Chd1 and Set2 negatively regulate DNA replication in

Saccharomyces cerevisiae

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ABSTRACT

Chromatin modifying factors regulate both transcription and DNA replication. The yFACT chromatin reorganizing complex is involved in both processes, and the sensitivity of some yFACT mutants to the replication inhibitor hydroxyurea (HU) is one indication of a replication role. This HU sensitivity can be suppressed by disruptions of the SET2 or CHD1 genes, encoding a histone H3(K36) methyltransferase and a chromatin remodeling factor, respectively. The additive effect of set2 and chd1 mutations in suppressing the HU sensitivity of yFACT mutants suggests these two factors function in separate pathways. The HU suppression is not an indirect effect of altered regulation of ribonucleotide reductase induced by HU. set2 and chd1 mutations also suppress the HU sensitivity of mutations in other genes involved in DNA replication, including CDC2, CTF4, ORC2, and MEC1. Additionally, a chd1 mutation can suppress the lethality normally caused by disruption of either MEC1 or RAD53 DNA damage checkpoint genes, as well as the lethality seen when a mec1 sml1 mutant is exposed to low levels of HU. The pob3 defect in S phase progression is suppressed by set2 or chd1 mutations, suggesting that Set2 and Chd1 have specific roles in negatively regulating DNA replication.
INTRODUCTION

Eukaryotic DNA is packaged into nucleosomes, which reduces access by DNA-binding proteins. Factors that modify chromatin structure therefore play essential roles in both transcription and DNA replication (BISWAS et al. 2005; BUDD et al. 2005; FORMOSA et al. 2001; FORMOSA et al. 2002; KROGAN et al. 2002; ORPHANIDES et al. 1999; SCHLESINGER and FORMOSA 2000). These factors include enzymes that modify histones post-translationally (BERGER 2007), ATP-dependent chromatin remodeling factors that reposition nucleosomes (CAIRNS 2005), and ATP-independent chromatin reorganizing factors such as the FACT complex (FORMOSA 2003). The mammalian FACT complex (facilitates chromatin transcription) was identified as a factor that promoted in vitro RNA polymerase II transcription using chromatin as a template (ORPHANIDES et al. 1998). The yeast FACT complex (yFACT) consists of two subunits, Spt16 and Pob3, and an associated HMG protein Nhp6 (BREWSTER et al. 2001; FORMOSA et al. 2001). In vitro studies show that yFACT alters the accessibility of DNA within nucleosomes without hydrolyzing ATP and without repositioning the histone octamer core relative to the DNA (BISWAS et al. 2005; FORMOSA et al. 2001; RHOADES et al. 2004).

Genetic and biochemical evidence indicate that yFACT has roles in both transcription and DNA replication. The SPT16 and POB3 genes are both essential for viability in Saccharomyces cerevisiae, and alleles with visible phenotypes have been isolated (MALONE et al. 1991; ROWLEY et al. 1991)(FORMOSA et al. 2001; SCHLESINGER and FORMOSA 2000). yFACT mutants show sensitivity to compounds that inhibit either transcriptional elongation or DNA replication, and they also display genetic interactions with both transcription and DNA replication mutants (BISWAS et al. 2005; BUDD et al. 2005; FORMOSA et al. 2001; FORMOSA et al. 2002; KROGAN et al. 2002; ORPHANIDES et al. 1999; SCHLESINGER and FORMOSA 2000). Evidence for a role for FACT in promoting transcription includes stimulation of transcription through a chromatin barrier in vitro
(ORPHANIDES et al. 1998), physical association of yFACT with elongation factors 
(KROGAN et al. 2002; SIMIC et al. 2003), binding of yFACT to transcribed regions of 
genes in vivo (MASON and STRUHL 2003; SAUNDERS et al. 2003), and reduced binding 
of TBP to promoters in yFACT mutants (BISWAS et al. 2005). A role for yFACT in 
replication is suggested by several observations, including physical interaction of yFACT 
with DNA polymerase alpha and Replication Protein A (VANDEMARK et al. 2006; 
WITTMEYER and FORMOSA 1997; WITTMEYER et al. 1999), delayed assembly of factors 
at a replication origin in a strain with mutations that cause reduced interaction between 
Pol alpha and Spt16 (Zhou and Wang 2004), decreased replication in Xenopus oocyte 
extracts from which FACT has been depleted, (OKUHARA et al. 1999), and sensitivity of 
some yFACT mutants to the replication inhibitor hydroxyurea (HU) (FORMOSA et al. 
2001; SCHLESINGER and FORMOSA 2000).

HU specifically blocks DNA synthesis by inhibiting ribonucleotide reductase (RNR) 
(EKlund et al. 2001), resulting in depletion of dNTP pools and stalled replication forks 
(KOC et al. 2004). A stalled replication fork triggers the DNA damage checkpoint 
pathway that requires the Mec1 and Rad53 kinases (homologous to human ATR and 
Chk2, respectively) (NEDELCHEVA-VELEVA et al. 2006). Checkpoint activation results in 
stabilization of stalled replication forks, inhibition of late-origin firing, blocking of 
mitosis, as well as increased expression of the RNR genes to compensate for decreased 
dNTP pools (PASERO et al. 2003). Yeast strains with mutations in DNA replication or 
checkpoint response genes often show sensitivity to HU in the growth medium (PARSONS 
et al. 2004). Additionally, deletion of either the MEC1 or RAD53 genes causes lethality, 
as these factors are absolutely essential to maintain the integrity of replication forks, even 
in the absence of any genotoxic or DNA replication stress (KAI and WANG 2003). 
Interestingly, the lethality caused by disruption of either MEC1 or RAD53 can be 
suppressed by a mutation in SML1, which encodes an inhibitor of ribonucleotide
reductase (ZHAO et al. 1998), as well as by overexpression of RNRI, encoding a subunit of ribonucleotide reductase (DESANY et al. 1998).

We have shown that transcriptional defects caused by yFACT mutation can be suppressed by mutations in two chromatin modifying factors, SET2 and CHD1 (BISWAS et al. 2006; BISWAS et al. 2007). Set2 encodes a histone methyltransferase that methylates K36 of histone H3 (STRAHL et al. 2002). Set2 is believed to play a role in transcriptional elongation, as Set2 associates with the elongating form of RNA polymerase II and modifies chromatin in transcribed regions (KROGAN et al. 2003; LI et al. 2003; LIU et al. 2005; POKHOLOK et al. 2005; RAO et al. 2005; XIAO et al. 2003). Additionally, set2 shows genetic interactions with genes implicated in elongation (BISWAS et al. 2006; KROGAN et al. 2003; LI et al. 2003). CHD1 encodes an ATP-dependent chromatin remodeler (TRAN et al. 2000) with a double chromodomain (FLANAGAN et al. 2007) and a Myb-related DNA-binding domain (WOODAGE et al. 1997). Like Set2, Chd1 associates with transcribed regions of genes (SIMIC et al. 2003) and shows physical and genetic interactions with elongation factors (BISWAS et al. 2007; KELLEY et al. 1999; KROGAN et al. 2002; SIMIC et al. 2003; TSUKIYAMA et al. 1999). Mutations in either SET2 or CHD1 suppress a variety of transcriptional defects caused by a yFACT mutation, including temperature sensitive growth, synthetic lethaliy betwen yFACT mutations and mutations of other transcription factors, defects in GALI gene induction, and defects in binding of TBP to promoters (BISWAS et al. 2006; BISWAS et al. 2007).

Because yFACT has a role in DNA replication, in this report we investigate the role of the Set2 and Chd1 chromatin modifying factors in regulating DNA replication. yFACT mutations can cause HU sensitivity, suggesting defects in DNA replication, and this can be suppressed by disruption of either SET2 or CHD1. Our results suggest that both Set2 and Chd1 have a role opposing that of yFACT in regulating DNA replication, and that Set2 and Chd1 act in two separate pathways. set2 and chd1 mutations can suppress the
HU sensitivity caused by other replication mutants, and *chd1* suppresses the lethality of *mec1* and *rad53* gene disruptions. Finally, the defect in S phase progression caused by *pob3* mutations is suppressed by *set2* or *chd1*, suggesting that Set2 and Chd1 may directly regulate DNA replication.

**MATERIALS AND METHODS**

Standard genetic methods were used for strain construction (SHERMAN 1991), which are listed in Suppl. Table S1. Cells were grown in YPD medium (SHERMAN 1991) at 30°C, except as noted, or in synthetic complete medium (SHERMAN 1991) with 2% glucose and supplemented with adenine, uracil and amino acids, as appropriate. Cell cycle synchronization was performed by alpha factor arrest and release using YM-1 medium as described (MITRA *et al.* 2006). RNA levels were determined with S1 nuclease protection assays as described (BHOITE and STILLMAN 1998; BISWAS *et al.* 2006) using oligonucleotides listed in Suppl. Table S2. mRNA levels were quantitated using a Molecular Dynamics phosphorimager and ImageQuant software. Western immunoblots were performed to detect Spt16 and Pob3 (VANDEMARK *et al.* 2007) and phosphorylated Rad53 (ALCASABAS *et al.* 2001); blots were scanned using a Li-Cor infrared scanner and quantitated using Odyssey software.

**RESULTS**

*set2* and *chd1* can suppress yFACT defects associated with DNA replication.

During DNA replication, newly deposited nucleosomes are acetylated at lysines 5 and 12 in histone H4 (GUNJAN *et al.* 2005). While substituting these two residues for non-acetylatable arginines results in viability in an otherwise wild type strain, the H4(K5R,K12R) mutant is lethal in yFACT mutant strains (FORMOSA *et al.* 2002; VANDEMARK *et al.* 2006), suggesting a role for yFACT in nucleosome deposition. We have previously shown that *set2* and *chd1* mutations can suppress transcriptional defects
caused by yFACT mutants (Biswas et al. 2006; Biswas et al. 2007), and we wondered whether deletion of SET2 or CHD1 would also suppress the DNA replication defects caused by combining a yFACT mutant and a histone H4(K5R, K12R) substitution. We constructed spt16-11 strains with the H4(K5R,K12R) mutant histone, along with either a set2 or chd1 mutation and analyzed growth (Fig. 1A). While the spt16-11 H4(K5R,K12R) strain grows at 25°C, viability is strongly decreased at 30°C or higher temperatures. A chd1 mutation strongly suppresses the synthetic lethality caused by combining spt16-11 with H4(K5R,K12R), whereas set2 weakly suppresses. This suggests that these two factors act in opposition to yFACT in promoting DNA replication.

HU reduces the pool of dNTPs available for DNA synthesis, resulting in reduced rates of DNA replication and increased risk of replication fork stalling and collapse. Mutations in many DNA replication genes or factors involved in the checkpoint response to DNA damage cause HU sensitivity (Parsons et al. 2004), as do some SPT16 and POB3 mutations (Formosa et al. 2002; O’Donnell et al. 2004; Schlesinger and Formosa 2000; Vandemark et al. 2006), consistent with a role for yFACT in DNA replication. Based on our earlier observations of suppression, we asked whether set2 or chd1 can suppress the HU sensitivity observed in spt16-11 or pob3(L78R) mutants. Deletion of either SET2 or CHD1 suppresses the HU sensitivities of both spt16-11 (Fig. 1B) and pob3(L78R) (Fig. 1C). We recently described pob3(Q308K), an allele that causes phenotypes suggesting defects in DNA replication (Vandemark et al. 2006). Deletion of either SET2 or CHD1 suppresses the HU sensitivity (Fig. 1D), as well as the temperature sensitivity (Fig. 1E) of a pob3(Q308K) mutant. Thus chd1 and set2 suppress the HU sensitivity for all three yFACT mutations tested.

It is possible that the mutations destabilized the yFACT proteins, and that the chd1 or set2 mutations suppress by stabilizing the yFACT proteins. To determine protein abundance, western immunoblots were performed and quantitated by infrared scanning (Fig. 2). The Pob3(L78R) protein is inherently unstable, accumulating to only about 20%
of wild type levels in cells grown at the permissive temperature of 25°C and somewhat lower levels at the nonpermissive temperature of 37°C (Fig. 2A and VANDEMARK et al. 2007). chd1 and set2 mutations do not result in a significant increase in Pob3(L78R) protein levels. The level of Pob3-Q308K protein is not appreciably affected by this mutation or by set2 or chd1 (Fig. 2B). The spt16-11 mutation has the most severe effect, with cells grown at the nonpermissive temperature showing about 11% of the wild type protein level (Fig. 2C). The chd1 and set2 proteins modestly suppress the instability of the Spt16-11 protein, with an approximately two-fold increase in Spt16-11 protein. We conclude that chd1 and set2 mutations do not suppress the yFACT defect by stabilizing unstable proteins, particularly for the pob3(L78R) and pob3(Q308K) alleles.

The HIR complex facilitates nucleosome assembly in vivo (GREEN et al. 2005; PROCHASSON et al. 2005; RAY-GALLET et al. 2002), and yFACT is proposed to facilitate reformation of the nucleosome following passage of either RNA or DNA polymerase (BELOTSEKOVSKAYA et al. 2003; FORMOSA et al. 2002). Consistent with both yFACT and the HIR complex having a role in nucleosome assembly, combining a yFACT mutation with disruption of any of the four genes encoding subunits in the HIR complex causes synthetic lethality (FORMOSA et al. 2002). In the W303 strain background, an spt16-11 hir2 double mutant is viable, but has a marked growth defect and is more sensitive to HU than single mutants (Fig. S1A, S1B). Importantly, disruption of either SET2 (Fig. S1A) or CHD1 (Fig. S1B) suppress the HU sensitivity of the spt16-11 hir2 double mutant. HTZ1 encodes the yeast H2A.Z histone variant of H2A (DRYHURST et al. 2004), and we showed that a spt16-11 htz1 double mutant is synthetically lethal at 33°C (BISWAS et al. 2006). The spt16-11 htz1 strain grows reasonably well at 25°C, but is quite sensitive to low levels of HU (Fig. S1C, S1D). This HU sensitivity is also suppressed by either a set2 (Fig. S1C) or a chd1 (Fig. S1D) mutation. Using HU sensitivity as a measure of a defect in DNA replication, we conclude that set2 and chd1 mutations suppress the replication defects caused by yFACT mutations.
set2 and chd1 have different phenotypes

The Spt- phenotype describes suppression of the histidine and lysine auxotrophies of strains with the his4-912δ and lys2-128δ alleles (SIMCHEN et al. 1984; WINSTON et al. 1984). While wild type strains with these alleles have His- and Lys- phenotypes, spt mutations change transcription start sites resulting in His+ Lys+ strains, which is the Spt- phenotype. Many spt16 and pob3 alleles, including spt16-11 and pob3(Q308K), display a Spt- phenotype (FORMOSA et al. 2001; VANDERMARK et al. 2006). As shown in Fig. S2, wild type strains are His- and Lys-, while the spt16-11 and pob3(Q308K) mutants grow in the absence of histidine and lysine (line 3 in each panel). A chd1 mutation reverses the Spt- phenotypes caused by spt16-11 (Fig. S2A) and pob3(Q308K) (Fig. S2B). In contrast, set2 fails to suppress the Spt- phenotype, as the spt16-11 set2 (Fig. S2C) and pob3(Q308K) set2 (Fig. S2D) strains show the same His+ Lys+ phenotypes as the SET2+ versions. Therefore, although set2 and chd1 mutations show similarity in their ability to suppress a wide range of yFACT phenotypes (BISWAS et al. 2006; BISWAS et al. 2007), only chd1 is able to suppress the Spt- phenotype. This difference in suppression of the Spt- phenotype suggests that the Set2 and Chd1 proteins function differently with regard to their ability to affect replication and transcription defects caused by yFACT mutations.

The failure of a set2 mutation to affect expression of the his4-912δ and lys2-128δ alleles is surprising. Kaplan et al. (2003) showed that a number of spt mutants also cause inappropriate transcription from ‘cryptic’ TATA elements within open reading frames. It was subsequently shown that preventing activation of these ‘cryptic’ TATA elements within genes requires methylation of K36 in histone H3 by Set2, and the methylated H3-K36 recruits the Rpd3(S) histone deacetylase complex (CARROZZA et al. 2005; JOSHI and STRUHL 2005; KEOGH et al. 2005). Importantly, either a set2 or a sin3 mutation (a sin3 mutation disrupts the Rpd3(S) complex) causes inappropriate transcription from these ‘cryptic’ TATA elements, similar to the effects seen with spt6 or spt16 mutations.
Because *spt16* and *set2* mutations both display transcriptional initiation from intragenic sites, one would predict that a *set2* mutation alone should cause an Spt- phenotype if the two phenotypes were mechanistically related. We also determined that a *sin3* mutation neither causes an Spt- phenotype, nor affects the Spt- phenotype of a *pob3(Q308K)* strain (*Fig. S2E*). Thus *set2* and *sin3* mutations, which both cause utilization of cryptic TATA elements, do not confer an Spt- phenotype in vivo. We conclude that assays of RNA from cryptic promoters and growth of *his4-912δ lys2-128δ* strains in the absence of histidine and lysine are not measuring the same phenotype.

**Set2 and Chd1 are additive in suppressing HU sensitivity of yFACT mutants.**

We showed that *set2* and *chd1* are additive in suppressing the temperature sensitive growth defect caused by a *pob3(L78R)* mutation (BISWAS et al. 2007). We now show that *set2* and *chd1* are also additive in suppressing the HU sensitive phenotypes of both *pob3(L78R)* (*Fig. 3A*) and *spt16-11* (*Fig. 3B*) mutants (growth of triple mutants in row 8 is stronger than for either double mutant in rows 6 and 7). This additivity is consistent with the idea that Chd1 and Set2 act in different pathways to regulate yFACT-mediated DNA replication.

**set2 and chd1 suppress replication mutants**

Because HU inhibits DNA replication, many strains with mutations in DNA replication factors are sensitive to HU (O’DONNELL et al. 2004). If Set2 and Chd1 play a role in DNA replication, then *set2* and *chd1* might suppress the HU sensitivity caused by a mutation in a DNA replication factor. We constructed double mutant strains with *set2* and a variety of replication mutations, including *cdc2-1*, *ctf4Δ*, *mcm2-1*, *mcm3-1*, *orc2-1*, *pol2-1*, and *pol1-17*. For most of these mutants, *set2* did not suppress. However *set2* shows strong suppression of the HU sensitivity in a *cdc2-1* mutant (*Fig. 4A*). *CDC2* encodes the catalytic subunit of DNA polymerase δ. A *set2* mutation shows weaker, but
still significant, suppression of a ctf4 gene disruption (Fig. 4B). CTF4 is required for efficient sister chromatid cohesion, and Ctf4 competes with yFACT for binding to DNA polymerase alpha (WITTMEYER and FORMOSA 1997). We also tested whether a chd1 mutation could suppress the HU sensitive phenotype of a number of replication mutations, including cdc2-1, mcm2-1, mcm3-1, orc2-1, and pol1-17, and found that chd1 suppresses orc2-1 but not the other mutations (Fig. 4C). ORC2 encodes a subunit of the origin recognition complex, required for formation of the pre-replication complex at origins. NHP10 encodes an HMG protein that is part of the Ino80 complex that participates in DNA repair (MORRISON et al. 2004). In the S288C strain background, nhp10 rad55 double mutants are HU sensitive, while nhp10 single mutants are not (MORRISON et al. 2004). In contrast, we find that nhp10 mutants in the W303 background are sensitive to HU, and that this defect can be suppressed by set2 (Fig. 4D). The fact that set2 and chd1 mutations can suppress the HU sensitivity of DNA replication mutants strongly suggests that these two chromatin factors play a role in DNA replication. Additionally, the distinct patterns of suppression by set2 and chd1 of the HU sensitivity of cdc2-1 and orc2-1 suggests that Set2 and Chd1 act through different mechanisms.

The pob3(Q308K) mutation does not affect RNR gene expression

HU is an inhibitor of the ribonucleotide reductase enzyme. This inhibition results in reduced dNTP pools and collapsed replication forks (KOC et al. 2004). Cells respond by inducing expression of the DNA damage regulon, including the four RNR genes encoding ribonucleotide reductase subunits (FOIANI et al. 2000). Thus, a mutant might display HU sensitivity because of a defect in RNR gene induction, instead of a direct defect in DNA replication. To test this explanation we grew wild type, pob3(L78R), and pob3(Q308K) cells for 2 hours in the presence of 100 mM HU, and compared the mRNA levels for the four RNR genes before and after induction (Fig. 5A). In wild type strains gene induction
ranged from 3 fold for \textit{RNR1} to nearly 200 fold for \textit{RNR3}. The \textit{pob3(L78R)} mutant showed significantly reduced ability to induce expression of \textit{RNR2}, \textit{RNR3}, and \textit{RNR4}, and a modest defect for \textit{RNR1}. Interestingly this transcriptional defect was partially suppressed by mutation of either \textit{SET2} or \textit{CHD1} (Supplemental Fig. S3, Fig. 5B). In contrast, the \textit{pob3(Q308K)} mutant showed normal induction of all four \textit{RNR} genes (Fig. 5A). This result shows that HU sensitivity can be caused by a yFACT mutation that supports normal transcriptional induction of the \textit{RNR} genes. This indicates that at least this yFACT mutant is defective directly in some aspect of DNA replication, so suppression of this allele cannot be an indirect effect of a change in RNR gene transcription.

Mutations in \textit{PAF1}, a component of the PAF transcriptional elongation complex, result in HU sensitivity (BETZ et al. 2002). The \textit{paf1} mutants show reduced expression of \textit{RNR1}, and a multicopy plasmid with \textit{RNR1} suppresses the HU sensitivity. Based on this result, we tested whether a multicopy plasmid with \textit{RNR1} would suppress the HU sensitivity of the \textit{spt16-11} and \textit{pob3(L78R)} mutants; however, the HU sensitivity of neither mutant was suppressed by YEp-\textit{RNR1} (data not shown). \textit{SML1} encodes an inhibitor of ribonucleotide reductase (ZHao et al. 1998). We therefore tested whether deletion of \textit{SML1} would suppress the HU sensitivity of yFACT mutants. \textit{spt16-11 sml1}, \textit{pob3(L78R) sml1}, and \textit{pob3(Q308K) sml1} double mutant strains showed the same HU sensitivity as the \textit{spt16-11}, \textit{pob3(L78R)}, and \textit{pob3(Q308K)} single mutants (data not shown), and we conclude that deletion of \textit{SML1} does not suppress the HU sensitivity of yFACT mutants.

In summary, our results show that the HU sensitivity of the \textit{pob3(Q308K)} mutant does not result from a defect in transcriptional induction of \textit{RNR} genes and the HU sensitivity caused by other yFACT mutants cannot be rescued by increasing RNR activity. The suppression of this HU sensitivity phenotype by \textit{set2} and \textit{chd1} therefore
suggests that these two factors act directly in opposition to yFACT in a pathway specific to DNA replication.

**Deletion of CHD1 bypasses the mecl and rad53 checkpoints**

Cells exposed to DNA damaging agents activate the Mec1 protein kinase, resulting in cell cycle arrest and transcriptional activation of targets such as the RNR genes. *MEC1* is essential for viability, but a *mec1 sml1* double mutant is viable (ZHANG et al. 1998) but very sensitive to HU. Importantly, this HU sensitivity is suppressed by a *chd1* mutation (Fig. 4E). Based on this result, we wondered whether *set2* or *chd1* mutations could suppress the lethality of a *mec1* null mutant. A *mec1 sml1* strain was mated to either a *chd1* or a *set2* strain, the diploid was sporulated, and tetrads were dissected. A *set2* mutation failed to allow viability of a *mec1 SML1* strain (data not shown). However, *mec1 chd1 SML1* spores are viable, although slow growing (Fig. 6A). The Rad53 kinase functions downstream of Mec1, and *RAD53* is also essential for viability. A cross shows that a *CHD1* gene disruption also suppresses the lethality of a *rad53* strain (Fig. 6B). A comparison of colony sizes indicates that *chd1* does not suppress *mec1* as strongly as *sml1* does (Fig. 6C). HU exposure activates the Mec1 kinase, resulting in phosphorylation of Rad53 (ALCASABAS et al. 2001). We find that *set2* or *chd1* mutations affect neither the degree of Rad53 phosphorylation in response to HU, nor the kinetics of appearance of activated Rad53 (Fig. 6D). We conclude that Chd1 functions in a pathway unrelated to or downstream of Rad53.

Mec1 is required for maintenance of replication fork integrity (CHA and KLECKNER 2002), and we wanted to investigate whether a *chd1* mutation could suppress replication fork instability caused by a *mec1* mutation. A brief exposure (2 to 4 hr) of *mec1 sml1* mutants to 10 mM HU results in more than 100 fold loss in viability (Fig. 6E), suggesting that maintenance of replication fork integrity by Mec1 is absolutely essential for cell viability. The *mec1 sml1 set2* triple mutant shows greater inviability after exposure to
HU, while a $chd1$ mutation suppresses the $mec1\ sml1$ lethality by 10 fold. The opposite effects of the $set2$ and $chd1$ mutations in this assay supports the idea that Set2 and Chd1 function in different pathways. Exposure of $mec1\ sml1$ mutants to HU results in replication fork collapse and cell death (LOPES et al. 2001; TERCERO and DIFFLEY 2001), and suppression of this lethality by a $CHD1$ gene disruption supports the idea that Chd1 has a negative role in replication fork stabilization.

$chd1$ and $set2$ restore S phase progression to a $pob3$ mutant.

$pob3(L78R)$ mutants are defective for progression through S phase at the non-permissive temperature (SCHLESINGER and FORMOSA 2000), and we wanted to determine whether $chd1$ or $set2$ mutants would suppress this defect. Cells were treated with $\alpha$-factor to arrest in G1, and then released from the block into media at the permissive temperature of 25°C, and at various time points after the release DNA content was determined by flow cytometry (Fig. 7A). With this protocol most wild type cells have completed replication by 30 min, and all by 40 min. In contrast, the $pob3(L78R)$ mutant has a severe defect in S phase progression, with few cells having replicated their DNA by 50 min. The $set2$ and $chd1$ mutations suppress the $pob3(L78R)$ defect in progressing through S phase, with many cells having replicated their genomes at 30 – 40 min following release from $\alpha$-factor arrest (Fig. 7A). A similar experiment was conducted with the $pob3(Q308K)$ mutant, except for cells were released from the $\alpha$-factor arrest at the semi-permissive temperature of 34°C. The $pob3(Q308K)$ mutation has a much more severe effect on DNA replication than $pob3(L78R)$, consistent with the previously described phenotypes (VANDEMARK et al. 2006); many of the cells are still in S phase 60 min after the release (Fig. 7B). The $set2$ and $chd1$ mutations also suppress the $pob3(Q308K)$ defect in S phase progression, with a majority of cells having completed replication within 50 min following release. These results clearly demonstrate negative roles for Chd1 and Set2 in S phase progression.
DISCUSSION

Replication of the genome is carefully regulated to ensure that each chromosome is duplicated once and only once. Problems at the replication fork can cause collapse of the fork, resulting in chromosome breaks, and the checkpoint machinery monitors both the replication fork and DNA integrity (CHA and KLECKNER 2002). The yFACT chromatin reorganizing complex binds to DNA polymerase alpha and Replication Protein A, and both genetic and biochemical studies suggest yFACT promotes DNA replication. We have shown that gene disruptions affecting either the Set2 histone methyltransferase or the Chd1 chromatin remodeling complex can suppress replication defective phenotypes, particularly the sensitivity to the HU replication inhibitor, caused by mutations in yFACT, MEC1, or other replication factors. Additionally, pob3 mutants have a marked defect in progressing through S phase, and this defect is effectively suppressed by mutations in either SET2 or CHD1. These observations suggest that Set2 and Chd1 have negative roles in regulating DNA replication, and that part of the function of yFACT is to overcome these barriers.

While the Set1 and Dot1 methyltransferases that modify H3(K4) and H3(K79), respectively, have been shown to have roles in DNA replication and repair (BOSTELMAN et al. 2007; CORDA et al. 1999; SCHRAMKE et al. 2001; SOLLIER et al. 2004; WYSOCKI et al. 2005), a role for the Set2 H3(K36) methyltransferase in replication has not been previously demonstrated. Similarly, the Ino80 and Swr1 ATP-dependent chromatin remodeling factors play important roles in repairing DNA breaks (BAO and SHEN 2007; VAN ATTIKUM and GASSER 2005), but Chd1 has not been previously implicated in either DNA replication or repair. Our results provide the first evidence for a negative role for Set2 and Chd1 in regulating DNA replication. It is not clear how set2 and chd1 suppress the HU sensitivity of yFACT mutants. One possibility is that these factors have a
negative role in regulating the yFACT-mediated recruitment of DNA replication factors which stabilize the replication fork and prevent fork collapse.

Hydroxyurea (HU) specifically blocks DNA synthesis by inhibiting ribonucleotide reductase (RNR), resulting in depletion of dNTP pools and stalling of replication forks (EKLUND et al. 2001; KOC et al. 2004). In reaction to the stalled replication forks the Mec1 - Rad53 checkpoint kinase cascade is activated, and one response is transcriptional induction of the RNR genes. We show that Set2 and Chd1 function downstream of Rad53 phosphorylation, and that that there is no defect in RNR gene transcription in a pob3(Q308K) strain in response to HU exposure. Thus, the HU sensitivity does not originate from defective transcriptional induction of RNR genes. Additionally, expression of RNR genes is not altered in chd1 or set2 mutant strains. Activity of the RNR enzymes is inhibited by the Sml1 protein, and sml1 mutations can suppress mutations in the replication checkpoint pathway (ZHAO et al. 1998). We therefore considered the possibility that altered transcriptional regulation of SML1 could result in the HU sensitivity of yFACT mutants or the suppression by set2 and chd1. Two experiments argue against this possibility. First, spt16-11, pob3(L78R), and pob3(Q308K) strains showed the same HU sensitivity whether the strain was SML1+ or sml1-. Second, a chd1 mutation suppresses the sensitivity of a mec1 mutant to HU despite the absence of SML1. Additionally, overexpression of RNR1 can suppress replication defects similar to an SML1 gene disruption (DESANY et al. 1998), but RNR1 overexpression does not suppress yFACT defects. We conclude that the effects of yFACT, set2, and chd1 mutations on HU sensitivity are independent of SML1.

Because of its dual role in transcription as well as replication, it is possible that the suppressive effects of set2 and chd1 mutations on yFACT replication defects are indirect, via transcriptional effects. If Set2 and Chd1 do play roles in regulating DNA replication, we would expect set2 and chd1 mutations to suppress replication defects caused by mutations in factors strictly involved in DNA replication. We find that a SET2 deletion
suppresses the HU sensitivity of cdc2-1 and ctf4 mutations and a CHD1 deletion suppresses the HU sensitivity in orc2-1 and mec1 sml1 strain. CDC2 encodes the catalytic subunit of DNA polymerase delta, CTF4 is required for efficient sister chromatid cohesion (HANNA et al. 2001) and competes with yFACT for binding to DNA polymerase alpha (WITTMEYER and FORMOSA 1997), ORC2 encodes a subunit of the origin recognition complex (DA-SILVA and DUNCKER 2007), and MEC1 encodes the checkpoint kinase that monitors replication fork integrity (NEDELCHEVA-VELEVA et al. 2006). Suppression of these replication defective mutations by set2 and chd1 strongly implies that these chromatin modifying factors have specific roles in negatively regulating DNA replication. The MEC1 and RAD53 checkpoint genes are normally essential for viability, but this can be suppressed by deletion of CHD1. Finally, pob3 mutants are defective for progression through S phase, but this defect can be suppressed by chd1 or set2 mutations. These results provide strong support for Chd1 and Set2 in negatively regulating DNA replication.

Chromodomains often bind methylated lysine residues (DANIEL et al. 2005), and one might expect Chd1 and Set2 to function in the same pathway, with Chd1 binding to H3(K36) methylated by Set2. However, structural work on yeast Chd1 suggests that it does not bind methylated lysines (FLANAGAN et al. 2007). We believe that Set2 and Chd1 function in separate pathways, as the two mutations show additivity in suppressing the HU sensitive phenotypes of yFACT mutants, and had different or even opposite effects in assays described here. Further experimental work is needed to decipher the mechanisms by which Set2 and Chd1 regulate DNA replication.
ACKNOWLEDGMENTS

We thank Jasper Rine and Rodney Rothstein for providing strains, John Diffley for providing Rad53 antibodies, and Craig Kaplan for discussions. This work was supported by grants from the National Institutes of Health.
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FIGURE LEGENDS

Fig. 1. *set2* and *chd1* replication defects caused by yFACT mutations.

A. Ten-fold dilutions of strains DY11848 (*spt16-11*), DY11852 (*spt16-11 set2*), DY11851 (*spt16-11 chd1*), DY11855 (*spt16-11 H4(K5R,K12R)*), DY11849 (*spt16-11 H4(K5R,K12R) set2*), and DY11853 (*spt16-11 H4(K5R,K12R) chd1*) were plated on complete medium for five days at 25°C or for three days at either 30°C or 33°C.

B. Ten-fold dilutions of strains DY150 (wild type), DY8690 (*set2*), DY6957 (*chd1*), DY8107 (*spt16-11*), DY8777 (*spt16-11 set2*), and DY9152 (*spt16-11 chd1*) were plated on complete or 50 mM HU medium for two days at 30°C.

C. Ten-fold dilutions of strains DY150 (wild type), DY8690 (*set2*), DY6957 (*chd1*), DY7379 (*pob3(L78R)*), DY8878 (*pob3(L78R) set2*), and DY9458 (*pob3(L78R) chd1*) were plated on complete or 50 mM HU medium for three days at 25°C.

D. In the left panel ten-fold dilutions of strains DY2860 (wild type), DY8898 (*set2*), DY10722 (*pob3(Q308K)*), and DY10723 (*pob3(Q308K) set2*) were plated at 30°C on complete medium for 3 days or on 150 mM HU medium for 4 days. In the right panel ten-fold dilutions of strains DY150 (wild type), DY9809 (*chd1*), DY10890 (*pob3(Q308K)*), and DY10897 (*pob3(Q308K) chd1*) were plated on complete or 50 mM HU medium for two days at 30°C.

E. In the left panel ten-fold dilutions of strains DY150 (wild type), DY8690 (*set2*), DY10308 (*pob3(Q308K)*), and DY12028 (*pob3(Q308K) set2*) were plated on complete medium at 30°C for 2 days or at 35°C for 3 days. In the right panel ten-fold dilutions of strains DY150 (wild type), DY9809 (*chd1*), DY10890 (*pob3(Q308K)*), and DY10897 (*pob3(Q308K) chd1*) were plated on complete medium for two days at 30°C or 35°C.

Fig. 2. *chd1* and *set2* mutations do not stabilize mutant yFACT proteins.

Strains were grown to logarithmic phase at 25°C, the culture was split, and then incubated for 3 hours at either 25°C or 37°C. Pob3 and Spt16 protein levels were
determined by western blotting. Identical gels stained with Coomassie blue verified equal protein loading. The blots were quantitated, and normalized to the wild type strain at that temperature.

A. The Pob3(L78R) protein has reduced abundance at both 25°C and 37°C. Strains DY150 (wild type), DY7379 (pob3(L78R)), DY9809 (chd1), DY9458 (pob3(L78R) chd1), DY8690 (set2), and DY8878 (pob3(L78R) set2) were used. The blot was probed with antibody to both Pob3 and Spt16, and the bottom band of the doublet in this experiment is a proteolytic fragment of Spt16.

B. The Pob3(Q308K) protein is at the same levels as wild type. Strains DY150 (wild type), DY10308 (pob3(Q308K)), DY10711 (chd1), DY10897 (pob3(Q308K) chd1), DY8795 (set2), and DY1228 (pob3(Q308K) set2) were used. In this experiment, only Pob3 was probed so both bands visualized are Pob3 protein.

C. The Spt16-11 protein has reduced abundance at 37°C, but normal levels at 25°C. Strains DY150 (wild type), DY8107 (spt16-11), DY10711 (chd1), DY12430 (spt16-11 chd1), DY8795 (set2), and DY8777 (spt16-11 set2) were used. The blot was probed with antibody to Spt16.

**Fig. 3.** set2 and chd1 are additive in suppressing HU sensitivity of yFACT mutants.

A. Ten-fold dilutions of strains DY150 (wild type), DY9809 (chd1), DY8690 (set2), DY9838 (chd1 set2), DY7379 (pob3(L78R)), DY9458 (pob3(L78R) chd1), DY8878 (pob3(L78R) set2), and DY9547 (pob3(L78R) chd1 set2) were plated at 25°C on complete medium for three days or 150 mM HU medium for six days.

B. Ten-fold dilutions of strains DY150 (wild type), DY9809 (chd1), DY8690 (set2), DY9838 (chd1 set2), DY8107 (spt16-11), DY12430 (spt16-11 chd1), DY8777 (spt16-11 set2), and DY9153 (spt16-11 chd1 set2) were plated at 30°C on complete medium for two days or 120 mM HU medium for four days.
**Fig. 4.** *set2* and *chd1* suppress HU sensitivity of DNA replication mutants.

**A.** Ten-fold dilutions of strains DY5662 (wild type), DY10055 (*set2*), DY10058 (*cdc2-1*), and DY10097 (*cdc2-1 set2*) were plated at 25°C on complete medium for three days or 100 mM HU medium for six days.

**B.** Ten-fold dilutions of strains DY5662 (wild type), DY10055 (*set2*), DY10056 (*ctf4*), and DY10092 (*ctf4 set2*) were plated at 25°C on complete medium for two days or 100 mM HU medium for nine days.

**C.** Ten-fold dilutions of strains DY150 (wild type), DY10711 (*chd1*), DY11082 (*orc2-1*), and DY11084 (*orc2-1 chd1*) were plated at 25°C on complete medium for two days or 50 mM HU medium for three days.

**D.** Ten-fold dilutions of strains DY150 (wild type), DY8825 (*set2*), DY12610 (*nhp10*), and DY12611 (*nhp10 set2*), were plated at 30°C on complete medium for 2 days or 150 mM HU medium for 5 days.

**E.** Ten-fold dilutions of strains DY150 (wild type), DY10675 (*chd1*), DY10665 (*mec1 sml1*), and DY10669 (*mec1 sml1 chd1*) were plated at 25°C on complete medium for two days or 2 mM HU medium for three days.

**Fig. 5.** *RNR* gene expression in *pob3(L78R)* and *pob3(Q308K)* mutants.

**A.** Strains DY150 (wild type) and DY7379 (*pob3(L78R)*)) were grown at 25°C in YPAD medium to O.D. 600 = 0.6, before a pre-induction sample (“−”) was taken, then HU was added to a concentration of 100 mM, and cultures were grown for an additional two hours before the post induction sample (“+ HU”) was taken. Strains DY10641 (wild type) and DY10642 (*pob3(Q308K)*)) were grown identically, expect at 30°C. RNA was isolated from the samples and expression of *RNR1, RNR2, RNR3,* and *RNR4* was determined by S1 nuclease protection, with tRNA serving as an internal control.

**B.** Graphs of RNR gene expression before and after HU induction using the quantitation from the S1 protection assay in *Fig. S3.*
Fig. 6. Deletion of CHD1 bypasses the MEC1 checkpoint.

A. DY6958 (chd1::LEU2) was crossed to DY10112 (mec1::TRP1 sml1::HIS3), yielding DY10671 (mec1::TRP1 chd1::LEU2 SML1). DY10671 was then mated to wild type strain DY1868, and haploid progeny are shown after seven days growth at 25°C. Symbols indicate selected genotypes.

B. Strains DY9809 (chd1::TRP1) and DY10689 (rad53::HIS3 YEp-LEU2-RNR1) were mated and the haploid progeny from sporulating that diploid are shown after five days growth. rad53 is lethal, but lethality can be suppressed by a multicopy plasmid with RNR1 (DESANY et al. 1998). Viable rad53 chd1 strains were recovered both with and without the YEp-LEU2-RNR1 plasmid, and the presence or absence of the YEp-LEU2-RNR1 plasmid did not affect the growth rate of the rad53 chd1 strains. Symbols indicate selected genotypes.

C. Ten-fold dilutions of strains DY10112 (mec1 CHD1 sml1) and DY10671 (mec1 chd1 SML1) were plated on complete medium at 25°C for three days.

D. Strains DY150 (wild type), DY9809 (chd1), and DY8690 (set2) were grown to logarithmic phase at 30°C and HU was added to a concentration of 200 mM. Samples were taken before HU addition, and at five minute intervals after, and examined on immunoblots probed with antibody to Rad53. Identical gels stained with Coomassie blue verified equal protein loading. The arrowhead indicates the position of phosphorylated Rad53.

E. 10 mM HU was added to cultures of strains DY150 (wild type), DY10670 (mec1 sml1 chd1), DY10148 (mec1 sml1), and DY10150 (mec1 sml1 set2) growing at 30°C, and at the indicated times samples were taken. Cells were sonicated and washed with water once before plating determine the fraction of viable cells. The experiment was also conducted with sml1, sml1 chd1, and sml1 set2 strains, and the results were similar to that seen for wild type.
**Fig. 7.** *set2* and *chd1* suppress the *pob3* defect in S phase progression.

A. Wild type (DY150), *pob3*(*L78R*) (DY7379), *pob3*(*L78R*) *chd1* (DY9458), and *pob3*(*L78R*) *set2* (DY8878) cells were grown to log phase at 25°C, arrested with α-factor for 2.5 hr, and released from the arrest by resuspending in fresh media containing protease at 25°C. Samples were taken at 10 min intervals and DNA content was determined by flow cytometry. The 1C and 2C positions are indicated.

B. As in part A, except cells released from the α-factor arrest at 34°C. Strains DY150 (wild type), DY10308 (*pob3*(*Q308K*)), DY10897 (*pob3*(*Q308K*) *chd1*), and DY12028 (*pob3*(*Q308K*) *set2*), were used.
Figure 1

A. spt16-11  H4(WT)  25°C  30°C  33°C
    spt16-11  H4(WT)  set2
    spt16-11  H4(WT)  chd1
    spt16-11  H4(K5R,K12R)
    spt16-11  H4(K5R,K12R)  set2
    spt16-11  H4(K5R,K12R)  chd1

B. complete  50 mM HU
    set2
    spt16-11  spt16-11
    set2
    spt16-11  spt16-11
    spt16-11  set2
    spt16-11  chd1

C. complete  50 mM HU
    set2  pob3(L78R)
    set2
    pob3(L78R)  set2
    pob3(L78R)
    pob3(L78R)  chd1

D. complete  150 mM HU
    set2  pob3(Q308K)
    set2
    pob3(Q308K)  set2
    pob3(Q308K)
    pob3(Q308K)  chd1

E. 30°C  35°C
    set2  chd1
    set2
    chd1
    set2
    chd1

A. set2  chd1
Figure 2

A. **pob3(L78R)**

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25°

α-Pob3

B. **pob3(Q308K)**

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37°

α-Pob3

C. **spt16-11**

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α-Spt16
Figure 3

A.  
chd1  
set2  
pob3(L78R)  
pob3(L78R) chd1  
pob3(L78R) set2  
pob3(L78R) chd1 set2  

B.  
chd1  
set2  
chd1 set2  
spt16-11  
spt16-11 chd1  
spt16-11 set2  
spt16-11 chd1 set2  

150 mM HU  
120 mM HU
Figure 4

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Figure 4
Figure 5

A. | wild type | pob3 \((L78R)\) | wild type | pob3 \((Q308K)\) | wild type | pob3 \((L78R)\) | wild type | pob3 \((Q308K)\) |
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RNR1
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tRNA control
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RNR2
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tRNA control
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```
RNR3
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tRNA control
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RNR4
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tRNA control
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B. | RNR1 mRNA | RNR2 mRNA | RNR3 mRNA | RNR4 mRNA |
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- Black bars: HU induced
- Gray bars: uninduced
Figure 6

A. WT chd1 mecl chd1 dead spore

B. WT chd1 rad53 YEp-RNR1 dead spore rad53 chd1

C. mecl sml1 mecl chd1

D. wild type chd1 set2

E. log cell number vs. time (hours)