Published in final edited form as:
Mol Cell. 2010 February 26; 37(4): 469–480. doi:10.1016/j.molcel.2010.01.020.

A role for Gcn5 in replication-coupled nucleosome assembly

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Summary

Acetylation of lysine residues at the H3 N-terminus is proposed to have a role in replication-coupled (RC) nucleosome assembly, a process critical for the inheritance of epigenetic information and maintenance of genome stability. However, the role of H3 N-terminal lysine acetylation and the corresponding lysine acetyltransferase (KAT) in RC nucleosome assembly are not known. Here we show that Gcn5, a KAT with a well-studied role in gene transcription, functions in parallel with Rtt109, the H3 lysine 56 KAT, to promote RC nucleosome assembly. Cells lacking both Gcn5 and Rtt109 are highly sensitive to DNA damaging agents. Moreover, cells lacking *GCN5* or expressing an H3 mutant with mutations at the H3 N-terminus result in compromised deposition of new H3 onto replicating DNA and a reduction in the binding of H3 with CAF-1, a histone chaperone involved in RC nucleosome assembly. These results demonstrate that Gcn5 regulates RC nucleosome assembly, in part, through promoting the association of H3 with CAF-1 via H3 acetylation.

Introduction

The nucleosome, the fundamental unit of chromatin, is comprised of 147 base pairs of DNA wrapped around a histone octamer of H2A, H2B, H3, and H4. During S phase of the cell cycle, parental nucleosomes are disassembled to facilitate access to DNA for the replication machinery. Replicated DNA must then be immediately reassembled into nucleosomes using parental histones as well as newly-synthesized histones in a process referred to as DNA replication-coupled nucleosome assembly. This process plays an important role in the inheritance of epigenetic states and the maintenance of genome integrity (Groth et al., 2007b; Morrison and Shen, 2009). While it is not well understood how parental histones are reassembled into nucleosomes following DNA replication, assembly of newly-synthesized histones into nucleosomes requires histone chaperones such as chromatin assembly factor 1 (CAF-1) (Stillman, 1986), Asf1 and Rtt106. These three proteins bind histone H3-H4 and function coordinately in nucleosome assembly during S phase of the cell cycle (Groth et al., 2007b; Li et al., 2008).

Newly-synthesized histone H3-H4 is acetylated by lysine acetyltransferases (KAT) before being assembled into nucleosomes (Roth et al., 2001). Histone H4 is acetylated at lysine residues 5 and 12 (K5, K12) by Hat1 (Ai and Parthun, 2004; Kleff et al., 1995), an acetylation pattern that is conserved from yeast to humans (Sobel et al., 1995). Patterns of acetylation on newly-synthesized H3 are not as conserved among species. In HeLa cells, acetylation of newly-synthesized histone H3.1 is barely detectable, while new H3 is diacetylated at K9 and K14 in

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Tetrahymena and K14 and K23 in *Drosophila* (Benson et al., 2006; Sobel et al., 1995). In yeast cells, newly-synthesized H3 is acetylated at lysine 56 (H3K56Ac) (Masumoto et al., 2005). We and others have shown that this modification is important for nucleosome assembly during DNA replication and DNA repair (Chen et al., 2008; Li et al., 2008). A recent study indicates that the function of this modification in nucleosome assembly appears to be conserved in mammalian cells (Das et al., 2009). In yeast cells, H3K56Ac is catalyzed by Rtt109 (Kat11) (Collins et al., 2007; Driscoll et al., 2007; Han et al., 2007a) and is dependent upon the histone chaperone Asf1 (Recht et al., 2006). We have shown that the binding of H3 with Rtt106 is barely detectable in cells lacking H3K56Ac, whereas the association of H3 with CAF-1 is reduced in cells lacking this modification (Li et al., 2008), suggesting that other modifications on H3 may also regulate the binding of H3 with CAF-1.

In addition to H3K56Ac, new H3 is predominantly acetylated at K9, followed by acetylation of K27 (Kuo et al., 1996). However, the yeast KAT that is responsible for acetylation of these lysine residues of newly-synthesized H3 is not well defined. Genetic evidence indicates that the N-terminus of H3, in particular the acetylation of five lysine residues (K9, K14, K18, K23, and K27), is important for nucleosome assembly (Li et al., 2008; Ma et al., 1998; Qin and Parthun, 2002). However, it is not known which KAT acetylates these five lysine residues and regulates nucleosome assembly.

Gcn5 is the catalytic subunit of three KAT complexes including SAGA, SLIK, and ADA. All of these Gcn5-containing complexes regulate transcription. *In vitro*, recombinant Gcn5 acetylates predominantly K14 of free H3 and shows little or no activity against nucleosomal H3 (Kuo et al., 1996). On the other hand, the SAGA and ADA complexes acetylate both free and nucleosomal H3. While ADA preferentially acetylates K14 and K18 of nucleosomal H3, SAGA acetylates K14 and K18 and to a lesser degree, K23 and K9 (Grant et al., 1999). Thus, the activity and specificity of Gcn5 is regulated by its associated proteins.

Cells lacking Gcn5 are sensitive to DNA damaging agents, suggesting that Gcn5, in addition to its role in gene transcription, may have a role in DNA replication and DNA repair (Choy and Kron, 2002; Tamburini and Tyler, 2005). However, how Gcn5 is involved in DNA replication or DNA repair is not well understood. Here we show that *gcn5*Δ *rtt109*Δ double mutant cells are highly sensitive to DNA damaging agents due to the loss of enzymatic activities of both enzymes. Moreover, *GCN5* genetically interacts with genes known to be involved in DNA replication, the DNA damage response, as well as nucleosome assembly. Furthermore, cells lacking *GCN5* or expressing an H3 mutant containing mutations at five lysine residues of the N-terminus of histone H3 exhibit compromised deposition of new H3 onto replicating DNA and reduced binding of H3 to CAF-1. Together, these results indicate that Gcn5 promotes nucleosome assembly, in part, by acetylating lysine residues at the H3 N-terminus which contributes to the binding of H3 with CAF-1.

Results

Gcn5 and Rtt109 function in parallel in cell growth and response to DNA damaging agents

Acetylation of N-terminal lysine residues of H3 is likely to function in parallel with H3K56Ac to resist DNA damage (Li et al., 2008). To find the lysine acetyltransferase (s) (KAT) that functions with H3K56Ac to promote genome stability, we tested the cell growth and DNA damage sensitivity of double mutant cells lacking *ASF1*, the H3K56Ac regulator (Han et al., 2007a), and one of the non-essential KATs in the budding yeast, *S. cerevisiae*. We found that mutations in *GCN5* and *ELP3*, but not mutations in other H3 KATs such as *SAS3*, resulted in severe growth defects and a high degree of DNA damage sensitivity when combined with the *asf1*Δ mutation (Table S1). The phenotypes observed in *gcn5*Δ *asf1*Δ double mutant cells are consistent with recently published reports showing that *gcn5*Δ mutant cells exhibit a synthetic

sick phenotype with the *rtt109*Δ mutant (Fillingham et al., 2008; Lin et al., 2008). We, therefore, tested whether Gcn5 was the KAT that functioned with H3K56Ac to resist DNA damaging agents. First, we asked whether the *gcn5*Δ mutant exhibited synthetic growth defects and sensitivity towards DNA damaging agents when combined with the *rtt109*Δ mutation or an H3 lysine 56 mutant with lysine 56 mutated to arginine (*H3K56R*). The *gcn5*Δ mutant cells were sensitive to the three DNA damaging agents camptothecin (CPT), hydroxyurea (HU), and methyl methane sulfonate (MMS) but to a lesser degree than *rtt109*Δ mutant cells (Figure 1A–B and Supplemental Figure 1). However, *gcn5*Δ *rtt109*Δ cells were more sensitive to DNA damaging agents than *gcn5*Δ or *rtt109*Δ single mutants alone (Figure 1B), and these double mutant cells exhibited an abnormal cell cycle profile with an increased amount of debris, an indication of cell death (Figure 1C). Furthermore, *gcn5*Δ *H3K56R* double mutant cells grew slowly and exhibited a similar pattern of DNA damage sensitivity to that of gcn5Δ *rtt109*Δ cells (Figure 1A and Supplemental Figure 1). Lastly, expression of wild-type Gcn5 or Rtt109 from a plasmid in *gcn5*Δ *rtt109*Δ double mutant cells rescued the synthetic growth defects and DNA damage sensitivity of the double mutant cells whereas expression of the Gcn5 catalytic dead mutant, *gcn5 E173Q* (Marmorstein, 2004), or Rtt109 catalytic dead mutant, *rtt109 D89N* (Han et al., 2007a), had no effect (Figure 1D and E). These results suggest that Gcn5 functions in parallel with Rtt109 in growth and response to DNA damaging agents and that the slow growth and DNA damage sensitivity of *gcn5*Δ *rtt109*Δ cells are due to loss of the enzymatic activity of both Rtt109 and Gcn5.

Both Gcn5 and Rtt109 acetylate histone H3 lysine 27

It is known that both Gcn5 and Rtt109 can acetylate lysine 9 and 23 of histone H3 (Berndsen et al., 2008; Fillingham et al., 2008). Recently, it was reported that Gcn5 is involved in H3K56Ac in human cells (Tjeertes et al., 2009). Because acetylation of lysine residues 9, 27 and 56 is present on newly-synthesized H3 (Kuo et al., 1996; Masumoto et al., 2005), we first asked whether Gcn5 is required for H3K56Ac in yeast cells. As reported (Han et al., 2007a), deletion of *RTT109* abolished H3K56Ac, whereas deleting *GCN5* had no detectable effect on H3K56Ac (Fig. 2A). Next, we analyzed how the *gcn5*Δ and *rtt109*Δ mutations affected acetylation of histone H3 lysine 27 (H3K27Ac). H3K27Ac was significantly reduced in *gcn5*Δ mutant cells compared to wild-type cells, whereas deletion of *RTT109* had no apparent effect on the level of H3K27Ac. Importantly, H3K27Ac was not detected in *gcn5*Δ *rtt109*Δ mutant cells, suggesting that both Gcn5 and Rtt109 are required for H3K27Ac *in vivo*.

Vps75 is a component of the Rtt109-Vps75 histone acetyltransferase complex (Han et al., 2007c). Therefore, we asked whether Vps75 was also required for H3K27Ac in yeast cells. Cells lacking *VPS75* had no apparent effect on H3K27 acetylation compared to wild-type cells (Figure 2B). However, H3K27Ac was not detected in *gcn5*Δ *vps75*Δ cells, whereas H3K56Ac was not affected in *gcn5*Δ *vps75*Δ mutant cells compared to wild-type cells (Figure 2B). These results indicate that both Gcn5 and the Rtt109-Vps75 complex are involved in H3K27 acetylation in yeast cells.

Lastly, we tested whether the Rtt109-Vps75 complex acetylated H3K27 *in vitro* and whether Asf1 enhanced the activity of the Rtt109-Vps75 complex towards H3K27. Recombinant Rtt109-Vps75 complex was incubated with recombinant H3-H4 and co-factor, acetyl-CoA, in the presence or absence of Asf1. The reaction mixtures were analyzed by Western blot using antibodies recognizing H3K27Ac. As shown in Fig. 2C, the Rtt109-Vps75 complex acetylated H3K27 *in vitro*, and Asf1 stimulated the activity of the Rtt109-Vps75 complex towards H3K27Ac (Figure 2C). Interestingly, replacement of H3K56 with arginine (H3K56R) had no apparent effect on the ability of the Rtt109-Vps75 complex to acetylate H3K27, suggesting that the acetylation of H3K56 by the Rtt109-Vps75 complex is not a prerequisite for this

complex to acetylate H3K27. Thus, the Rtt109-Vps75 complex acetylates both H3K27 and H3K56 *in vitro* and *in vivo*.

A Gcn5-containing complex works together with Rtt109 in a common process

Gcn5 is the catalytic subunit of three KAT complexes, SAGA, ADA and SLIK, with well-known roles in gene transcription. To identify which known Gcn5-containing complex functions in parallel with Rtt109, we deleted all of the non-essential subunits of the SAGA, SLIK and ADA complexes in *rtt109*Δ mutant cells and compared growth and DNA damage sensitivity of each of the double mutants with that of *gcn5*Δ *rtt109*Δ cells (Figure 3A, Supplemental Figure 2). We found that deletion of five subunits, *ADA1*, *ADA2*, *ADA3*, *SPT7*, and *SPT20*, in *rtt109*Δ mutant cells phenocopied *gcn5*Δ *rtt109*Δ cells. In contrast, deletion of the unique components of the SAGA (*SPT8*), SLIK (*RTG2*) or ADA (*AHC1* and *AHC2*) complexes, as well as genes shared among the Gcn5-containing complexes, *SGF29*, *CHD1*, *UBP8*, *SGF73*, *SGF11*, and *SPT3*, had no apparent effect on the growth and DNA damage sensitivity of *rtt109*Δ mutant cells (Figure 3A and Supplemental Figure 2). These results demonstrate that Ada1, Ada2, Ada3, Spt7, and Spt20 function with Gcn5 to maintain cell growth and resist DNA damage in the absence of Rtt109.

Cells lacking Ada1, Spt7 and Spt20 exhibit sensitivity towards DNA damaging agents (Supplemental Figure 2) as well as defects in transcription (Sterner et al., 1999), suggesting that Ada1, Spt7 and Spt20 function together in a complex. Ada2, Ada3 and Gcn5 are known to form the core subunits of the SAGA, ADA and SLIK complexes (Balasubramanian et al., 2002; Candau and Berger, 1996; Grant et al., 1997). Therefore, in an attempt to characterize the complex containing Gcn5, Ada1, Ada2, Ada3, Spt7 and Spt20, tagged Ada1 (Ada1-TAP) was first purified from yeast cells, and the resulting proteins were further purified using Gcn5 tagged with the Flag epitope (see Experimental Procedures) (Ada1-Gcn5). As a comparison, we also purified Gcn5 from yeast cells using tandem affinity purification (Gcn5-all). The Gcn5-containing complex purified using the Ada1-Gcn5 procedure acetylates predominantly H3 of core histones, with limited activity against nucleosomal H3. On the other hand, the Gcn5-all complex acetylated both nucleosomal H3 as well as free H3 (Figure 3D and E). Because the SAGA and ADA complexes acetylated nucleosomal H3 (Grant et al., 1997), the Ada1-Gcn5 complex we purified may be distinct from the SAGA and ADA complex. To test this idea further, we followed the same Ada1-Gcn5 purification procedure and purified a complex from cells lacking both Ahc1 (the structural component of the ADA complex (Eberharter et al., 1999) and Spt8 (the unique subunit of SAGA). Deletion of *AHC1* and *SPT8* did not affect co-purification of Ada2, Ada3 and Spt7 with Ada1 and Gcn5 (Figure 3F) or the activity profile of the Ada1-Gcn5 complex (Figure 3G). Based on these genetic and biochemical studies, we suggest that a Gcn5-containing complex acetylates the N-terminal lysine residues of newly-synthesized histone H3.

Cells lacking Gcn5 or expressing the H3 *5KR* mutant exhibit defects in cell cycle progression

The complex integrity of SAGA, SLIK and ADA, as well as their role in transcription, is mediated partly by their unique subunits (Eberharter et al., 1999). The fact that loss of the unique components of these complexes had no apparent effect on the cell growth and DNA damage sensitivity of *rtt109*Δ mutant cells suggests that the DNA damage sensitivity observed in *gcn5*Δ *rtt109*Δ (as well as *ada1*Δ *rtt109*Δ, *ada2*Δ *rtt109*Δ and *ada3*Δ *rtt109*Δ, *spt7*Δ *rtt109*Δ, and *spt20 rtt109*Δ) cells is not likely due to the loss of function of any of the three previously characterized Gcn5-containing complexes in gene transcription. Instead, we suggest that Gcn5 may function in parallel with Rtt109 to maintain genome stability in a manner that is independent of the transcriptional function of the ADA, SAGA and SLIK complexes. To test this idea further, we first asked whether the *gcn5*Δ mutant affected cell cycle progression. Because Gcn5 acetylates the H3 N-terminus, we also analyzed the cell cycle progression of

cells expressing the H3 mutant containing mutations at five lysine residues (H3 *5KR*: K9R, K14R, K18R, K23R and K27R) of the H3 N-terminus. Wild-type, *gcn5*Δ and H3 *5KR* cells were arrested at G1 using α-factor and then released into fresh media to allow cell cycle progression. Every 15 minutes, aliquots of cells were removed for analysis of DNA content by FACS and H3K56Ac by Western blot. As shown in Figure 4A and C, wild-type, *gcn5*Δ and H3 *5KR* mutant cells entered S phase with similar kinetics. However, both *gcn5*Δ and H3 *5KR* mutant cells entered the next cell cycle 15 minutes later than wild-type cells. This result is consistent with previous observations that *gcn5*Δ mutant cells accumulate at G2/M phase of the cell cycle (Zhang et al., 1998). Like wild-type cells, H3K56Ac started to peak at 45 minutes in *gcn5*Δ and H3 *5KR* mutant cells. However, the decline of this modification was 15 minutes later in the *gcn5*Δ and H3 *5KR* mutant cells compared to wild-type cells (Figure 4B and D). Together, these results suggest that *gcn5*Δ and H3 *5KR* mutant cells exhibit abnormal cell cycle progression and deregulation of H3K56Ac during the cell cycle.

Gcn5 is required for the maintenance of genome stability

Next, we asked whether the *gcn5*Δ mutant had a higher level of spontaneous chromosome breaks than wild-type cells using the Rad52 foci assay (Lisby et al., 2001). Rad52, a mediator of DNA double strand break repair, is localized throughout the nucleus in the absence of chromosome breaks. In the presence of chromosome breaks, Rad52 molecules accumulate and form foci at chromosome break sites. As reported, about 10% of wild-type cells formed Rad52 foci, and all these cells were in S/G2/M phase of the cell cycle (Figure 5A) (Lisby et al., 2001). Moreover, significantly more *rtt109*Δ mutant cells displayed Rad52 foci than wild-type cells (Driscoll et al., 2007; Han et al., 2007b). About two-fold (20% vs 10%) more *gcn5*Δ mutant cells formed Rad52 foci than wild-type cells under normal growth conditions (Figure 5A). Thus, like *rtt109*Δ mutant cells, *gcn5*Δ mutant cells exhibit spontaneous chromosome breaks during normal growth conditions that may contribute to abnormal H3K56Ac and cell cycle progression.

Like *gcn5*Δ mutant cells, significantly more *ada2*Δ and *ada3*Δ cells formed Rad52 foci than wild-type cells. In contrast, the *ahc1*Δ mutant, which did not exaggerate growth defects or DNA damage sensitivity of *rtt109*Δ mutant cells, did not exhibit a significant change in cells with Rad52 foci compared to wild-type cells (Figure 5A). Lastly, we observed that significantly more *gcn5*Δ mutant cells had Rad52 foci than wild-type cells when both cells were challenged with zeocin, a drug that induces double strand breaks (Figure 5B). These results suggest that Gcn5 functions to maintain genome stability during normal S phase as well as when under insult from DNA damaging agents. Consistent with this idea, *gcn5*Δ mutant cells exhibited synthetic growth defects with mutations in the checkpoint kinase *RAD53* as well as exaggerated sensitivity towards the DNA damaging agent CPT when combined with mutations in *RAD53* and *MEC1* (another checkpoint kinase) (Figure 5C–D and Supplemental Figure 3A–C). These results provide additional support for the idea that Gcn5 is required to maintain genome stability and that the synthetic phenotypes of *gcn5*Δ *rtt109*Δ cells are likely due to the compromised ability of these double mutant cells to maintain genome stability.

GCN5 genetically interacts with genes involved in DNA replication and replication-coupled nucleosome assembly

RTT109 genetically interacts with genes involved in DNA replication (Collins et al., 2007; Han et al., 2007a). Therefore, we tested whether *GCN5* genetically interacted with *CDC7* and *CDC17* using the temperature sensitive (ts) mutants of *CDC7* and *CDC17*, *cdc7-1* and *cdc17-1*, respectively. Cdc7 is a protein kinase required for initiation of DNA replication, and Cdc17 is the catalytic subunit of the DNA polymerase α –primase complex. The *gcn5*Δ *cdc7-1* mutant grew more slowly at permissive and semi-permissive temperatures compared to either single mutant alone. The same was true for the *gcn5*Δ *cdc17-1* double mutant at the

semi-permissive temperature (33°C) (Supplemental Figure 3D). The genetic interactions among *GCN5*, *CDC7* and *CDC17* suggest that Gcn5, like Rtt109, has a role in DNA replication.

Recently, we have shown that CAF-1 and Rtt106 are two effectors that bind H3K56Ac and promote nucleosome assembly during S phase (Li et al., 2008). Therefore, we examined whether *GCN5* genetically interacted with *CAC1* (the large subunit of the histone chaperone CAF-1) and *RTT106* (Figure 5E and supplemental Figure 3E). The *gcn5*Δ *cac1*Δ and *gcn5*Δ *rtt106*Δ cells were more sensitive to at least two DNA damaging agents compared to the *cac1*Δ, *rtt106*Δ or *gcn5*Δ single mutants. Moreover, when compared to *gcn5*Δ *asf1*Δ double mutant cells, *gcn5*Δ *cac1*Δ and *gcn5*Δ *rtt106*Δ cells exhibited lesser growth defects and were less sensitive to DNA damaging agents (Figure 5E and supplemental Figure 3E). This web of genetic interactions (Figure 5F) between *GCN5* and genes involved in DNA replication, replication-coupled nucleosome assembly and the DNA damage response suggests that Gcn5, like Rtt109, plays a role in DNA replication and/or replication-coupled nucleosome assembly.

Gcn5 and acetylation of five lysine residues at the H3 N-terminus promote efficient nucleosome assembly

To determine whether Gcn5 is needed for efficient nucleosome assembly during S phase, we used the chromatin immunoprecipitation assay (ChIP) to analyze how the *gcn5*Δ mutation affected the deposition of newly-synthesized H3, marked by H3K56Ac (Masumoto et al., 2005), onto replicating DNA. Briefly, wild-type and *gcn5*Δ mutant cells were synchronized at G1 using α-factor and then released into fresh media containing HU for different periods of time. ChIP assays were performed using antibodies recognizing H3K56Ac or H3 (Figure 6A). The ChIP DNA was analyzed using primers amplifying the early replication origin *ARS607* and a DNA fragment 14 Kb from *ARS607* (ARS607+14kb). While HU has no effect on the firing of early replication origins such as *ARS607*, it prevents firing of late replication origins and impedes progression of the DNA replication fork (Tercero and Diffley, 2001). Therefore, the replication fork originating from *ARS607* cannot reach ARS607+14 Kb in the presence of HU. Significantly more H3K56Ac was detected at replicating DNA (*ARS607*) than at ARS607 +14 kb in wild-type cells, confirming earlier results indicating that replication fork passage is required for deposition of H3K56Ac onto replicating DNA (Li et al., 2008). Importantly, H3K56Ac was significantly reduced at replicating DNA (*ARS607*) in *gcn5*Δ mutant cells compared to wild-type cells (Figure 6B and C). Because the overall level of H3K56Ac was not affected in *gcn5*Δ mutant cells (Figure 4 and Supplemental Figure 4A), the reduction of H3K56Ac at replicating DNA was not likely due to reduction of the overall level of H3K56Ac in *gcn5*Δ mutant cells. Lastly, compared to extracts prepared from wild-type cells, cell extracts prepared from *gcn5*Δ mutant cells exhibited a compromised ability to assemble plasmid DNA into nucleosomes (Supplemental Figure 4B). These results provide direct evidence supporting the idea that Gcn5 is required for efficient deposition of H3 onto replicating DNA.

Next we asked whether H3 acetylated at lysine 9 (H3K9Ac) or lysine 27 could be detected on replicating DNA during S phase of the cell cycle using the ChIP assay described above. The antibodies against H3K9Ac and H3K27Ac were specific because more DNA was precipitated from wild-type cells than from H3 mutant cells where lysine 9 and lysine 27 were mutated to arginine (Supplemental Figure 4C). In wild-type cells, the level of H3K9Ac and H3K27Ac at replicating DNA (*ARS607*) increased when cells entered S phase, whereas the level of H3K9Ac and H3K27Ac at ARS607+14kb did not change significantly (Figure 6D and E). These results confirm that H3K9Ac and H3K27Ac are present on newly-synthesized H3 and demonstrate that replication fork passage is required for the deposition of these two modified forms of H3 onto replicating DNA.

Compared to wild-type cells, deposition of H3K9Ac was significantly compromised in *gcn5*Δ mutant cells (Figure 6D). Because the level of H3K27Ac was low in G1 (Time 0) in

*gcn5*Δ mutant cells compared to wild-type cells, it was not clear whether deposition of H3K27Ac was also compromised in *gcn5*Δ mutant cells. We also detected Gcn5 at early replication origins (*ARS305* and *ARS607*) at G1 and at replicating DNA during early S phase. Moreover, the level of Gcn5 at these two origins did not change significantly when cells were released from G1 to early S phase (Supplemental Figure 4D–E). Together, these results are consistent with the idea that Gcn5 acetylates lysine residues of the N-termini of H3 molecules prior to their assembly into nucleosomes.

Lastly, we determined whether cells expressing the H3 *5KR* mutant affected the deposition of H3K56Ac onto replicating DNA. Compared to wild-type cells, the deposition of H3K56Ac was significantly reduced in cells expressing the H3 *5KR* mutant. The reduction of H3K56Ac on replicating DNA was not due to a reduced level of H3K56Ac in H3 *5KR* mutant cells (Supplemental Figure 4A and Figure 4). Thus, acetylation of some or all five lysine residues at the H3 N-terminus, possibly by Gcn5 and Rtt109, is important for the deposition of H3K56Ac onto replicating DNA.

Gcn5 and acetylation of five lysine residues at the H3 N-terminus are important for efficient association of H3 with CAF-1

Deposition of newly-synthesized H3, marked by H3K56Ac, requires the histone chaperones CAF-1 and Rtt106 (Li et al., 2008). To understand how Gcn5 and acetylation of lysine residues at the H3 N-terminus impact H3K56Ac deposition, we tested whether Gcn5 and acetylation of five lysine residues at the H3 N-terminus are required for efficient binding of H3 with CAF-1, Asf1 and Rtt106. First, Cac2-, Asf1- or Rtt106-TAP was purified from wild-type and *gcn5*Δ mutant cells, and co-purified H3 molecules were detected by Western blot using antibodies against H3. As shown in Fig. 7A–B, the association of H3 with Asf1 and Rtt106 was slightly reduced in *gcn5*Δ mutant cells compared to wild-type cells, whereas the binding of H3 with CAF-1 was reduced dramatically in *gcn5*Δ mutant cells compared to wild-type cells. The reduction of H3 binding with CAF-1 was not likely due to the reduced level of Cac2, H3 or H3K56Ac in *gcn5*Δ mutant cells (Fig. 7A, right panel). Thus, Gcn5 is required for the efficient binding of H3 with CAF-1.

Using the same approach, we also observed that mutation of five lysine residues at the H3 N-terminus also resulted in a significant reduction in the binding of H3 with CAF-1 and a modest effect on the association of H3 with Asf1 and Rtt106 (Figure 7C and D). These results are consistent with our previous studies indicating that in cells lacking H3K56Ac, the binding of H3 with Rtt106 is reduced to an undetectable level by Western blot, and the association of H3 with CAF-1 is significantly reduced (Li et al., 2008). Together, these results indicate that acetylation of five lysine residues at the H3 N-terminus, possibly by Gcn5 and Rtt109, is important for the binding of H3 with CAF-1.

Our results appear to contradict a previously published report showing that deletion of the H3 N-terminus has no apparent effect on human CAF-1's ability to assemble the H3-H4 mutant onto replicating DNA using the *in vitro* SV40 DNA replication-coupled nucleosome assembly assay (Shibahara et al., 2000). There are two potential explanations for this discrepancy. First, it is possible that human CAF-1 and yeast CAF-1 are regulated differently by acetylation of H3 N-termini. In this regard, we notice that unlike the acetylation pattern on newly-synthesized H4, acetylation patterns on newly-synthesized H3 appear to diverge from yeast to human (Benson et al., 2006; Sobel et al., 1995). Alternatively, the *in vivo* assays used in this study are more sensitive towards the detection of the contribution of the H3-N-terminus to CAF-1-mediated nucleosome assembly than the *in vitro* assay used previously. Future studies are needed to address these two possibilities. Nonetheless, our studies are consistent with yeast genetic studies suggesting that the N-terminus of yeast H3 has a role in nucleosome assembly (Li et al., 2008; Ma et al., 1998).

Discussion

Here we show that Gcn5 functions in parallel with Rtt109 to promote DNA replication-coupled nucleosome assembly. Cells lacking *GCN5* exhibit synthetic growth defects and sensitivity to DNA damaging agents in combination with mutations that perturb H3K56 acetylation (*rtt109*Δ, *asf1*Δ, and *H3K56R)*, DNA replication (*CDC7* and *CDC17*), the DNA damage response (*RAD53* and *MEC1*), and two histone chaperones (*CAC1* and *RTT106*) that are involved in DNA replication-coupled nucleosome assembly. This web of genetic interactions suggests that Gcn5 has a role in DNA replication independent of its role in gene transcription. Moreover, we have shown that Gcn5 and five lysine residues of the H3 N-terminus are required for efficient deposition of new H3 onto replicating DNA and for efficient binding of H3 with CAF-1. Together, these results demonstrate that Gcn5 promotes DNA replication-coupled nucleosome assembly, partly through acetylation of lysine residues at the N terminus of new H3 molecules prior to their deposition onto replicating DNA.

A role for Gcn5 in DNA replication-coupled nucleosome assembly

In yeast and mammalian cells, Gcn5-containing complexes are known for their roles in transcriptional activation and elongation (Sterner et al., 1999). Therefore, it is possible that the *gcn5*Δ mutant phenotype displayed in the absence of Rtt109 may be an indirect effect of mis-regulated transcription of genes involved in nucleosome assembly following DNA replication. However, two lines of evidence argue against this idea. First, gene expression profiling using microarrays indicates that the expression level of genes critical for DNA replication-coupled nucleosome assembly such as PCNA, CAF-1 and Rtt106 are not affected to a detectable degree in *gcn5*Δ cells (Lee et al., 2000). Second, loss of ten genes, including *SPT8* and *UBP8*, known to function with Gcn5 in transcriptional regulation did not display the same phenotype as the *gcn5*Δ mutant when combined with the *rtt109*Δ mutation. Because genes whose transcription are regulated by Gcn5 overlap with or depend on these ten genes (Grant et al., 1997; Grant et al., 1999; Lee et al., 2000; Sterner et al., 1999), it is not likely that the defects in nucleosome assembly observed in *gcn5*Δ mutant cells, as well as the synthetic defect in response to DNA damaging agents of *gcn5*Δ *rtt109*Δ mutant cells, are solely due to consequences of impaired gene transcription. Because Rtt109 promotes nucleosome assembly following DNA replication and DNA repair (Chen et al., 2008; Li et al., 2008), we propose that Gcn5 and Rtt109 function in parallel to promote efficient DNA replication-coupled nucleosome assembly. We provide the following data to further support this conclusion. First, like other genes involved in replication-coupled nucleosome assembly, Gcn5 is required for maintaining genome stability. Second, *GCN5* genetically interacts in a negative way with genes involved in DNA replication (*CDC7* and *CDC17*), the DNA damage response (*RAD53* and *MEC1*) as well as three histone chaperones involved in DNA replication-coupled nucleosome assembly (CAF-1, *ASF1* and *RTT106*). More importantly, deposition of newly-synthesized H3, marked by H3K56Ac, onto replicating DNA is compromised in *gcn5*Δ mutant cells, and cell extracts prepared from *gcn5*Δ mutant cells are less efficient at assembling DNA into nucleosomes. Together, these results reveal a role for Gcn5 in replication-coupled nucleosome assembly.

How is Gcn5 involved in nucleosome assembly? We presented several lines of evidence supporting a model in which Gcn5 promotes nucleosome assembly in part by acetylating lysine residues at the H3 N-terminus that contribute to the association of H3 with CAF-1. CAF-1 is the primary histone chaperone that promotes replication-coupled nucleosome assembly in yeast and mammalian cells (Stillman, 1986). First, cells lacking Gcn5 exhibit similar defects in cell cycle progression as those expressing the H3 *5KR* mutant that contains mutations at five lysine residues of the H3 N-terminus. Second, *gcn5*Δ and H3 *5KR* mutant cells exhibit similar defects in the deposition of new H3 onto replicating DNA and a similar reduction of H3 binding

to CAF-1. Thus, we propose that Gcn5 acetylates the N-terminus of H3, promoting replication-coupled nucleosome assembly, in part by increasing the binding of H3 with CAF-1.

However, cells lacking Gcn5 or expressing the H3 *5KR* mutant are more sensitive to DNA damaging agents than cells lacking Cac1, the large subunit of CAF-1. Therefore, the DNA damage sensitivity of *gcn5*Δ or H3 *5KR* mutant cells is not solely due to the reduced binding of H3 with CAF-1. In budding yeast, Gcn5 is also involved in DNA repair by unknown mechanisms (Tamburini and Tyler, 2005). It has been shown that Gcn5 is recruited to the break site, and acetylation of the H3 N-terminus increases following generation of chromosome breaks (Tamburini and Tyler, 2005). Thus, analogous to H3K56Ac that promotes nucleosome assembly following DNA replication as well as DNA repair (Chen et al., 2008; Li et al., 2008), Gcn5 may also promote nucleosome assembly following repair of damaged DNA. Thus, the DNA damage sensitivity observed in *gcn5*Δ or H3 *5KR* mutant cells may be a manifestation of the combined defects in both DNA replication and DNA repair of these mutant cells.

In mammalian cells, it has been reported recently that Gcn5 and p300/CBP are the mammalian H3K56Ac KATs (Das et al., 2009; Tjeertes et al., 2009). Moreover, H3K56Ac is important to maintain genome stability (Yuan et al., 2009) and promote replication-coupled nucleosome assembly in mammalian cells (Das et al., 2009). Therefore, it would be interesting to determine whether Gcn5 also has a role in replication-coupled nucleosome assembly in mammalian cells, and if so, whether Gcn5 functions in this process through acetylation of lysine residues at the H3 N-terminus and/or H3K56.

Gcn5 functions with other proteins to promote nucleosome assembly

Gcn5 is a component of three distinct complexes, ADA, SAGA and SLIK. Other components regulate the integrity and activity of the Gcn5-containing complexes and modulate Gcn5's role in gene transcription. For instance, Ahc1, one of the unique components of the ADA complex, is required for the structural integrity of the ADA complex (Eberharter et al., 1999). Ada2 and Ada3, two components of the SAGA complex, target Gcn5 to acetylate nucleosomal histones (Grant et al., 1997). Ada1, Spt7 and Spt20 are required for the structural integrity of the SAGA complex (Sterner et al., 1999). We have utilized a genetic approach to identify five genes, *ADA1*, *ADA2*, *ADA3*, *SPT7* and *SPT20*, that when mutated in *rtt109*Δ mutant cells, displayed a similar cell growth and DNA damage sensitivity phenotype as deletion of *GCN5*. These genetic interactions are consistent with recent genome-wide studies indicating that *RTT109* exhibits synthetic growth defects with mutation of the core subunits of the SAGA complex, including *ADA1*, *ADA2*, *ADA3*, and *GCN5* (Lin et al., 2008). Moreover, we have shown that cells lacking *ADA1*, *ADA2*, *ADA3*, *SPT7*, or *SPT20* alone were also sensitive to DNA damaging agents. These results suggest that these five gene-products function with Gcn5 to promote nucleosome assembly. Supporting this idea, we purified a complex using Ada1 and Gcn5 as bait, and this complex acetylates H3 in core histones more efficiently than nucleosomal H3. While it remains to be determined how these five gene products function with Gcn5 to promote replication-coupled nucleosome assembly, it is possible that these five gene products regulate the ability of Gcn5 to acetylate newly-synthesized H3 for efficient deposition by CAF-1.

Involvement of multiple acetylation sites and KATs in replication-coupled nucleosome assembly

Acetylation at multiple sites on H3 and H4 has been implicated in replication-coupled nucleosome assembly. These include acetylation of lysine residues 5, 8, and 12 of newly-synthesized H4, acetylation of the five lysine residues at the H3 N-terminus and H3K56Ac. Because H3K56Ac promotes nucleosome assembly by increasing the binding affinity of H3 with CAF-1 and Rtt106 (Li et al., 2008), and acetylation of lysine residues of the H3 N-terminus contributes to the binding of H3 with CAF-1, it is likely that acetylation of lysine residues on

H4 also regulate the binding of histones with histone chaperones involved in replication-coupled nucleosome assembly. A recent study shows that acetylation of both K5 and K8 of H4 cooperate to bind one binding pocket of a single bromodomain of Brdt (Moriniere et al., 2009). Therefore, it is tempting to speculate that acetylation of multiple sites on H3 and H4 may coordinate the binding of H3-H4 with CAF-1 and other histone chaperones.

In addition to Gcn5, several KATs including Rtt109, Elp3 (Table 1, (Li et al., 2009)) and Hat1 have implicated roles in replication-coupled nucleosome assembly. This provides a possible explanation for why assembly of new H3-H4 is essential for cell viability (Kim et al., 1988), yet none of these KATs are. Moreover, recent studies suggest that histone acetyltransferases may participate in distinct steps of replication-coupled nucleosome assembly. In addition to acetylating new histone H3-H4 to promote nucleosome assembly, some of these lysine acetyltransferases may acetylate parental nucleosomes. Following DNA replication, parental nucleosomal histones must be transferred to newly-synthesized DNA. It is proposed that human Asf1 can disrupt parental nucleosomes and promote the transfer of parental H3-H4 to DNA behind replication forks (Groth et al., 2007a). How this process occurs is still an enigma. Analogous to the role of acetylation in the promotion of nucleosome remodeling during gene transcription, it is possible that some of these KATs acetylate parental nucleosomes for nucleosome disassembly by Asf1 or remodeling by chromatin remodeling complexes. In this regard, we found that Gcn5 can be detected at replication origins in both G1 and early S phase. Thus, it is possible that in addition to its role in acetylating new H3, Gcn5 may regulate acetylation of parental nucleosomes, facilitating their disassembly. Future studies are needed to address how these lysine acetyltransferases coordinate to promote efficient nucleosome assembly and to determine the molecular mechanisms by which Gcn5 functions in replication and repair.

Experimental Procedures

Yeast strains and Plasmids

All yeast strains were derived from W303-1 or BY4741 and are listed in the supplemental yeast strain table. Strains were made using standard yeast procedures and media. All plasmids were constructed using standard molecular biology procedures and were confirmed by sequencing.

DNA damage sensitivity assays

Freshly grown cells were taken and diluted to 0.6 (unless otherwise indicated) at OD_{600}. Then 10-fold serial dilutions of each yeast stain were performed. Five microliters of yeast cells at different dilutions were then dotted on YPD media or YPD media containing different concentrations of drugs, camptothecin (CPT), methyl methane sulfonate (MMS) and hydroxyurea (HU). To select plasmids, SCM dropout media containing different concentrations of DNA damaging agents was used. Images were taken after incubation for 2–5 days. Unless indicated, plates were incubated at 30°C.

Detection of Rad52-YFP foci

Cells expressing Rad52 tagged with yellow fluorescent protein (YFP) at its C-terminus were grown to exponential phase at 25 °C, harvested and fixed in PBS with 4% formaldehyde. To test the effect of the DNA damaging agent zeocin, cells were treated with 100 μg/ml zeocin for varying amounts of times and then fixed. After washing and sonication, cells were placed on a glass slide with 1% agarose. A single DIC image and 11 YFP images obtained at 0.3-μm intervals along the *z*-axis were captured for each frame using a Zeiss LSM10 confocal microscope. Rad52-YFP foci were counted by inspecting images of all focal planes. Percentages of cells in S/G2/M phase containing YFP foci were calculated. For each strain, about 200–1000 cells were scored for Rad52-YFP foci.

Gcn5 complex purification

Gcn5 alone was purified using the TAP purification procedures as described (Li et al., 2008). We also designed a two-step strategy to enrich Ada1-Gcn5 containing complex. First, we made a yeast strain (*ada1-TAP, gcn5*Δ*. GCN5-Flag*), purified Ada1 first using the IgG sepharose. After elution of proteins using the TEV protease, proteins were further purified using antibodies against the Flag epitope and eluted with Flag peptides (see Supplemental Experimental Procedures for details). Eluted proteins were used for HAT assays and analyzed by Western blot.

Chromatin immunoprecipitation (ChIP) Assays

To determine the forms of histones deposited onto replication forks, ChIP assays were performed as previously described (Huang et al., 2007; Li et al., 2008). Following collection of ChIP DNA, quantitative PCR was performed to quantify ChIP DNA as well as whole cell DNA using primer pairs amplifying replication origins or fragments of downstream of the replication origins as indicated in Figure legends. The data were presented either as percentage of ChIP DNA over while cell DNA or ratio of ChIP signals from samples using antibodies against a modified form of histones over those using antibodies against histone H3.

Western blot analysis of modified forms of histone H3 and whole histone H3 was described in detail in the Supplemental Experimental Procedures. The HAT assays were performed as described (Han et al., 2007a).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Drs. Alain Verreault, Shelly Berger, Michael Weinreich, Jessica Tyler and Steve Kron for reagents and Dr. Alabert for the protocol to visualize Rad52 foci. This work is supported by grants from the NIH (GM719729 and GM 81838). RB is supported by the Mayo Clinic Sydney Luckman Family Predoctoral Fellowship, and JH is supported by Kendall-Mayo postdoctoral fellowship. We would also like to thank Brian Davies, Qing Li and Michael Guy for their critical reading of the manuscript.

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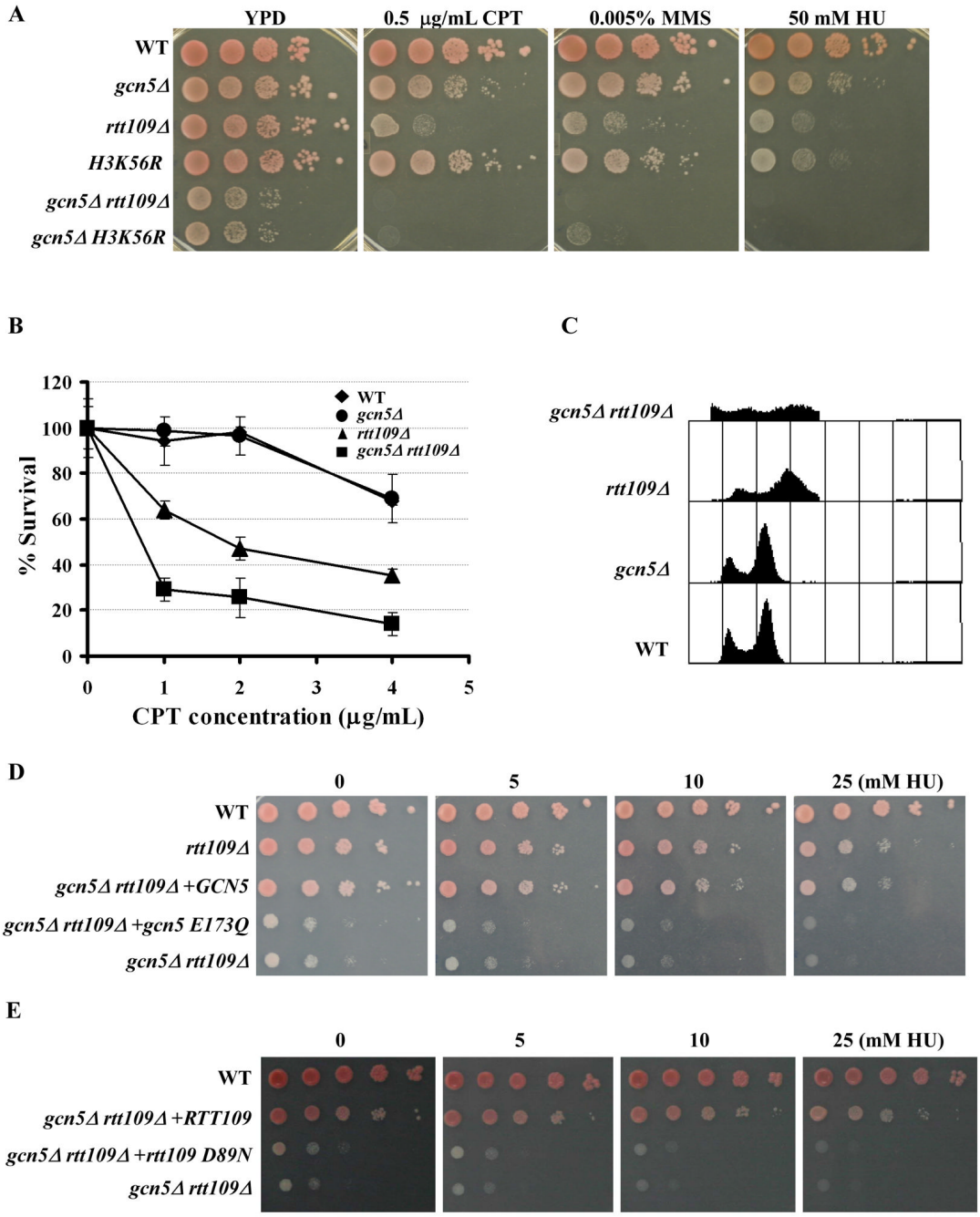

Figure 1.

Gcn5 and H3 lysine 56 acetylation function in parallel in growth and response to DNA damaging agents. Data presented in Table S1 indicate the presence of synthetic genetic interactions between the HAT, *GCN5*, and *ASF1*, a regulator of H3K56Ac. (A) The *gcn5*Δ mutant exhibits synthetic phenotypes with *rtt109*Δ and *H3K56R* mutants. Ten fold serial dilutions of wild-type (WT) or mutant yeast cells with relevant genotype indicated were spotted onto normal growth media, YPD, or media containing the indicated concentration of the DNA damaging agents hydroxyurea (HU), methyl methane sulfonate (MMS), or camptothecin (CPT). Full data presented in Figure S1. (B) The *gcn5*Δ *rtt109*Δ double mutant cells are more sensitive to CPT than either single mutant. Yeast cells were treated with the indicated

concentration of CPT for 2 hours, and the percentage of surviving cells was reported. (C) FACS analysis of the DNA content of unsynchronized yeast cells. (D–E) The catalytic activity of Gcn5 (D) and Rtt109 (E) is required for cell growth and sensitivity towards DNA damaging agents. Mutant cells transformed with either plasmid for wild-type *GCN5*, *gcn5 E173Q*, or empty vector (*gcn5*Δ *rtt109*Δ) were spotted onto media containing different concentrations of HU (D). Similar experiments were also performed for wild-type *RTT109* or the *rtt109 D89N* (E).

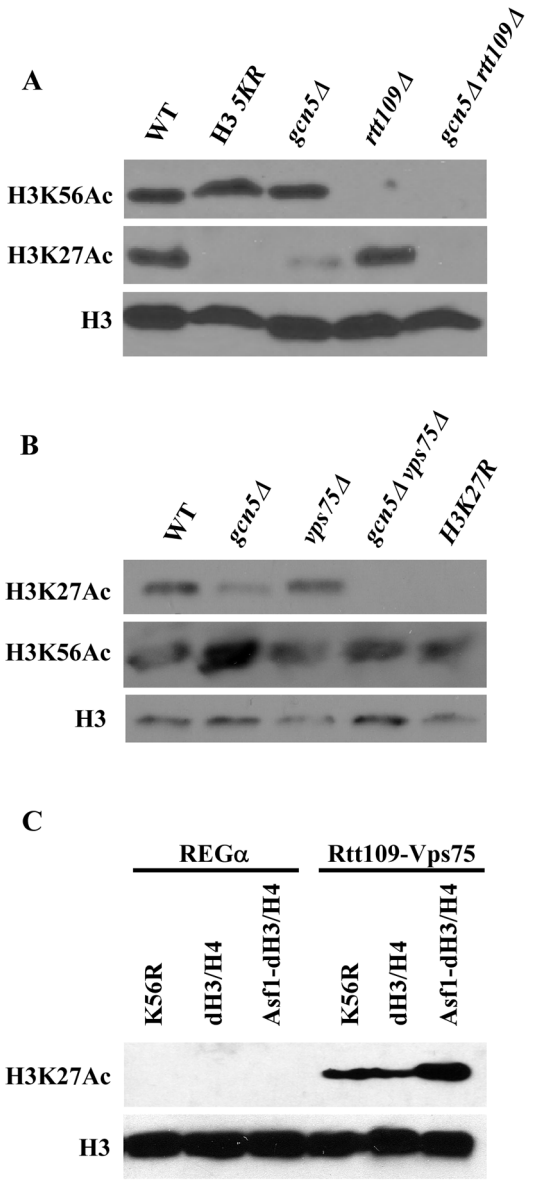

Figure 2.

Both Gcn5 and Rtt109 acetylate H3 lysine 27 (H3K27Ac). (A) Cells lacking both *GCN5* and *RTT109* result in a significant loss of H3K27Ac. The H3 *5KR* mutant contains mutations at five lysine residues of the N-terminus of H3 (9, 14, 18, 23 and 27). (B) Vps75 is essential for H3K27Ac in the absence of Gcn5. Western blot analysis of whole cell extracts showing loss of H3K27Ac in *gcn5*Δ *vps75*Δ cells. *H3K27R*: lysine 27 of H3 was mutated to arginine. (C) The Rtt109-Vps75 complex acetylates H3K27 *in vitro*. HAT assays were performed using recombinant histone H3-H4 in the presence or absence of the histone chaperone Asf1. Western blot was performed using antibodies against H3 or H3K27Ac.

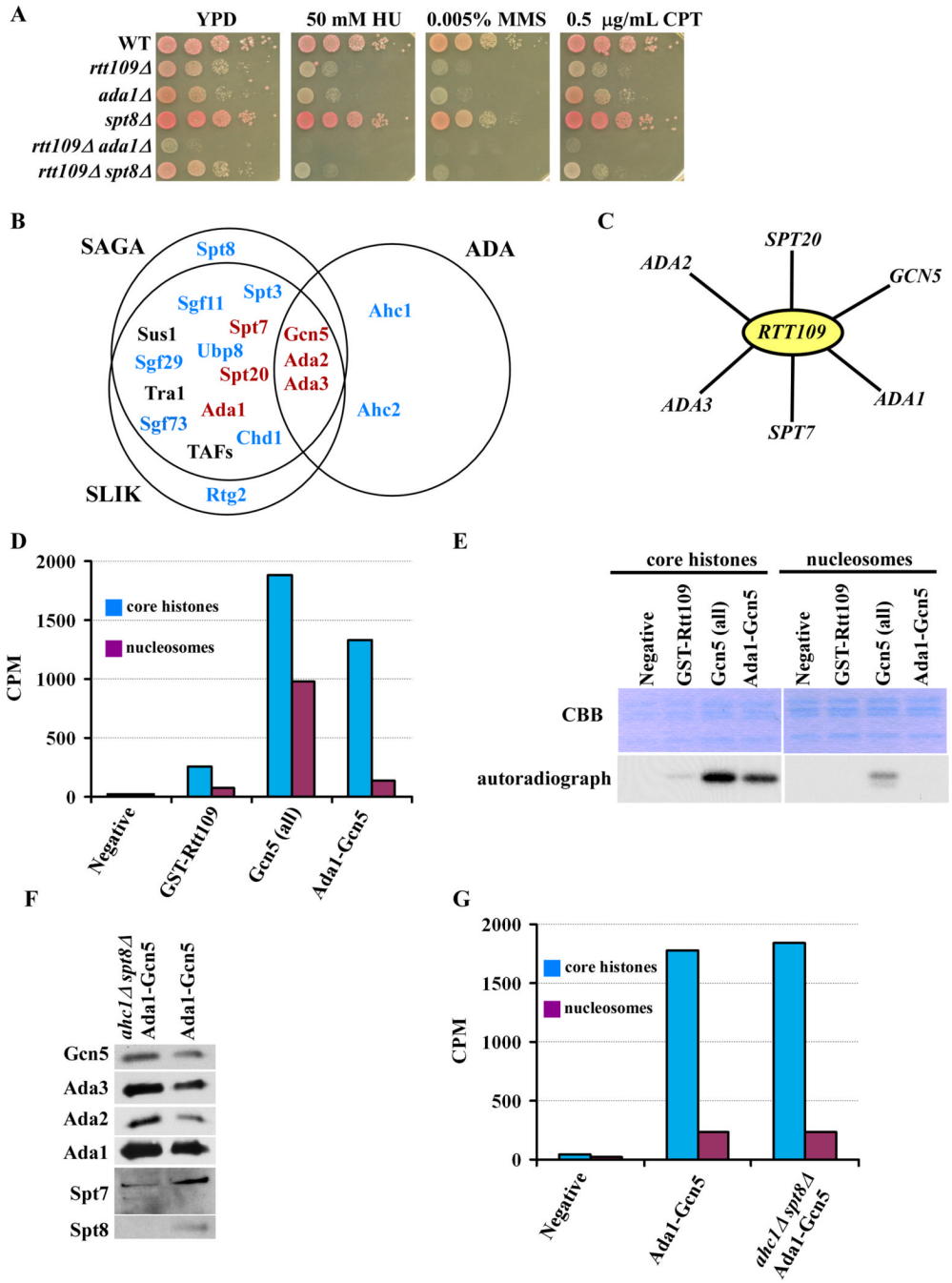

Figure 3.

A Gcn5-containing complex functions in parallel with Rtt109 in a common process. (A) Mutations in *ADA1* but not *SPT8* phenocopy the *gcn5*Δ mutant in the absence of Rtt109. (B) Venn diagram depicting the subunits of the SAGA, SLIK, and ADA complex. Genes in black represent those that were not tested, those in blue are genes that did not show synergistic phenotypes, and in red, are genes that exhibited synthetic phenotypes when combined with deletion of *RTT109*. Complete genetic data is in Figure S2. (C) Web depicting genetic interactions with *RTT109*. (D and E) The proteins that co-purified with Ada1 and Gcn5 (Ada1-Gcn5) have HAT activities different from those purified with Gcn5-TAP (Gcn5-all). Core histones and mono-nucleosomes were used as substrates for the HAT assays and

incorporated ^{3}H-CoA acetate was quantified (D). The HAT assay samples were also resolved on SDS-PAGE and revealed by Coomassie Brilliant Blue staining (CBB) (E, top) or by fluorography (E, bottom). (F) Western blot analysis of indicated components of the Ada1-Gcn5 protein complex purified from wild-type (Ada1-Gcn5, Ada1-TAP Gcn5-Flag) cells or cells lacking Ahc1 and Spt8 (*ahc1*Δ *spt8*Δ). (G) The HAT activity of complexes purified from wild-type (Ada1-Gcn5) cells and *ahc1*Δ *spt8*Δ cells.

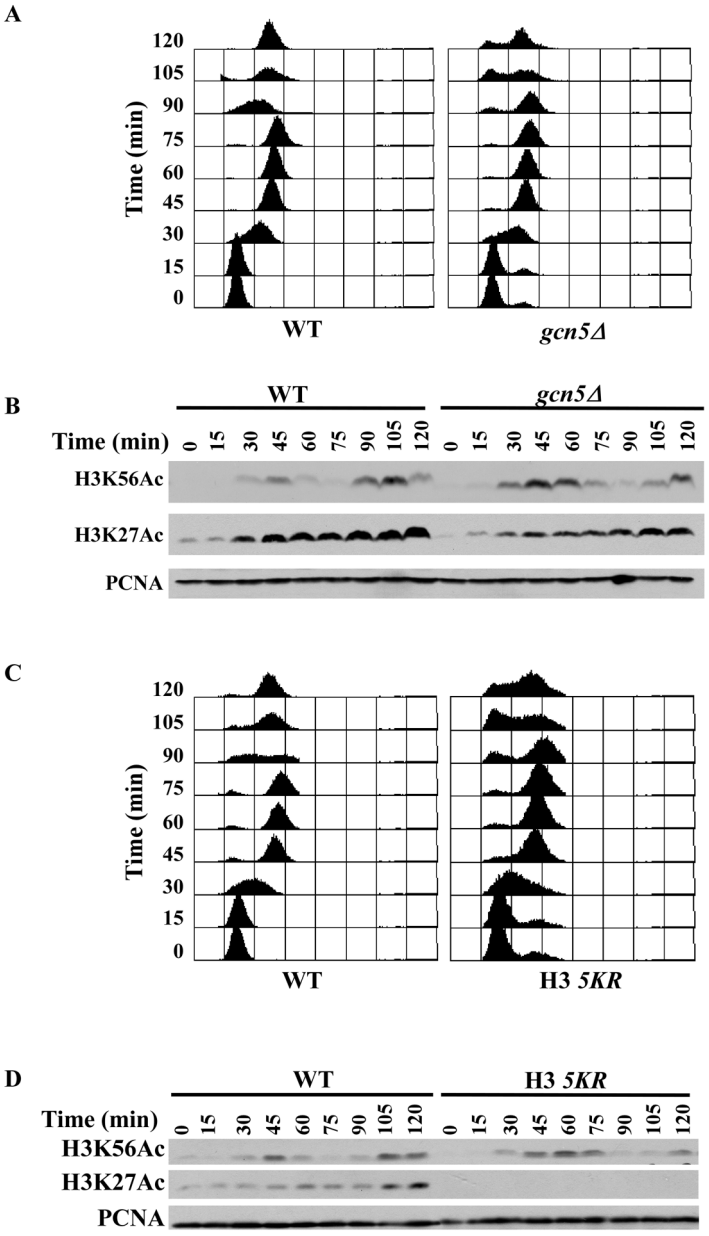

Figure 4.

The *gcn5*Δ and H3 *5KR* mutants affect cell cycle progression and cell cycle dynamics of H3K56Ac. (A-B) The *gcn5*Δ cells exhibit a prolonged G2/M phase and persistence of high levels of H3K56Ac compared to wild-type cells. Every 15 minutes after release from G1, aliquots of cells were removed for analysis of DNA content (A) or analysis of H3K56Ac and H3K27Ac by Western blot (B). (C-D) Cells expressing the H3 *5KR* mutant showed a prolonged G2/M phase and persistence of a high level of H3K56Ac compared to wild-type cells. The experiments were performed as described in A and B.

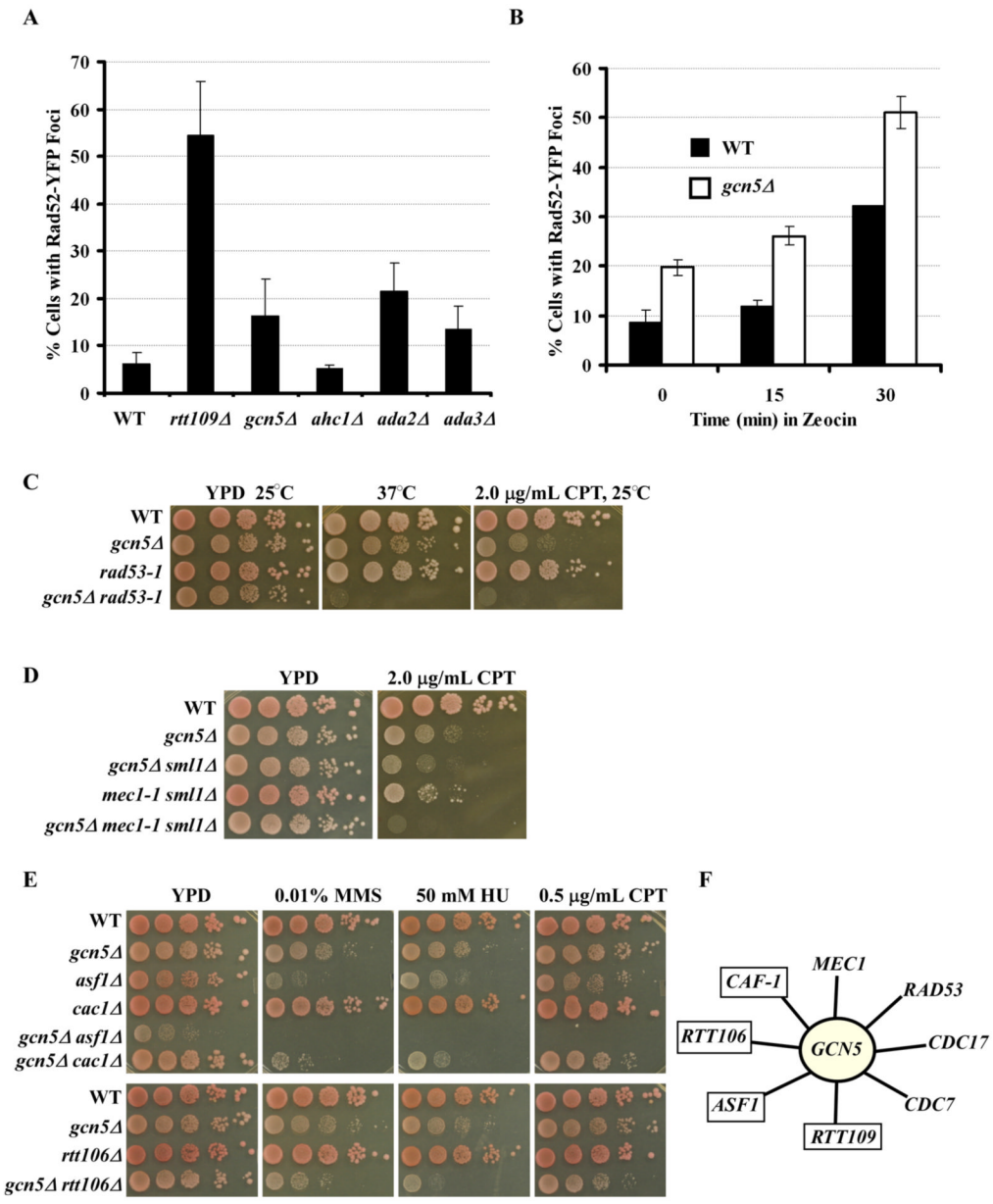

Figure 5.

GCN5 is involved in DNA replication-coupled nucleosome assembly. (A) Cells lacking *GCN5*, *ADA2* or *ADA3* exhibited a higher level of Rad52 foci compared to wild-type cells. The percentage of cells with Rad52-YFP foci was determined as described in Experimental Procedures. Data represent the mean percentage ± SEM. Mock experiments were also performed in which the genotype of each strain was unknown to the person who performed the experiment. (B) The *gcn5*Δ mutant cells exhibited higher levels of chromosome breaks than wild-type cells when challenged with a DNA damaging agent. Wild-type or *gcn5*Δ cells expressing Rad52-YFP were treated with zeocin for 0, 15, or 30 minutes and the percentage of cells containing Rad52 foci was determined. (C–D) The *gcn5*Δ mutation exhibited a synthetic genetic interaction with mutations in the checkpoint kinases *RAD53* (C, *rad53-1*) and *MEC1* (D, *mec1-1*). (E) The *gcn5*Δ mutant exhibited slow growth and increased DNA damage sensitivity with mutations in *ASF1*, *CAC1*, and *RTT106*. (F) A summary of genetic interactions

observed for *GCN5*. Those in blue boxes indicate genes that are known to be involved in nucleosome assembly. Complete genetic data is presented in Figure S3.

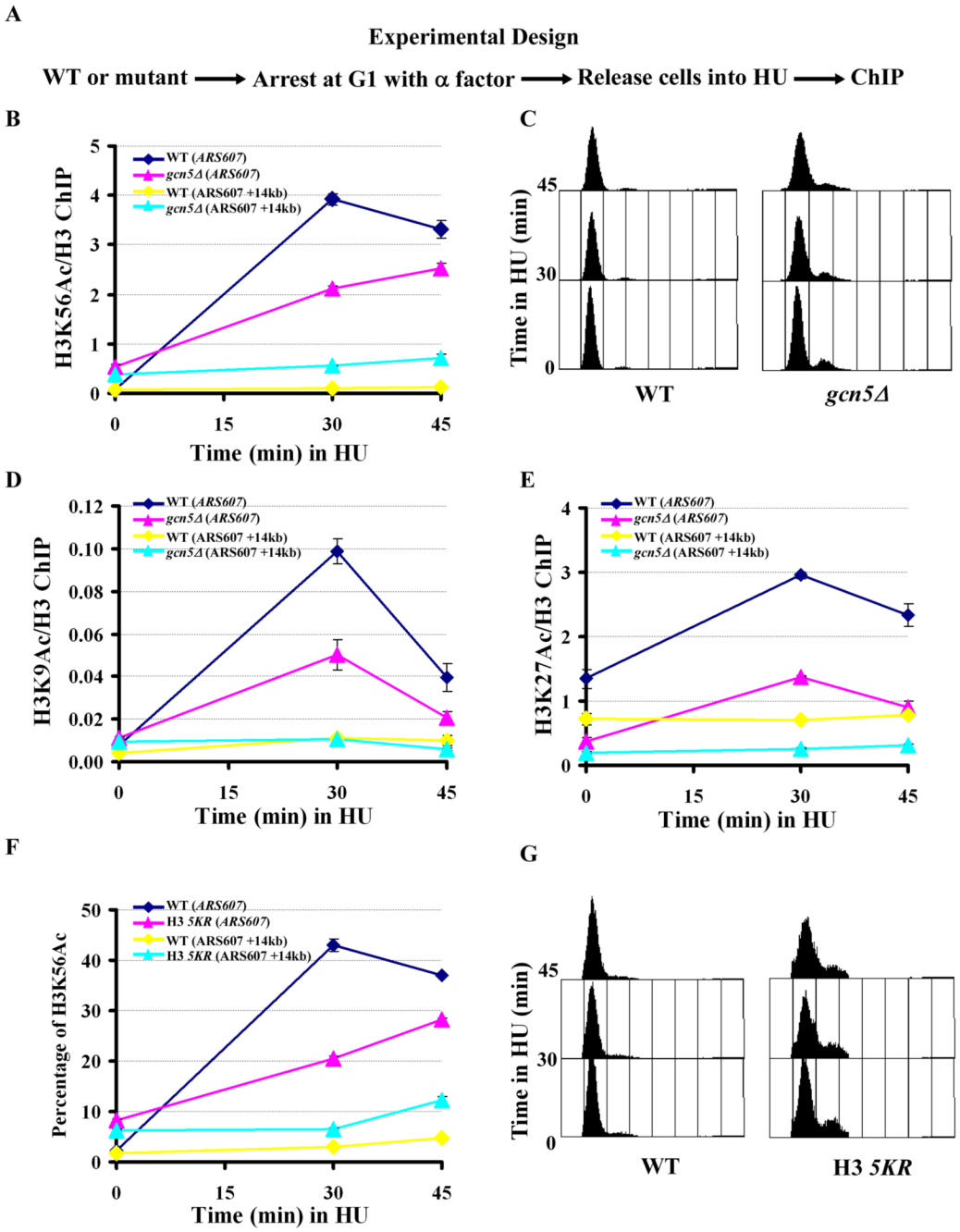

Figure 6.

Gcn5 and five lysine residues at the N-terminus of H3 are required for efficient deposition of newly-synthesized H3 onto replicating DNA. (A) A schematic diagram showing ChIP experimental design. Wild-type or mutant cells (*gcn5*Δ or H3 *5KR*) were arrested in G1 using α-factor and then released into HU. At different time points, samples were removed for FACS analysis and ChIP assays using antibodies against H3 or H3K56Ac. (B) The deposition of H3K56Ac onto replicating DNA is compromised in *gcn5*Δ mutant cells. The ChIP DNA was analyzed using primers that amplify the replication origin *ARS607* or a fragment 14 kb away from *ARS607* (ARS607+14 kb). The ratio of H3K56Ac ChIP signal over that of H3 was calculated. (C) FACS analysis of the DNA content of wild-type and *gcn5*Δ mutant cells used

in B. (D–E) H3K9Ac (D) and H3K27Ac (E) were detected at replicating DNA. The experiment was performed as described in A and B except that antibodies against H3K9Ac or H3K27Ac were used. (F) Deposition of H3K56Ac was compromised in cells expressing the H3 *5KR* mutant cells. The experiment was performed as described in A and B except that the percentage of DNA that was precipitated by antibodies against H3K56Ac was calculated by analyzing both ChIP DNA and DNA from whole cells using real-time PCR. (G) FACS analysis of the DNA content of yeast strains used in experiments described in F. Each ChIP experiment was performed independently at least twice. The data represent mean ± SEM of three real-time PCR samples from one experiment. See also Figure S4.

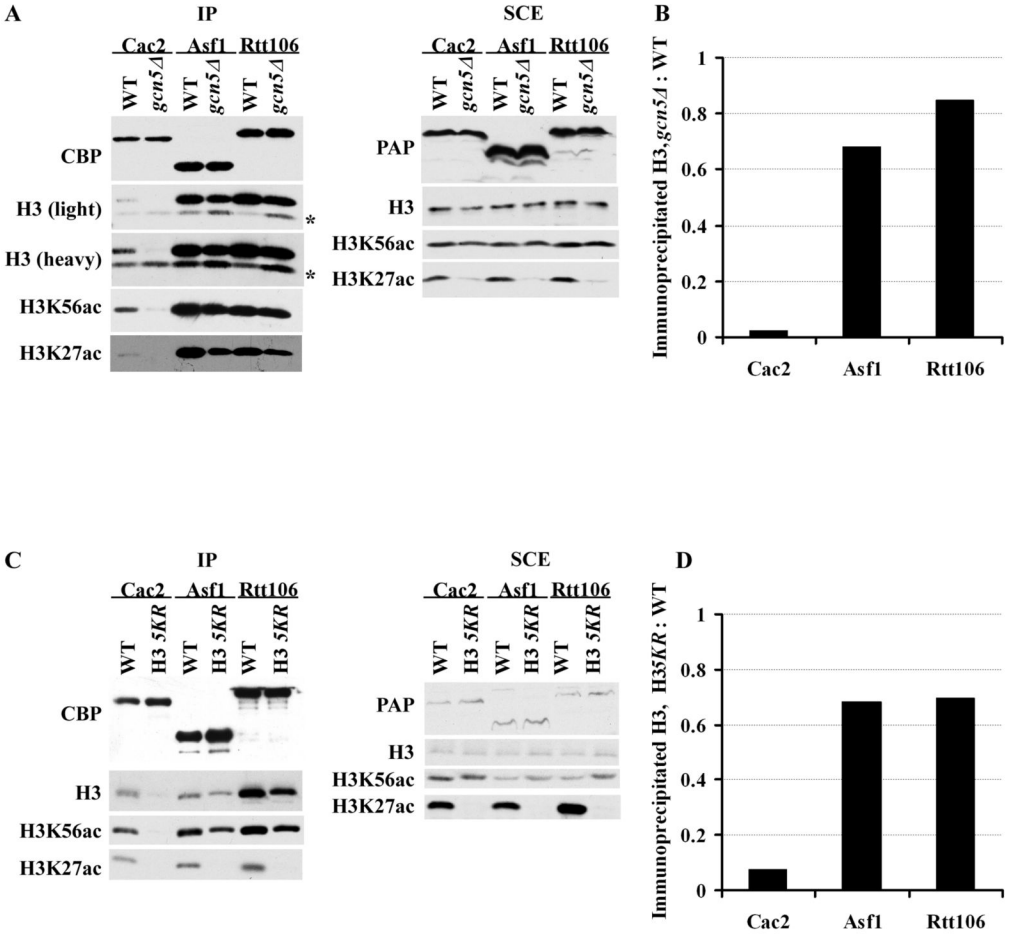

Figure 7.

Gcn5 and acetylation of five lysine residues of the H3 N-terminus regulate the binding of H3 with CAF-1. (A–B) The binding of H3 with CAF-1 is significantly reduced in *gcn5*Δ mutant cells. (A) Cac2-, Asf1- and Rtt106-TAP were purified from wild-type and *gcn5*Δ mutant cells, and co-purified proteins as well as proteins in soluble cell extracts (SCE) were detected by Western blot using antibodies against H3, H3K56ac, H3K27ac, calmodulin binding peptide (CBP) and IgG (PAP). * indicates non-specific band. (B) Quantification of the co-purified H3. The H3 intensity shown in A was quantified, and the ratio of the H3 that co-purified from each protein from the *gcn5*Δ cells over wild type cells was reported. (C–D) The association of H3 with CAF-1 is significantly reduced in cells expressing the H3 *5KR* mutant cells. The experiment was performed as described in A and quantification in D was performed as described in B.