MOLECULAR MECHANISMS THAT COORDINATE HISTONE AND DNA SYNTHESIS IN *S. CEREVISIAE*

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INTRODUCTION

During S-phase, the newly replicated DNA is packaged into chromatin with the help of histone proteins immediately behind the replication fork. It is crucial to strictly coordinate DNA and histone synthesis as any imbalance can lead to deleterious effects such as genomic instability. Cells have evolved numerous strategies to achieve this coordination and the bulk of histone synthesis occurs only during DNA replication in S-phase. Drugs that interfere with DNA replication elicit an evolutionarily conserved response that leads to a rapid decrease in histone transcript levels. However, the molecular mechanisms responsible for this down regulation of histone transcripts upon replication arrest or DNA damage are not completely understood.

OBJECTIVE

Our research is aimed at understanding how DNA damage checkpoint kinases orchestrate the balance between histone synthesis and DNA replication following replication arrest or DNA damage using budding yeast as a model system. Here we dissect the molecular mechanisms that lead to the down regulation of histone transcript levels upon replication arrest and DNA damage.

Figure 1

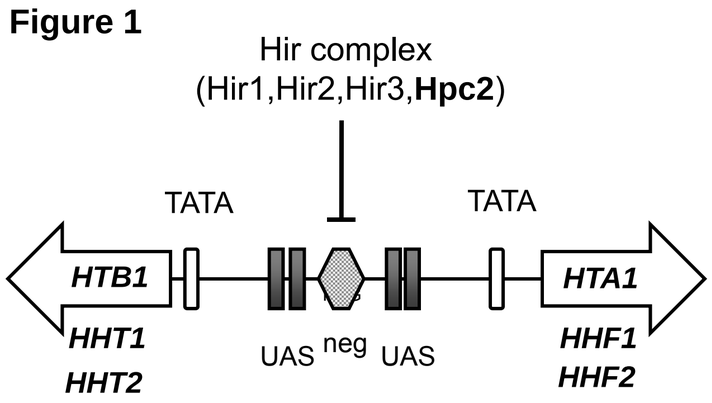

Fig. 1. Organization of histone gene pairs in the budding yeast. All yeast histone genes exhibit the same organization, except *HTA2-HTB2*, which lacks the neg element and hence is regulated in a Hir independent manner**.** *HTB1* = Histone Two B copy 1; *HTA1* = Histone Two A copy 2; TATA = tata box; UAS= upstream activating sequence; neg = Negative element involved in the Histone Regulator (Hir) complex-mediated transcriptional repression of histone genes outside of S-phase and in response to genotoxic reagents.

METHODS

- Analyze the role of the checkpoint kinase Rad53 in the down-regulation of histone mRNAs in response to replication arrest with hydroxyurea (HU) or DNA damage with Methyl Methane Sulfonate (MMS) by Northern blotting.
- Evaluate the role of upstream kinases Mec1/Tel1 in the Rad53 mediated down regulation of histone mRNAs in response to replication arrest or DNA damage.
- Use Chromatin immunoprecipitation assays to determine if the RSC chromatin remodeling complex is recruited to histone promoters following DNA damage and replication arrest.
- Test if Mec1/Rad53 phosphorylates the Hpc2 subunit of the Histone regulator complex (Hir) in response to DNA damage and replication arrest to repress histone gene transcription. Use mass spectrometric approaches and site directed mutagenesis to identify phosphorylation sites on Hpc2 that are essential for histone gene repression.

RESULTS

Figure 2

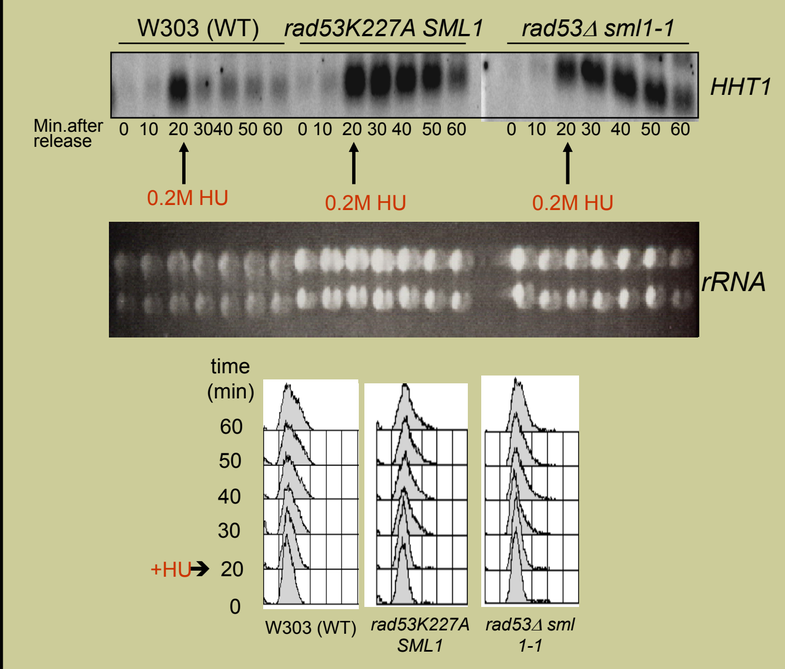

Figure 3

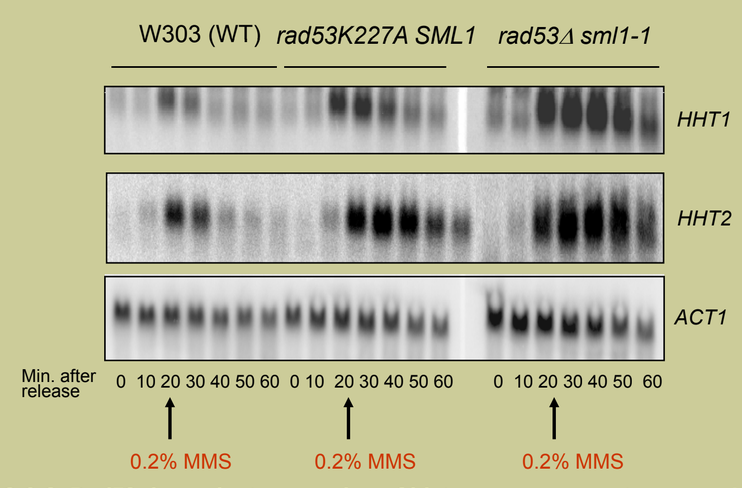

Fig. 2 & 3. Rad53 dependent repression of histone gene promoters upon HU or MMS mediated replication arrest. Wild type (W303-WT), *rad53* kinase dead mutant (*rad53K227A*) and *rad53* deletion mutant (*rad53*Δ) strains were arrested in G1 with a-factor. Cells were released from G1 arrest to allow progression into S-phase and were treated with HU or MMS 20 minutes after release from G1 arrest. Cells were harvested every 10 minutes and Northern blots were performed to quantitate the levels of both copies of histone H3 (*HHT1* and *HHT2*) transcripts. Ethidium bromide stained rRNA or actin mRNA is shown as a loading control. The FACS analysis of the cells shows the arrest of all the strains in S-phase upon the addition of HU. The *sml1-1* allele allows the viability of *rad53* deletion mutant cells.

Figure 4

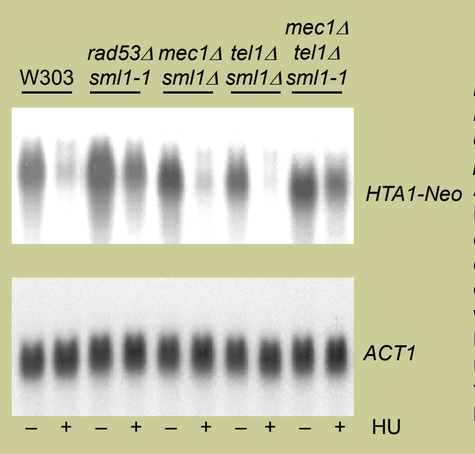

Fig. 4. Histone gene repression in response to hydroxyurea is dependent on the activity of the protein kinases Mec1 and Tel1. Asynchronous populations of the indicated strains carrying a plasmid encoding a neomycin reporter gene driven by the *HTA1* promoter were either left untreated or incubated with 0.2M HU for 25min. Northern blots were performed to detect the neomycin mRNAs. *ACT1* transcripts were monitored as loading controls.

Figure 5

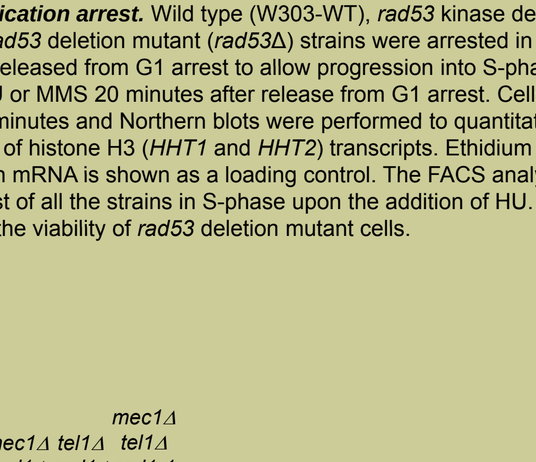

Fig. 5. Histone gene repression in response to replication arrest is dependent upon the continuous activity of the upstream kinases Mec1 and Tel1. Histone gene transcription is rapidly activated following release from HU arrest and concomitant Rad53 dephosphorylation. Asynchronously growing wild type W303 strain carrying a plasmid encoding a neomycin reporter gene driven by the *HTA1* promoter was treated with 0.2M HU for 90min following which the HU was washed away and cells were grown in the absence of HU for the indicated time periods. The upper panel shows *Neo* reporter transcript levels whereas the middle panel depicts the 26S rRNA levels as a loading control. The bottom panel shows a Western blot performed to detect Rad53 phosphorylation in the same samples. The FACS profile of the cells at the point of harvesting is shown on the right.

Figure 6

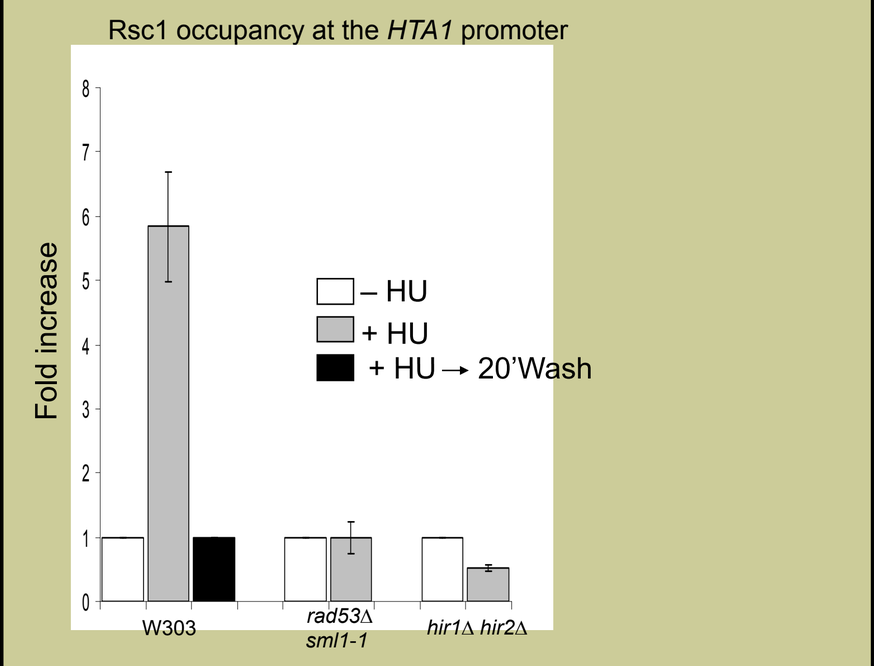

Fig.6. HU-induced repression of the HTA1-HTB1 promoter is triggered by Rad53-dependent recruitment of the RSC chromatin remodeling complex to the promoter. (A) Rsc1 is recruited to the *HTA1* promoter in response to HU. Asynchronously growing wild type (W303), *rad53* and *hir1 hir2* mutant cells expressing Rsc1-Myc were fixed with formaldehyde either before (0 min) or 40min after HU addition. The HU-induced recruitment of Rsc1-Myc to the *HTA1-HTB1* promoter was quantitated using chromatin immunoprecipitation assays and real time PCR. For each immunoprecipitated DNA sample, PCR was performed in triplicate and the signal for the *HTA1-HTB1* promoter was normalized to background signal derived from the *MATa* locus. The ratio of the two signals before HU addition was assigned a value of 1. The bar graph shows an average of data from four independent experiments for each strain.

Figure 7

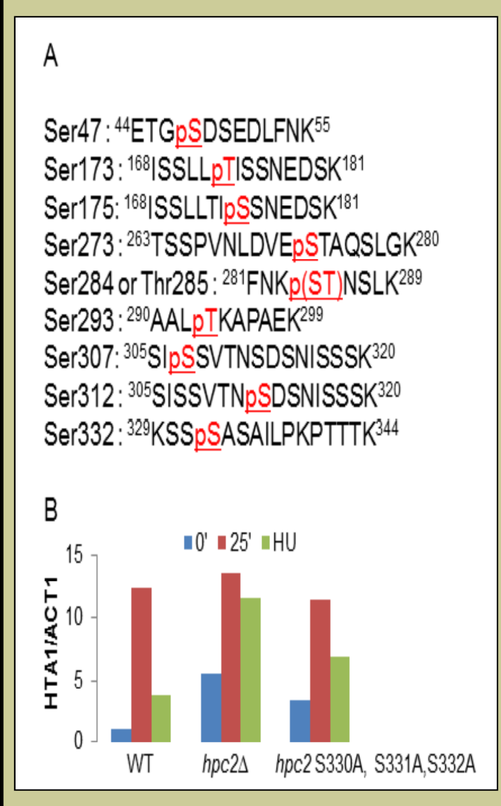

Fig. 7. Hpc2 residues are phosphorylated in vitro by Rad53 and (B) hpc2 phosphorylation mutants exhibit a defect in histone gene repression upon replication arrest.
As a first step towards confirming the published Rad53 dependent phosphorylation sites on Hpc2 (Smolka, et al., 2007; Chen et al, 2010) and detecting additional phosphorylation sites on Hpc2, we have carried out mass spectrometric analyses of Hpc2 following *in vitro* phosphorylation by Rad53 using purified recombinant proteins. A *hpc2* S330A, S331A, S332A phosphorylation mutant with the previously identified S332 was created by site directed mutagenesis. Exponentially growing *hpc2-phos*Δ cells were arrested with α–factor for two hours and released for 25 minutes at which time 0.2M HU was added for 20 minutes. Samples were analysed by (RT)-qPCR. Real Time PCR was performed with *HTA1* specific primers and TaqMan probes and *HTA1* expression levels were normalized to the expression level of *ACT1* and related to the WT 0 min sample which was arbitrary set to 1.

Figure 8

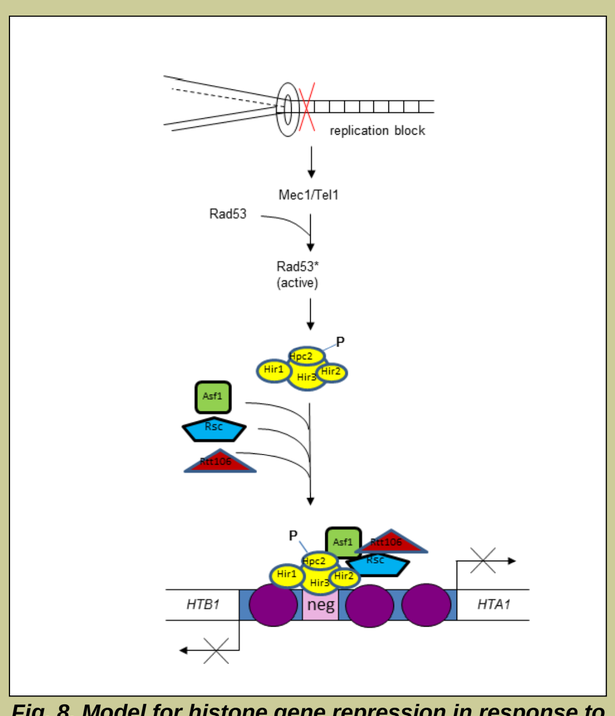

Fig. 8. Model for histone gene repression in response to replication arrest in budding yeast.

CONCLUSION

In this study we demonstrate that: 1.) checkpoint kinases are necessary for transcriptional repression of histone genes upon DNA damage and replication arrest. 2.) Rad53 mediates this repression by recruiting the RSC complex to the histone gene promoters and phosphorylating the Hpc2 subunit of the Hir complex.