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Transcriptional Regulation by Asf1

*NEW MECHANISTIC INSIGHTS FROM STUDIES OF THE DNA DAMAGE RESPONSE TO REPLICATION STRESS**[S]

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Asf1 is a conserved histone H3/H4 chaperone. We find that Asf1 in budding yeast promotes an essential cellular response to replication stress caused by the ribonucleotide reductase inhibitor hydroxyurea. That is, Asf1 stimulates derepression of DNA damage response (DDR) genes during the S phase. Derepression of DDR genes strongly correlates with Asf1 binding to their promoters. Having identified the C terminus and histone-binding domains of Asf1 as molecular determinants of its constitutive and inducible association with chromatin, we tested whether Asf1 binding to DDR genes is mechanistically important for their derepression. Our results provide little support for this hypothesis. Rather, the contribution of Asf1 to DDR gene derepression depends on its ability to stimulate H3K56 acetylation by lysine acetyltransferase Rtt109. The precise regulation of H3K56 acetylation in the promoters of DDR genes is unexpected: DDR gene promoters are occupied by H3K56-acetylated nucleosomes under repressing conditions, and the steady state level of H3K56 promoter acetylation does not change upon derepression. We propose that replication-coupled deposition of Lys^{56}-acetylated H3 poises the DDR genes in newly synthesized daughter duplexes for derepression during the S phase. In this model, the presence of a histone mark that destabilizes nucleosomes is compatible with suppression of transcription because in the uninduced state, DDR gene promoters are constitutively occupied by a potent repressor-corepressor complex.

The conserved histone H3/H4 chaperone Asf1 has multiple functions in chromatin metabolism. It directly contributes to replication-independent incorporation of H3 and H4 into nucleosome core particles in a pathway involving the HIR proteins (1) and delivers H3/H4 to other chaperones (minimally CAF-I and Rtt106) for incorporation into nucleosomes during DNA replication (2). In addition to promoting nucleosome assembly, Asf1 can affect the post-translational modification state of histones. It stimulates the activity of lysine acetyltransferases that predominantly modify Lys^9 and Lys^{56} of newly synthesized H3, and it can promote Set2-dependent trimethylation of H3K36 in chromatin (3–7).

Asf1 contributes to the regulation of transcription by virtue of its effects on chromatin metabolism. Studies in budding yeast suggest that Asf1 destabilizes promoter nucleosomes during periods of high transcription in a way that accommodates initiation and elongation (8–10). Stimulation of promoter activity by Asf1-dependent chromatin destabilization has been particularly well characterized at *PHO5*, which is induced when cells are starved for phosphate (9, 11). In current models, *PHO5* chromatin is destabilized by Asf1 mainly because: 1) Asf1 is in the supply line that provides Lys^{56}-acetylated H3 for incorporation into promoter nucleosomes, and 2) H3K56 acetylation may facilitate nucleosome eviction (10, 12). Hence, Asf1 functions to ensure a constant supply of Lys^{56}-acetylated H3 under inducing conditions. Incorporation of this H3 into the active *PHO5* promoter by histone turnover drives H3K56 acetylation higher at this location, which favors transcription (10). In addition to potentiating H3K56 acetylation of chromatin, Asf1 may also partly contribute to *PHO5* induction by directly removing histones from promoter chromatin (10, 13, 14). This hypothesis is supported by the observation that Asf1 can dissociate histone H3/H4 dimers from H3/H4 tetramers *in vitro* (10, 15). Furthermore the Tyler lab (16) has reported that Asf1 occupies *PHO5* under inducing conditions.

It is currently unclear whether the mechanisms by which Asf1 stimulates transcription of *PHO5* are important for high transcription of other Asf1-dependent genes. Here we address this issue in work focused on the regulation of two DNA damage response (DDR)[4] genes in budding yeast: *RNR3* and *HUG1*. Under normal conditions, these genes are repressed by a *trans*-acting factor, Crt1 (17), which recruits a corepressor complex comprised of Ssn6 and Tup1. Ssn6-Tup1 shifts the chromatin in DDR gene promoters toward an inactive state by multiple, redundant mechanisms (18, 19). Derepression of the DDR genes is a critical step in the physiological response of cells to the appearance of abnormal DNA structures in the nucleus. The mechanism of derepression has been

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental text, references, Table S1, and Figs. S1–S10.

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[4] The abbreviations used are: DDR, DNA damage response; HU, Hydroxyurea; MMS, methyl methanesulfonate.

intensively studied: it involves the loss of Crt1 and Ssn6/Tup1 from the promoter, eviction of promoter nucleosomes, and induction of acetylation in the tails of H3 and H4 (17, 19, 20).

Although regulation of the DDR genes shares some features in common with the regulation of *PHO5* (for example, involvement of some of the same chromatin-regulating protein complexes), there are important differences. First, unlike *PHO5*, the DDR genes are kept in the off state by a repressor bound to their promoters (17). Second, whereas hyperacetylation of the N-terminal tail of H4 readies *PHO5* promoter chromatin for activation (21), there is no evidence that DDR genes are poised for derepression by enrichment of a histone mark normally linked to high transcription. Finally, in *asf1*Δ cells we observe modest derepression of the DDR genes during normal growth but no change in *PHO5* expression (see below). Collectively, these results suggest that Asf1 may regulate DDR genes using a novel mechanism. The findings reported in this study support this contention and shed new light on the structure-function relationships of yeast Asf1.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Media—The strains used in this study are listed in supplemental Table S1. All of the strains used are derived from BY4741 (22) unless otherwise specified. Single deletion mutants from the *Saccharomyces cerevisiae* haploid nonessential gene deletion library (23) were verified to be correct by PCR using multiple primer sets. Chromosomal mutations were generated by one-step integration using PCR products obtained from previously described plasmids (24, 25). The addition of sequence encoding the 13-Myc epitope tag was verified by PCR using three primer sets: primers flanking the target gene (upstream, downstream), primers specific to the Myc epitope, and a primer flanking the target gene plus a primer specific for the *HIS3* selection marker. An *asf1*^{V94R}*-3HA*::*kanMX* strain, constructed by H. Mewhort, was used to generate *asf1*^{V94R}*-13MYC*::*HIS3*. All of the media were prepared as described previously, and standard genetic methods were used throughout this study (26).

RNA Isolation and Analysis—Total RNA was isolated by hot phenol extraction (27) from cells grown as described in the figure legends. DNA probes for Northern blotting were prepared by random primed labeling of PCR products (sequences available upon request). cDNA was generated from isolated RNA using Quanta qScript cDNA SuperMix and subjected to RT-PCR on a Bio-Rad iCycler. *RNR3* and *HUG1* expression was normalized to an internal region of *RDN18-1*. The oligonucleotides used were: *RNR3* (+151/+315), *HUG1* (+2/+156), and *RDN18-1* (+756/+866).

Immunoblotting—Total proteins were prepared by trichloroacetic acid precipitation (28). Identical cell equivalents of protein were compared between samples (except for the subcellular fractionation experiment, in which all lanes were loaded with the same amount of protein; see Fig. 2*C*). The antibodies were as follows: α-Rad53 (yC-19; Santa Cruz sc-6749), α-H3 (Abcam ab1791), α-penta-acetylated H4 (Upstate 06-946), α-actin (Millipore MAB1501), α-Myc (Millipore 9E10), and α-H3K56ac (Upstate 07-677).

ChIP—Cells for ChIP were grown at 30 °C in YPD to an A_{600} of 0.4 and then grown in either the absence or presence of 0.2 M hydroxyurea (HU) for 1 h before cross-linking with formaldehyde. See supplemental data for information regarding cross-linking in the absence of HU. The steps after cell harvesting were performed as described previously (29) with minor changes as outlined in the supplemental data.

Chromatin Fractionation—The cells were grown in the presence or absence of 0.2 M HU for 1 h. Chromatin was purified through a sucrose cushion as described (30), except that KCl was used at 50 rather than 100 mM in buffer EB. Ten μg of total protein from each fraction was analyzed by immunoblotting. The experiment was performed three times and yielded results identical to those shown in Fig. 2*C*.

RESULTS

Full Derepression of a DNA Damage Response Gene by Replication Stress Signals Requires Asf1—Microarrray and Northern blotting experiments revealed abnormally high expression of a subset of DDR genes in *asf1*Δ cells grown under normal conditions (Fig. 1*A* and supplemental Fig. S1). To decipher the implications of this finding for possible regulation of DDR genes by Asf1, we studied *RNR3* and *HUG1* in more detail. *RNR3* and *HUG1* encode unrelated proteins (Rnr3 is a subunit of ribonucleotide reductase; the function of Hug1 is unknown (31)). These well characterized genes (18–20, 31–34) have similar promoter structures (20) (supplemental Fig. S2). Whereas they differ slightly in some steps of regulation (20, 31), *RNR3* and *HUG1* are both controlled by mechanisms that involve the *trans*-acting factor Crt1 and the corepressor Ssn6/Tup1 (17, 31). Their derepression also involves similar steps of promoter chromatin reconfiguration triggered by checkpoint kinase Rad53 (31, 35).

Our observation that a subset of DDR genes is derepressed in cells lacking *ASF1* is consistent with the fact that Rad53 is partially activated in unperturbed *asf1*Δ cells (36). This effect has been attributed to a higher level of spontaneous DNA damage in cells lacking *ASF1*. Therefore, we hypothesized that DDR genes are induced in *ASF1* null mutants by a Rad53-dependent pathway. To address this hypothesis, we measured *RNR3* and *HUG1* transcription in cells lacking both *ASF1* and *RAD53*. Indeed, deletion of *RAD53* suppressed the modest derepression of *RNR3* and *HUG1* in an *asf1*Δ strain (Fig. 1*B*).

Some non-DDR genes depend on Asf1 for full promoter activity (8, 14). To test whether Asf1 is similarly required for full derepression of *RNR3* and *HUG1*, we measured the expression of their mRNAs in asynchronous cultures treated with 0.2 M HU to induce replication stress. Despite the fact that both genes were partly derepressed to start with, further *RNR3* induction by HU was delayed in *asf1*Δ cells, and *HUG1* induction was severely dampened (Fig. 1, *C* and *D*). Because Rad53 was activated similarly in wild type and *asf1*Δ cells treated with HU (Fig. 1*E*, compare *lanes 2* and *4*), the latter dampening is not due to inefficient checkpoint signaling (37). As noted above, Rad53 is partially activated in *asf1*Δ cells grown under normal conditions, consistent with the fact that the DDR pathway is partly activated in these cells (36) (Fig.

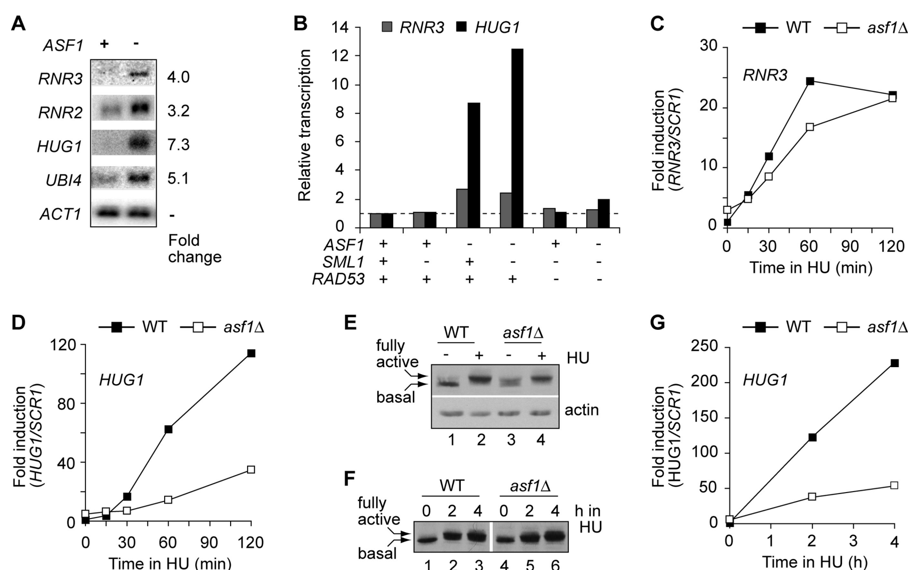

FIGURE 1. **Asf1 dependence of *HUG1* and *RNR3*.** *A*, Northern blotting analysis of selected genes during normal growth. *ACT1* is the loading control. *B*, graph showing the effects of selected mutations on transcription of *RNR3* and *HUG1*, both of which were normalized to the *SCR1* control. *SML1* is deleted to allow viability of *rad53*Δ cells. The transcripts were detected by Northern blotting. *C*, Northern blotting analysis of HU induction of *RNR3* transcription. *D*, Northern blotting analysis of HU induction of *HUG1* transcription. *E*, immunoblotting analysis of Rad53 modification state (same cells as in *C* and *D*). Fully active Rad53 is retarded in its migration compared with basal state Rad53. Actin is the loading control. *F*, immunoblotting analysis of Rad53 modification state. *Lanes 1–3* and *4–6* are from different gels. *G*, Northern blotting analysis of *HUG1* transcription after prolonged exposure to HU (same cells as in *F*).

1*E*, compare *lanes 1* and *3*). Importantly, Rad53 was fully activated in *asf1*Δ cells after prolonged treatment with HU, but *HUG1* transcription was still blocked (Fig. 1, *F* and *G*). Therefore, cells lacking *ASF1* are unable to fully derepress *HUG1* in response to replication stress, even though the checkpoint can be fully engaged.

Asf1 Association with Chromatin Is Globally Induced under Conditions of Replication Stress—It has been proposed that Asf1 can stimulate transcription by disassembling nucleosomes in the course of its untargeted association with chromatin (see Introduction) (10, 16). Asf1-dependent nucleosome disassembly can occur in promoters and coding regions and might require physical association of Asf1 with genes under its control (8, 38, 39). In addition to genes, Asf1 has been localized to an origin of replication in HU-treated cells and a region downstream of that origin (38). These observations led us to explore the possibility that Asf1 might be present at the promoters of *RNR3* and *HUG1* to directly facilitate transcriptional derepression. To address this possibility, we performed ChIP of Asf1-Myc using a modified version of a protocol developed in the Struhl lab (29). Validation of this modified protocol is shown in supplemental Fig. S3.

Consistent with the idea that Asf1 is present at the promoters of DDR genes to directly reconfigure nucleosomes, cross-linking of Asf1 to the promoters of *RNR3* and *HUG1* was readily detected by ChIP in wild type cells grown under normal conditions (Fig. 2*A* shows the raw data from a representative experiment). However, Asf1 also cross-linked to all other tested loci, namely a gene-free region (TELV amplicon), and the promoters of *PHO5*, *DSE1*, and *SCR1*. The ChIP results for *RNR3* and *HUG1* are quantitated in Fig. 2*B*; Asf1 was enriched at each promoter by ~20-fold over a control with no antibody.

Asf1 was also detected in the chromatin fraction obtained from unfixed cells by a conventional biochemical approach (Fig. 2*C*, *lane 3*). These data support the idea that random association of Asf1 with chromatin in normally cycling cells makes it available for direct reconfiguration of nucleosomes (16) in the course of checkpoint induction of DDR genes.

The fact that Asf1 randomly associates with chromatin under noninducing conditions does not preclude the possibility that its association with target promoters is responsive to signals that affect transcription. Indeed, there is some evidence that association of Asf1 with the promoters of target genes is regulated by signals that affect transcription. For example, glucose availability more strongly affects Asf1 occupancy of glucose-responsive promoters than a gene-free region (supplemental Fig. S2*A* in Ref. 8).

Therefore, we used ChIP to test whether HU induces Asf1 cross-linking to the promoters of DDR genes. As anticipated (38), Asf1 could be cross-linked to chromatin in HU-treated cells. Given the possibility of formaldehyde quenching by HU (40) (Fig. S4, *A* and *B*), we performed cross-linking in medium lacking HU (see supplemental data). Using an HU washout procedure, we found that treatment with HU caused a 2–3-fold increase in Asf1 cross-linking at *RNR3* and *HUG1* (Fig. 2*D* and supplemental S4, *B* and *C*). This increase in Asf1 association with chromatin upon HU treatment is similar in magnitude to induction of Asf1 cross-linking by other physiological stimuli (8).

Hydroxyurea induction of Asf1 cross-linking to chromatin, however, was not specific to DDR genes (Fig. 2, *A* and *D*); it occurred at a gene-free region (TELV), an Asf1-dependent gene that is not induced under any of the conditions we use (*PHO5*), a gene that is repressed by HU (*DSE1* (41)), and an RNA polymerase III-transcribed gene (*SCR1*). Immunodeple-

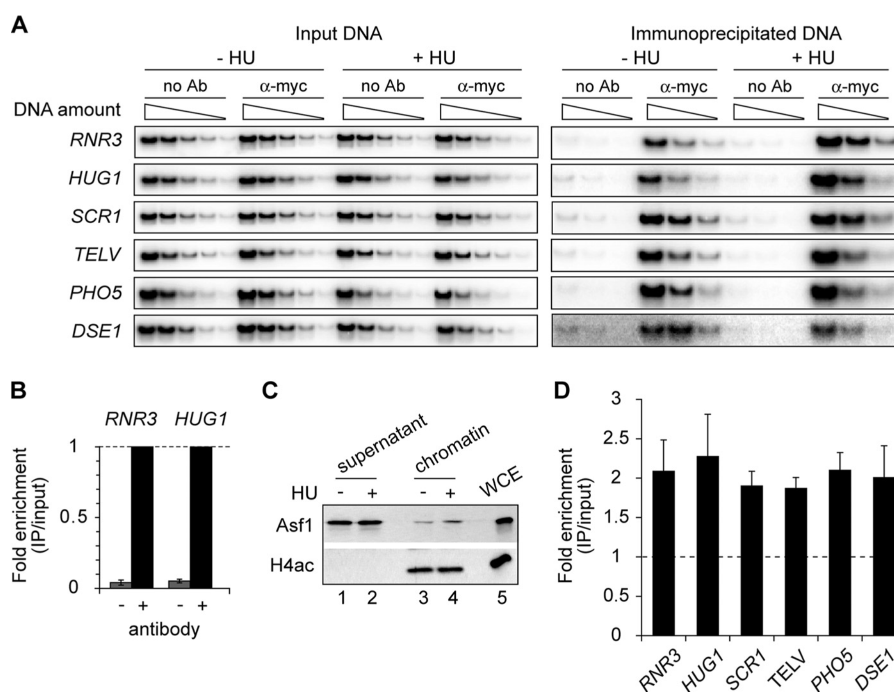

FIGURE 2. **Constitutive and inducible association of Asf1 with chromatin.** *A*, Asf1-Myc cross-linking to specific genomic locations, as detected by ChIP. *B*, quantitative comparison of the Asf1-Myc signal at *HUG1* and *RNR3* from ChIP experiments performed with or without anti-Myc antibody (*Ab*). PCR signal from immunoprecipitations that included antibody was set to 1 for each primer pair. The experiment was performed in triplicate; the *error bars* indicate standard deviations. *C*, immunoblotting analysis of Asf1-Myc in the chromatin and nonchromatin fractions from unfixed cells. As expected, tail-acetylated H4 (H4ac) is enriched in the chromatin fraction. *D*, quantitation of ChIP analysis of Asf1 occupancy at specific loci in HU-treated cells relative to untreated cells (the latter was set to 1). The experiments were performed at least in triplicate; the *error bars* indicate standard deviations from the mean.

tion of Asf1-Myc was essentially identical from whole cell extracts prepared from untreated or HU-treated cells (supplemental Fig. S5*A*), suggesting that previous treatment with HU does not influence the efficiency of Asf1-Myc immunoprecipitation from whole cell extracts. Importantly, bulk expression of Asf1-Myc did not change upon HU treatment (supplemental Fig. S5*B*). Therefore, the observed recruitment of Asf1-Myc to DNA during HU treatment is authentic. Furthermore, fixation was not required to detect inducible association of Asf1 with bulk chromatin (Fig. 2*C*, *lanes 3* and *4*; note that the bulk chromatin fractionation protocol is less stringent than the ChIP method for detection of Asf1 on chromatin). These results indicate that stimulation of Asf1 association with chromatin, as revealed by ChIP, is due to *de novo* recruitment rather than increased cross-linking of Asf1 molecules that reside permanently on chromatin.

Asf1 association with chromatin also increased when cells were treated with methyl methanesulfonate (MMS), another inducer of replication stress that triggers derepression of the DDR genes (42) (supplemental Fig. S6). Thus, Asf1 is recruited to chromatin when cells experience replication stress caused by HU or MMS. Because MMS lacks a free amine group, it is not expected to quench formaldehyde. Indeed, the processing protocol that revealed MMS induction of Asf1 association with chromatin (supplemental Fig. S6) did not include a washout step. This finding supports our conclusion that HU induction of Asf1 cross-linking to chromatin observed in experiments that involved HU washout is a true response to replication stress. Considering our transcriptional data (Fig. 1), and current intense interest in the function of Asf1 in human cells treated with HU (43–45), we chose to further study the effects of HU on Asf1 function in DDR gene regulation.

The experiments outlined above were performed in the BY4741 strain background often used for large scale genetic analysis in yeast (23). The results are not restricted to this strain background: HU also induced Asf1 cross-linking in a W303 (46) derivative harboring Asf1-Myc (supplemental Fig. S7). Additionally, although Asf1 recruitment to chromatin during HU treatment seemingly occurs in a nonspecific fashion, this effect is not generally observed for proteins involved in histone regulation. In particular, chromatin association of the Rtt109 lysine acetyltransferase, which requires Asf1 for H3K56-directed enzymatic activity, did not increase in response to HU treatment (supplemental Fig. S8).

Collectively, our transcription and ChIP experiments show that derepression of *RNR3* and *HUG1* occurs concomitantly with increased recruitment of Asf1 to their promoters during replication stress. However, given that HU seems to globally induce the association of Asf1 with chromatin, HU-induced recruitment of Asf1 to the promoters of DDR genes may or may not be required for their transcriptional derepression. These observations raise two main possibilities: 1) Asf1 recruitment to the promoters of DDR genes is needed for their subsequent derepression or 2) Asf1 recruitment occurs at the same time as, but is dispensable for, gene derepression.

Asf1 Association with Chromatin: New Functions for Known Asf1 Motifs—To directly test whether Asf1 association with chromatin is important for derepression of DDR genes, we first identified Asf1 mutations that dampen its cross-linking

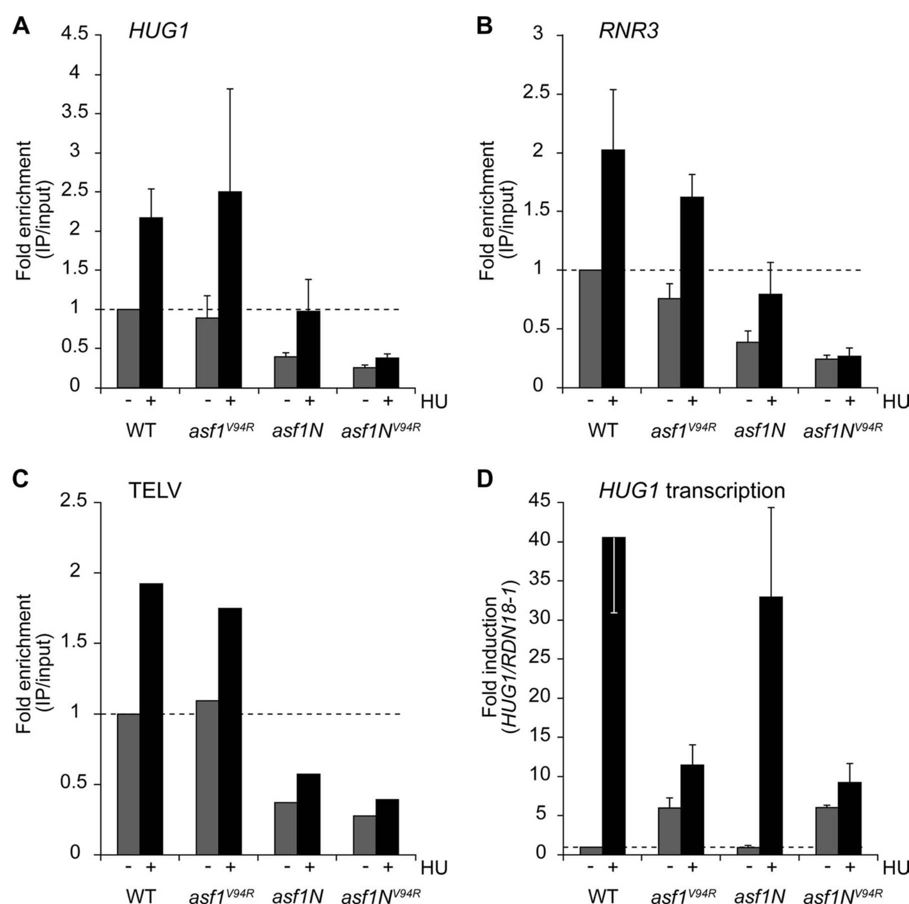

FIGURE 3. **Association of Asf1 with chromatin is not needed for its ability to promote derepression of the DDR genes.** Wild type and mutant versions of Asf1 were assayed for cross-linking to *HUG1* (*A*), *RNR3* (*B*), and the TELV geneless region (*C*) by ChIP. Protein occupancy is normalized to the signal for wild type Asf1 in untreated cells (set to 1). *A* and *B* were performed in triplicate; the *error bars* indicate standard deviations from the mean. *C* shows the results of a single experiment. *D*, relative transcription of *HUG1* normalized to *RDN18-1*. The RT-PCR signal obtained from untreated wild type cells is set to 1. The *bars* represent the averages of two independent experiments. The *error bars* indicate the range.

to chromatin. Yeast Asf1 consists of two domains (supplemental Fig. S9*A*). Amino acids 1–155 comprise its conserved N terminus (asf1N), whereas amino acids 156–279 comprise an acidic C-terminal region, which is restricted to *Saccharomyces* (although phosphorylation might provide a similar negatively charged region in human Asf1) (47). The conserved N-terminal domain binds tightly to the H3-H4 dimer to form a complex that is possibly stabilized by the C-terminal domain (48, 49).

We predicted that association of Asf1 with chromatin would depend primarily on binding of its N-terminal domain to H3 and H4 because mutation of Asf1 valine 94 to arginine nearly abolishes the ability of Asf1 to bind to histones (50). The V94R mutation confers phenotypes observed in the null mutant but does not affect overall protein expression (50–52) (supplemental Fig. S9*B*). In particular, like *asf1*Δ cells, $asf1^{V94R}$ mutants are sensitive to HU and accumulate with a G_2/M DNA content when grown under normal conditions (supplemental Fig. S9, *C* and *D*). Surprisingly, this mutation had no effect on constitutive or HU induction of Asf1 binding to chromatin. That is, the $asf1^{V94R}$ and wild type Asf1 proteins associated with *HUG1*, *RNR3*, and TELV at comparable levels both under normal conditions and during HU treatment (Fig. 3, *A*–*C*). Therefore, Asf1 can associate with chromatin both constitutively and under conditions of replication stress, even when it is unable to robustly bind the H3/H4 dimer. This finding indicates that binding of H3/H4 by the N-terminal domain of Asf1 is not necessary and sufficient for HU induction of Asf1 association with chromatin.

We next tested whether deletion of the C-terminal domain of Asf1 affects its binding to chromatin. asf1N, which lacks the C-terminal domain, was expressed at a slightly higher level than wild type Asf1 during normal growth and grew similarly to wild type cells in the presence of HU (supplemental Fig. S9, *B* and *C*). Nonetheless, asf1N occupancy at *HUG1*, *RNR3*, and TELV was on average 60% lower than wild type (Fig. 3, *A*–*C*). The wild type and *asf1N* strains had almost identical cell cycle profiles (supplemental Fig. S9*D*), so the low ChIP signal for asf1N is not an indirect consequence of abnormal cycling. We conclude that the C-terminal domain of Asf1, on its own, contributes to constitutive, untargeted association of Asf1 with chromatin. Even though the total level of asf1N association with chromatin after HU treatment was decreased compared with wild type, binding of asf1N to *HUG1* and *RNR3* was induced by the same fold as wild type when cells are treated with HU (Fig. 3, *A* and *B*). Therefore, the C-terminal domain of Asf1 is dispensable for its further

recruitment to the promoters of DDR genes under conditions of replication stress.

Based on the results for strains expressing asf1N and asf1^{V94R}, we hypothesized that histone binding by the core domain of Asf1 accounts for the residual capacity of asf1N to associate with chromatin. Consistent with this hypothesis, asf1N harboring the V94R mutation had very low chromatin binding activity, even in HU-treated cells (Fig. 3, *A–C*; note that this protein is expressed at the wild type level (supplemental Fig. S9*B*)). This effect could not be explained by perturbed cell cycling because the asf1N^{V94R} mutant exhibited the same DNA flow cytometry profile as cells expressing asf1^{V94R} (supplemental Fig. S9*D*), which cross-linked normally to chromatin (Fig. 3, *A–C*). In addition, the asf1N^{V94R} double mutant was not more HU-sensitive than the asf1^{V94R} single mutant (supplemental Fig. S9*C*). Given these results, and the fact that the human equivalent of yeast asf1N^{V94R} folds normally (50), we conclude that in the absence of the acidic C-terminal tail, the conserved histone-binding domain of Asf1 becomes more important for the ability of Asf1 to associate with chromatin.

The Relationship between Transcription and Association of Asf1 with Chromatin—We next determined how the regulation of *HUG1* is affected by the capacity of Asf1 to associate with chromatin. *HUG1* was selected for this analysis because of its strong dependence on Asf1 for derepression (Fig. 1, *D* and *G*). Given that cells expressing asf1^{V94R} phenocopy *asf1*Δ cells, we were not surprised that *HUG1* was modestly derepressed in the *asf1*^{V94R} mutant under normal conditions (Fig. 3*D*; compare with Fig. 1*D*). However, asf1^{V94R} was not more strongly cross-linked to the promoter of *HUG1* than the wild type protein under these conditions (Fig. 3*A*). Deletion of the C-terminal domain of Asf1 strongly inhibited its association with *HUG1* but had little effect on basal transcription (Fig. 3, *A* and *D*). These results suggest that constitutive association of Asf1 with chromatin, on its own, is not important for *HUG1* transcription under normal conditions. The same is likely true under inducing conditions. The V94R mutation, even in the context of full-length Asf1, severely compromised *HUG1* induction by HU (Fig. 3*D*) without affecting recruitment upon HU treatment (Fig. 3*A*). It follows that the V94R mutation confers a defect in transcriptional induction that is unrelated to the capacity of Asf1 to bind to chromatin. Importantly, asf1N supported almost normal induction of *HUG1* transcription (Fig. 3*D*), despite the fact that its association with chromatin in HU-treated cells increased only to the baseline observed for wild type protein in unstimulated cells (Fig. 3*A*). Collectively, our studies of Asf1 mutants suggest that Asf1 promotes transcriptional induction of *HUG1* by a mechanism that involves binding of its core domain to H3-H4 but not its inducible association with chromatin.

H3K56 Acetylation Is Important for Derepression of HUG1 under Conditions of Replication Stress—Although Asf1 association with chromatin is probably not a major driving force in transcriptional derepression of the DDR genes under conditions of replication stress, cells lacking *ASF1* are strongly impaired for derepression of *HUG1* and delayed for derepression of *RNR3* (Fig. 1, *C* and *D*). This suggests that Asf1 promotes derepression of the DDR genes via a mechanism that does not require Asf1 to act directly at these promoters. In further investigating the mechanism used by Asf1 to promote DDR gene transcription in response to replication stress, we considered the recent evidence from the Tyler lab that H3K56 acetylation is important for the induction of *PHO5* upon phosphate limitation (10). Because Asf1 is absolutely required for H3K56 acetylation and H3K56 acetylation increases the breathability of nucleosomal DNA on/off the histone octamer (12), it seemed likely that Asf1 might promote derepression of DDR genes through its positive effect on the H3K56 acetylation reaction catalyzed by Rtt109.

To explore this possibility, we first compared *HUG1* and *RNR3* transcription in *asf1*Δ and *rtt109*Δ mutants, both of which lack H3K56 acetylation. As in *asf1*Δ cells, *HUG1* and *RNR3* were moderately derepressed under normal conditions in *rtt109*Δ cells (Fig. 4, *A* and *B*). This result was expected because both mutants show chronic checkpoint activation (4, 36), as revealed by partial Rad53 activation (Fig. 1*E*). Under replication stress, *HUG1* derepression was severely compromised in cells lacking *ASF1* or *RTT109*, and *RNR3* derepression was delayed in both mutants. This suggests that H3K56 acetylation may be needed for optimal derepression of *HUG1* and *RNR3*.

To more directly explore this possibility, we examined the regulation of DDR genes in H3K56Q, K56A, and K56R mutants, which lack H3K56 acetylation (supplemental Fig. S10). While the K56Q and K56A mutations mimic constitutive acetylation of residue 56, the K56R mutation mimics permanent deacetylation (53).

Under normal conditions, *HUG1* and *RNR3* were partially derepressed in cells harboring the H3K56Q or H3K56A mutation (Fig. 4, *C* and *D*). This finding is consistent with the observation of Celic *et al.* (54) that *HUG1* and *RNR3* are upregulated in *hst3*Δ *hst4*Δ cells, which lack the histone deacetylases responsible for deacetylating H3K56. Therefore, during normal proliferation the DDR genes are partly activated in cells containing or mimicking high levels of H3K56 acetylation. These findings are consistent with the possibility that transcription of DDR genes is favored by H3K56 acetylation.

Our studies of DDR gene regulation in H3K56 mutants under conditions of replication stress in fact suggest direct dependence of derepression on H3K56 acetylation. Derepression of *HUG1* and *RNR3* by HU is more robust in H3K56A and H3K56Q mutants than in the parental wild type strain (Fig. 4, *C* and *D*). Because this mutant phenotype is not associated with hyperinduction of Rad53 (Fig. 4, *E* and *F*; see also Ref. 55), H3K56 acetylation stimulates derepression of DDR genes by a mechanism that does not depend on its possible effects on checkpoint signaling.

The conclusion that H3K56 acetylation potentiates DDR gene depression independently of its effects on checkpoint signaling is supported by the results obtained for the H3K56R mutant. Derepression of *HUG1* is severely compromised in this mutant (Fig. 4*C*). Derepression of *RNR3* is also compromised but only delayed (by 1 h; Fig. 4*D*). Because *RNR3* is eventually induced to the wild type level in H3K56R cells,

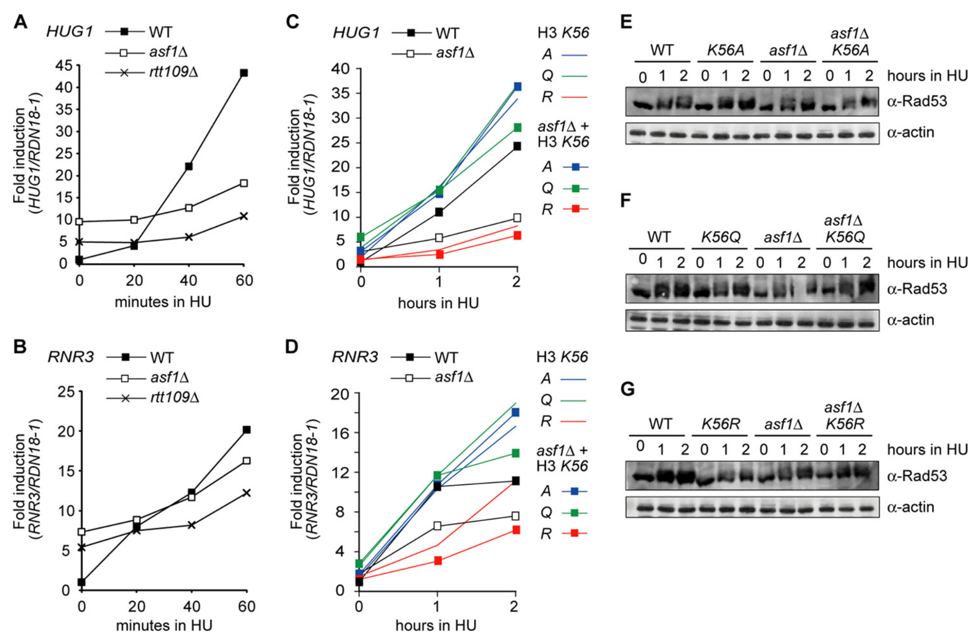

FIGURE 4. **Effects of chromatin regulators and H3K56 acetylation on transcription of two DDR genes.** *A*, *HUG1* derepression is compromised in *asf1*Δ and *rtt109*Δ cells. *HUG1* transcription in wild type and mutant strains, with or without HU treatment, was normalized to *RDN18-1*; RT-PCR signal in wild type, untreated cells was set to 1. Time points represent the averages from two independent experiments. *B*, analysis of *RNR3* transcription was performed as in *A*. *C*, analysis of *HUG1* derepression in the indicated strains was performed as in *A*. *D*, analysis of *RNR3* transcription (performed as in *B*). *E–G*, H3K56 mutants activate Rad53. Immunoblotting analysis of Rad53 modification state in the indicated mutants with or without HU treatment. The antibodies used were anti-Rad53 and anti-actin (loading control).

checkpoint signaling supported by the mutant is sufficient for full derepression of *RNR3* (even though Rad53 activation might be slightly dampened; Fig. 4*G*). It follows that the failure to fully derepress *HUG1* in H3K56R cells is not due to inadequate checkpoint signaling. We conclude that H3K56 acetylation by Rtt109 is important for derepression of *HUG1* and *RNR3* by replication stress checkpoint signals.

Because efficient acetylation of H3K56 by Rtt109 requires Asf1, it follows that Asf1 contributes to derepression of the DDR genes by stimulating H3K56 acetylation. This conclusion is strongly reinforced by our finding that the K56Q and K56A mutations suppress the transcriptional defects of the *ASF1* null strain (Fig. 4, *C* and *D*). Furthermore, the K56R mutation did not produce additive transcriptional defects in combination with a deletion of *ASF1*, indicating that *ASF1* and H3K56 acetylation function in the same pathway to contribute to derepression of DDR genes during replication stress.

H3K56 Acetylation at DDR Genes during Their Derepression—Studies of the *PHO5* gene have revealed that increased H3K56 acetylation favors induction of transcription (10). It was proposed that, under inducing conditions, an increased proportion of Lys^{56}-acetylated H3 molecules in the promoter facilitates high transcription because this modification may weaken nucleosomal histone-DNA contacts, thereby promoting chromatin disassembly. Therefore, we next tested whether the correlations between H3K56 acetylation and the genetic requirements for derepression reflect a similar role for H3K56 acetylation in the regulation of the DDR genes.

Initially, we determined how H3 occupancy at the promoters of *RNR3* and *HUG1* is affected under derepressing conditions. The bulk H3 antibody used for this ChIP experiment (Abcam ab1791) has been widely employed by others (10, 20), and in our hands on average yielded a 2,250-fold higher ChIP signal than control immunoprecipitations with no antibody. At both *RNR3* and *HUG1*, there was a modest trend toward decreased promoter cross-linking of H3 in wild type cells in the presence of HU (Fig. 5, *A* and *B*). This result is consistent with previous evidence that H3 and H4 are lost from the *RNR3* promoter when cells are treated with MMS (20, 33), although the effect of MMS at *RNR3* was much larger (*i.e.* histone loss was greater) than we observed using HU. We suspect that this difference may be due to the longer drug treatment times used by others or to differences between MMS and HU in the way that they affect overall cellular physiology. For example, MMS causes damage to lipids and RNA that HU is incapable of generating (56). Nevertheless, our results show minimal histone loss from the promoter of *RNR3*, even when it is maximally induced by HU treatment (Figs. 1*C* and 5*A*; 60 min). Similarly, only 25% of H3 is lost from *HUG1* at 1 h after HU addition (Fig. 5*B*), when transcription is induced by 40–50-fold (Figs. 1*D* and 3*D*). This may be the upper limit of H3 eviction at *HUG1*, because in one further experiment we observed only 18% lower H3 occupancy at 4 h after HU addition when *HUG1* is fully derepressed (~225-fold induction; Fig. 1*G*).

H3K56 acetylation was monitored by ChIP using a commercial H3K56ac antibody extensively validated in the literature (Upstate 07-677). We readily detected Lys^{56}-acetylated H3 at *RNR3* and *HUG1* under repressing and derepressing conditions. In the absence of HU, the ChIP signal for Lys^{56}-acetylated H3 was ~10-fold higher at the promoters of *RNR3* and *HUG1* in wild type cells than in *asf1*Δ cells, which lack H3K56 acetylation. Although Lys^{56}-acetylated H3 was also present at the *RNR3* and *HUG1* promoters in HU-treated cells, its enrichment was lower than in untreated cells (Fig. 5,

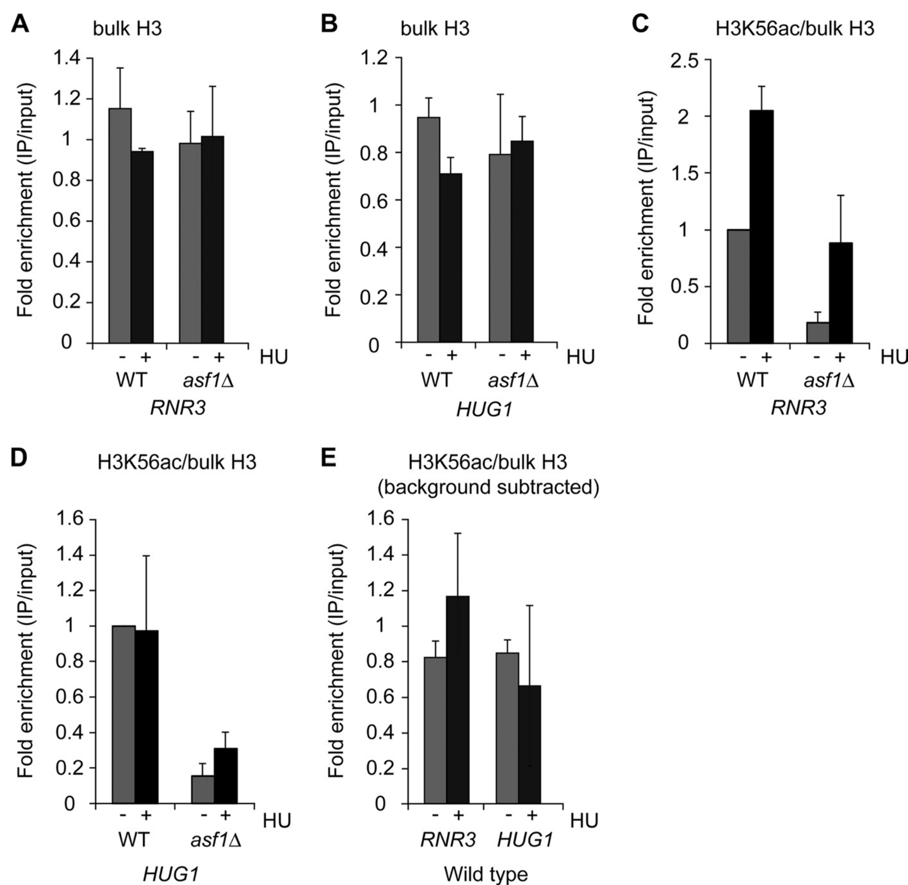

FIGURE 5. **Regulation of H3K56 acetylation in the promoters of *RNR3* and *HUG1*.** *A*, H3 cross-linking to the promoter of *RNR3*, as measured by ChIP. Immunoprecipitated DNA was normalized to input DNA and signal obtained in untreated cells was set to 1. *B*, H3 cross-linking to the promoter of *HUG1* was analyzed as in *A*. *C* and *D*, ChIP signals were obtained using the H3K56ac antibody to probe lysates from a strain lacking *ASF1*. Normalization was performed as in *A*, and the H3K56ac signal was normalized to bulk H3. *E*, H3K56 acetylation in the promoters of *RNR3* and *HUG1* was normalized to bulk H3, and then the HU-induced background signal revealed in *C* and *D* (also normalized to bulk H3) was subtracted. The *bars* represent the averages of at least three independent experiments. The *error bars* indicate the standard deviation.

C and *D*, compare fold differences in −*HU* (*gray bars*) with fold differences in +*HU* (*black bars*)). We determined that this reduction in enrichment was caused by variations in background binding of the antibody to chromatin. Specifically, we found that background binding of the H3K56ac antibody to chromatin in *asf1*Δ cells increased ~5-fold at *RNR3* and 2-fold at *HUG1* upon HU treatment (Fig. 5, *C* and *D*, +*HU*). This change will dampen real reductions and artificially inflate real increases in H3K56ac occupancy after HU treatment. Therefore, in further analyzing the H3K56ac ChIP results, we subtracted the background signal calculated for *asf1*Δ cells (−/+ HU) from the H3K56ac signal obtained for wild type cells (both normalized to bulk H3 cross-linking). Others have also reported that nonspecific binding of the H3K56ac antibody to chromatin can vary in *asf1*Δ cells. For example, in the careful study of Williams *et al.* (supplemental Fig. S3*B* in Ref. 10), the background H3K56ac signal at *PHO5* was found to fluctuate up to 3.7-fold between induction time points. Together, these findings point out the importance of performing all H3K56 acetylation ChIPs not only in the strain of interest but also in a congenic *ASF1* null strain or K56R mutant.

When effects on background binding are taken into account, the data reveal a slight increase or slight decrease in the proportion of Lys^{56}-acetylated H3 at the promoters of *RNR3* and *HUG1*, respectively, upon HU treatment (Fig. 5*E*). Therefore, derepression of the DDR genes under conditions of replication stress is not associated with a large increase in H3K56 acetylation, despite the fact that overall H3K56 acetylation is induced (by 5-fold after 3 h in 0.2 M HU (57)). In other words, whereas *HUG1* strongly requires H3K56 acetylation for full derepression (Fig. 4, *A* and *C*), its regulation differs from that of *PHO5*, where a transcription-coupled mechanism establishes a higher steady state condition of H3K56 acetylation concomitantly with transcriptional induction. Conversely, H3K56 acetylation increases slightly at the promoter of *RNR3* under derepressing conditions, even though H3K56 acetylation has a less important role at this promoter (it affects the kinetics of derepression but not the ability to fully derepress transcription). The results for *HUG1* and *RNR3* indicate that in the case of the DDR genes, an increase in the proportion of H3 that is Lys^{56}-acetylated in the promoter region is not needed for high transcriptional derepression.

DISCUSSION

Here we have shown that histone chaperone Asf1 is important for transcriptional derepression of two DDR genes in budding yeast under conditions of replication stress. In cells

lacking *ASF1*, derepression of *HUG1* in response to replication stress caused by treatment with HU is severely compromised, whereas derepression of *RNR3* is delayed (Fig. 1). Although Asf1 association with the promoters of *HUG1* and *RNR3* increased under these same conditions, we determined that HU induces Asf1 binding to chromatin nonspecifically (Fig. 2). That is, Asf1 associates constitutively with all regions of the genome tested thus far, and this association is globally induced by treatment with HU. Importantly, the identification of Asf1 mutants that are compromised in their ability to bind chromatin revealed that Asf1 association with the promoters of the DDR genes was not needed for their transcriptional derepression (Fig. 3).

The functional significance of Asf1 association with chromatin in budding yeast remains unknown. Our mapping of the determinants of this association, however, raises some interesting possibilities. We find that the acidic C-terminal tail of Asf1 is important for its binding to chromatin (Fig. 3). This domain stabilizes the interaction of Asf1 with replication factor C, which loads proliferating cell nuclear antigen onto DNA (38), and may strengthen the interaction between Asf1 and histones (48, 51) by a mechanism that does not involve H3K56 acetylation (57). Furthermore, a mutation that virtually eliminates Asf1 binding to the H3/H4 dimer (50) ($asf1^{V94R}$) abolishes residual chromatin binding by the C-terminal tail mutant. Collectively, these findings are consistent with the notion that chromatin-associated Asf1 has a role in the control of replication (38) that is directly tied to nucleosome metabolism at forks. Because HU treatment causes S phase arrest, it could be that HU induction of Asf1 cross-linking to chromatin in mixed populations of cells is a reflection of the increased proportion of replicating cells in such populations.

The fact that Asf1 promotes transcription of the DDR genes even when its binding to their promoters is impaired led us to hypothesize that Asf1 contributes to derepression of the DDR genes other than by direct chromatin disassembly. Indeed, we show that H3K56 acetylation, which is catalyzed by Rtt109 and requires Asf1 as a cofactor, is important for derepression of the DDR genes upon treatment with HU (Fig. 4). Mutations that perturb the H3K56 acetylation reaction (*rtt109*Δ, *asf1*Δ, and $asf1^{V94R}$) or mimic the unacetylated state (H3K56R) severely compromise derepression of *HUG1* and cause *RNR3* derepression to be delayed. Conversely, derepression of the DDR genes is normal or better than normal in cells expressing mutations that mimic constitutive H3K56 acetylation (H3K56Q and H3K56A).

In an important model based on studies of *PHO5*, one step in the pathway of transcriptional induction by H3K56 acetylation is induction of promoter acetylation (relative to H3 occupancy) (10). We find that H3K56 acetylation is only slightly increased at the promoter of *RNR3* and slightly decreased at the promoter of *HUG1* under derepressing conditions (Fig. 5). Therefore, regulation of DDR genes does not seem to conform to the model described previously for *PHO5*. That is, the promoters of *RNR3* and *HUG1* do not shift from a state of low to high H3K56 acetylation upon derepression.

This difference between *PHO5* and the DDR genes raises two possibilities. On the one hand, the dependence of *RNR3* and *HUG1* gene derepression on H3K56 acetylation might reflect an indirect effect of H3K56 acetylation on transcriptional regulation of these genes. We have not ruled out all of the potential mechanisms by which abnormal regulation of H3K56 acetylation might indirectly impact transcriptional control of the DDR genes. However, because Rad53 activation remains quite robust in mutants compromised for H3K56 acetylation and derepression of *HUG1* (Figs. 1, *E* and *G*, and 4, *C* and *G*), it is unlikely that abnormal regulation of H3K56 acetylation has a detrimental effect on DDR gene expression by virtue of interference with checkpoint signaling.

A plausible alternative to indirect regulation of DDR gene derepression by H3K56 acetylation is direct regulation by similar mechanisms that apply at other genes, including *PHO5*. Perhaps most importantly, H3K56 acetylation in the promoters of DDR genes may increase the plasticity of nucleosomes and therefore the permissiveness of promoter chromatin for transcription (12).

If this scenario is correct, then why is derepression of the DDR genes not associated with increased H3K56 acetylation of promoter chromatin (as observed upon induction of *PHO5*)? A simple answer to this question could be that the promoters of DDR genes are already marked by high H3K56 acetylation when they receive the HU-dependent signal for derepression. In this model, chromatin marking by H3K56 acetylation is due to replication-coupled nucleosome assembly, and high H3K56 acetylation in the promoters of DDR genes facilitates their immediate activation upon dissociation of the Crt1 and Ssn6-Tup1 repressors in response to replication stress in the S phase.

The idea that Lys^{56}-acetylated H3 deposited by replication-coupled chromatin assembly is maintained in the promoters of DDR genes, thereby poising them for derepression, is supported by several facts. First, HU elicits derepression of DDR genes only in cells that are in the S phase. *PHO5* induction, however, can occur outside of the S phase (58). Second, overall H3K56 acetylation of chromatin is dramatically induced during the S phase, and all newly synthesized H3 molecules used for replication-coupled nucleosome assembly are acetylated at Lys^{56} (53, 59). Therefore, derepression of the DDR genes occurs at a time when replication-coupled chromatin assembly increases the probability that the promoters of newly replicated DDR genes will harbor one or more H3K56-acetylated nucleosomes. Third, high H3 acetylation in the promoters of *RNR3* and *HUG1* is insufficient for their derepression (19), indicating that high levels of acetylation can be tolerated at these promoters. Similarly, an H3K56Q mutant that mimics permanent acetylation shows only partial derepression of the DDR genes under normal conditions (Fig. 4, *C* and *D*). We therefore propose that H3K56 acetylation poises newly replicated DDR genes for derepression in the event that replication interference triggers the checkpoint response.

An inherent risk in maintaining genes in a poised state is an increased likelihood of spurious transcription under repressing conditions. In this regard, poising by H3K56 acetylation might be well tolerated at DDR genes because, unlike *PHO5*,

the DDR genes are actively repressed by a *trans*-acting factor (Crt1) during normal growth (17). Because *PHO5* transcription is not blocked by binding of a transcriptional repressor to its promoter, H3K56 acetylation at this location may be kept low to prevent spurious transcription during normal growth. Upon phosphate removal, increased incorporation of Lys^{56}-acetylated H3 into the *PHO5* promoter would facilitate high levels of *PHO5* transcription. Therefore, previous evidence for dissimilar regulation of *PHO5* and DDR gene transcription (*de novo* activation *versus* derepression) may provide an explanation for the difference in regulation of H3K56 acetylation at the DDR genes compared with *PHO5*. To summarize, we propose that the DDR genes are maintained in a poised but repressed state during normal proliferation by the combined action of the replication-coupled chromatin assembly machinery, which reconstitutes Lys^{56}-acetylated H3 into new nucleosomes, and Crt1, which recruits the Ssn6-Tup1 corepressor complex.

This model predicts that derepression of the DDR genes by HU will be dampened in mutants in which the activity of replication-dependent H3/H4 chaperones is compromised. Because deposition of Lys^{56}-acetylated H3 into nucleosomes is reduced in cells lacking both Rtt106 and a component of CAF-I (2), it is possible that DDR gene repression will be compromised in mutants lacking Rtt106 and a component of CAF-I. However, additional histone chaperones are also thought to contribute to replication-coupled H3/H4 deposition into nucleosomes. Once the full complement of replication-coupled H3/H4 chaperones has been identified (this work is ongoing), it should be feasible to rigorously test whether disruption of replication-coupled H3/H4 assembly into nucleosomes compromises derepression of DDR genes by HU.

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