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ATP-dependent chromatin remodeling and histone acetyltransferases in 5-FU cytotoxicity in *Saccharomyces cerevisiae*

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ABSTRACT. Chromatin is thought to modulate access of repair proteins to DNA lesions, and may be altered by chromatin remodelers to facilitate repair. We investigated the participation of chromatin remodelers and DNA repair in 5-fluorouracil (5-FU) cytotoxicity in *Saccharomyces cerevisiae*. 5-FU is an antineoplastic drug commonly used in clinical settings. Among the several strains tested, only those

with deficiencies in ATP-dependent chromatin remodeling (CR) and some histone acetyltransferases (HAT) exhibited sensitivity to 5-FU. CR and HAT double-mutants exhibited increased resistance to 5-FU in comparison to the wild-type mutant, but were still arrested in G2/M, as were the sensitive strains. The participation of Htz1p in 5-FU toxicity was also evaluated in single- and double-mutants of CR and HAT; the most significant effect was on cell cycle distribution. 5-FU lesions are repaired by different DNA repair machineries, including homologous recombination (HR) and post-replication repair (PRR). We investigated the role of CR and HAT in these DNA repair pathways. Deficiencies in Nhp10 and CR combined with deficiencies in HR or PRR increased 5-FU sensitivity; however, combined deficiencies of HAT, HR, and PRR did not. CRs are directly recruited to DNA damage and lead to chromatin relaxation, which facilitates access of HR and PRR proteins to 5-FU lesions. Combined deficiencies in HAT with defects in HR and PRR did not potentiate 5-FU cytotoxicity, possibly because they function in a common pathway.

Key words: 5-FU; ATP-dependent chromatin remodelers; Histone acetyltransferases; Homologous recombination repair; Post-replication repair

INTRODUCTION

Most antineoplasic drugs target the DNA of cancer cells to create cytotoxic effects. This cytotoxicity may be related to DNA damage induction as single- (SSBs) and double-strand breaks (DSBs), inter- and intra-strand crosslinks, and interference in purine and pyrimidine metabolism (Ding et al., 2006). Since the DNA damage may be repaired by different cellular DNA repair machineries, efficient repair of these lesions could result in drug resistance and chemotherapy failure (Wyatt and Wilson III, 2009). Therefore, therapies targeting DNA repair have emerged as promising approaches in anticancer research.

Efficient DNA repair depends on many factors, including how the DNA is packaged with histones and non-histone proteins into chromatin. Highly condensed structures likely do not allow access of the DNA-associated repair factors to the lesions (Ataian and Krebs, 2006; Escargueil et al., 2008; Huertas et al., 2009). The first step in chromatin packaging consists of 146 bp of DNA wrapped around a histone octamer containing 2 copies each of the core histones H2A, H2B, H3, and H4. This structure may be modified by chromatin-modifying enzymes that act as i) covalent post-translational histone tail modifiers (acetylation, methylation, phosphorylation, and ubiquitylation) or ii) ATP-dependent remodelers (Altaf et al., 2007; Osley et al., 2007). Covalent modifications alter the charge of specific residues, affecting histone-histone and histone-DNA interactions, and signaling to other protein complexes. Chromatin remodeling depends on multi-protein complexes, which employ ATP hydrolysis energy to alter the histone-DNA interaction. These complexes act by sliding nucleosomes in the DNA molecule, restricting access to specific sequences (for review, see Gangaraju and Bartholomew, 2007; Escargueil et al., 2008) or inserting the histone variants into nucleosomes, which may alter the higher-order chromatin structure (Henikoff and Ahmad, 2005; Altaf et al., 2007).

Our study was designed to investigate the involvement of DNA repair and chromatin structure modifiers in response to the antineoplasic drug 5-fluorouracil (5-FU). We employed *Saccharomyces cerevisiae* as a genetically tractable model organism with many features of chromatin and DNA repair that are common to human cells. 5-FU is an antimetabolite analog of uracil that must be converted to its active metabolites for cytotoxic effect (for review, see Wyatt and Wilson III, 2009). These metabolites could be misincorporated into DNA (Kufe et al., 1981) and RNA (Kufe and Major, 1981) or result in nucleotide pool imbalance (Noordhuis et al., 2004). Many aspects of the 5-FU action mechanism have already been reported (Noordhuis et al., 2004; Matuo et al., 2009); however, the involvement of DNA repair associated with chromatin remodelers for 5-FU cytotoxicity has not been described.

MATERIAL AND METHODS

Yeast strains and growth conditions

Yeast strains deficient in chromatin remodelers and post-translational chromatin modifiers were kindly provided by Dr. Lisiane Meira (Biological Engineering Division, MIT, Cambridge, USA), acquired from Euroscarf (European *S. cerevisiae* Archive for Functional Analysis), or constructed by gene replacement. Relevant genotypes of the *S. cerevisiae* strains used in this study are indicated in Table 1. For routine growth, complete liquid medium (YPD) containing 1% (w/v) yeast extract, 2% (w/v) bacto-peptone, and 2% (w/v) glucose was employed. Medium containing 2% (w/v) bacto-agar was used for plates. Synthetic medium containing 1.7 g/L yeast nitrogen base, 5 g/L ammonium sulfate, 20 g/L glucose, supplemented with the appropriate amino acids (40 g/mL) was employed for selection of transformants.

Experiments were performed in exponential phase (Log). Log cultures were obtained by inoculation of 5 x 10^6 cells/mL YPD overnight cultures into 5 mL fresh YPD medium. After 3-h incubation at 30°C with aeration, the cultures contained 1 to 2 x 10^7 cells/mL. The number of cells was determined by counting in a Neubauer chamber.

5-FU sensitivity assays

In order to pre-determine 5-FU sensitivity and optimal drug range, logarithmic cultures were serially diluted by 1:10 between 10^7 to 10^3 cells/mL and 4-µL aliquots were spotted onto rich medium plates with or without 5-FU. Plates were incubated at 30°C for 2 days. Experiments were performed at least twice for each dose and on independent days.

Cytotoxicity and cytostatic effect evaluation by poissoner quantitative drop test (PQDT)

The PQDT protocol was described by Poletto et al. (2009) and was employed with minor modifications. Cytotoxicity was measured by survival assays after 5-FU treatments on YPD plates and cytostatic activity was evaluated by colony area measurement estimated from scanned images of Petri plates, using the ImageJ Analysis Software (version 1.39; National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/). Graphics represent the average of three independent experiments.

Table 1. *Saccharomyces cerevisiae* strains used in this study.

Strains	Relevant genotypes	Pathway affected	Source
BY4741 (WT)	*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*	-	Euroscarf
*hho1*Δ	BY4741; with *hho1::kanMX4*	H1 histone	Euroscarf
*hta1*Δ	BY4741; with *hta1::kanMX4*	H2A histone	Euroscarf
*htb2*Δ	BY4741; with *htb2::kanMX4*	H2B histone	Euroscarf
*hht1*Δ	BY4741; with *hht1::kanMX4*	H3 histone	Euroscarf
*hhf1*Δ	BY4741; with *hhf1::kanMX4*	H4 histone	Euroscarf
*nhp10*Δ	BY4741; with *nhp10::kanMX4*	HMG	L. Meira
*arp4*Δ	BY4741; with *arp4::kanMX4*	CR	L. Meira
*ino80*Δ	BY4741; with *ino80::kanMX4*	CR	L. Meira
*swr1*Δ	BY4741; with *swr1::kanMX4*	CR	L. Meira
*elp3*Δ	BY4741; with *elp3::kanMX4*	HAT	L. Meira
*esa1*Δ	BY4741; with *esa1::kanMX4*	HAT	L. Meira
*gcn5*Δ	BY4741; with *gcn5::kanMX4*	HAT	L. Meira
*hat1*Δ	BY4741; with *hat1::kanMX4*	HAT	L. Meira
*hat2*Δ	BY4741; with *hat2::kanMX4*	HAT	L. Meira
*hpa2*Δ	BY4741; with *hpa2::kanMX4*	HAT	L. Meira
*hpa3*Δ	BY4741; with *hpa3::kanMX4*	HAT	L. Meira
*sas2*Δ	BY4741; with *sas2::kanMX4*	HAT	L. Meira
*sas3*Δ	BY4741; with *sas3::kanMX4*	HAT	L. Meira
*ubc4*Δ	BY4741; with *ubc4::kanMX4*	UB	Euroscarf
*ubc5*Δ	BY4741; with *ubc5::kanMX4*	UB	Euroscarf
*hda1*Δ	BY4741; with *hda1::kanMX4*	HDAC	L. Meira
*sin3*Δ	BY4741; with *sin3::kanMX4*	HDAC	L. Meira
*sir2*Δ	BY4741; with *sir2::kanMX4*	HDAC	L. Meira
*hos1*Δ	BY4741; with *hos1::kanMX4*	HDAC	L. Meira
*hos2*Δ	BY4741; with *hos2::kanMX4*	HDAC	L. Meira
*hos3*Δ	BY4741; with *hos3::kanMX4*	HDAC	L. Meira
*hst1*Δ	BY4741; with *hst1::kanMX4*	HDAC	L. Meira
*hst2*Δ	BY4741; with *hst2::kanMX4*	HDAC	L. Meira
*hst3*Δ	BY4741; with *hst3::kanMX4*	HDAC	L. Meira
*hst4*Δ	BY4741; with *hst4::kanMX4*	HDAC	L. Meira
*rpd3*Δ	BY4741; with *rpd3::kanMX4*	HDAC	L. Meira
*dot1*Δ	BY4741; with *dot1::kanMX4*	HML	L. Meira
*msi1*Δ	BY4741; with *msi1::kanMX4*	HMT	Euroscarf
*set2*Δ	BY4741, with *set2::kanMX4*	HMT	Euroscarf
*rph1*Δ	BY4741; with *rph1::kanMX4*	HDML	Euroscarf
*htz1*Δ	BY4741; with *htz1::LEU2*	HV	This study
*arp4*Δ*htz1*Δ	BY4741; with *arp4::kanMX4*, *htz1::LEU2*	CR/HV	This study
*hat1*Δ*arp4*Δ	BY4741; with *hat1::kanMX4*, *arp4::URA3*	HAT/CR	This study
*hat1*Δ*esa1*Δ	BY4741; with *hat1::kanMX4*, *esa1::URA3*	HAT	This study
*hat1*Δ*htz1*Δ	BY4741; with *hat1::kanMX4*, *htz1::LEU2*	HAT/HV	This study
*esa1*Δ*htz1*Δ	BY4741; with *esa1::kanMX4*, *htz1::LEU2*	HAT/HV	This study
*rad6*Δ	BY4741; with *rad6::LEU2*	PRR	This study
*rad52*Δ	BY4741; with *rad52::LEU2*	HR	This study
*xrs*Δ	BY4741; with *xrs2::LEU2*	HR	This study
*nhp10*Δ*rad6*Δ	BY4741; with *nhp10::kanMX4*, *rad6::LEU2*	HMG/PRR	This study
*nhp10*Δ*rad52*Δ	BY4741; with *nhp10::kanMX4*, *rad52::LEU2*	HMG/HR	This study
*nhp10*Δ*xrs2*Δ	BY4741; with *nhp10::kanMX4*, *xrs2::LEU2*	HMG/HR	This study
*hat1*Δ*rad6*Δ	BY4741; with *hat1::kanMX4*, *rad6::LEU2*	HAT/PRR	This study
*hat1*Δ*rad52*Δ	BY4741; with *hat1::kanMX4*, *rad52::LEU2*	HAT/HR	This study
*hat1*Δ*xrs2*Δ	BY4741; with *hat1::kanMX4*, *xrs2::LEU2*	HAT/HR	This study
*gcn5*Δ*rad6*Δ	BY4741; with *gcn5::kanMX4*, *rad6::LEU2*	HAT/PRR	This study
*gcn5*Δ*rad52*Δ	BY4741; with *gcn5::kanMX4*, *rad52::LEU2*	HAT/HR	This study
*gcn5*Δ*xrs2*Δ	BY4741; with *gcn5::kanMX4*, *xrs2::LEU2*	HAT/HR	This study
*ino80*Δ*rad6*Δ	BY4741; with *ino80::kanMX4*, *rad6::LEU2*	CR/PRR	This study
*ino80*Δ*rad52*Δ	BY4741; with *ino80::kanMX4*, *rad52::LEU2*	CR/HR	This study
*ino80*Δ*xrs2*Δ	BY4741; with *ino80::kanMX4*, *xrs2::LEU2*	CR/HR	This study
*swr1*Δ*rad6*Δ	BY4741; with *swr1::kanMX4*, *rad6::LEU2*	CR/PRR	This study
*swr1*Δ*rad52*Δ	BY4741; with *swr1::kanMX4*, *rad52::LEU2*	CR/HR	This study
*swr1*Δ*xrs2*Δ	BY4741; with *swr1::kanMX4*, *xrs2::LEU2*	CR/HR	This study

HMG = high-mobility group non-histone protein; CR = ATP-dependent chromatin remodeling; HAT = histone acetyltransferase; UB = ubiquitin; HDAC = histone deacetylase; HML = histone methylase; HMT = histone methyltransferase; HDML = histone demethylase; HV = histone variant; PRR = post-replication repair; HR = homologous recombination.

Construction of double-mutants

Double-mutants were obtained by disruption of *ARP4*, *ESA1*, *HTZ1*, *RAD6*, *RAD52*, and *XRS2* by homologous recombination. The bifunctional yeast/*Escherichia coli* vector YcpLac33 was used as template for amplification of *arp4*::URA3 and *esa1*::URA3 disruption cassettes. pGadT7 was the template for *htz1*::LEU2, *rad6*::LEU2, *rad52*::LEU2, and *xrs2*::LEU2 cassettes. Primers are listed in Table 2. Cassettes were amplified with Platinum Taq DNA polymerase High Fidelity (Invitrogen), purified with a PureLink™ gel extraction kit (Invitrogen), and used for yeast transformation according to the LiAc/PEG protocol (Gietz and Woods, 2002). Disruption was confirmed by PCR and restriction analysis of purified genomic DNA from transformant yeast colonies selected in synthetic media lacking uracil or leucine.

Table 2. Primers used in this study.

Name and sequence	Product length (bp)
arp4::URA3	
5'-ATGTCCAATGCTGCTTTGCAAGTTTATGGCGGCGACGAAGGCAGTTGGACGATCGATGAT-3'	1404
5'-CTATCTAAACCTATCGTTAAGCAATCTTTCGACGCCCACC CAGGGTTATTGTCTCATGAG-3'	
esa1::URA3	
5'-ATGTCCCATGACGGAAAAGAAGAACCTGGTATTGCCAAAAGCAGTTGGACGATCGATGAT-3'	1404
5'-TTACCAGGCAAAGCGTAACTGAGAGGCAGTAAATACCGGTCAGGGTTATTGTCTCATGAG-3'	
htz1::LEU2	
5'-ATGTCAGGAAAAGCTCATGGAGGTAAAGGTAAATCCGGCG GGCCGGTCGAAATTCCCCTA-3'	1489
5'-TTATTTCTTACTTCCCTTTTTTTCCACTTTCAATAATAAT GCCGGAACCGGCTTTTCATA-3'	
rad6::LEU2	
5'-ATGTCCACACCAGCTAGAAGAAGGTTGATGAGAGATTTTACTTAACTTCTTCGGCGACAG-3'	1489
5'-TCAGTCTGCTTCGTCGTCGTCGTCGTCATCATCATCA TAGCAACCATTATTTTTTTC-3'	
rad52::LEU2	
5'-ATGAATGAAATTATGGATATGGATGAGAAGAAGCCCGTTT CTTAACTTCTTCGGCGACAG-3'	1489
5'-TCAAGTAGGCTTGCGTGCATGCAGGGGATTGATCTTTGGT CACAGGAAACAGCTATGACC-3'	
xrs2::LEU2	
5'-ATGTGGGTAGTACGATACCAGAATACATTGGAAGATGGCTCTTAACTTCTTCGGCGACAG-3'	1489
5'-TTATCCTTTTCTTCTTTTGAACGTAAACTTCGGACCGTCG ATGTCTGCCCCTAAGAAGAT-3'	

Cell cycle analysis

Cells were monitored by distribution in G1/S (unbudded), S (small-budded), and G2/M (large-budded) phase. About 200 to 300 cells (treated with 5-FU or untreated controls) were counted in a Neubauer chamber. Large-budded cells were defined as those in which the bud was >50% the size of the mother cell (Cardone et al., 2006). Graphics represent the average of three independent experiments.

Analysis of 5-FU in DNA

Cells were lysed with lyticase (Sigma) and genomic DNA was isolated using 10% SDS, 5 M KOAc, and 70% ethanol. DNA (1.5 µg) was digested at 37°C for 3 h with 1 U uracil DNA glycosylase (UDG) and 1 U human AP endonuclease (APE1) in buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, and 10 mM $MgCl_2$. Reaction products were separated on a 0.8% agarose gel and stained with ethidium bromide. Graphics represent the average of three independent experiments. Images were acquired with a Kodak Gel Logic 200 Imaging System and DNA band intensity was measured with the Kodak Molecular Imaging Software.

RESULTS AND DISCUSSION

Chromatin structure affects DNA-related processes such as replication, transcription, and recombination (Altaf et al., 2007). More recently, its influence on DNA repair has been described (Ataian and Krebs, 2006; Escargueil et al., 2008). Since most antineoplasic drugs act by inducing DNA damage, efficient repair of these lesions may influence clinical response (Ding et al., 2006). Given that chromatin structure modulates access of repair proteins to the site of damage, chromatin remodelers are emerging as a promising target for cancer therapy (Escargueil et al., 2008).

5-FU is an antimetabolite antitumor drug whose cytotoxicity is largely based on creating DNA damage; it has been employed to treat carcinomas arising in the gastrointestinal tract, ovary, breast, head, neck, and esophagus. Several potential modes of action have been proposed, but the participation of chromatin modifiers in 5-FU toxicity has not been described.

Strains deficient in chromatin remodeling and histone acetyltransferases (HATs) presented sensitivity towards 5-FU

To investigate the importance of chromatin remodeling factors for 5-FU cytotoxicity, we screened a large panel of *S. cerevisiae* strains with deletion mutations in genes with roles in canonical histones, non-histone proteins related to the high-mobility group (HMG), ATP-dependent chromatin remodeling (CR), and covalent post-translational modifications by HAT, histone deacetylases, histone ubiquitinization, histone methylation, and histone demethylation. Strains deficient in HMG, CR, and some HATs were more sensitive to 5-FU in comparison to the wild-type (WT) strain (Figure 1).

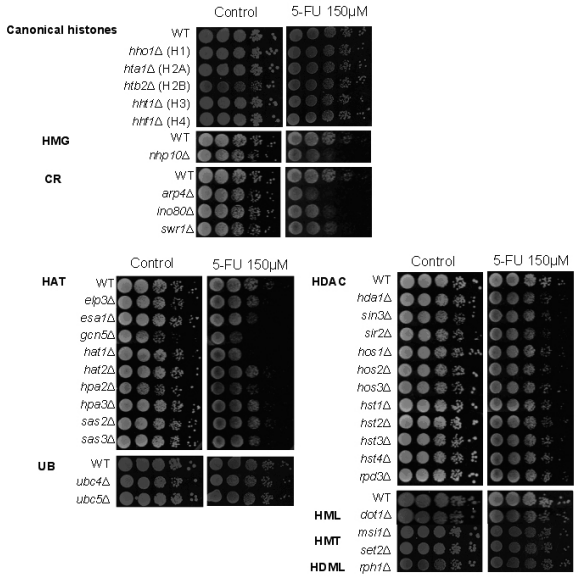

Figure 1. 5-Fluorouracil (5-FU) sensitivity in *Saccharomyces cerevisiae*-deficient strains. Logarithmic cultures were serially dilluted 10-fold and spotted onto YPD medium plates with 5-FU. HMG = high mobility group; CR = ATP-dependent chromatin remodeling; HAT = histone acetyltransferase; UB = ubiquitin; HDAC = histone deacetylase; HML = histone methylase; HMT = histone methyltransferase; HDML = histone demethylase.

The data presented in Figure 1 show that strains deficient in Nhp10 (an HMG-1-like protein), Arp4, Ino80, or Swr1 (ATP-dependent CR proteins) were more sensitive to 5-FU than the WT control. Interestingly, all of these proteins belong to the INO80 subfamily, which is composed of the INO80 and SWR1 complexes. The INO80 complex slides nucleosomes into DNA regions to promote chromatin relaxation and includes several proteins such as Ino80, Arp4, and Nhp10 (for review, see Bao and Shen, 2007). The Ino80 subunit is a functional ATPase and nucleosome spacing factor with 3'- to 5'-DNA helicase activity *in vitro* (van Attikum and Gasser, 2005a). Arp4 (actin-related protein 4) is present in chromatin-modifying complexes such as INO80, SWR1, and NuA4 and can bind histones, modified histones, and nucleosomes, and possesses ATP-binding activity (Hargreaves and Crabtree, 2011). Nhp10 is a subunit of the INO80 complex that can bind to DNA or nucleosomes; its absence leads to reduced binding activity by the INO80 complex, but the complex retains the ability to mobilize nucleosomes (Bao and Shen, 2007). Nhp10 is also necessary for the specific interaction of INO80 at DSB sites in response to DNA damage (Morrison et al., 2004). The SWR1 complex is composed of 14 polypeptides including Swr1, Vps71, Act1, and Arp4 (for review, see Bao and Shen, 2007); the major function of this complex is to exchange histone H2A in nucleosomes for its variant Htz1 (H2AZ in mammals), replacing the preexisting H2A-H2B dimer with Htz1-H2B (Mizuguchi et al., 2004). This substitution of canonical histones with histone variants generates a structurally and functionally distinct region in the chromatin (Henikoff and Ahmad, 2005).

5-FU also sensitized strains deficient in Esa1, Gcn5, and Hat1 (Figure 1). All of these proteins are involved in DSB recombinational repair (Tamburini and Tyler, 2005; Ataian and Krebs, 2006). Esa1 is the catalytic subunit of the NuA4 HAT complex and mutations in this protein reduce recruitment of INO80 and SWR1 to damage sites (Downs et al., 2004). It is required for cell cycle progression (Doyon and Côté, 2004) and it preferentially acetylates H4 histone (Clarke et al., 1999) within coding regions, suggesting a role in global acetylation. Gcn5 is a nuclear HAT that belongs to the Ada and SAGA complexes; it preferentially acetylates H3 histone and plays important roles in histone acetylation during transcription activation (Clarke et al., 1999). Gcn5 also has a separate and independent role in nucleotide excision repair and is essential for efficient repair of UV damage at repressed loci (Yu et al., 2005). It is responsible for most of the post-UV increase in histone acetylation at lysines 9/14; these are seen throughout much of the yeast genome and are not related to transcriptional activation. Hat1 is an HAT-type B; it acetylates cytoplasmic non-chromatin-associated histones that will be transported to the nucleus. At the nucleus, it may play a role in chromatin assembly with Hif1, a histone H3/H4 chaperone (Qin and Parthum, 2006; Benson et al., 2007); it is also important for telomeric silencing and the Hat1 mutant is deficient in recombination repair (Benson et al., 2007).

5-FU effects on the interaction between chromatin remodeling factors

Based on the preliminary data from the 5-FU sensitivity screen (Figure 1), the most sensitive strains were selected to investigate the cellular effects of these deletions. The cytotoxic and cytostatic activities of 5-FU were evaluated by the PQDT method. The *arp4Δ* and *ino80Δ* CR-single mutants were sensitive to 5-FU, although deletion of Arp4 resulted in more pronounced toxicity (Figure 2A). The WT strain presented a potent and similar cytostatic

activity for all 5-FU concentrations tested. In contrast, strains defective in Arp4 or Ino80 presented stronger, dose-dependent cytostatic activity (Figure 2B). The *esa1Δ*, *hat1Δ*, and *gcn5Δ* HAT single-mutants were sensitive to 5-FU (Figure 2C) and showed a strong cytostatic dose-response in *esa1Δ* and *hat1Δ* (Figure 2D).

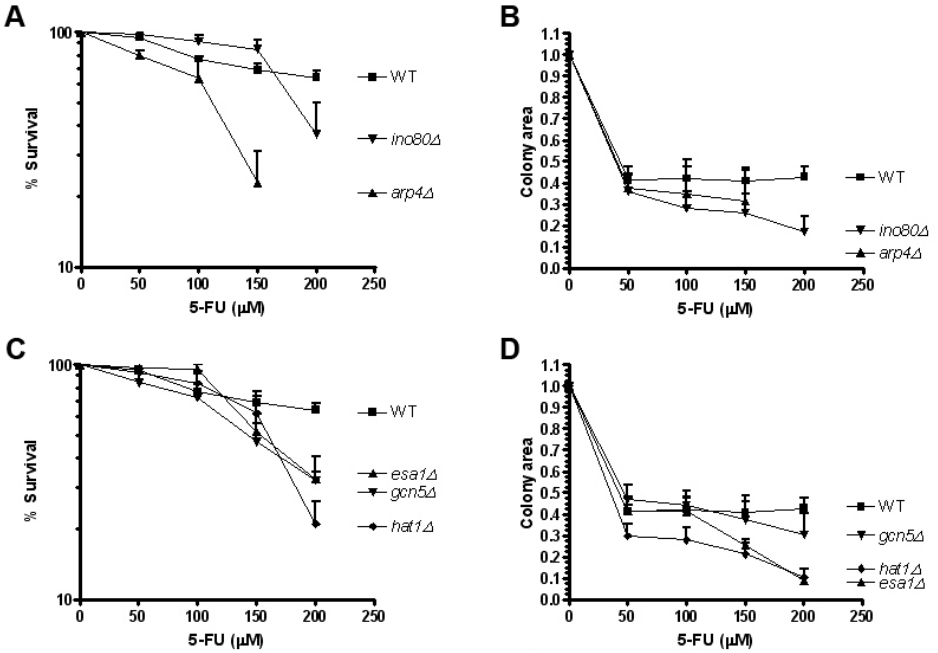

Figure 2. Cytotoxicity (**A**, **C**) and cytostatic (**B**, **D**) effect of 5-fluorouracil (5-FU) in *Saccharomyces cerevisiae*-deficient strains. Data are reported as means ± standard deviation for three independent experiments.

The possible interaction between chromatin remodeling factors in 5-FU toxicity was evaluated by constructing double-mutants. Deficiencies in CR/HAT, such as *arp4Δesa1Δ* and *hat1Δarp4Δ*, did not produce sensitivity to 5-FU (Figure 3A and B) and showed a cytostatic effect similar to WT (Figure 3C and D). Surprisingly, the HAT double-mutant *hat1Δesa1Δ* was not sensitive to 5-FU (Figure 3E) and exhibited a cytostatic effect similar to WT (Figure 3F).

Since we observed that Swr1 was important for 5-FU cytotoxicity, we investigated the possible role of the histone variant Htz1 and its interactions with CRs and HATs. Htz1 incorporation into nucleosomes prevents spreading of silent chromatin into euchromatin regions (Raisner and Madhani, 2006). It also ensures efficient initiation of transcription and cooperates with other components to repel silencing factors (Campos and Reinberg, 2009). The data presented in Figure 4A, B, and C show that defects in Htz1 did not result in significant sensitivity to 5-FU, and that the double-mutants involving *htz1Δ* and *arp4Δ*, *esa1Δ* or *hat1Δ* showed no sensitivity to this drug in comparison to WT. The single- and all double-mutants involving Htz1 showed a cytostatic effect similar to WT (Figure 4D, E and F).

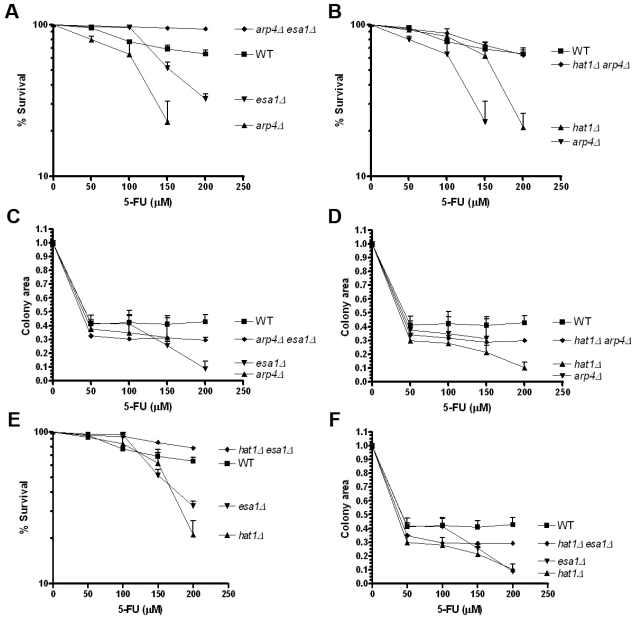

Figure 3. 5-Fluorouracil (5-FU) sensitivity (**A**, **C** and **E**) and cytostatic (**B**, **D** and **F**) activity in yeast defective strains in chromatin remodeling double-mutants. Data are reported as means ± standard deviation for three independent experiments.

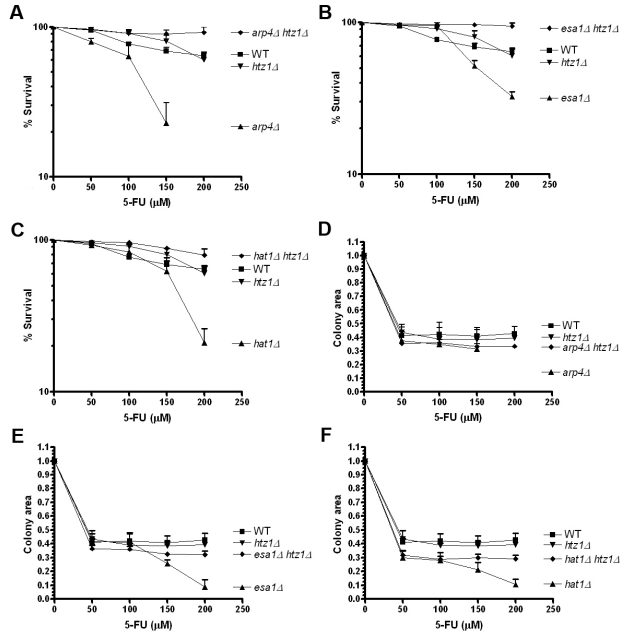

Figure 4. Cytotoxicity (**A**, **C** and **E**) and cytostatic (**B**, **D** and **F**) effect of 5-fluorouracil (5-FU) in *Saccharomyces cerevisiae*-deficient strains involving the histone variant Htz1. Data are reported as means ± standard deviation for three independent experiments.

5-FU induces cell cycle arrest in chromatin remodeling-deficient strains

The data presented in Figures 2, 3, and 4 indicate that 5-FU has potent cytostatic activity; thus, we investigated the cell cycle distribution in chromatin remodeling-deficient strains. After 5-FU exposure, WT, *esa1Δ*, and *htz1Δ* strains showed an increase in the S-phase population, while strains defective in Arp4, Hat1, Ino80, and Swr1, as well as the double-mutants *arp4Δesa1Δ*, *hat1Δarp4Δ*, *hat1Δesa1Δ*, *arp4Δhtz1Δ*, *esa1Δhtz1Δ*, and *hat1Δhtz1Δ* were arrested in G2/M (Figure 5). The single-mutants were more sensitive and presented pronounced cytostatic activity in response to 5-FU, leading to G2/M arrest, except *esa1Δ*, probably because of its role in cell cycle progression. Interestingly, although the double-mutants did not exhibit cytotoxic and cytostatic responses to 5-FU, they did exhibit G2/M arrest.

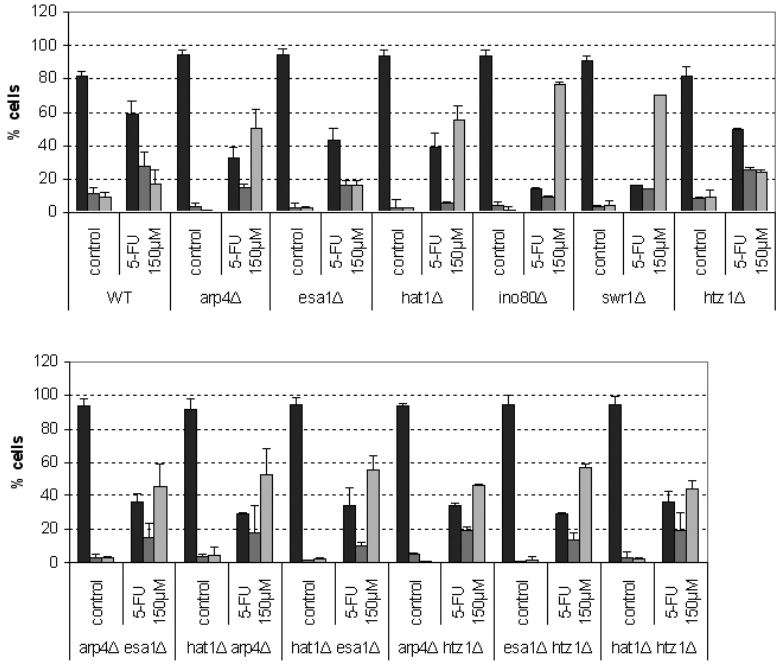

Figure 5. Cell cycle analysis. Distribution of G1/S (black columns), S (gray columns) and G2/M (light-gray columns) cells in single- and double-mutants after 5-fluorouracil (5-FU) exposure. Data are reported as means ± standard deviation for three independent experiments.

Deficiencies in chromatin remodeling did not interfere in the excision of 5-FU misincorporated into DNA

Considering that chromatin remodelers alter chromatin structure to allow access of repair proteins, this aspect of our study aimed to investigate if strains deficient in CR, HATs, and Htz1 were able to repair 5-FU lesions. 5-FU is an antimetabolite that may be misincorporated into DNA; it is removed from DNA by the uracil glycosylase Ung1 (UDG in human) via base

excision repair (BER) (Matuo et al., 2010). The resulting AP site is processed by Apn1 (APE1 in humans) and, if not properly repaired, it is converted to SSB and DSB. Several chromatin remodeling mutants were exposed to 5-FU, the DNA extracted, and digested with UDG and APE1. In the presence of misincorporated 5-FU, DNA breaks are generated, thus reducing the molecular weight of DNA on denaturing agarose gels. Our data showed that defects in CR, HATs, and Htz1 did not influence 5-FU excision from DNA, since there was no difference between digested and undigested DNA migration (Figure 6). Therefore, 5-FU misincorporated into DNA is effectively removed by BER enzymes in chromatin remodeling-defective strains, including the CR/HAT and HAT double-mutants.

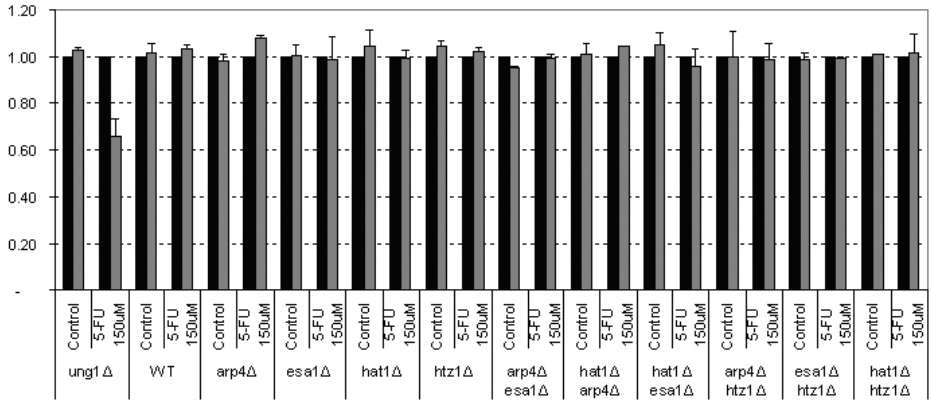

Figure 6. Presence of 5-fluorouracil (5-FU) and abasic sites in yeast genome DNA. DNA from strains treated with or without 5-FU were digested with uracil DNA glycosylase and APE1, and fractionated by agarose gel electrophoresis. The strain *ung1Δ* exposed to 5-FU was employed as a positive control, since this strain accumulates fluoronucleotides, and when its DNA is digested with both base excision repair enzymes, 5-FU is removed and double-strand break may be formed, which decrease the amount of DNA band in comparison to control without enzymes. The graph represents the relative value of DNA band quantified by the Kodak Molecular Imaging Software. Black columns = DNA without digestion; gray columns = DNA digested with enzymes. Data are reported as means ± standard deviation for three independent experiments.

Chromatin modifiers influence the repair of 5-FU by homologous recombination (HR)

Since we observed that deficiencies in CR, HATs, and Htz1 did not interfere with 5-FU removal from DNA by BER, the influence of chromatin remodeling on other DNA repair pathways was investigated. 5-FU lesions are processed by several DNA repair pathways, such as BER, mismatch repair, post-replication repair (PRR) and HR (Matuo et al., 2010). HR repairs DSBs and is the prevalent mechanism in yeast (Aylon and Kupiec, 2004). Considering the observed G2/M arrest in strains deficient in chromatin modifiers, the participation of chromatin remodelers in HR repair was investigated by employing double-mutants. Double-mutants of Rad52 with HMG, CR, and HAT, as well as Xrs2 with the same chromatin modifiers were constructed. Xrs2 acts with Mre11 and Rad50 (MRX complex) at the initial steps of HR, recognizing and processing the broken ends, while Rad52 works in the intermediate steps, stimulating strand exchange by facilitating Rad51 binding to single-stranded DNA (Ataian and Krebs, 2006). Results showed that deletion of *RAD52* or *XRS2* in *nhp10*Δ, *ino80*Δ, and

*swr1*Δ strains increased 5-FU sensitivity in comparison to the single-mutants, but not in the HAT-deficient strains *gcn5*Δ and *hat1*Δ (Figures 7 and 8). Therefore, both HATs may work together in the HR pathway. These data suggest that HMG and CRs are important promoters of chromatin relaxation at DSB damage sites during HR repair, and HATs may contribute to this process.

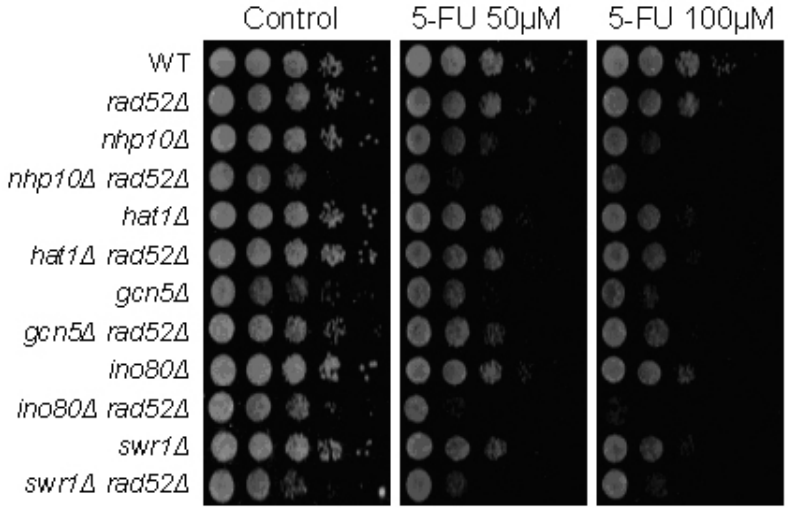

Figure 7. Participation of chromatin remodelers in homologous recombination repair of 5-fluorouracil (5-FU) lesions. Sensitivity was evaluated in single- and double-mutants involving *rad52*Δ and high-mobility group non-histone protein, ATP-dependent chromatin remodeling or histone acetyltransferase.

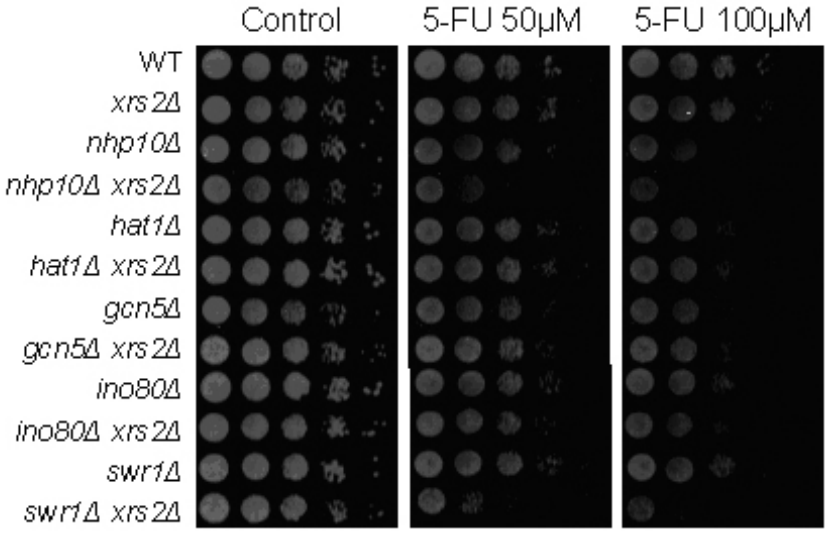

Figure 8. Participation of chromatin remodelers in homologous recombination repair of 5-fluorouracil (5-FU) lesions. Sensitivity was evaluated in single- and double-mutants involving *xrs2*Δ and high-mobility group non-histone protein, ATP-dependent chromatin remodeling or histone acetyltransferase.

Indeed, previous studies have reported that several chromatin modifiers are involved in DSB repair by HR (Tamburini and Tyler, 2005; Ataian and Krebs, 2006). At the lesion site, the Mec1 and Tel1 enzymes phosphorylate histone H2AX at serine-129 (referred as γH2AX). Mec1, through its binding partner Ddc2 and Tel1 via interaction with the Mre11/Rad50/Xrs2 complex, recruits multiple-chromatin-modifying complexes that allow DNA repair factors access to the lesion (van Attikum and Gasser, 2005b). γH2AX recruits the HAT complex NuA4 (yeast homologue of mammalian TIP60) via its Arp4 subunit and Nhp10 facilitates Arp4-γH2AX interaction (van Attikum and Gasser, 2005a). Esa1 (catalytic component of NuA4) acetylates the N-terminal tail of H4 histone surrounding the break site (van Attikum and Gasser, 2005a; Loizou et al., 2006); then, INO80 and SWR1 CR complexes, as well as HATs Hat1 and Gcn5, are recruited to facilitate efficient repair of DNA damage (Downs et al., 2004; Qin and Parthum, 2006; Bao and Shen, 2007). The INO80 complex promotes the removal or nucleosome slide to facilitate processing of newly broken DNA ends, which enables conversion of double-strand DNA ends into 3'-single-strand overhangs by the MRX complex (van Attikum et al., 2005a) and controls the rate at which Rad51 displaces replication protein A during HR (Ataian and Krebs, 2006; Bao and Shen, 2007). The SWR1 complex is also recruited for DNA repair; it possesses ATP-dependent CR activities, which deposit the histone variant Htz1 (homolog of H2AZ) in specific locations *in vivo* and exchange modified histones after repair has been completed (Loizou et al., 2006). The histone variant Htz1 has roles in transcriptional activation, antagonization of gene-silencing and chromosome stability (Raisner and Madhani, 2006), and when incorporated at DSB sites, it contributes to local chromatin relaxation. Other histone modifications are also important for DNA repair by HR (van Attikum and Gasser, 2005b), such as acetylation by Hat1 and Gcn5 (Ataian and Krebs, 2006). Hat1 is recruited at DSB after H2AX phosphorylation concomitant with Rad52, which suggests that Hat1 may act facilitating DNA repair by HR and/or act in the restoration of chromatin structure following recombinational repair, since Hat1 catalyzes acetylation of amino-terminal tails of newly synthesized histones (Qin and Parthum, 2006). Gcn5 is required for transcriptional activation and separately participates in the nucleotide excision repair of ultraviolet light-induced DNA lesions (Yu et al., 2005).

Chromatin remodeling interferes in 5-FU repair by PRR

5-FU lesions are also repaired by PRR (Matuo et al., 2010). In order to investigate the influence of chromatin modifiers in PRR, we constructed double mutants of Rad6 with HMG, CR, and HATs. Rad6p is a ubiquitin-conjugating enzyme that exists in a complex with Rad18. Rad6-Rad18 form a stable complex with single-stranded DNA (Broomfield et al., 2001) and mediate ubiquitin conjugation of the DNA polymerase processivity factor PCNA (proliferating cell nuclear antigen), which promotes replication through DNA lesions by mutagenic or error-free translesion synthesis (TLS) (Minesinger and Jinks-Robertson, 2005; Prakash et al., 2005). Mono-ubiquitinylation of PCNA mediates error-prone TLS, while poly-ubiquitinylation triggers the error-free pathway. TLS occurs in S-phase to ensure replication completion, but also operates in G2/M (Karras and Jentsch, 2010). Our data demonstrated that deletion of *RAD6* in *nhp10*Δ, *ino80*Δ, and *swr1*Δ increased sensitivity to 5-FU in comparison to the single-mutants, but the same was not observed for strains defective in HATs such as *hat1*Δ and *gcn5*Δ (Figure 9).

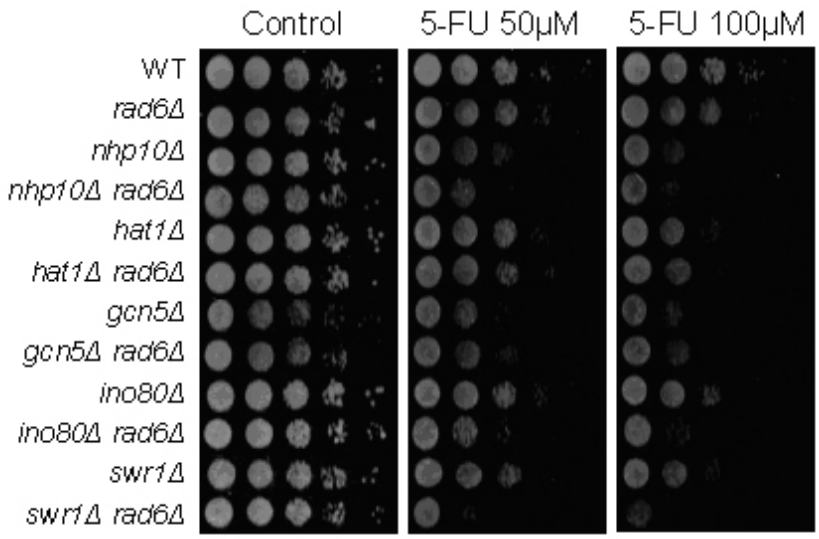

Figure 9. Involvement of chromatin modifiers in post-replication repair of 5-fluorouracil (5-FU). Sensitivity was investigated in single- and double-mutants combining *rad6Δ* and high-mobility group non-histone protein, ATP-dependent chromatin remodeling or histone acetyltransferase.

Few studies have investigated the participation of chromatin modifiers in PRR. Besides the activity of INO80 in HR, it also plays roles in DNA damage tolerance during replication: this complex binds to replication forks during S-phase and allows access of the RAD6 and RAD51 pathways to process obstructed replication forks. INO80 regulates ubiquitination of PCNA and Rad51-mediated processing of recombination intermediates at blocked replication forks by allowing proper recruitment of Rad18 and Rad51 (Falbo et al., 2009).

CONCLUDING REMARKS

These data demonstrate that some ATP-dependent CR factors and specific HATs may influence 5-FU cytotoxicity, probably due to interference with DNA repair. Ino80 and Swr1 CR are directly recruited to DNA damage and lead to chromatin relaxation, which facilitates access of HR and PRR proteins to 5-FU lesions, as well as Nhp10. Deficiencies in Rad52 and Xrs2 (HR) or Rad6 (PRR), combined with Ino80, Swr1, and Nhp10 yielded increased sensitivity to 5-FU. However, deficiencies in Gcn5 and Hat1 combined with defects in HR and PRR did not potentiate 5-FU cytotoxicity, possibly because they work in a common pathway. Figure 10 summarizes the participation of chromatin remodeling in 5-FU lesion repair by HR and PRR.

Chromatin structure influences many biological processes and may modulate DNA repair, which then directly interferes in the activity of antineoplasic drugs. Thus, identification of new targets that improve the efficacy of anticancer agents can provide new possibilities for cancer treatment and possibly overcome drug resistance and side effects.

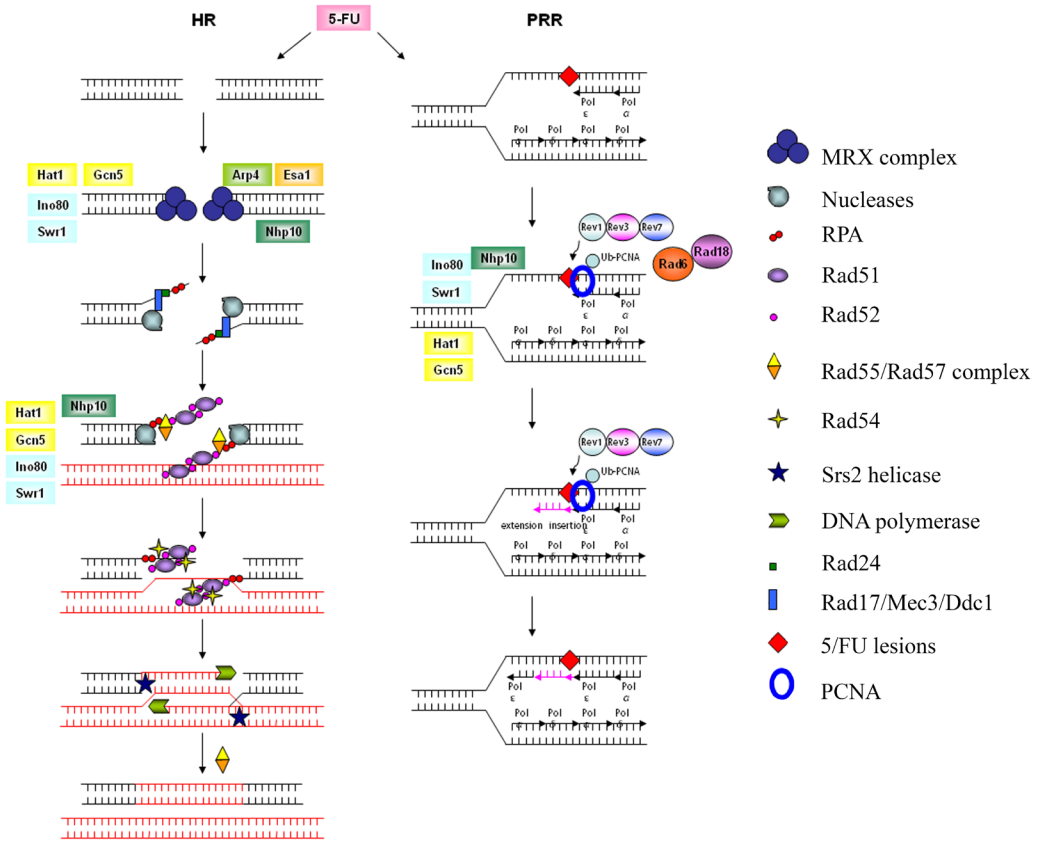

Figure 10. Chromatin remodeling and DNA repair of 5-fluorouracil (5-FU) lesions. 5-FU may induce different types of DNA damage, such as double-strand breaks (DSB) and replication inhibition, which are repaired by homologous (HR) or post-replication repair (PRR), respectively. Several types of high-mobility group non-histone protein, ATP-dependent chromatin remodeling and histone acetyltransferase participate in different steps of HR and PRR. In HR pathway (left side): after DSB formation, the Mre11/Rad50/Xrs2 (MRX complex) recognize the damaged DNA. At this step, several chromatin modifiers (Arp4, Esa1, Nhp10, Ino80, Swr1, Gcn5, and Hat1) may act. Nucleases bind to DNA and generate single-stranded DNA tails, which are coated by the replication protein A (RPA). Then, Rad51 filament is formed, displacing RPA from resected DNA. Rad52 and Rad55/Rad57 complex mediate filament formation, and Rad54 may also participate at this step. During this step, chromatin remodelers may work. Concomitantly, the filament search for homologous sequences and when they are found, the resection ceases and Rad51 filament is disassembled, mediated by Srs2 helicase. DNA polymerase restore the DNA sequency followed by religation. Rad24; Rad17/Mec3/Ddc1 (HR pathway modified from Aylon and Kupiec, 2004). In PRR pathway (right side): 5-FU lesions may stall replication forks. DNA polδ and polε replicate genomic DNA, but they are unable to bypass lesions. In response to stalled replication fork, Rad6/Rad18 complex ubiquitinates proliferating cell nuclear antigen (PCNA), which causes the dissociation of DNA replication polymerases and the association of damage bypass polymerases: pol zeta (Rev3/Rev7) associated to Rev1. At this step, chromatin modifiers (Nhp10, Ino80, Swr1, Hat1, and Gcn5) may act. Pol zeta or Rev1 inserts a nucleotide in opposite to the lesion and then pol zeta extends the DNA. The lesion bypass complex (Rev3/Rev7 and Rev1) dissociates from the template and normal replication polymerases reassociate to continue replication (PRR pathway adapted from Gan et al., 2008).

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