, inefficient origins can act as backups to early-firing, efficient origins if replication forks collapse in early S-phase (Barberis et al., 2010; Blow and Ge, 2009).

If replication from efficient origins is inhibited, dormant origins may fire. Dormant origins have the ability to act as functional replication origins, yet do not fire in their chromosomal context. Dormant origins can fire with replication fork arrest of nearby origins and inactivation of the Mec1/Rad53 checkpoint (Santocanale et al., 1999).

Dormant origins are also activated when efficient origins are deleted on an episomal fragment of the *S. cerevisiae* chromosome III (Dershowitz et al., 2007). Interestingly, a chromosome fragment with no functional ARS sequences, including all known dormant origins, had an elevated loss rate compared to a chromosome with functional origins, but was still stably maintained (Dershowitz et al., 2007). The replication checkpoint protein Rad9 and components of the DNA damage

pathway have been shown to be important in propagating this and other origin deficient episomes (Theis et al., 2007; Theis et al., 2010; van Brabant et al., 2001)

In this study, we aimed to test the premise that multiple origins are required for efficient replication of an endogenous chromosome by systematically deleting ARS sequences to create a chromosome that is essentially devoid of replication origins for approximately 200 kilobases. We found that similar to other studies, the strain was surprisingly healthy. Upon further examination, we detected *de novo* origin firing from deleted ARS sites, suggesting that origins in *S. cerevisiae* may possess unexpected plasticity.

Methods

Strains, plasmids, and genetic methods

Strains used in this study were created using standard yeast manipulations and are listed in Table 5. Cells were grown in standard YPD medium or synthetic selective medium as appropriate.

The *7oriΔ* strain was constructed by deleting confirmed ARS sequences identified by the Origin Database (OriDb) (Siow et al., 2011) and on the Saccharomyces Genome Database (www.yeastgenome.org).

BrdU IP

DNA was harvested from cells harboring a single integrated BrdU incorporation vector (Viggiani and Aparicio, 2006) and replicated DNA was immunoprecipitated as described (Viggiani et al., 2010) with the following modifications: Lysis buffer contained 100mM Tris-HCl pH8.0 with 20% glycerol, cell lysate was sonicated using a Bioruptor sonicator bath (Diagenode), and BrdU immunoprecipitation was performed using Dynabeads Protein G (Novex). Input (G1) and immunoprecipitated DNA was amplified using whole genome amplification (GenomePlex, Sigma-Aldrich), and then UDG fragmented and terminally labeled as previously described (Yadon et al., 2010). Samples were competitively hybridized to custom tiling arrays (Roche NimbleGen) as previously described (Yadon et al., 2010). Arrays contain 50-mer probes overlapping by an average of 42 bp, covering both strands of chromosomes III, VI, and XII and representing 14% of the yeast genome. Array usage and data manipulation were performed as described (Rodriguez and Tsukiyama, 2013). The sequences of microarray probes are available upon request.

2D gel electrophoresis

DNA was prepared from synchronously growing cells (30˚C) of the appropriate strains using the CTAB procedure (Liberi et al., 2006). 5 µg DNA was digested with BspHI and ClaI (*ARS607*), SphI (*ARS606*), or SphI and AvaI (*ARS606* adjacent) (NEB). 2D gels were performed as described (Friedman and Brewer, 1995). First dimension gels were 0.4% agarose run at 30V for 23 hours, and second

dimension gels were 1.0% agarose run at 160V for 7 hours. DNA was transferred onto nylon GeneScreen Plus membranes (PerkinElmer) and hybridized with ^{32}P-labeled PCR probes corresponding to each ARS. Signals were detected using a Storage Phosphor Screen (Molecular Dynamics) and Typhoon Trio imager (GE Healthcare).

Results

A chromosome VI origin deficient strain is surprisingly healthy

It has been hypothesized that the presence of multiple origins with different properties is essential for cell survival during replication stress and DNA damage. To test this premise, we created the *7oriΔ* strain, a yeast mutant in which the seven known active origins on the left arm of chromosome VI were deleted to create a 200 kb region presumed to be devoid of active origins (Figure 10a). Origins were deleted in the following order: *ars600::LEU* and *ars606::ADE* (crossed together to make a double deletion mutant), *ars601/2::loxP*, *ars605::loxP*, *ars603::loxP*, *ars604::loxP*, and finally *ars603.5::KANMX*. Origins were deleted by homologous recombination and replacement with the indicated marker, and ARSs replaced by loxP were first deleted with the KanMX marker, which contains two loxP homologous recombination sites on either end, then cells were treated with the Cre/Lox enzyme to induce marker loop out. Two ARS sequences, *ARS602.5* and *ARS603.1*, have only recently have been identified and confirmed, and therefore remained intact.

ARS604 overlaps with the non-essential proteasome activator *BLM10*, and *ARS605* overlaps with *MSH4*, which is involved in meiotic recombination. As we performed our experiments in haploid cells, *MSH4* disruption was not a problem, and although Blm10 has been reported to contribute to resistance to DNA damaging reagents in other strain backgrounds (Febres et al., 2001), we never detected any growth defects when *ARS604* was deleted (Figure 10b).

We hypothesized that the *7oriΔ* strain would encounter difficulty fully replicating chromosome VI, and as a result, would have slow growth or cell death, especially when grown in the presence of replication inhibitors or DNA damaging agents. Surprisingly, the *7oriΔ* chromosome was perfectly healthy, even when exposed to relatively high levels of replication inhibiting drugs (Figure 10b).

Orc2 is not enriched at deleted origins

To confirm that origin deletion did eliminate functional origins, we performed Orc2 ChIP-ChIP. Orc2 is one of six subunits in the essential ORC complex, which is the first protein complex to bind to origins in the replication initiation process. As shown in Figure 11, subtraction of *7oriΔ* Orc2 signal from wild-type signal clearly demonstrated that Orc2 levels were diminished specifically at the deleted origins in the *7oriΔ* strain.

Because portions of sequence had been deleted from the chromosome in the *7oriΔ* strain, it was possible that Orc2 was bound to the markers replacing them but because the marker sequences do not hybridize to our tiling arrays, gave the

appearance of depletion. However, when we zoomed in on Orc2 levels at these loci, Orc2 depletion extended beyond the deleted region (Figure 12). Additionally, depletion was seen at *ars605Δ* in which only a 34 base pair loxP site remains. If Orc2 remained bound at these deleted origins, the signal should have been detected using our arrays. Because we did not find such localization, the simplest interpretation is that no residual Orc2 was bound to these loci.

De novo origins fire after ARS sequences are deleted

To determine how replication of the *7oriΔ* chromosome VI is accomplished without ORC localization at origins, we performed a replication profiling analysis using BrdU (Viggiani et al., 2010) (Figure 13). This revealed significant contribution of *ARS602.5* and *ARS603.1*, which have been recently identified as functional origins (Knott et al., 2009; Siow et al., 2011). In addition, unexpectedly robust BrdU signals were detected around most of the origins that were deleted, except for *ARS603.5*. This result suggests that the low levels of residual ORC bound around deleted origins are sufficient to support initiation of DNA replication and shows surprising flexibility in the yeast replication system.

To determine if *de novo* origins (*neo*-origins) arose immediately after ARS deletion or was gained gradually during *7oriΔ* construction, we performed site-specific PCR of BrdU incorporated DNA on the intermediate strains generated during the construction of *7oriΔ*. *ARS606* was the first ARS deleted, and we detected strong BrdU enrichment at the remaining *ARS606* locus in the single *ars606::ADE*

strain (Figure 14). Similarly, *ARS605* showed strong BrdU signal after it was replaced with a loxP site in the *4oriΔ* intermediate strain (Figure 14).

To determine the mechanisms that contribute to *neo*-origin firing, we performed 2D gel analysis of the region adjacent to *ARS606*. This origin was deleted by replacement with the ADE marker, and we tested for replication bubble structures in both wild-type and *7oriΔ* strains (Figure 15). We tested two fragments, one that included the ARS sequence and one that excluded it. In wild-type cells, because the origin is located toward the end of the restriction fragment, the replication bubbles "pop" and form Y-structures before cells are harvested (see schematics in Figure 15). The fragment that does not include *ARS606* also shows only Y-structures, as it is replicated passively. Interestingly, we saw signal corresponding to replication bubbles only in the *7oriΔ* strain with the fragment that includes the ADE marker. Because bubble structures are only detectable if the origin is central to the replication fragment, this suggests that the *ars606::ADE neo*-origin is originating either from the left end of the ADE marker or the endogenous sequence close to it. Indeed, the pRS402 plasmid, which was used as a template for replacing *ARS606,* contains a fragment of *ARS1516*, which is likely contributing to *neo-origin* firing at this locus.

Neo-origin firing is dependent on replacement marker

Not many endogenous origins have been tested for activity after deletion. We wanted to test if some origins have the innate capacity to make *neo*-origins upon

deletion, or if it is situation-dependent. We constructed another *ars606Δ* allele by deleting *ARS606* with the *KANMX* marker. After confirming knockout by PCR (data not shown), we tested this strain for BrdU enrichment. The results clearly showed that unlike the *ars606::ADE* allele, a *neo*-origin does not arise when *ARS606* is deleted with the KAN marker (Figure 16). This result suggests that the *ARS1516* fragment present in the pRS402 plasmid is able to confer replication origin activity when used to replace *ARS606.*

7oriΔ genetically interacts with origin firing and lysine acetyltransferase mutants

Strains with origin-depleted chromosomes have shown synthetic sickness with replication checkpoint and DNA damage pathway genes (Theis et al., 2007; Theis et al., 2010; van Brabant et al., 2001). We tested the *7oriΔ* strain for interaction with genes known to contribute to these and other pathways (Table 6). Interestingly, we found no genetic interaction with DNA damage pathway genes, including *RAD9*.

We hypothesized that replication initiation of the *7oriΔ* chromosome requires low levels of ORC complex bound to non-canonical, low affinity sites around deleted origins, and would therefore be sensitive to further decreases in levels of ORC protein. To test this possibility, we introduced the *orc2-1* temperature-sensitive mutation into the *7oriΔ* strain. It has been shown that the level of Orc2 protein in the orc2-1 mutant gradually decreases as growth

temperature increases (Shimada et al., 2002). Consistent with our model, *7oriΔ* and *orc2-1* mutations exhibit synthetic growth defects (Figure 17). If H3 and H4 KATs facilitate origin firing, as evidence in the first section of this thesis shows, we expect KAT mutations to exhibit synthetic growth defects with *7oriΔ* as well. Indeed, both the *gcn5Δ eaf5Δ* double deletion mutant and the *esa1-1851* mutant exhibit strong synthetic growth defects with *7oriΔ* mutations (Figure 17).

Discussion

We developed the *7oriΔ* chromosome to test the consequence of deleting origins on an endogenous chromosome. Other studies have tested origin depleted episomal chromosomes (Dershowitz and Newlon, 1993; Dershowitz et al., 2007; van Brabant et al., 2001), but there is evidence that aneuploid cells are unstable (Sheltzer et al., 2011), and we were interested in understanding origin deletion in its chromosomal context. We found that *7oriΔ* cells were healthy even when grown in the presence of replication inhibitors, yet interact with mutations that affect origin firing, suggesting that deleting origins can be detrimental to the cell in certain circumstances.

Although many studies have contributed to origin mapping, not all functional ARSs have yet been identified. ORC and Mcm2-7 binding is not enriched at all functional origins, and the ARS consensus sequence (ACS) is extremely degenerate. Since the time we constructed the *7oriΔ* strain, two ARS sequences have been

identified and confirmed. We are now in the process of attempting to create a *9oriΔ* strain, but we cannot ignore the possibility that other ARSs will be identified yet again. It is possible that in the absence of canonical origins, other ACS sites will almost always become functional.

The most surprising finding was discovering *de novo* origin firing from deleted ARS sequences. This almost certainly contributed to the robust growth of the *7oriΔ* strain, and to our knowledge has never been reported before. Interestingly, the *neo*-origin at *ARS606* was dependent on the ADE marker, and no origin firing was detected when replaced with the KAN marker, suggesting that there were elements in the ADE plasmid that can act as ARSs. However, the *neo*-origin at *ARS605* has a 34 base pair loxP sequence as a replacement, and is unlikely to act as an ARS, suggesting that there is also an innate ability of the endogenous sequence surrounding *ARS605* that can support *neo*-origin firing.

Because the *7oriΔ* chromosome VI relies on inefficient *neo*-origins for DNA replication, *7oriΔ* cells become sensitive to mutations that decrease origin activities. Indeed, the *orc2-1, gcn5Δ eaf5Δ,* and *esa1-1851* mutations all show synthetic sickness with *7oriΔ*. Therefore, the *7oriΔ* strain will be a useful tool to characterize mutations that affect origin activities, and to investigate how inefficient origins are activated.

Our results suggest that the sequence surrounding ARSs possesses a plasticity never reported before. Interestingly, replication origins in higher eukaryotes initiate from loosely defined initiation regions that could be described as

similar in plasticity to the *neo*-origins in *7oriΔ*. A tempting hypothesis is that the *7oriΔ* strain sensitizes *S. cerevisiae* to defects in factors that are important for mammalian replication. In the future, we hope to use this and other origin deficient chromosome strains to discover the pathways that are required for cell viability in these circumstances.

Table 5: Yeast strains used to analyze an origin deficient chromosome

Strain	Genotype	Reference
W1588-4C	*MATa or MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5+*	Zhao et al. 1998
YTT4024	*MATa ars600Δ::LEU2 ars601/2Δ::loxP ars603Δ::loxP ars603.5Δ::KAN ars604Δ::loxP ars605Δ::loxP ars606Δ::ADE2*	This study
YTT4977	*MATa mec1Δ::KAN sml1Δ::NAT*	This study
YTT5158	*MATa Orc2-2XLinker-3XFlag-klTRP1*	This study
YTT5160	*MATa Orc2-2XLinker-3XFlag-klTRP1 ars600Δ::LEU2 ars601/2Δ::loxP ars603Δ::loxP ars603.5Δ::KAN ars604Δ::loxP ars605Δ::loxP ars606Δ::ADE2*	This study
JRY4125	*MATα orc2-1*	Suter et al. 2004
YTT5401	*MATa orc2-1 ars600Δ::LEU ars600Δ::LEU2 ars601/2Δ::loxP ars603Δ::loxP ars603.5Δ::KAN ars604Δ::loxP ars605Δ::loxP ars606Δ::ADE2*	This study
MSY2431	*MATa esa1-1851::klURA3*	Bird et al. 2002
YTT4934	*MATa esa1-1851::klURA3 ars600Δ::LEU2 ars601/2Δ::loxP ars603Δ::loxP ars603.5Δ::KAN ars604Δ::loxP ars605Δ::loxP ars606Δ::ADE2*	This study
MSY2432	*MATa esa1-L357H::klURA3*	Bird et al. 2002
YTT4937	*MATa esa1-L357H ars600Δ::LEU2 ars601/2Δ::loxP ars603Δ::loxP ars603.5Δ::KAN ars604Δ::loxP ars605Δ::loxP ars606Δ::ADE2*	This study
YTT4032	*MATa gcn5Δ::KAN*	This study
YTT4783	*MATa gcn5Δ::HYG ars600Δ::LEU2 ars601/2Δ::loxP ars603Δ::loxP ars603.5Δ::KAN ars604Δ::loxP ars605Δ::loxP ars606Δ::ADE2*	This study

Continued on next page

Table 5: Yeast strains used to analyze an origin deficient chromosome (cont.)

Strain	Genotype	Reference
YTT3174	*MATa eaf5Δ::HYG*	This study
YTT4950	*MATa eaf5Δ::NAT ars600Δ::LEU2 ars601/2Δ::loxP ars603Δ::loxP ars603.5Δ::KAN ars604Δ::loxP ars605Δ::loxP ars606Δ::ADE2*	This study
YTT4953	*MATa gcn5Δ::HYG eaf5Δ::NAT*	This study
YTT4955	*MATa gcn5Δ::HYG eaf5Δ::NAT ars600Δ::LEU2 ars601/2Δ::loxP ars603Δ::loxP ars603.5Δ::KAN ars604Δ::loxP ars605Δ::loxP ars606Δ::ADE2*	This study
YTT5260[a]	*MATa URA3::pRS306-BrdUinc ADE2+ LEU2+*	This study
YTT5262[a]	*MATa URA3::pRS306-BrdUinc ars600Δ::LEU2 ars601/2Δ::loxP ars603Δ::loxP ars603.5Δ::KAN ars604Δ::loxP ars605Δ::loxP ars606Δ::ADE2*	This study
YTT5387[a]	*MATa URA3::pRS306-BrdUinc ars606Δ::ADE2*	This study
YTT5388[a]	*MATa URA3::pRS306-BrdUinc ars600Δ::LEU2 ars601/2Δ::loxP ars605Δ::loxP ars606Δ::ADE2*	This study
YTT5396[a]	*MATa URA3::pRS306-BrdUinc ars606Δ::KAN*	This study

All strains are isogenic to W1588-4C, a RAD5+ strain of W303. Only alleles that differ from W303 are listed.

a BrdU incorporation plasmid provided by O. Aparicio (Viggiani et al. 2006).

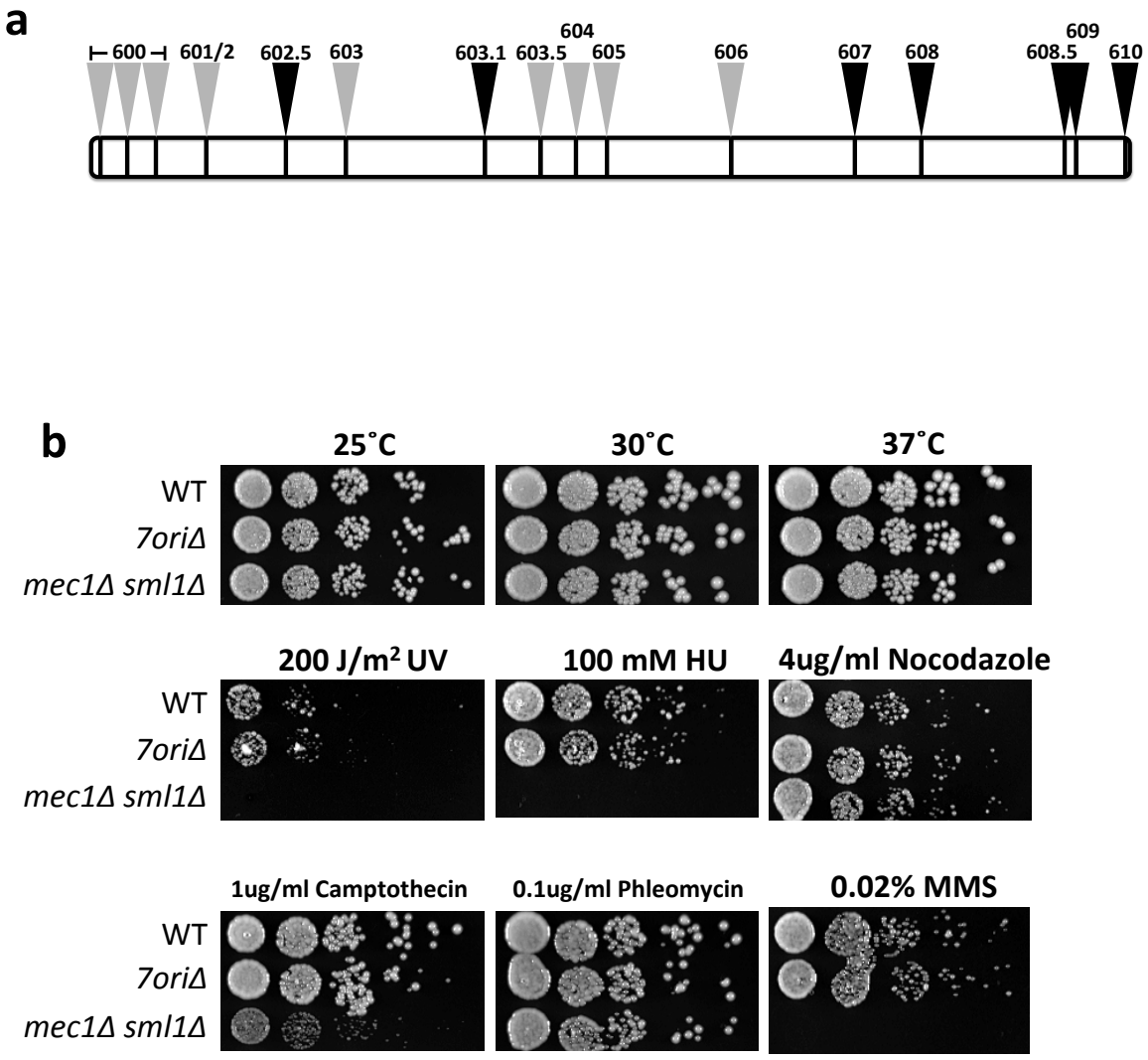

Figure 10: Chromosome VI *7oriΔ* ARS deletion strain is not sensitive to replication inhibitors. (a) Schematic of ARS locations on chromosome VI. ARS sequence positions are represented by a black bar and labeled with an arrow. Black arrows represent ARSs that remain unperturbed in *7oriΔ* cells, and grey arrows are ARSs deleted in *7oriΔ*. (b) Five-fold serial dilution assay of wild-type and *7oriΔ* on indicated media. The *mec1Δ sml1Δ* S phase checkpoint mutant is shown as a replication inhibitor positive control.

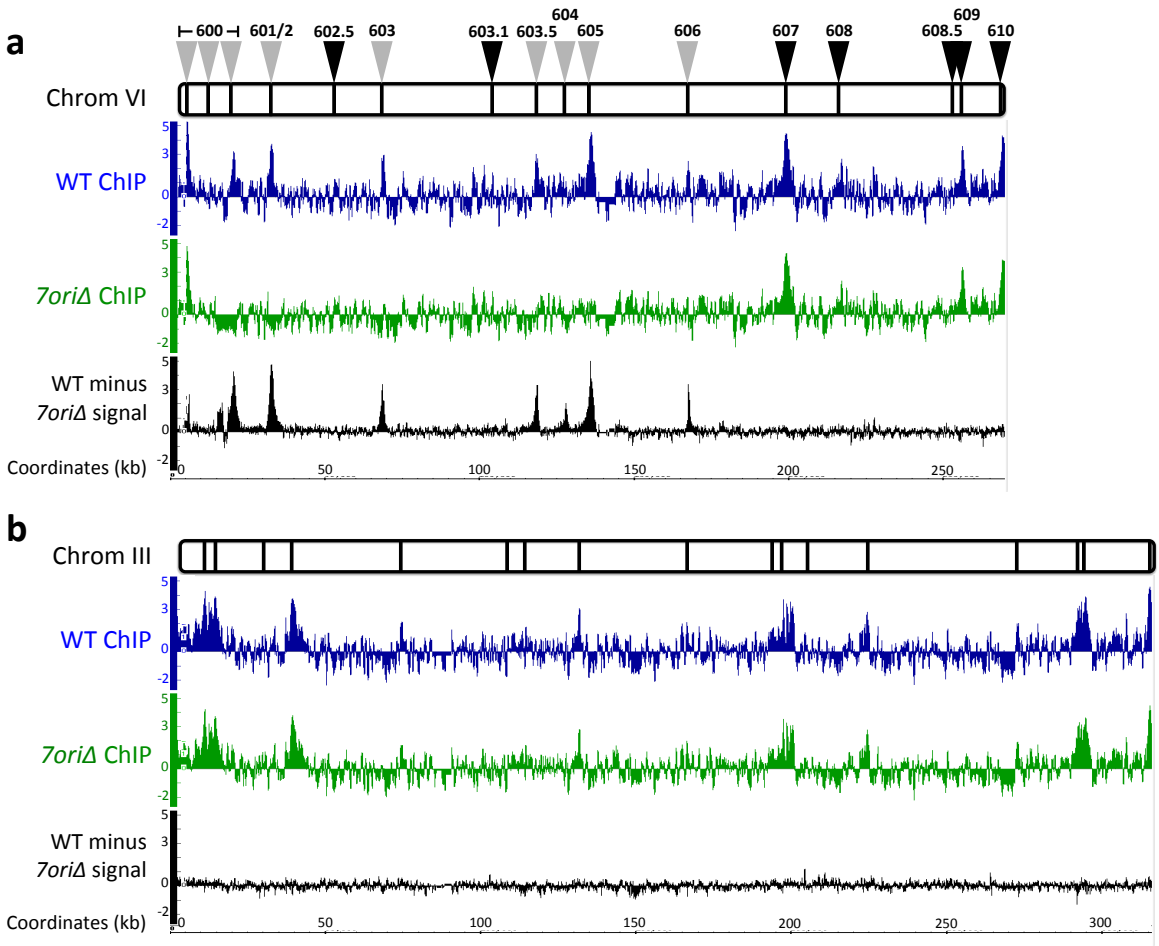

Figure 11: Deleted origins do not recruit Orc2. Orc2-3XFlag chromatin ChIP-Chip of (a) chromosome VI and (b) chromosome III in G2/M arrested cells. Wild-type (blue) and *7oriΔ* (green) Orc2 ChIP signals are shown along with the difference between the wild-type and *7oriΔ* signal (black) to show level of Orc2 depletion in *7oriΔ*. ARS positions are shown as black bars above each panel. Deleted ARSs on the *7oriΔ* chromosome VI are labeled with grey arrows and ARSs that remained intact are labeled with black arrows. The y axes show Orc2 signal ratio in log2 scale, and the x axes show chromosomal coordinates.

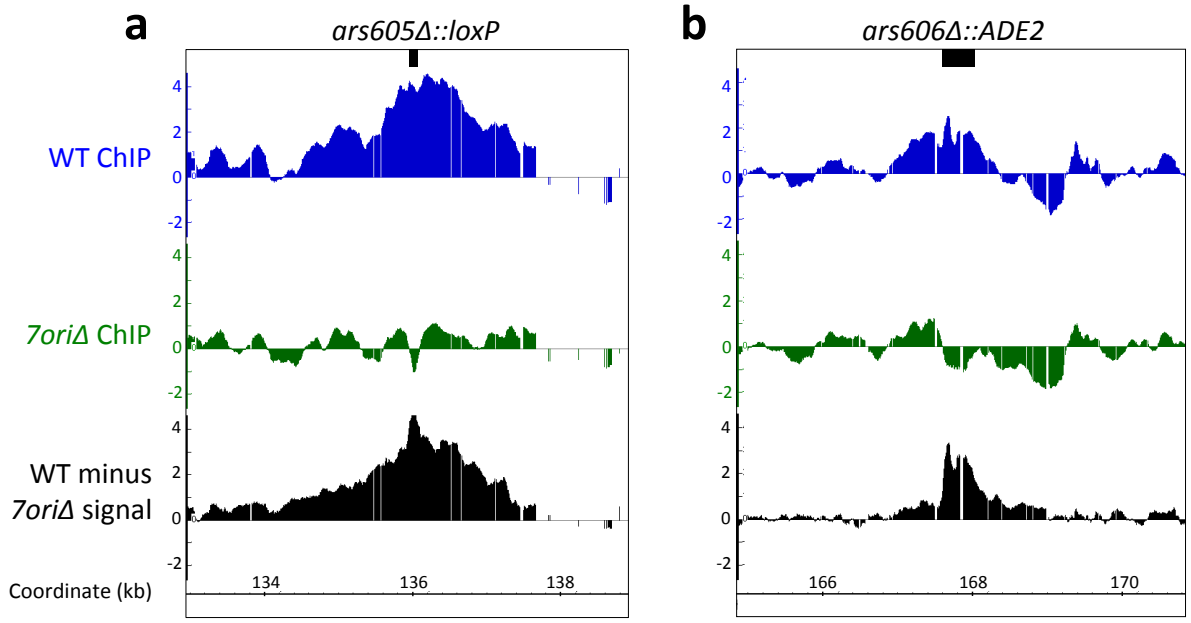

Figure 12: Orc depletion is not due to sequence deletion alone. Zoomed in view of Orc2-3XFlag ChIP-Chip data from Figure 13 at (a) ARS605 and (b) ARS606. The y axes show Orc2 signal ratio in log2 scale, and the x axes show chromosomal coordinates. Positions of ARS sequences are labeled with a black bar and labeled with replacement sequence.

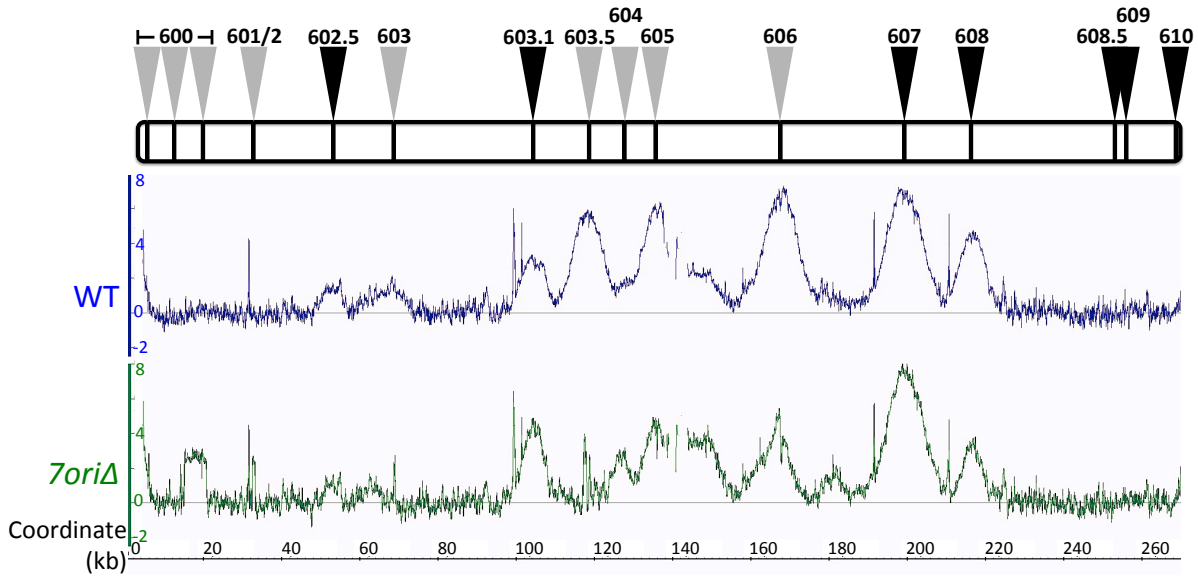

Figure 13: Neo-origns form at deleted ARS sites. BrdU IP-ChIP replication profile analysis of chromosome VI. Wild-type and *7oriΔ* cells were arrested in G1 and released into media containing BrdU and 200mM HU for 60 min. The y axes show BrdU signal ratio in log2 scale using an unreplicated sample (G1) as a reference, and the x axes show chromosomal coordinates in kilobases. Positions of ARS sequences are labeled with a black bar and labeled with the replacement sequence if deleted in *7oriΔ*.

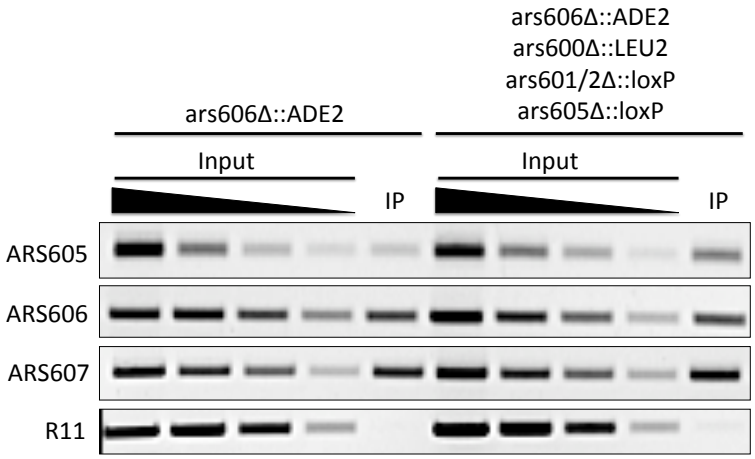

Figure 14: Neo-origins acquire activity immediately after endogenous origin deletion. BrdU ChIP in indicated strains followed by PCR at specified origins. Input dilutions are 1, 1:10, 1:50, 1:250. ARS607 is shown as a positive origin-firing control, and the late replicating region R11 is shown as a negative control.

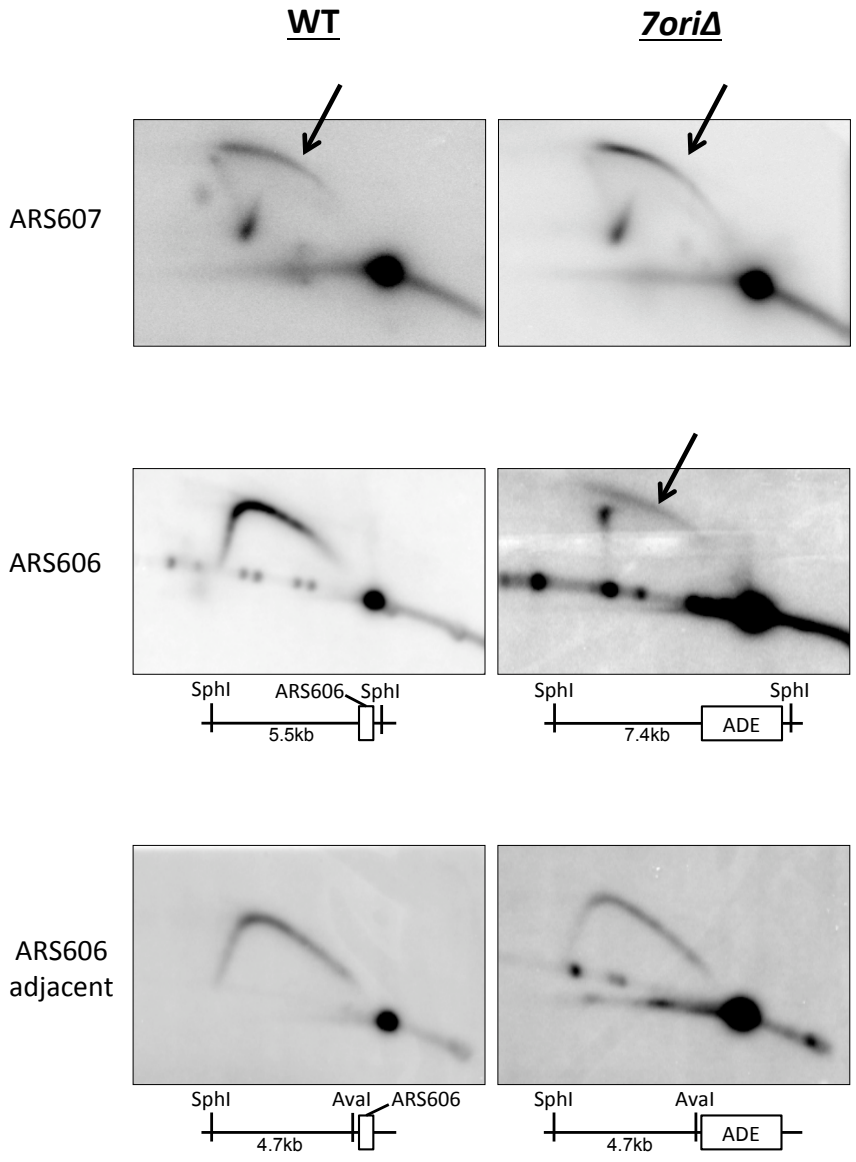

Figure 15: ARS606 *neo*-origin fires from right flank of ADE marker. 2D gel analysis of origin firing at ARS606 in wild-type and *7oriΔ* cells. Schematic of fragments is located under each 2D gel. ARS607 is shown as a positive origin-firing control. Black arrows point to replication bubble signal.

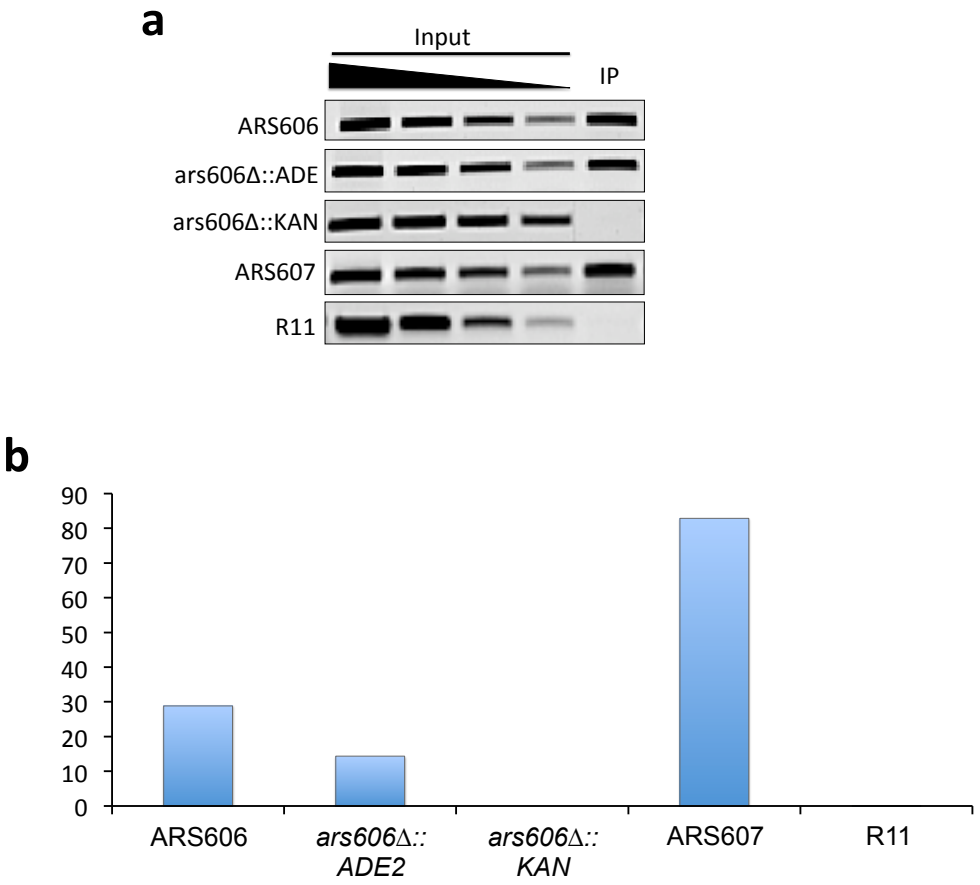

Figure 16: ARS606 *neo*-origin formation is dependent on replacement sequence. **(a)**BrdU ChIP at indicated loci followed by PCR at specified origins. Input dilutions are 1, 1:10, 1:50, 1:250. ARS607 is shown as a positive origin-firing control, and the late replicating region R11 is shown as a negative control. (b) Quantitation of data shown in (a). Y-axis shows IP signal as a percentage of input.

Table 6: Genetic interactions with *7oriΔ*

Gene allele	Interaction with *7oriΔ*
rad9Δ	--
mec1Δ sml1Δ	--
rad53Δ sml1Δ	--
clb5Δ	--
mag1Δ	--
sic1Δ	--
mad3Δ	--
isw1Δ	--
mrc1Δ	--
tof1Δ	--
asf1Δ	--
cac1Δ	--
hat1Δ	--
hap1Δ	--
rrm3Δ	--
trf4Δ	--
hho1Δ	--
htz1Δ	--
rsc1Δ	--
rsc2Δ	--
esa1-L357H	--
esa1-1851	+
yng2Δ	+
eaf1Δ	+
orc2-1	+
yng2Δ	+
gcn5Δ eaf5Δ	+

+ growth defect of *deg-gcn5 eaf5*Δ is partially rescued
- growth defect is worse when mutations are combined

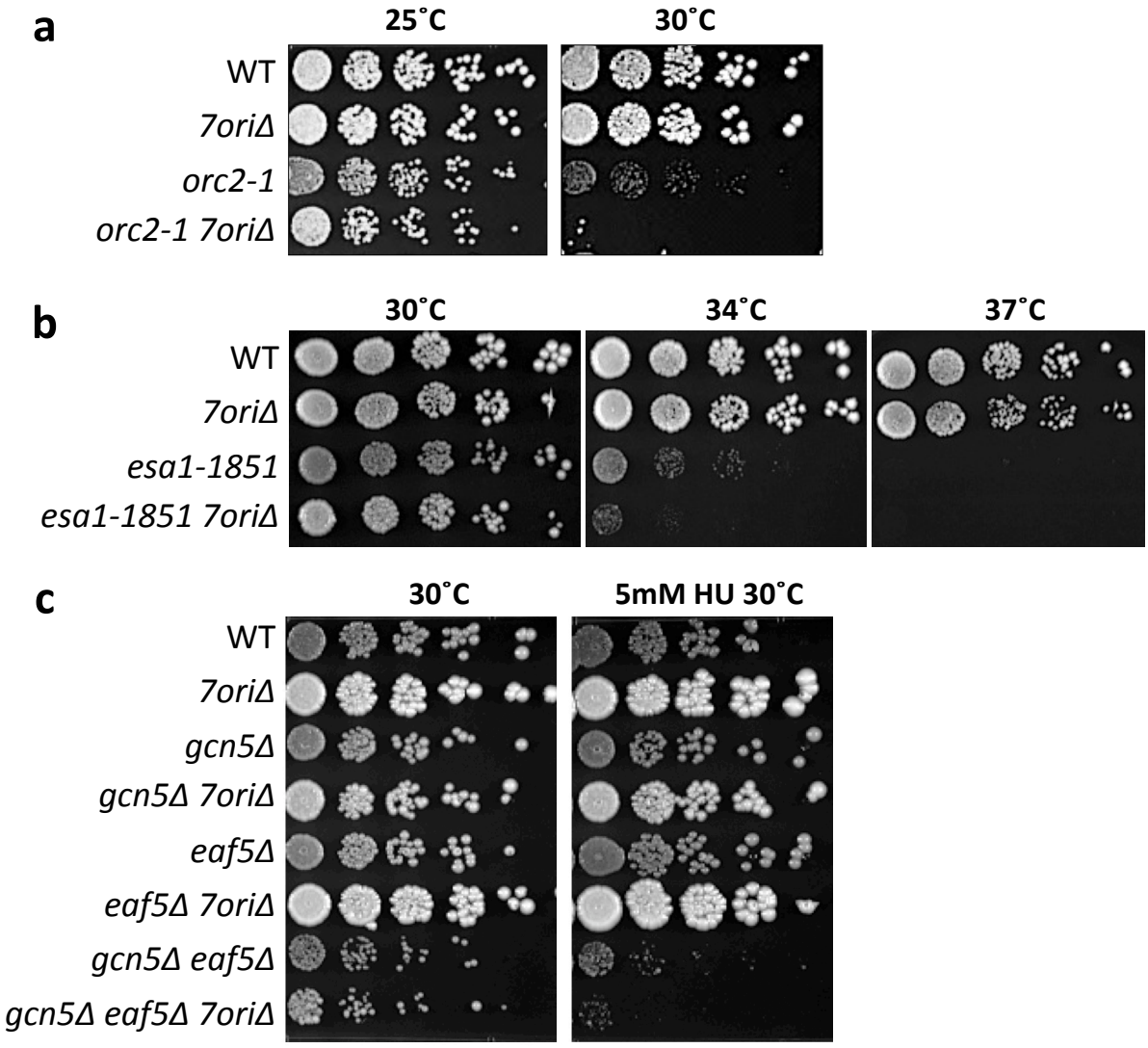

Figure 17: *7oriΔ* interacts with mutations affecting origin firing and histone lysine acetylation. Fivefold serial dilutions of *7oriΔ* combined with the (a) *orc2*-1 temperature-sensitive allele, (b) *esa1*-1851 temperature-sensitive allele, and (c) *gcn5Δ* and ea*f5Δ* deletion mutants. Cells were spotted onto indicated media and grown for 2 days at the indicated temperature either with or without the addition of the replication inhibitor hydroxyurea (HU).

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