Universidade Federal do Rio Grande do Sul
Instituto de Biociências
Programa de Pós-Graduação em Genética e Biologia Molecular

Estudo da interação entre remodeladores de cromatina e vias de reparação de DNA em resposta ao dano induzido por agentes antineoplásicos e ditelureto de difenila em *Saccharomyces cerevisiae*

Tese de Doutorado

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LISTA DE ABREVIATURAS E SIGLAS

3-CPS: 3-carbetoxipsoraleno

4-NQO: 4-Nitroquinolina 1-oxido

5-Aza-CR: 5-azacitidina

5-Aza-CdR: 5-aza-2-deoxicitidina

5-FU: 5-Fluorouracil

8-HQ: 8-hidroxiquinolina

8-MOP: 8-metoxipsoraleno

ATM: Ataxia telangiectasia mutada

ATR: Ataxia telangiectasia mutada e relacionada à Rad3

BER: "*Base Excision Repair*", Reparação pela via de Excisão de Bases

CDDP: *cis*-diaminodicloroplatina II

DECH: doença enxerto-contra-hospedeiro

DNA-PK: proteína cinase dependente de DNA

DSBs: "*Double-strand breaks*", quebras de fita dupla de DNA

DTDF: ditelureto de difenila

dTMP: "*deoxythymidine monophosphate"*

dTTP: "*deoxythymidine triphosphate"*

dUMP: "*deoxyuridine monophosphate"*

dUTP: desoxiuridina trifosfato

ERO: espécies reativas de oxigênio

FdUMP: 5-Fluoro-2'-desoxiuridina-5'-monofosfato

FdUTP: 5'-fluoro-2'-desoxiuridina trifosfato

FUDP: 5-fluorouridina-5'-difosfato

FUMP: 5-fluorouridina-5'-monofosfato

FUrd: 5-fluorouridina

FUTP: 5-fluorouridina-5'-trifosfato

GGR: "*Global Genome Repair*", reparo global do genoma pelo NER

GPx: glutationa peroxidase

HAT: "*Histone Acetyltransferase*", Acetil-transferase de histonas

HDAC: "*Histone Deacetylase*", Desacetilase de histonas

HMG: "*high-mobility group"*

HML: "*Histone Methylase*", metilase de histonas

HMT: "*Histone Methyltransferase*", metiltransferase de histonas

HR: "*Homologous Recombination*", Reparação pela via de Recombinação Homóloga

ICLs: "*interstrand DNA crosslinks",* Pontes cruzadas intercadeias

MMR: "*Mismatch Repair*", Reparação de bases mal-emparelhadas

MNNG: N-metil-N'-nitro-N-nitrosoguanidina

MRN: complexo MRE11/RAD50/NBS1

MRX: complexo Mre11p/Rad52p/Xrs2p

NDEA: N-nitrosodietilamina

NER: "*Nucleotide Excision Repair*", Reparação por Excisão de Nucleotídeos

NHEJ: "*Non-homologous end joining*", Reparação pela via de Recombinação Não-Homóloga ou ilegítima

PI3K: Fosfatidilinositol 3-cinase

PRR: "*Post-replication Repair*", Reparação pós-replicacional

PUVA: psoraleno + UVA (terapia)

RNR: ribonucleotídeo redutase

RR: Ribonucleotídeo Redutase

SAHA: "*suberoylanilide hydroxamic acid",* Ácido suberoilanide hidroxâmico

Sítios AP: sítios apurínicos/apirimídicos

SSB: "*Single-strand breaks*", quebras de fita simples

ssDNA: "*single-stranded DNA*", DNA de fita simples

TK: "*Thymidine kinase",* Timidina cinase

TLS: "*Translesion synthesis*", Reparação por Síntese Translesão

TMP: 4, 5', 8 trimetoxipsoraleno

TS: Timidilato sintetase

UV: Luz ultra-violeta

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RESUMO

Interstrand DNA crosslinks (ICLs) são importantes lesões genotóxicas que podem causar morte celular se não for devidamente reparadas. Diversas drogas antitumorais atuam através da indução de ICLs no DNA, e a resistência a essas drogas está muitas vezes relacionada à maior capacidade de células tumorais para reparar tais lesões. As ICLs são lesões complexas que requerem o envolvimento de diferentes vias de reparação, tais como BER NER, TLS e vias de reparação de DSBs (RH e NHEJ). Além disso, foi demonstrado que Pso2p desempenha um papel importante na reparação de ICLs. Muitos estudos também apontam para as alterações epigenéticas como essenciais para a sobrevivência da célula em resposta ao dano genotóxico, através da indução de *checkpoints* de ciclo celular e da modulação da eficiência (e, possivelmente, da escolha) das vias de reparação de DNA. Assim, a primeira parte desta Tese apresenta uma revisão dos mecanismos envolvidos na citotoxicidade dos ICLs induzidos pela furocumarina bifuncional 8-metoxipsoraleno (8-MOP) em *Saccharomyces cerevisiae*. A abordagem adotada discute a ação integrada de proteínas que atuam em vias de reparação de DNA envolvidas na remoção de ICLs, em combinação com fatores envolvidos no remodelamento da cromatina dependente de ATP.

A fim de investigar o papel do remodelamento da cromatina em resposta a danos ao DNA induzidos pelo tratamento com furocumarinas mono e bifuncionais fotoativadas e ditelureto de difenila (DTDF), foram utilizadas linhagens de *S. cerevisiae* deficientes em diferentes proteínas relacionadas com a reparação do DNA e o remodelamento da cromatina. DTDF é um composto organotelurado proposto como um protótipo potencial para o desenvolvimento de novas drogas antitumorais. A sensibilidade do mutante *pso2Δ* ao DTDF, semelhante à observada para o 8-MOP, indica que este composto pode induzir lesões capazes de bloquear a replicação do DNA, tais como ligações cruzadas ou DSBs *hairpin capped*. Os resultados obtidos mostraram que os duplos mutantes *pso2Δswr1Δ* e *rad52Δswr1Δ* não são mais sensíveis aos agentes testados, DTDF, 3-CPS e 8-MOP, do que o mais sensível dos simples mutantes (*pso2Δ* e *rad52Δ*, respectivamente). Tendo em mente que Rad52p e Pso2p não têm interação epistática (aditiva ou sinergística) na reparação das lesões fotoinduzidas por 3-CPS e 8-MOP, sugere-se que Pso2p pode fornecer substrato para vias de reparação diferentes de HR, que poderiam ser NHEJ canônica e/ou MMEJ. O fato de que *swr1Δ* (deficiente em NHEJ) não aumenta a sensibilidade dos duplos mutantes, além da baixa sensibilidade

observada para o mutante *yku80Δ* ao DTDF e à fotoadição de psoralenos, sugere que as lesões induzidas podem ser direcionadas para as vias HR ou MMEJ, ao invéz de NHEJ canônica. O envolvimento da via MMEJ sujeita a erros na reparação de lesões induzidas por DTDF pode explicar a indução de mutação *frameshift*, observada para este agente.

Além disso, investigou-se a citotoxicidade induzida pelas drogas antitumorais cisplatina (CDDP), 5-fluorouracil (5-FU) e sua combinação, em *S. cerevisiae*. Os resultados obtidos com os mutantes de reparação do DNA mostraram que a sensibilidade à CDDP + 5-FU parece refletir a sensibilidade de linhagens específicas a cada droga utilizada individualmente. Este resultado sugere que o efeito intensificado do tratamento combinado na terapia do câncer pode ser devido à ação de cada agente isoladamente, conforme observado em clones diferentes da população heterogênea de células transformadas. Os resultados referentes aos mutantes em remodelamento da cromatina demonstraram que o tratamento com CDDP + 5-FU provoca uma resposta mais eficaz na indução de morte celular, nos mutantes deficientes em CR (*ino80Δ*), HATs (*elp3Δ, gcn5Δ*, *hat2Δ* e *hpa2Δ*), HDACs (*sin3Δ, sir2Δ*, *hos2Δ* , *hst1Δ, hst2Δ*, e *hst3Δ*), HML (*dot1Δ*) e HMT (*erg6Δ*), enquanto CDDP induz sensibilidade em mutantes HAT, além do mutante de HDAC *hos3Δ*, e o tratamento com 5-FU induz sensibilidade em HAT (*gcn5Δ*) e HDAC (*sin3Δ* e *hos3Δ*). Esses resultados apontam para o envolvimento de proteínas de remodelamento da cromatina como importantes alvos terapêuticos em resposta a agentes genotóxicos. Portanto, os resultados obtidos podem abrir novas perspectivas para a compreensão dos mecanismos envolvidos na resposta ao tratamento com CDDP + 5-FU, bem como auxiliar na escolha dos melhores protocolos terapêuticos.

ABSTRACT

Interstrand DNA crosslinks (ICLs) are important genotoxic lesions that can cause cell death if not properly repaired. Several antitumoral drugs act by inducing ICLs in DNA, and the resistance to these drugs is often related to an increased capacity of tumor cells to repair such lesions. The ICLs are complex lesions requiring involvement of different pathways for repair, such as BER, NER, TLS and DSB repair pathways (HR and NHEJ). In addition, Pso2p was shown to play an important role in ICLs repair. Many studies also point to the epigenetic changes as essential for cell survival in response to genotoxic damage, through induction of cell cycle checkpoints and modulating the efficiency (and possibly, the choice) of the DNA repair pathways. So, the first part of this Thesis gives a review of the mechanisms involved in the cytotoxicity of the ICLs induced by the bifunctional furocumarin 8-methoxypsoralen (8-MOP) in *Saccharomyces cerevisiae*. The adopted approach discusses the integrated action of proteins that work in the DNA repair pathways involved in ICLs removing in combination with factors involved in ATP-dependent chromatin remodeling.

In order to investigate the role of the chromatin remodeling in response to DNA damage induced by treatment with mono- and bifunctional photoactivated furocumarins and diphenyl ditelluride (DPDT), we used *S. cerevisiae* strains deficient in different proteins related to DNA repair and chromatin remodeling. DPDT is organotellurium compound proposed as a potential prototype for development of new antitumoral drugs. The sensitivity of *pso2Δ* to DPDT, similar to that observed for 8-MOP, indicates that this compound could induce lesions able to block DNA replication, such as crosslinks or hairpin-capped DSBs. The obtained results showed that the double mutants *pso2Δswr1Δ* and *rad52Δswr1Δ* are not more sensitive to the tested DPDT, 3-CPS and 8-MOP agents than the more sensitive single mutant (*pso2Δ* and *rad52Δ,* respectively). Having in mind that Rad52p and Pso2p have non-epistatic interaction (additive or synergistic) in the repair of 3-CPS and 8-MOP photoinduced lesions, we suggest that Pso2p could provide substrate for repair pathway different from HR, which could be canonical NHEJ and/or MMEJ. The fact that *swr1Δ* (deficient in NHEJ) do not enhance the sensitivity of the double-mutants, in addition to the observed low sensitivity of the *yku80Δ* mutant to DPDT and psoralens photoaddition, suggests that the induced lesions could be directed to HR or MMEJ pathways rather than to canonical NHEJ. The

involvement of the error-prone MMEJ in repair of DPDT-induced lesions could explain the observed frameshift mutation induction by this agent.

Moreover, we investigated the cytotoxicity induced by the antitumoral drugs cisplatin (CDDP), 5-fluorouracil (5-FU), and their combination in *S. cerevisiae*. The results obtained with DNA repair mutants showed that the sensitivity to cisplatin + 5-FU appears to reflect the sensitivity of the specific strains to each individual drug used. This finding suggests that the enhanced effect of the combined treatment in cancer therapy could be due to the action of each agent alone, as observed on different clones from the heterogeneous population of transformed cells. The results concerning chromatin remodeling mutants demonstrated that CDDP + 5-FU treatment causes a more effective response in the induction of cell death, in CR (*ino80Δ*), HATs (*elp3Δ, gcn5Δ, hat2Δ* and *hpa2Δ*), HDACs (*sin3Δ, sir2Δ, hos2Δ , hst1Δ, hst2Δ,* and *hst3Δ*), HML (*dot1Δ*) and HMT (*erg6Δ*) mutants, while CDDP induces sensitivity in HAT mutants, besides the HDAC mutant *hos3Δ*, and 5-FU treatment induces sensitivity in HAT (*gcn5Δ*) and HDACs (*sin3Δ* and *hos3Δ*). These results point to involvement of chromatin remodeling proteins as important therapeutic targets in response to genotoxic agents. Therefore, our findings could provide new insights for understanding the mechanisms involved in the response to treatment with CDDP + 5-FU, as well as to help in the choice of optimal therapeutic protocols.

INTRODUÇÃO

1. Introdução Geral

Células eucarióticas são continuamente expostas a uma série de fatores que ameaçam a estabilidade do seu genoma e a manutenção da vida. Desse modo, a evolução deve-se, em grande parte, a um complexo sistema integrado, capaz de coordenar a progressão do ciclo celular e a detecção e reparação de danos estruturais na molécula de DNA. Assim, as células previnem a transmissão de aberrações cromossômicas ao longo das gerações (Hoeijmakers, 2001; Lobrich and Jeggo, 2007; Singh *et al.*, 2009). Agentes sintéticos, fatores ambientais químicos e físicos, como fumaça de tabaco, derivados petroquímicos, radiação ultravioleta (UV) e radiação ionizante, são importantes indutores de danos ao DNA eucariótico (Batista *et al*., 2009). Além disso, produtos originários do metabolismo celular, como espécies reativas de oxigênio (ERO), também constituem uma importante fonte de danos ao DNA, de modo que alterações no equilíbrio redox celular aumentam a incidência destes danos. Assim, a exposição das células a agentes genotóxicos, bem como a sua habilidade em reagir adequadamente aos danos por eles causados, são fatores críticos para o desenvolvimento do câncer (Ishikawa *et al.*, 2006).

Também podem ocorrer danos ao DNA no genoma de determinadas células, por processos biológicos distintos, promovendo variabilidade genética, como ocorre na meiose eucariótica, ou gerando diversidade biológica como forma de aumentar a sobrevivência e a resposta do organismo a agentes patogênicos. Tais modificações são observadas no sistema imunológico adaptativo de vertebrados mandibulados, por exemplo (Bonatto *et al*., 2005; Kato *et al*., 2012). Defeitos nas vias de reparação de danos ao DNA estão relacionadas ao envelhecimento precoce, desenvolvimento de câncer, disfunções imunológicas e até deficiências neurológicas (Dinant *et al.*, 2008). O processo de reparação de danos ao DNA é bioquimicamente complexo, ocorre em várias etapas, e envolve diversas proteínas cujas funções são distintas. Conforme a natureza ou extensão do dano, diferentes complexos proteicos são mobilizados, constituindo as vias de reparação de DNA, responsáveis pela manutenção da integridade do genoma. As diferentes vias de reparação de DNA apresentam uma sobreposição funcional das suas proteínas, de modo que vários complexos proteicos atuam simultaneamente, na reparação de diferentes tipos de dano (Brugmans *et al*., 2007; Coppedè and Migliore,2010).

Um tipo importante de lesão, capaz de dar início a processos deletérios para as células, é a formação de quebras na molécula de DNA. Estas quebras podem ocorrer em uma única cadeia do DNA, sendo denominadas *single-strand breaks* (SSBs), ou nas duas cadeias, quando são chamadas de *double-strand breaks* (DSBs). Embora capazes de causar câncer, a indução de DSBs é paradoxalmente utilizada no tratamento desta e outras doenças, através do emprego de agentes terapêuticos capazes de introduzir DSBs nas células de câncer, em número suficiente para ativar vias de morte celular (Helleday *et al*. 2008).

A resposta das células para as lesões geradas por agentes genotóxicos inicia-se pela detecção do dano, com subsequente ativação de vias de sinalização que conduzem a paradas no ciclo celular, remodelamento da cromatina e recrutamento e ativação de fatores de reparação (Figura 1). No caso de danos muito severos, as células dispõem ainda de mecanismos indutores de morte celular por apoptose.

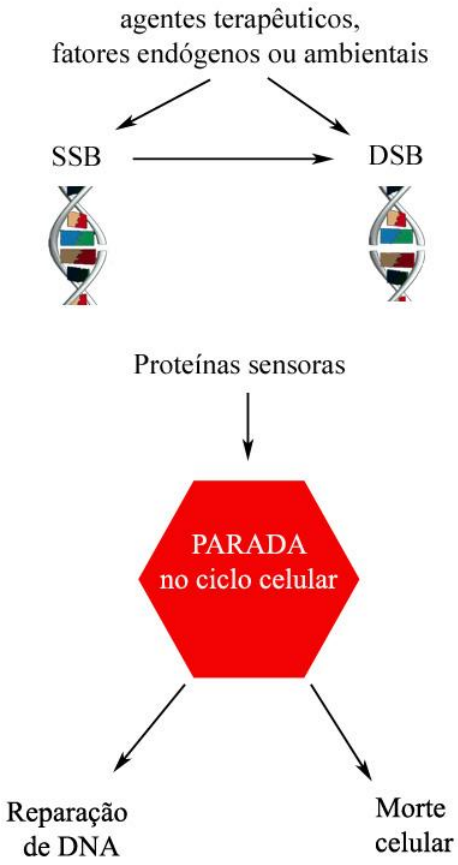

Figura 1. Esquema ilustativo das respostas celulares às lesões genotóxicas

Drogas antitumorais, como cisplatina, mitomicina C, derivados de mostardas nitrogenadas e psoralenos fotoativados, têm seus efeitos genotóxicos atribuídos à sua capacidade de introduzir pontes intercadeias, ou *interstrand crosslinks* (ICLs) no DNA das células eucarióticas (Hinz, 2010; Legerski, 2010; Wood, 2010). As ICLs unem covalentemente ambas as cadeias de DNA, impedindo a sua separação e bloqueando processos de replicação ou transcrição, levando à formação de DSBs. Além disso, SSBs também podem ser convertidas a DSBs, durante o processo de reparação do DNA. Proteínas sensoras reconhecem as quebras na molécula de DNA e desencadeiam uma cascata de sinalização, que leva à parada no ciclo celular e reparação do dano. No caso de danos muito severos, ou na ausência ou inativação de proteínas de reparação de DNA, a célula se direciona para vias apoptóticas.

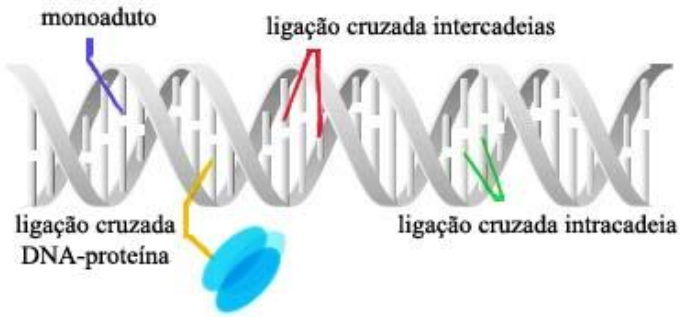

Figura 2. Esquema ilustrativo dos tipos de interações entre a molécula de DNA e drogas que formam adutos (Adaptado de McHugh, Spanswick, and Hartley 2001).

Diversos trabalhos procuram também desvendar o papel das modificações de histonas nos processos de recrutamento e propagação da sinalização de dano ao DNA (Suganuma and Workman, 2010; Sarkar *et al*., 2010; Crea *et al*., 2011; Symington and Gautier, 2011; Parthun, 2012) e na regulação da transcrição gênica (Hou *et al*. 2010; Xue-Franzén *et al*. 2010; Morillo-Huesca *et al*. 2010; Marques *et al*. 2010). Além disso, surgem a cada dia novas evidências que apontam para a utilização do código de histonas na busca de novos alvos terapêuticos que possam ser úteis no combate ao câncer.

Os remodeladores de cromatina dependentes de ATP da família SWI/SNF encontram-se inativos em alguns cânceres humanos, o que sugere sua função como supressores tumorais (Luijsterburg and Attikum, 2011). O silenciamento epigenético, observado em diversos tipos de câncer, é potencialmente reversível, constituindo um

importante alvo terapêutico (Kwa *et al*. 2011). Modificações epigenéticas podem ocasionar a ativação de oncogenes, o silenciamento de supressores tumorais e até o descontrole da proliferação de células tumorais. Estas alterações podem decorrer de anormalidades nos níveis de enzimas que atuam no remodelamento da cromatina, bem como da distribuição da metilação do DNA pelo genoma, uma vez que o padrão de metilação observado durante a progressão tumoral encontra-se alterado. Assim, durante a tumorigênese, a desmetilação pode ocasionar instabilidade genômica, ou pela ativação de retrotransposons silenciados, ou pelo aumento de rearranjos cromossômicos (Cortez and Jones 2008; Ellis *et al*., 2009). Existem atualmente drogas capazes de inibir a metilação DNA, como a 5-azacitidina (5-Aza-CR ou Vidaza®) e 5-aza-2-deoxicitidina (5-Aza-CdR ou decitabine), assim como drogas inibidoras de desacetilases, como a tricostatina A e o *suberoylanilide hydroxamic acid* (SAHA), empregadas no tratamento de síndrome mielodisplástica, além de leucemia mielóide aguda e crônica (Kristensen *et al*., 2009).

Alterações na atividade de acetil transferases de histonas (*histone acetyltransferases*, HATs) são observadas em tumores sólidos e hematológicos, seja por mutações que causam a inativação de HATs ou pela ação de oncoproteínas virais (Ellis *et al*., 2009). Nesse contexto, a redução na expressão da HAT Tip60 (que atua na modulação da sinalização via ATM, p53 e Myc) causa hipoacetilação do genoma, o que resulta em defeitos na indução de apoptose (Ellis *et al*., 2009).

2. Agentes genotóxicos

2.1. Cisplatina

A cisplatina é um agente antitumoral amplamente utilizado na quimioterapia, mostrando-se eficaz em diversas neoplasias humanas e de animais, principalmente quando a droga é empregada no tratamento de melanomas malignos, além de tumores de testículo, de ovário, da cabeça e do pescoço e também nos carcinomas da bexiga e do pulmão (Wang and Lippard, 2005; Boulikas *et al*., 2007; Michalke, 2010). Por outro lado, a administração da cisplatina, feita por via intraperitoneal ou intravenosa,

apresenta limitações decorrentes de sua elevada toxicidade, responsável por efeitos colaterais desagradáveis como náuseas e vômitos, além de hipomagnesemia, supressão da medula óssea, ototoxicidade, neurotoxicidade e nefrotoxicidade. Além disso, também pode ocorrer o desenvolvimento de resistência à droga por parte das células tumorais, o que constitui mais um importante obstáculo ao seu uso clínico (Wang and Lippard, 2005; Crea *et al*., 2011). Em uma tentativa de superar essas limitações e ampliar a gama de tumores tratáveis, milhares de análogos de platina foram sintetizados e avaliados quanto a sua atividade anticâncer, sendo que apenas três compostos platinados foram aprovados para comercialização: a carboplatina, a oxaliplatina e a nedaplatina (Yonezawa *et al*., 2006; Boulikas *et al*., 2007). Diferenças no reconhecimento e processamento das lesões genotóxicas causadas por esses compostos contribuem para as diferenças observadas na sua citotoxidade e atividades antitumorais (Desoize and Madoulet, 2002; Yonezawa *et al*., 2006; Boulikas *et al*., 2007; Michalke, 2010).

A molécula de cisplatina (*cis*-diaminodicloroplatina II) é formada por um complexo de platina, com dois átomos de cloro e duas moléculas de amônia na posição *cis*, em uma estrutura planar quadrada. A cisplatina pode reagir com muitas estruturas celulares, como membranas, proteínas e ácidos nucleicos, sendo seu principal alvo a molécula de DNA (Figura 3) (Michalke, 2010; Guainazzi and Schärer, 2010; Crea *et al*., 2011). A molécula de cisplatina forma adutos covalentes com o DNA, na posição N7 da base guanina, os quais podem ocorrer entre guaninas adjacentes (pontes intracadeias de DNA), localizadas na mesma cadeia de DNA, guaninas localizadas em cadeias opostas (pontes intercadeias de DNA), ou ainda formar monoadutos ou pontes cruzadas entre DNA e proteínas nucleares. A cisplatina provoca grandes distorções na estrutura do duplex de DNA, uma vez que as pontes intercadeias de DNA dobram a molécula em direção ao sulco maior, expondo a superfície do sulco menor e permitindo a ligação de diversas classes de proteínas. Estas incluem proteínas do grupo de alta mobilidade, ou *high-mobility group* (HMG), além de proteínas de reparação, fatores de transcrição e a histona H1, que reconhece preferencialmente adutos platina – DNA (Noll *et al*., 2006; Vasquez, 2010; Wood, 2010; Davey *et al*., 2010; Deans and West, 2011).

A formação de adutos de cisplatina na molécula de DNA pode ocasionar quebras, especialmente DSBs, durante o processo de replicação. Além disso, essas DSBs também podem surgir como consequência da atuação de proteínas de reparação de DNA, uma vez que essas lesões envolvem interações complexas entre diferentes vias de reparação, incluindo *nucleotide excision repair* (NER), *recombination repair* (HR),

non-homologous end-joining (NHEJ) e *translesion synthesis* (TLS) (Moura *et al*., 2010).

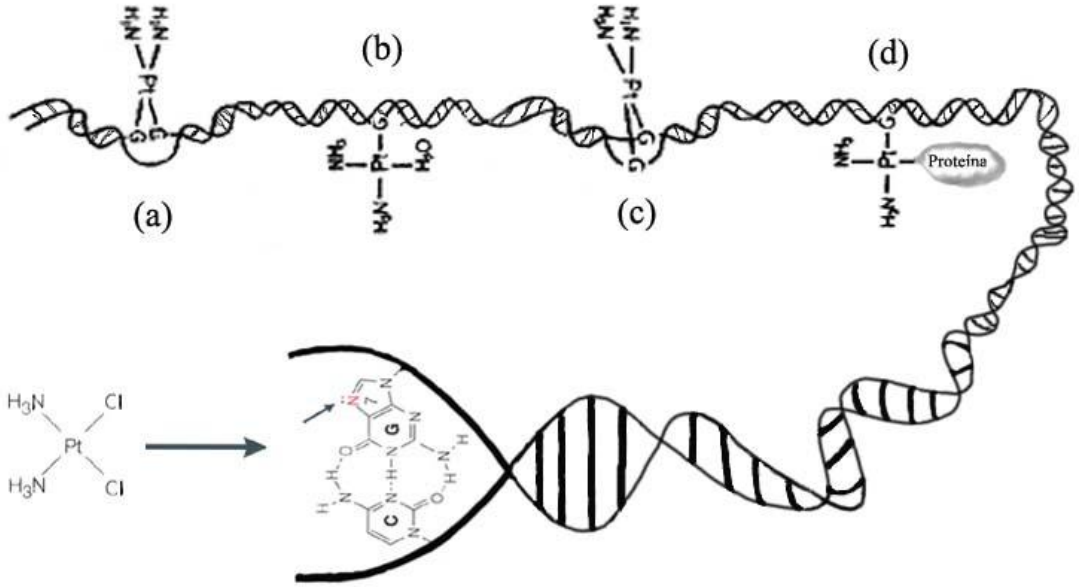

Figura 3. Estrutura da cisplatina e suas interações com o DNA. A molécula de estrutura planar quadrada da cisplatina forma adutos covalentes com o DNA, na posição do N7 da base guanina. Estes adutos podem: (a) ocorrer entre guaninas adjacentes, localizadas na mesma cadeia de DNA, originando pontes intracadeia; (b) formar monoadutos com o DNA; (c) ocorrer entre guaninas localizadas em cadeias opostas do DNA; ou ainda (d) formar ligações cruzadas entre a molécula de DNA e proteínas nucleares.

2.2. 5-fluoruracil

O agente antitumoral 5-fluorouracil (5-FU), é um análogo de pirimidina amplamente empregado em oncologia, particularmente no tratamento do câncer colo-retal, além de tumores de ovário, mama, cabeça e pescoço (Rahman *et al*., 2006; Seiple *et al*., 2006), geralmente em terapias combinadas com outros agentes antineoplásicos como leucovorin (Longley *et al*., 2003; Kuebler and Gramont, 2003; Álvarez *et al*., 2012), irinotecan (Grivicich *et al*., 2005), metotrexato (Longley *et al*., 2003) e

compostos platinados como cisplatina (Grem, 1997) e oxaliplatina (Kuebler and Gramont, 2003; Pera *et al*., 2012), ou à radioterapia (Rich *et al*., 2004).

O 5 - FU é um análogo da base uracil, com um átomo de flúor no lugar do hidrogênio, na posição do carbono-5 do anel de pirimidina. A droga é Internalizada pelas células por difusão e sistema de transporte facilitado de nucleobase, e requer a conversão para a sua forma ativa antes de exercer seus efeitos citotóxicos. Quando o 5-FU é convertido a 5-fluorouridina-5'-trifosfato (FUTP) e 5'-fluoro-2 'desoxiuridina-5'-trifosfato (FdUTP), estes podem ser incorporados ao RNA ou ao DNA, respectivamente, alterando a sua síntese e estabilidade (Meyers *et al.*, 2003).

Em eucariotos superiores, O metabólito ativo da droga, FdUMP, forma um complexo estável com a enzima timidilato sintetase (TS), que catalisa a conversão de desoxiuridina monofosfato (dUMP) para desoxitimidina monofosfato (dTMP), levando à inibição da enzima pela formação de um complexo ternário covalente entre esta e o 5,10-metileno-tetrahidrofolato. Como consequência, ocorre um desequilíbrio no *pool* de nucleotídeos, com redução nos níveis de dTMP e aumento nas concentrações de dUMP (Longley *et al*., 2004; Meyers *et al*., 2003). Como a maioria das polimerases de DNA têm baixa discriminação por dTTP e dUTP, o aumento na concentração de dUMP pode ocasionar uma incorporação significativa de uracil ao DNA (Grem, 1997; Meyers *et al*., 2003; Dornfeld and Johnson, 2005; Fischer *et al.*, 2007). Além disso, FdUMP pode ser fosforilado a FdUTP, e incorporado ao DNA. Em leveduras, a enzima timidina cinase (*thymidine kinase*, TK) está ausente, impedindo a conversão direta de 5-FU em FdUMP, o que sugere que a toxicidade do tratamento com 5-FU neste microorganismo se dê por meio da incorporação errônea de fluoropirimidinas ao DNA e RNA (Matuo *et al*., 2010) (Figura 4). O tratamento com 5-FU causa mutações e codificação errada de proteínas (Rosen *et al*., 1969), inibe tanto o processamento do pré-RNAr (Ghoshal and Jacob, 1994) como as modificações pós transcricionais de RNAt, além de induzir poliadenilação e *splicing* de RNAm revisado em Matuo *et al*., 2010). Um importante estudo realizado em nosso laboratório revelou que as vias BER e MMR são importantes na sensibilidade ao tratamento com 5-FU em leveduras, além das vias HR e PRR, também necessárias na reparação destas lesões (Matuo *et al.*, 2010).

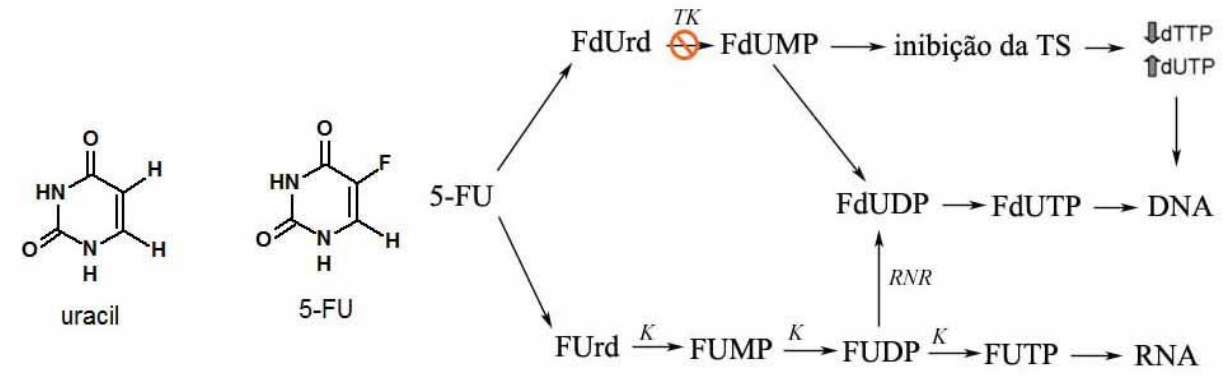

Figura 4. Estrutura e metabolismo do 5-FU . TS = timidilato sintetase; timidina cinase (*thymidine kinase*) = TK; FUrd = 5-fluorouridina; FUMP, FUDP e FUTP = 5-fluorouridina-5'-mono, di e trifosfato; FdUDP = 5'-fluoro-2'-desoxiuridina difosfato; FdUTP = 5'-fluoro-2'-desoxiuridina trifosfato; dUTP = desoxiuridina trifosfato; K = *kinases*; RNR: ribonucleotídeo redutase (Adaptado de Matuo *et al*., 2010).

2.3. Psoralenos: 8-MOP e 3-CPS

Psoralenos ou furocumarinas são compostos aromáticos tricíclicos presentes em uma ampla variedade de plantas comestíveis, como salsa, aipo, cenoura e frutas cítricas (Manderfeld *et al*., 1997; Smith *et al*., 2004). Trata-se de metabólitos secundários, isolados de plantas das famílias Umbelliferae, Rutaceae, Moraceae e Leguminosae (Guo and Yamazoe, 2004). A utilização destes compostos com fins medicinais data dos tempos do Egito Antigo, por meio do emprego de extratos da planta *Ammi majus*, que crescia às margens do rio Nilo, no tratamento de doenças da pele.

A furocumarina bifuncional 8-metoxipsoraleno (8-MOP) é hoje uma droga bem estabelecida na PUVA terapia de doenças inflamatórias da pele, como vitiligo, psoríase, eczema, dermatite atópica (Tominaga *et al*., 2009; Reuter *et al*., 2010; Archier *et al*., 2012), sendo também empregada no tratamento do linfoma cutâneo de células T (Toyooka and Ibuki, 2009), na doença enxerto-contra-hospedeiro (DECH) (Rubegni *et al*., 2005), doenças auto-imunes, como artrite reumatóide, esclerose sistêmica, lupus eritematoso sistêmico, pênfigo vulgar, doença de Crohn, além de ser utilizada na

prevenção de rejeição e indução de tolerância em transplantes de órgãos sólidos, como coração, pulmão e rim (Valdez and Andersson, 2010; Morelli and Larregina, 2010). O potencial genotóxico do 8-metoxypsoraleno (8-MOP, Figura 5) deve-se à sua capacidade em intercalar-se, reversivelmente, entre os pares de bases da molécula de DNA, por forças de Van der Waals e pontes de hidrogênio. Em presença de radiação UVA (365nm), o 8-MOP liga-se covalentemente a bases pirimídicas, principalmente timinas, formando adutos, em um processo denominado fotoadição. Quando a dupla ligação C5-C6 da base pirimídica liga-se à dupla ligação C3-C4 do núcleo cumarínico, chamamos monoadições do tipo 3,4. Se a reação ocorrer entre a dupla ligação C5-C6 da base pirimídica e a dupla ligação C4-C5 do grupo furano, chamamos monoadições do tipo 4',5'. As monoadições do tipo 3,4 não absorvem novos fótons cujo comprimento de onda seja superior a 320nm (Zarebska *et al*., 2000). Contudo, as monoadições do tipo 4',5' podem absorver um fóton adicional, de 365nm, e ainda pode ocorrer uma segunda fotoadição, esta entre a dupla ligação C3- C4 do anel cumarínico e outra pirimidina, na cadeia oposta do DNA, resultando na formação de ICLs.

Quando as furocumarinas são capazes de formar tanto monoadições como biadições, são denominadas bi-funcionais. Estas furocumarinas possuem o sítio C3-C4 do anel cumarínico e o sítio C4-C5 do anel furano disponíveis, e apresentam uma conformação planar. São exemplos de furocumarinas bi-funcionais os agentes 8-MOP e TMP (4, 5', 8 trimetoxipsoraleno). No caso de furocumarinas cuja estrutura molecular permita apenas monoadições, estas são denominadas monofuncionais, como é o caso dos agentes 3-carbetoxipsoraleno (3-CPs, Figura 5) e da Angelicina (Guainazzi and Schärer, 2010). Os efeitos genotóxicos das fotoadições por furocumarinas são muitos, e resultam em letalidade, mutagênese e recombinogênese, em procariotos e eucariotos (Dardalhon *et al*., 2007; Lehoczký *et al*., 2007; Ho and Schärer, 2010). A extensão da reação de fotoadição pode ser controlada pela dose e comprimento de onda da radiação UV, de modo que mais de 40% dos monoadutos formados podem ser convertidos em ICLs. Estima-se que a DL37 do 8-MOP corresponde a cerca de 60 monoadutos e 30 ICLs por genoma, em células haploides de levedura (do tipo selvagem) em divisão (Bankmann and Brendel, 1989). O espectro de UVA (320-400 nm) induz ICL eficientemente, e comprimentos de onda mais longos (UVA em 400 nm) produzem principalmente monoadutos (Noll *et al*., 2006). Em células de levedura, ICLs causadas pelo tratamento com 8-MOP/UVA são reconhecidas por proteínas da via NER. A excisão das ICLs ou mesmo o colapso da forquilha de replicação ocasionado pelas

mesmas resulta na formação de DSBs (Magaña-Schwencke *et al*., 1982; Mladenov and Iliakis, 2011). As principais vias envolvidas na reparação de ICLs em *S. cerevisiae* são HR, TLS e uma via não completamente compreendida, mediada por Pso2p, que apresenta tanto atividade 3'5' exonuclease como também atividade endonuclease, esta específica para estruturas de DNA tipo *hairpins* (Henriques *et al*., 1997; Brendel and Henriques, 2001; Callebaut *et al*., 2002; Brendel *et al*., 2003; Bonatto *et al*., 2005; Tiefenbach and Junop, 2012).

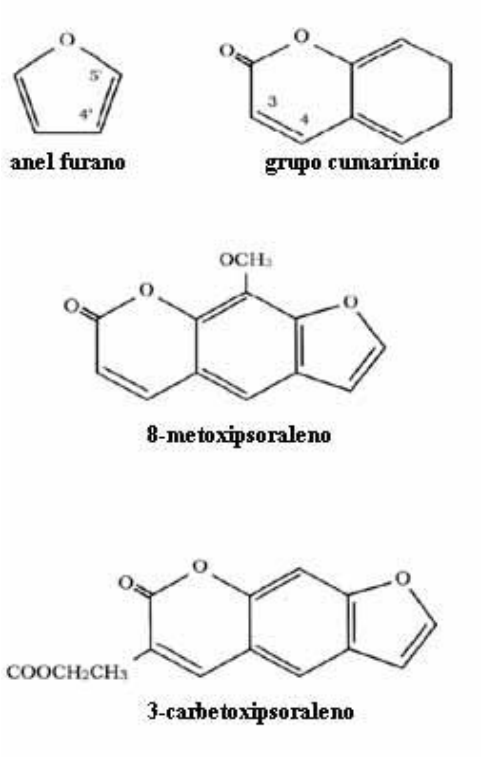

Figura 5. Estrutura molecular das furocumarinas.

2.4. Ditelureto de difenila

Encontrado com frequência em vegetais, particularmente em membros da Família Alliaceae, o telúrio metálico pode estar presente em fluidos corporais, como sangue e urina, embora sua função fisiológica em mamíferos ainda não esteja completamente esclarecida (Siddik and Newman, 1988; Newman *et al*., 1989; Řezanka and Sigler, 2008). Esse elemento também se faz presente em muitas proteínas de

bactérias (Ogra *et al*., 2008), leveduras e outros fungos (Yu *et al*., 1993), sob a forma de telurocisteína e telurometionina.

Compostos organotelurados apresentam propriedades imunomoduladoras e antiinflamatórias, que possibilitam o seu emprego como agente antitumoral e antiviral (Frei *et al*., 2008; Sredni-Kenigsbuch *et al*., 2008; Friedman *et al*., 2009), e já foram utilizados no tratamento da sífilis e da tuberculose. Estes compostos podem se apresentar como antioxidantes, por mimetizar a atividade da enzima glutationa peroxidase (GPx) (Ren *et al*., 2002; Huang and Hu, 2008). Seu efeito antiproliferativo também foi relatado em diferentes tecidos (revisado em Degrandi *et al*., 2010).

O ditelureto de difenila (DTDF) é um composto organotelurado sólido e altamente hidrofóbico, com peso molecular de 409,2 g (Figura 6). É um importante intermediário em reações de síntese orgânica, em especial de drogas contendo telúrio (Muñiz, 2005). A exposição ao DTDF causa teratogênese (Stangherlin *et al*., 2006), induz efeitos tóxicos em plaquetas, leucócitos, eritrócitos e promielócitos (Sailer *et al*., 2003; Borges *et al*., 2004; Nogueira *et al*., 2004) de ratos, além de causar citotoxidade, genotoxidade e mutagenicidade *in vitro* (Degrandi *et al*., 2010).

O DTDF induz apoptose em células HL-60 em cultura, em concentrações da ordem de 1M (Sailer *et al*., 2003), e pode induzir à ativação de caspases em concentração de apenas 1μM (Iwase *et al*., 2004). Alguns compostos organotelurados inibem seletivamente a atividade cisteíno-proteásica da catepsina B, constituindo-se candidatos potenciais a drogas antimetastáticas (Cunha *et al*., 2005). O DTDF também inibe a atividade da enzima sulfidrílica δ-ALA-D *in vitro*, em sobrenadante de fígado, com IC50 em torno de 10μM (Barbosa *et al*., 1998; Meotti *et al*., 2003; Nogueira *et al*., 2003). Como consequência, a síntese de grupamentos heme é interrompida, comprometendo a formação de novas moléculas de hemoglobina, citocromos e catalase. Além disso, o acúmulo de ácido aminolevulínico, substrato da δ-ALA-D, promove uma condição pró-oxidante (Emanuelli *et al*., 2001; Gonçalves *et al*., 2009). Embora o tipo de lesão genotóxica causada pelo tratamento com DTDF ainda não esteja precisamente esclarecida, sabe-se que o tratamento com DTDF induz DSBs em células de mamíferos, e experimentos realizados em leveduras apontam para o papel de proteínas da via de excisão de bases e vias recombinacionais na reparação destas lesões. Adicionalmente, a oxidação de GSH é fortemente aumentada em presença de DTDF, em leveduras e células de mamíferos, e este efeito é dose-dependente, sugerindo que o DTDF pode reagir com tióis biológicos (Degrandi *et al*., 2010). A depleção de GSH representa uma

diminuição nas defesas antioxidantes não enzimáticas, que pode levar a estresse oxidativo, com conseqüente indução de lesões oxidativas. Se GSH está relacionada aos efeitos do tratamento com DTDF, o pré-tratamento com compostos que estimulam aumento no GSH celular devem reduzir ou até abolir este efeito. De fato, o pré-tratamento das células com *N*-ac (molécula antioxidante que atua promovendo a síntese de GSH e removendo ERO) reduziram o declínio dos níveis de GSH na célula (Degrandi *et al*., 2010).

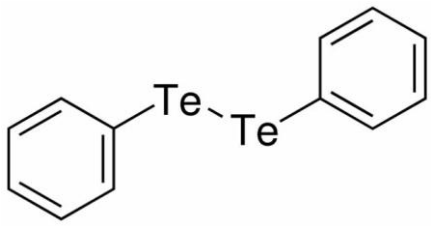

Figura 6. Fórmula estrutural do ditelureto de difenila.

3. Reparação de danos ao DNA no contexto da cromatina

Conforme anteriormente mencionado, a molécula de DNA está constantemente sujeita a sofrer alterações na sua estrutura, ocasionadas tanto por fatores endógenos como exógenos. Consequentemente, as células eucarióticas desenvolveram mecanismos complexos de resposta a danos genotóxicos, com a finalidade de preservar a estabilidade do seu genoma. Nesse contexto, os danos à molécula de DNA desencadeiam, primeiramente, a detecção do sítio lesionado, com posterior amplificação do sinal, que se dá por meio de uma cascata de proteínas cinases. Por fim, ocorre a ativação de uma série de proteínas efetoras, as quais promovem uma parada no ciclo celular, seguida da reparação do DNA (Méndez-Acuña *et al*., 2010).

Os mecanismos de reparação de DNA são alvos terapêuticos promissores no tratamento do câncer, constituindo-se em importantes ferramentas para o desenvolvimento de novos protocolos, ou mesmo proporcionando o aprimoramento daqueles já existentes, com a finalidade de aumentar a especificidade tecidual e reduzir os efeitos colaterais (Helleday *et al*., 2008; Smith *et al*., 2010). As proteínas recrutadas para o reconhecimento, processamento ou tolerância aos danos variam conforme a

natureza das lesões ao DNA, de modo que as principais vias de reparação de DNA atualmente descritas incluem: reparação de bases mal-emparelhadas, *mismatch repair* (MMR, Figura 7); reparação por excisão de bases, *base excision repair* (BER, Figura 8); reparação por excisão de nucleotídeos, *nucleotide excision repair* (NER, figura 9); reparação por síntese translesão, *translesion synthesis* (TLS, Figura 10); reparação por recombinação homóloga, *homologous recombination* (HR, Figura 11) e, finalmente, reparação por recombinação não-homóloga ou ilegítima, *non-homologous end joining* (NHEJ, Figura 12) (Coppedè and Migliore, 2010; Guainazzi and Schärer, 2010; Mladenov and Iliakis, 2011).

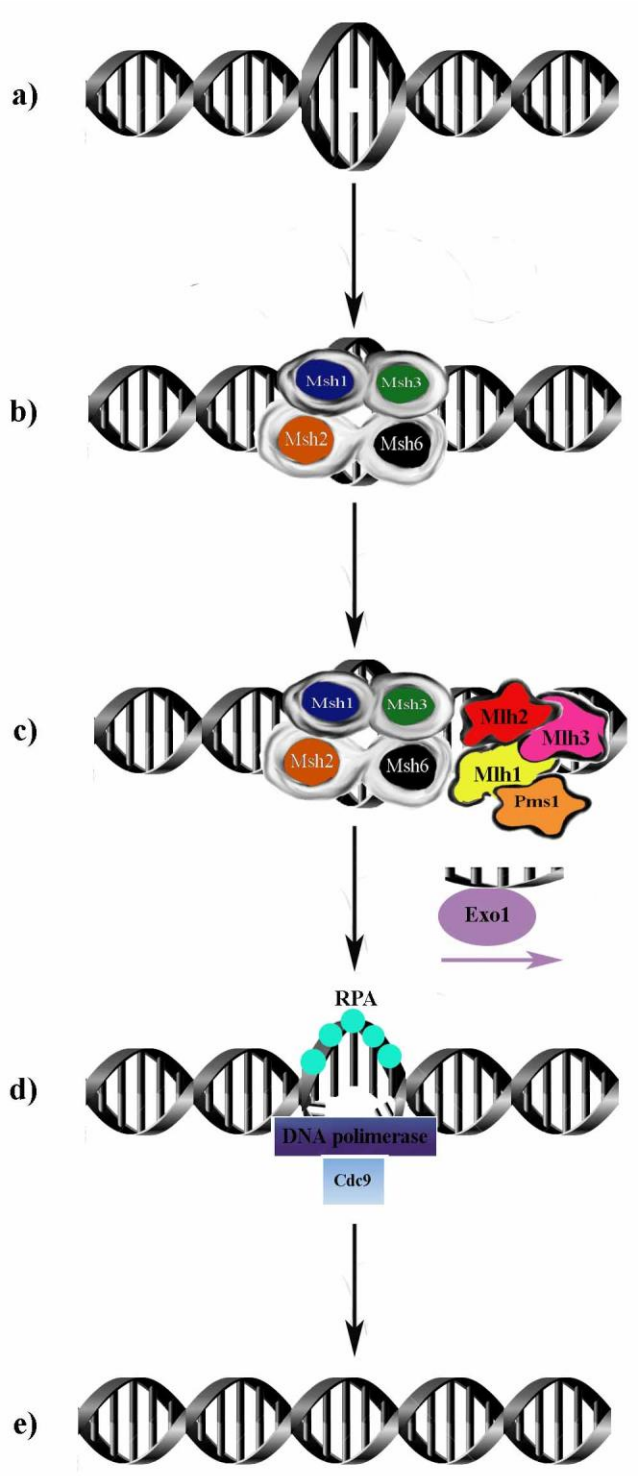

Figura 7. Representação esquemática da reparação de DNA pela via MMR, em leveduras. As proteínas Msh1, Msh2, Msh3 e Msh4 reconhecem as bases mal emparelhadas na molécula de DNA (a) e (b). Posteriormente, Mlh1, Mlh2, Mlh3 e Pms1 ligam-se às proteínas de reconhecimento, promovendo a clivagem do oligonucleotídeo contendo a base mal emparelhada (c), com a remoção do fragmento de DNA pela exonuclease Exo1. A RPA liga-se às regiões de DNA de cadeia simples, enquanto a

enzima DNA polimerase re-sintetiza o fragmento de DNA, e a ligase Cdc9 religa as extremidades (d), recompondo completamente a molécula (e) (adaptado de Ataian and Krebs, 2006).

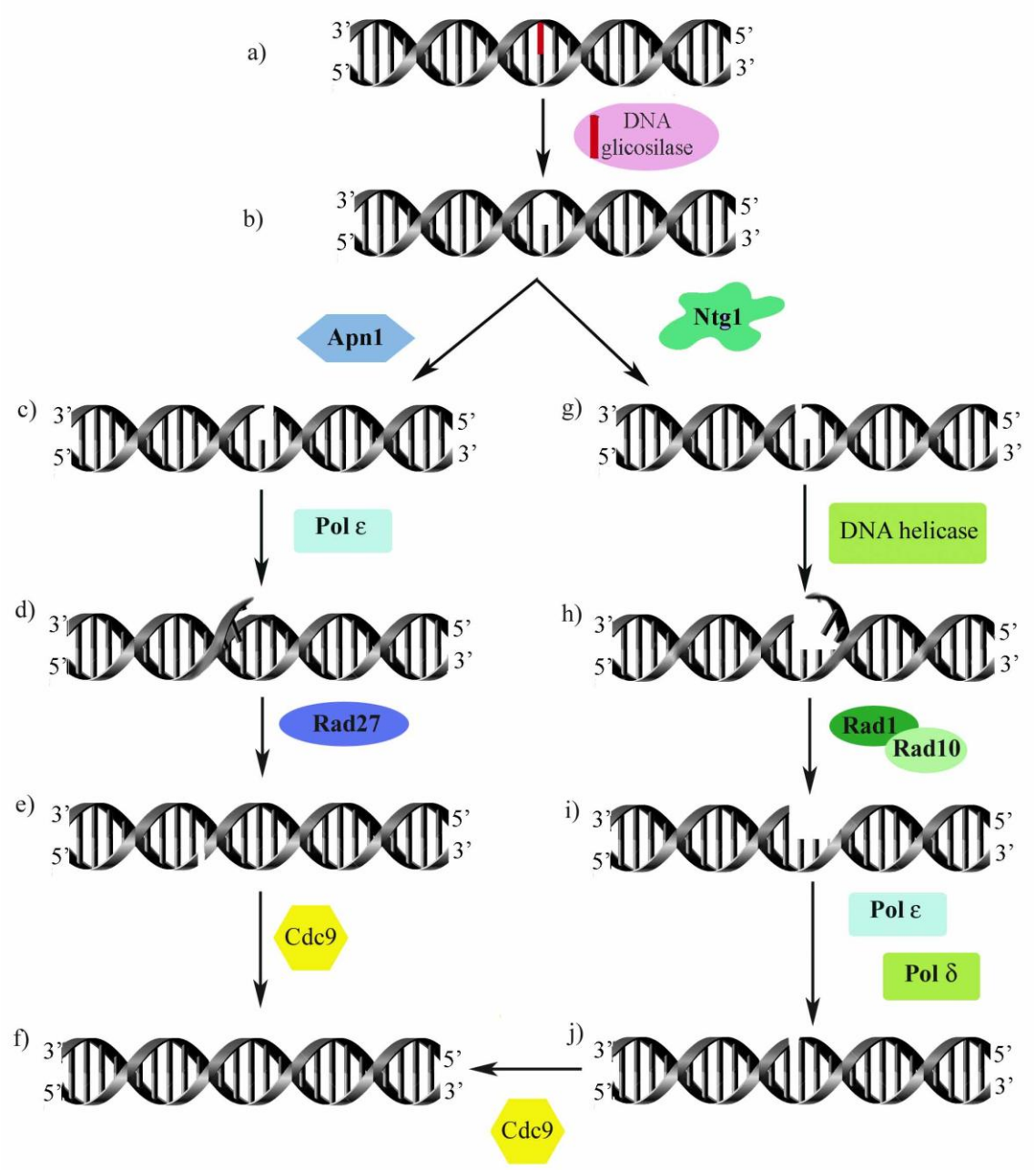

Figura 8. Esquema ilustrativo da via de reparação de DNA por excisão de bases (BER) em leveduras. A base danificada (a) é removida da molécula de DNA pela enzima DNA glicosilase, o que resulta na formação de um sítio AP (b). As enzimas AP endonuclease (Apn1), DNA polimerase (Polε), 5'-flap endonuclease (Rad27) e DNA ligase (Cdc9) efetuam a reparação da maioria dos sítios AP. Uma fração menor

destes sítios é clivada pelas AP liases Ntg1, Ntg2 ou Ogg1, seguido pela clivagem por Rad1-Rad10 (Boiteux and Guillet, 2004).

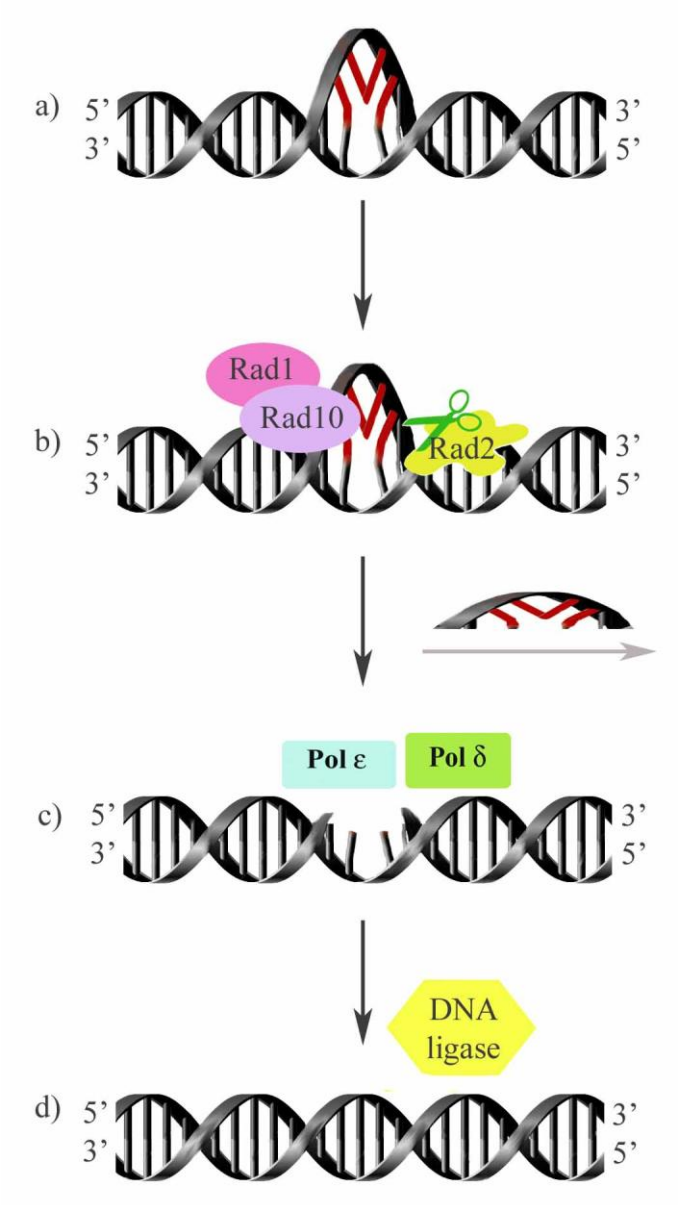

Figura 9. Representação esquemática da reparação de DNA por excisão de nucleotídeos (NER). Após o reconhecimento da lesão ao DNA (a), o complexo de excisão formado por Rad1-Rad10 e Rad2 cliva as extremidades do oligonucleotídeo que contém o dano (b). As enzimas DNA polimerase ε e DNA polimerase δ preenchem a lacuna (c), e a DNA ligase sela as extremidades, reconstituindo a molécula de DNA (d).

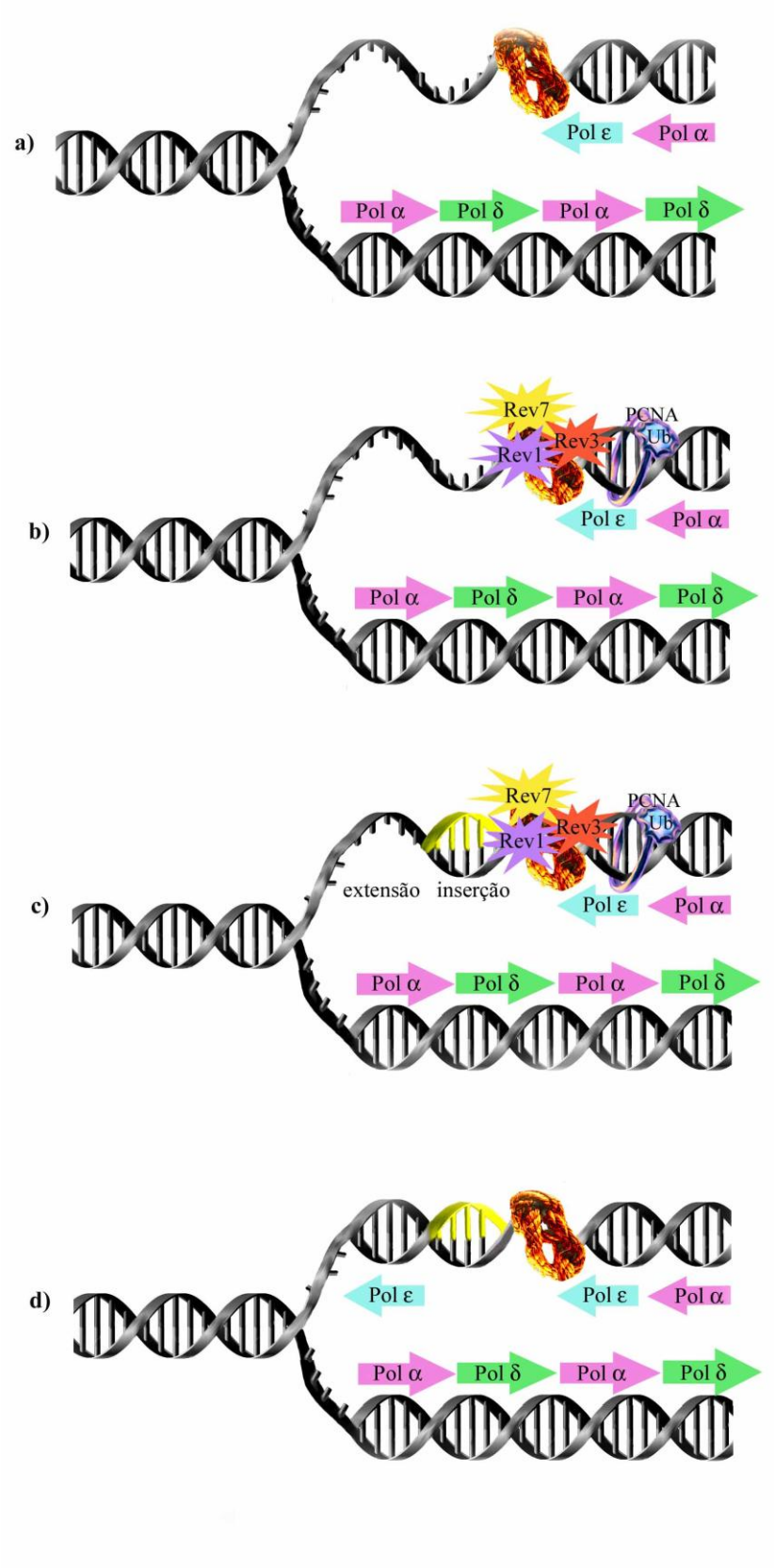

Figura 10. Representação esquemática da reparação de DNA por síntese translesão (TLS) em leveduras. A lesão ao DNA é representada por um nó (a). As DNA polimerases replicativas polδ e polε, responsáveis pela replicação do DNA

genômico, são incapazes de ultrapassar lesões. Em face do bloqueio da forquilha de replicação, o complexo PCNA-ubiquitina dissocia as polimerases replicativas e recruta outras polimerases (pol zeta, Rev3-Rev7, associadas a Rev1), estas capazes de ultrapassar lesões (b). Como consequência da ação destas polimerases translesão, ocorre a inserção de um oligonucleotídeo no filamento oposto ao que contém a lesão, possibilitando a extensão da cadeia pela pol zeta (c). Ultrapassada a lesão, o complexo Rev3-Rev7 e Rev1 é dissociado e as polimerases replicativas são reassociadas, dando continuidade à síntese de DNA (d) (adaptado de Gan *et al*., 2008).

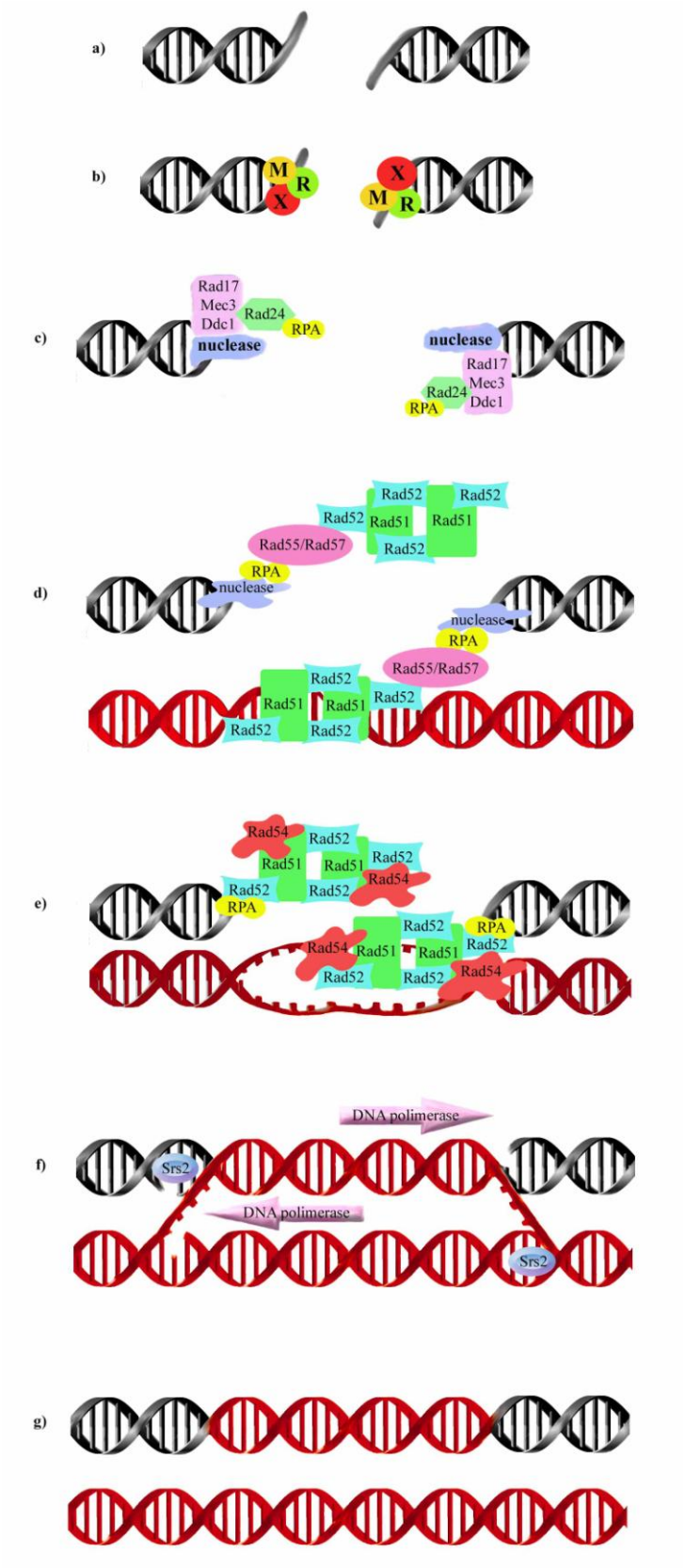

Figura 11. Representação esquemática da reparação de DNA pela via de recombinação homóloga (HR), em leveduras. Uma vez estabelecida uma DSB (a), o complexo formado por Mre11p/Rad52p/Xrs2p (MRX) reconhece a quebra e liga-se a

esta nas extremidades (b), seguido por nucleases que formam caudas de DNA de cadeia simples, as quais, por sua vez, são cobertas por RPAs (c). Em seguida, forma-se um filamento de Rad51p, ocupando o lugar das RPAs. Rad52p e o complexo Rad55p/Rad57p mediam a formação do filamento (d), com a possível participação de Rad54p. Concomitantemente, ocorre invasão do filamento na busca por seqüências homólogas de DNA que, uma vez encontradas, cessam o processo de resecção, e Rad51p dissocia-se (e). A helicase Srs2p media a dissociação de Rad51p, e a DNA polimerase sintetiza as novas cadeias de DNA (f). Finalmente, as extremidades quebradas são religadas (g) (adaptado de Aylon and Kupiec, 2004).

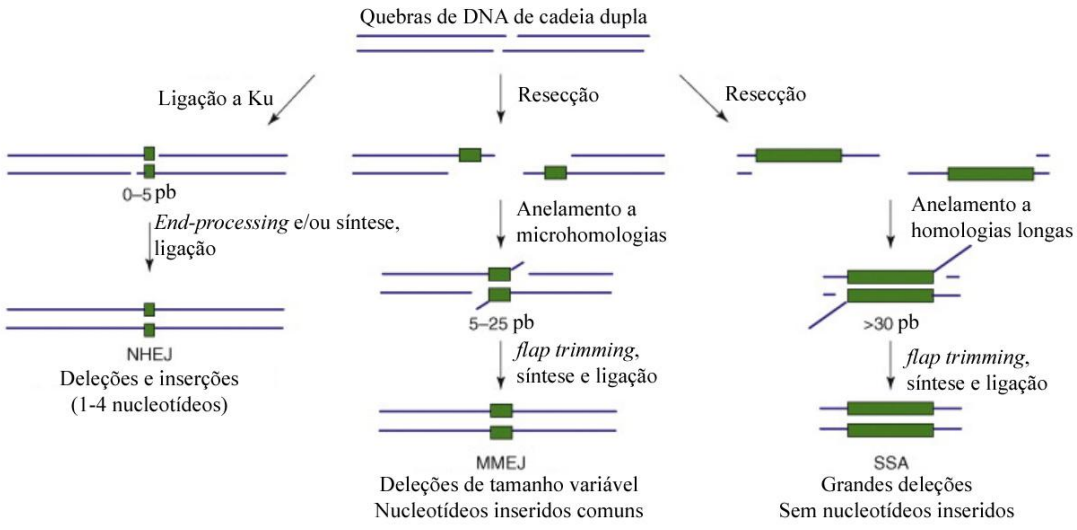

Figura 12. Representação esquemática da reparação de DNA pelas vias NHEJ, MMEJ e SSA em *S. cerevisiae*. Durante a reparação de DSB pela via NHEJ, ocorre ligação do heterodímero Ku70-Ku80, impedindo a ressecção de *DNA-ends*. A reparação se dá pelo anelamento a microhomologias (caixas verdes), com preenchimento por Pol4 e ligação pela DNA ligase IV, resultando em pequena deleção e produtos de inserção. MMEJ e SSA necessitam de ressecção terminal do DNA ou o seu relaxamento, para revelar sequências homólogas. Contudo, o comprimento da homologia necessária para a via MMEJ (5-25 pb) é mais curto do que para SSA. SSA e MMEJ também exigem a clivagem da extremidade 3' antes da etapa de síntese, preenchimento e ligação.

Considerando que os produtos da via MMEJ podem conter nucleotídeos inseridos, estes nunca são observados na via SSA (adaptado de McVey and Lee, 2008).

A estrutura nucleossomal da cromatina forma uma barreira, que impede a interação direta de proteínas com a molécula de DNA, bloqueando o acesso de enzimas de transcrição, replicação ou reparação de DNA (Dinant *et al*., 2008; Sarkar *et al*., 2010). Para transpor esta barreira, as células contam com enzimas capazes de alterar a estrutura da cromatina, remodelando-a. Esse processo se dá por meio de dois mecanismos principais: o primeiro consiste em modificar covalentemente os resíduos localizados nas caudas N- ou C-terminais das histonas, por fosforilação, acetilação, metilação, ubiquitilação e ADP-ribosilação (Kouzarides, 2007; Altaf *et al*., 2007; Sharma *et al*., 2010). Essas modificações rompem as interações da histona com o DNA, e servem como sítios de ligação para fatores associados à cromatina (Palomera-Sanchez and Zurita, 2011). O segundo mecanismo, por sua vez, consiste em utilizar a energia da hidrólise do ATP para reposicionar ou remover os nucleossomas da cromatina, ou, ainda, trocar variantes de histona, sem necessariamente remover o nucleossoma (Lusser and Kadonaga, 2003; Eberharter and Becker, 2004). Em presença de danos à molécula de DNA, o remodelamento da cromatina se processa, inicialmente, por meio da fosforilação de uma variante da histona H2A, denominada H2AX (Fernandez-Capetillo *et al*., 2004). Essa fosforilação é induzida por um grupo de proteínas evolutivamente conservadas, pertencentes à família das fosfatidilinositol-3-cinases (PI3K), denominadas de ATM (*Ataxia Telangiectasia Mutated*) e ATR (*ATM-/Rad3-related proteins*) em mamíferos, ou Mec1p/Tel1p, em leveduras. As enzimas da família PI3K induzem uma cascata de fosforilação em diferentes substratos, como mediadores, transdutores e efetores. Uma vez fosforilada, H2AX (agora chamada de γ-H2AX) ativa outras cinases, também pertencentes à família das PI3K, que induzem paradas no ciclo celular e coordenam a reparação do DNA danificado (revisado em Faucher and Wellinger, 2010; Palomera-Sanchez and Zurita, 2011) (Figura 13). Assim, a formação de γ-H2AX resulta no recrutamento de muitos complexos de remodelamento ao local do dano ao DNA, causando alterações estruturais na cromatina, de modo que as proteínas envolvidas na reparação do DNA tenham acesso à lesão.

γ-H2AX também é capaz de recrutar complexos de coesina às DSBs, o que pode explicar a formação de um amplo domínio de cromatina contendo γ-H2AX nas suas proximidades. Esse efeito previne a perda de regiões cromossômicas inteiras, bem

como quebras na molécula de DNA, resultantes da progressão para quebras cromossômicas e translocações, além de manter as extremidades terminais do DNA em estreita proximidade, aumentando a eficiência do reparo (Altaf *et al*., 2007). Em leveduras, a relação de ligantes de γ-H2A inclui a acetiltransferase de histona (HAT, *histone acetyltransferase*) NuA4, bem como os complexos de remodelamento da cromatina INO80 e SWR1. O complexo de HAT NuA4 é o primeiro a surgir na região da quebra do DNA, e liga-se a γ-H2A pela sua subunidade Arp4p, também presente nos complexos INO80 and SWR1. A subunidade Nhp10p do complexo INO80 também é necessária para a interação com γ-H2A, e é possível que as subunidades Nhp10p e Ies3p facilitem essa interação (revisado por Attikum and Gasser, 2009). SWR1 possivelmente atua na remoção de γ-H2A da cromatina, ocasionando a liberação dos fatores de reparação do DNA dos sítios de dano. Outra possibilidade para a finalização da sinalização via γ-H2AX é a existência de fosfatases capazes de desfosforilar a histona, e, de fato, a fosfatase de levedura Pph3p regula a fosforilação de H2AX *in vivo* (Altaf *et al*., 2007). Contudo, acredita-se que o complexo INO80 atua promovendo a manutenção de altos níveis de γ-H2AX nas proximidades das DSBs, e o complexo SWR1 substitui γ-H2AX por H2AZ.

Em células de mamíferos, o complexo Mre11p–Rad50p–Nbs1p (MRN), que atua no reconhecimento, sinalização e reparação de DSBs, além do seu papel na recombinação e no controle de *checkpoint* nas fases S ou G2/M do ciclo celular, liga-se a γ-H2AX pela subunidade Nbs1p. Esta interação entre Nbs1p e γ-H2AX é necessária para a retenção do complexo MRN no sítio de dano, embora estudos recentes demonstrem que o complexo MRN pode dar início à ressecção da extremidade do DNA e ao reparo por HR, em uma via independente de H2AX, a qual também começa com a ativação de ATM e *checkpoint* de ciclo celular (Yuan and Chen, 2010).

Conforme mencionado, a reparação de DSBs em células eucarióticas se dá principalmente pelas vias HR e NHEJ. A via HR se utiliza da informação da cromátide irmã não danificada ou do cromossoma homólogo para a reparação, enquanto a via NHEJ liga diretamente as terminações da cadeia de DNA após a quebra. Além disso, sabe-se que embora a formação de γ-H2AX não seja necessária para as etapas iniciais da reparação de DNA, esta é importante para concentrar a maquinaria de reparação nas proximidades das lesões, bem como para o recrutamento de remodeladores de cromatina, como a HAT NuA4, cuja atividade promove o relaxamento da estrutura da cromatina que circunda a lesão genotóxica (Escargueil *et al*., 2008).

A histona H2AX pode ser fosforilada tanto por ATM/ATR (envolvido no reconhecimento de lesões ao DNA pela via HR) como por DNA-PK (envolvida no reconhecimento de lesões ao DNA pela via NHEJ), sugerindo que γ-H2AX desempenhe um papel central nestas duas vias de reparação de DNA. Tanto HR como NHEJ podem de alguma forma competir para a reparação, e seus componentes podem se ligar à mesma lesão no DNA e, eventualmente, encontram-se co-localizados, o que dificulta a determinação do quanto as modificações específicas de histonas podem associar-se, ou até mesmo determinar a escolha por vias específicas de reparação de DNA (Escargueil *et al*., 2008).

Em termos gerais, enquanto a eficiência da via BER não requer modificações na estrutura da cromatina, a via NER e vias envolvidas na reparação de DSBs exigem eventos de relaxamento global e local. Na via NER, a inibição transcricional leva (a) ao recrutamento de fatores de reparação acoplada à transcrição, após o bloqueio da enzima RNA polimerase II; e (b) à percepção da presença de danos ao DNA, desencadeando eventos que resultam no relaxamento global da estrutura da cromatina, permitindo o processo de reparação do genoma global. A detecção de DSBs inicialmente induz a fosforilação de H2AX, que consiste em uma resposta inicial comum para as vias HR e NHEJ. Este evento leva ao relaxamento global e local da estrutura da cromatina, por meio do recrutamento de modificadores de histonas e da atividade de modificadores de cromatina. O relaxamento global e local da cromatina aumenta a acessibilidade de marcadores epigenéticos, levando à ativação de *checkpoints* específicos. A via MMR, ao contrário de outras vias de reparação de DNA, é dependente de alterações na estrutura da cromatina, estas induzidas pela maquinaria de replicação.

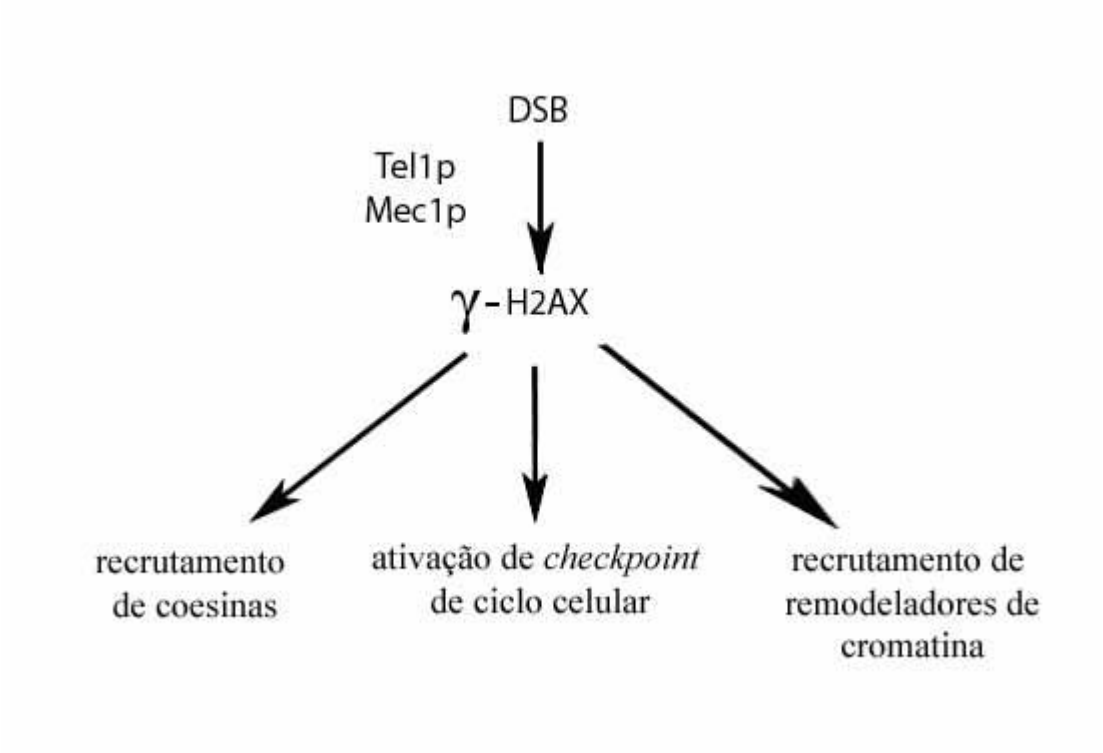

Figura 13. Sinalização γ-H2AX e reparação de DSBs em leveduras.

4. Papel da proteína Pso2p na reparação de ICLs em *S. cerevisiae*

Mutantes de leveduras hipersensíveis a agentes indutores de ICLs foram identificados no início da década de 80 (Tabela 1) por dois diferentes processos de triagem em *Saccharomyces cerevisiae*, os quais mostraram sensibilidade a psoralenos fotoativados (*pso*2-1) (Henriques and Moustacchi, 1980) e sensibilidade a mostardas nitrogneadas (*snm*1-1) (Siede and Brendel, 1981). Mais tarde, Cassier-chauvat e Moustacchi (1988) demonstraram que os genes PSO2 e SNM1 eram alélicos, e a nomenclatura *PSO2* foi adotada para unificá-los (Brendel and Henriques, 2001). Os mutantes de levedura *pso2* apresentam uma caracterítica única, que é a hipersensiblidade a psoralenos fotoativados e a agentes alquilantes bi-ou poli-funcionais, enquanto exibem uma sensibilidade praticamente igual às linhagens selvagens, a agentes monofuncionais, radiações ionizantes, radiação UVC (λ = 254 nm) (revisado em Henriques and Brendel, 1990; Henriques *et al.*, 1997; Brendel and Henriques, 2001) e endonuclease HO (Li and Moses, 2003). A seguir, foram caracterizados fenotipicamente e molecularmente 10 genes PSO (Tabela 1), sendo que oito destes genes (*PSO1*, *PSO2*, *PSO3*, *PSO4*, *PSO5*, *PSO8*, *PSO9* e *PSO10*) estão

envolvidos na reparação de danos ao DNA ou na tolerância a estes, enquanto *PSO6* e *PSO7* influenciam processos fisiológicos que não são relacionados ao metabolismo de ácidos nucleicos.

Tabela 1. Características das proteínas codificadas pelos genes *PSO* e os fenótipos dos seus alelos mutantes[1].

Gene/Alelo	Proteína (kDa)/Função	Fenótipo do mutante
PSO1/REV3	173; subunidade catalítica da DNA polimerase zeta	Sensível a radiação e a mutágenos químicos, baixa mutabilidade.
PSO2/SNM1	72; 5'-exonuclease e clivagem endonucleolítica de estruturas de DNA tipo *hairpin*	Sensível a todos os tratamentos indutores de ICL, baixa mutabilidade somente com mutágenos de ICL.
PSO3/RNR4	40,1; pequena subunidade (y4) de ribonucleotídeo redutase	Baixa mutabilidade e recombinação induzidas, sensível a baixas temperaturas de crescimento e a ERO.
PSO4/PRP19	56,7; proteína associada à spliceossoma	Sensível a mutágenos, sem recombinação mitótica, baixa mutabilidade, sem esporulação a 30°C.
PSO5/RAD16	91,3; DNA helicase do NER global	UVC-, sensível a danos oxidativos, envolvido no controle do envelhecimento, mutabilidade normal.
PSO6/ERG3	43; desaturase de ergosterol	Sensível a ERO, a nistatina, a calcoflúor branco, superprodução e má distribuição de chitina.
PSO7/COX11	28; citocromo C oxidase	Células em crescimento sensíveis a 4-NQO, 8HQ e NDEA.
PSO8/RAD6	19,5; reparação de DNA conjugado a ubiquitina	Baixa mutabilidade, esporulação variável, crescimento lento a 16°C.
PSO9/MEC3	53,1; *checkpoint* de reparação de DNA (G2)	Sensível a UVC, esporulação normal e baixa mutabilidade.
PSO10/MMS21	SUMO ligase e componente	Sensível a PUVA, MNNG, UVC, baixa

Abreviaturas: ERO = espécies reativas de oxigênio; 4-NQO = 4-Nitroquinolina 1-oxido; 8HQ = 8-hidroxiquinolina; NDEA = N-nitrosodietilamina; PUVA = psoralenos + UVA (PUVA terapia); MNNG = N-metil-N'-nitro-N-nitrosoguanidina. [1]Tabela baseada em (Henriques *et al*., 1997; Brendel and Henriques, 2001; Brendel *et al*., 2003; Bonatto *et al*., 2005; Cardone *et al*., 2006; Hoch *et al*., 2008; Tiefenbach and Junop, 2012).

A proteína Pso2p pertence a um pequeno grupo de proteínas que atuam predominantemente durante a reparação de ICLs (Brendel *et al*., 2003), e apresenta atividade 5'-exonuclease de DNA (Li *et al*., 2005), além de atividade endonucleásica específica para estruturas de DNA tipo *hairpin* (Tiefenbach and Junop, 2012). Sua atividade nucleásica deve-se a presença de um domínio metalo-β-lactamase hidrolase, associado a um domínio β-CASP (CPSF Artemis SNM1/PSO2) (Callebaut *et al*. 2002; Bonatto *et al*. 2005), sendo este último exclusivo para a família de genes *PSO2*, previsto para ser um domínio de ligação de ácido nucléico (Callebaut *et al*., 2002; Li *et al*., 2005; Bonatto *et al*., 2005; Lenain *et al*., 2006; Hejna *et al*., 2007). Foram identificados cinco ortólogos humanos para o gene *PSO2*: CPSF73 e ELAC2 (envolvidos no processamento de RNA) e SNM1A, SNM1B/Apollo, SNM1C/Artemis (envolvidos no metabolismo do DNA e regulação do ciclo celular, Tabela 2) (revisado por Bonatto *et al*., 2005; Cattell *et al*., 2010; Yan *et al*., 2010).

Tabela 2. Ortólogos humanos *SNM1* para o gene *PSO*.

Gene	Função
SNM1A	- atua como 5'-exonuclease, sendo o homólogo funcional de Pso2p de leveduras em mamíferos (Bonatto *et al*., 2005; Hazrati *et al*., 2008). - Não possui função de endonuclease (Hejna *et al*., 2007). - Atua mediando a resistência a algumas drogas indutoras de cross-links e na manutenção da estabilidade genômica após a formação de ICLs (Dronkert *et al*., 2000; Akhter *et al*., 2004; Ahkter *et al*., 2005; Akhter and Legerski, 2008).
SNM1B/Apollo	-5'-exonuclease; atua na manutenção

	telomérica (Lenain *et al*., 2006).
SNM1C/Ártemis	Apresenta atividade 5'-exonuclease (individualmente). - endonuclease - complexo Artemis: DNA-PKcs clivam as extremidades 5' e 3' do DNA e apresenta atividade de abertura de *hairpin* (Ma *et al*., 2002). - atua na recombinação V(D)J e na imunocompetência, e sua deficiência resulta em severa imunodeficiência associada com elevada radiossensibilidade celular (Moshous *et al*., 2001).

Linhagens de leveduras deficientes em Pso2p apresentam defeitos na reparação de DSBs resultantes da clivagem das forquilhas de replicação colapsadas pelo ICL (McHugh *et al*., 2000; Cattell *et al*., 2010). Mutações no gene *PSO2* demonstram relações epistáticas com genes que codificam proteínas da via NER, no que se refere à sensibilidade a agentes indutores de ICL, mas mutantes *pso2Δ* incisam ICL com cinética normal, entretanto, o DNA de alto peso molecular não é reconstituído (Magaña-Schwencke *et al*., 1982; Cattell et al., 2010). Estudos *in silico* e genético-bioquímicos indicam que Pso2p possivelmente atua na restauração do DNA de alto peso molecular, por meio da limpeza das extremidades de DNA geradas após a remoção do ICL, embora não se conheça o modo pelo qual a função endonúcleásica de Pso2p seja ativada, ou mesmo as proteínas responsáveis por essa ativação. Sabe-se que Pso2p pode ser direcionado para o local da incisão do ICL para a ressecção das estruturas intermediárias da reparação, fornecendo substratos para as vias TLS (pelas polimerases Rev1, Polζ, Polη, Polκ, Polι and Polν), na fase G1 do ciclo celular, ou HR, nas fases S e G2 (revisado em Cruz *et al*., 2012). Pso2p também demonstrou um papel de sobreposição com Exo1p, durante a reparação de forquilhas de replicação colapsadas (Yan *et al*., 2010; Sarkar, Kiely, and McHugh, 2010). Recentemente, Tiefenbach e Junop (2012) demonstraram que Pso2p apresenta uma função de endonuclease de

abertura de *hairpin*, cuja atividade é necessária para a reparação de quebras cromossomais que comportam extremidades fechadas do tipo *hairpin*. Esse achado confirma as análises de bioinformática *in silico* de Brendel *et al.*, (2003), que sugeriram que Pso2p pudesse ser uma endonuclease específica relacionada à abertura de estruturas do tipo *hairpin*, as quais surgem durante a replicação do DNA, em presença de ICLs. Desse modo, Pso2p pode atuar no processamento das extremidades de DNA, gerando substratos adequados para o processo de reparação, embora a sua função precisa ainda apresente muitos aspectos passíveis de elucidação.

5. Complexos de remodelamento de cromatina e seu papel na reparação de DNA

5.1. Complexo NuA4

A acetiltransferase de histona (HAT) NuA4 é um complexo multiproteico responsável pela acetilação das histonas H4 e H2A, e exerce um importante papel na reparação de DNA (Figura 14). Mutações nas suas subunidades Esa1p ou Yng2p, ou ainda nos resíduos de lisina da histona H4 (H4K5, 8, 12, 16), resultam em hipersensibilidade a agentes indutores de DSB, e prejudicam a reparação da DSB por não "relaxar" a cromatina nas proximidades da lesão. O complexo NuA4 também interage com γ-H2AX por meio da sua subunidade Arp4p, e é o primeiro complexo a surgir no local da lesão (Altaf, Saksouk, and Côté, 2007; Attikum and Gasser, 2009). A presença de NuA4 na região da DSB promove o recrutamento de fatores de reparação de DNA e outros complexos de remodelamento de cromatina, como SWR1 e INO80, talvez pelo aumento na acessibilidade à estrutura da cromatina, promovido pela acetilação de H4 (Altaf, Saksouk, and Côté, 2007). Além disso, a acetilação de H2AZ por NuA4 talvez promova a substituição de H2A por γ-H2A, de modo dependente de SWR1, controlando a taxa destas duas formas de H2A em torno do sítio da DSB, e influenciando o fenômeno conhecido como adaptação de *checkpoint* (Osley, Tsukuda, and Nickoloff, 2007). Durante a adaptação de *checkpoint*, as células podem escapar de uma parada estendida de *checkpoint*, e retornar ao ciclo celular, mesmo em presença de uma DSB não reparada (Papamichos-Chronakis, Krebs, and Peterson, 2006).

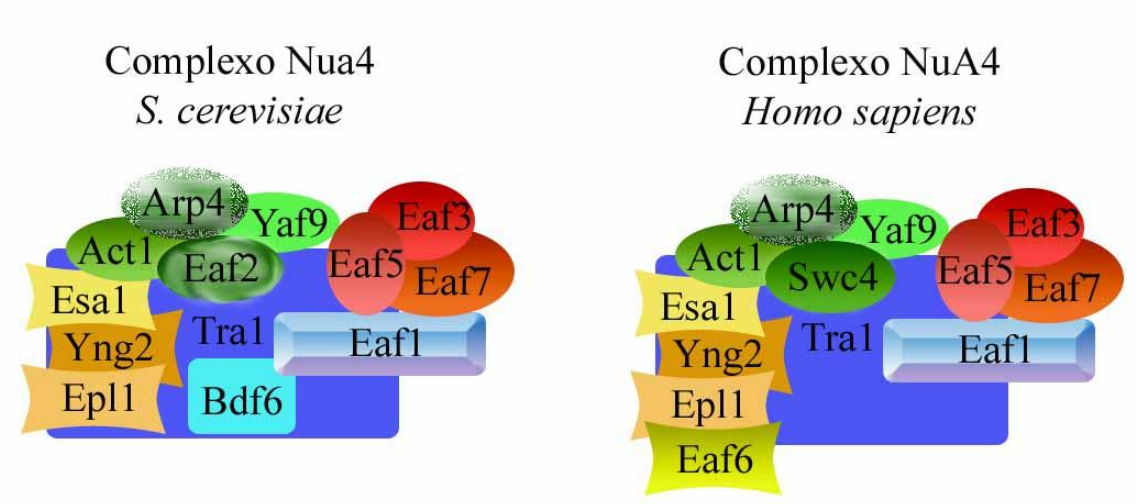

Figura 14. Representação esquemática das subunidades do complexo NuA4 de *S. cerevisiae* e *Homo sapiens*.

5.2. Complexo INO80

O complexo INO80 é uma ATPase pertencente a uma subfamília da família de remodeladores de cromatina SWI/SNF, com ortólogos e homólogos identificados em leveduras, moscas, plantas e mamíferos. Os complexos de remodelamento de cromatina da subfamília INO80 são INO80 and SWR1, em *S. cerevisiae*; INO80, p400 e *Snf2-related CBP activator protein* (SRCAP), em mamíferos; e INO80 e p400, em *Drosophila melanogaster* (Morrison and Shen, 2009). O complexo INO80 de leveduras tem aproximadamente 12 subunidades, com diferentes atividades enzimáticas, como a ATPase Swi2p/Snf2p-*like*, representada pela subunidade Ino80p, e proteínas com homologia com a helicase bacteriana RuvBp.

Muitas evidências conectam o remodelamento de cromatina dependente de INO80 à reparação de DSBs (Osley, Tsukuda, and Nickoloff, 2007). O complexo INO80 é recrutado à DSB pela sua interação com γ-H2A, possivelmente por meio das subunidades Nhp10p e Arp4p, e a subunidade Arp8p influencia a taxa de carregamento de Rad51p na DSB, talvez atuando no deslocamento nucleossomal, de maneira independente da fosforilação de H2A (Sarkar, Kiely, and McHugh, 2010). INO80 é necessário para a disrupção dos nucleossomas posicionados no locus MAT de leveduras (*mating-type*), após a indução de DSB *in vivo*, e esta atividade de remodelamento

resulta na evicção de nucleossomas inteiros, em regiões de ~ 5–6 kb ao redor da DSB, talvez fornecendo o substrato para a fosfatase de γ-H2AX, Pph3p (Osley, Tsukuda, and Nickoloff, 2007). Por outro lado, INO80 pode atuar como aceptor de nucleossoma, seqüestrando temporariamente os nucleossomas deslocados, ou estabilizando os nucleossomas alterados durante a reparação, e cooperando com outros fatores para a restauração da estrutura da cromatina após a conclusão do reparo (Sarkar, Kiely, and McHugh, 2010).

Muitos estudos relatam o papel do complexo INO80 na retomada da replicação, após estresse replicativo, e nas vias de tolerância ao dano durante a replicação. Defeitos na montagem da cromatina em DNA nascente afetam negativamente a progressão da forquilha, na fase S do ciclo celular (Sarkar, Kiely, and McHugh, 2010). Embora existam evidências da participação de INO80 no reparo de DSB por HR em plantas e leveduras, a etapa da via HR que depende de INO80 ainda não foi esclarecida (Osley, Tsukuda, and Nickoloff, 2007), mas mutantes da subunidade Arp do complexo INO80 de *S. cerevisiae* apresentam defeitos na via NHEJ, bem como na via HR (Morrison and Shen, 2009). A ausência do complexo INO80 causa defeitos na adaptação a *checkpoint*, e não se sabe se os papéis postulados para INO80 durante os estágios iniciais da via HR e da adaptação a *checkpoint* estão relacionados, ou se INO80 tem diferentes papéis no repado de DSB e adaptação de *checkpoint* (Osley, Tsukuda, and Nickoloff, 2007).

O complexo INO80 opera na mesma direção do NER, e também contribui para a remoção de fotoprodutos UV em regiões de alta incidência de nucleossomas (Sarkar, Kiely, and McHugh, 2010). Adicionalmente, INO80 interage com o complexo Rad4p–Rad23p, um complexo de reconhecimento de danos da via NER, após seu recrutamento à cromatina por Rad4p, de forma dependente de dano por UV. Após a ativação da resposta ao dano, INO80 é fosforilado em sua subunidade Ies4p por cinases ATM e ATR *in vivo* e *in vitro*, e isso modula a resposta celular a agentes causadores de danos ao DNA, sem alterar a composição do complexo ou sua habilidade para localizar sítios de dano. A subunidade Ies4p é também fosforilada pelas cinases Tel1p e Mec1p, modulando respostas de *checkpoint* de replicação de DNA, sem alterar o processo de reparo de DSB (Morrison and Shen, 2009).

5.3. Complexo RSC

O complexo de remodelamento de cromatina de leveduras RSC é membro da classe dos remodeladores SWI/SNF, e é composto por 15 subunidades. Mutações nas suas subunidades Rsc1p, Rsc2p e Sth1p causam hipersensibilidade a agentes indutores de quebras no DNA (Attikum and Gasser, 2005). RSC tem homólogos em outros eucariotos e atua em ambos, ativação e repressão transcricional, bem como na manutenção da viabilidade celular, e também tem papel no reparo de DSB (Osley, Tsukuda, and Nickoloff, 2007).

A subunidade Rsc8p e a subunidade catalítica Sth1p (ambas pertencentes ao complexo RSC) são recrutadas às DSBs, e ligam-se rapidamente à lesão, onde se acumulam em regiões imediatamente adjacentes ao dano (Attikum and Gasser, 2005). Defeitos na atividade do complexo RSC causam hipersensibilidade à DSB, o que pode ser atribuído a defeitos nas vias NHEJ e HR. Além disso, RSC é recrutado à DSB ao mesmo tempo que o complexo MRX, envolvido nas vias NHEJ, HR, e na sinalização de *checkpoint*, o que pode levar ao remodelamento da cromatina próximo ao sítio da quebra, facilitando o acesso de fatores da via NHEJ (Osley, Tsukuda, and Nickoloff, 2007). O complexo RSC também pode carregar coesinas nos braços cromossomais, mantendo as cromátides irmãs próximas, e durante o reparo de DSB, promovendo a ligação das terminações proximais, na finalização do NHEJ, e distais, na HR (Osley, Tsukuda, and Nickoloff, 2007). A importância deste carregamento de coesinas reflete o fato de que defeitos no estabelecimento da coesão durante a fase S ou carregamento *de novo* de coesinas na DSB durante a fase G2 reduz a eficiência da reparação pós-replicativa (*post-replicative repair*, PRR) (Attikum and Gasser, 2005).

5.4. Complexo SWI/SNF

O complexo SWI/SNF de levedura é formado por 11 subunidades, e a sua subunidade catalítica, Swi2p/Snf2p, pertencem à classe de proteínas de remodelamento de cromatina dependentes de ATP, conservada em todos os eucariotos SWI/SNF atua na transcrição gênica e na reparação de DSB (Lee *et al*., 2010; Chen *et al*., 2010). Leveduras mutantes com deficiência na atividade de SWI/SNF são sensíveis a agentes

genotóxicos que causam DSB, e fortes evidências sugerem seu papel na via HR, por sua associação com ambos, o locus receptor MATa e o locus doador HMLα..

SWI/SNF aumenta a acessibilidade ao DNA no core nucleossomal, embora as proteínas do NER também aumentem a atividade de remodelamento, sugerindo uma relação cooperativa entre o reparo de danos ao DNA e o remodelamento da cromatina. O complexo SWI/SNF altera os contatos histona-DNA, deslocando ou remodelando nucleossomas, ou transferindo dímeros de histonas *in vitro* (Tomar *et al*., 2009; Albini and Puri, 2010). Acerca do papel do complexo SWI/SNF na reparação por HR, este pode ser o de "relaxar" ou modificar a estrutura da ordem maior da cromatina no loci doador HM, facilitando as interações entre proteínas e DNA e DNA-DNA durante a formação dos filamentos sinápticos (Chai *et al*., 2005). Como os complexos INO80 e SWI/SNF são recrutados para o MAT DSB praticamente ao mesmo tempo, pode ocorrer a compartimentalização das atividades de remodelamento de cromatina dependente de ATP, com INO80 disruptando a cromatina no lócus receptor para HR, e SWI/SNF modificando a estrutura da cromatina no lócus doador (Osley, Tsukuda, and Nickoloff, 2007).

5.5. Complexo SWR1

O complexo multimérico SWR1 de leveduras possui uma subunidade ATPase relacionada a Swi2p/Snf2p (Swr1p), e pertence à classe INO80 de remodeladores de cromatina. Esse complexo catalisa a troca da histona canônica H2A pela variante Htz1, que é incorporada à cromatina, evitando a propagação de regiões de heterocromatina na eucromatina (Attikum and Gasser, 2005b). Esta dinâmica de alteração da composição nucleossomal regula os processos de transcrição gênica, silenciamento de genes, segregação cromossômica e reparação de DNA (Morillo-Huesca *et al*., 2010).

Embora a variante de histona Htz1p de levedura seja amplamente difundida na cromatina, essa localiza-se, principalmente, próximo aos sítios de início da transcrição de genes inativos, e na região limítrofe entre heterocromatina e eucromatina (Osley, Tsukuda, and Nickoloff, 2007). A biologia de SWR1 e Htz1p vem sendo intensamente estudada quanto ao seu papel na regulação transcricional, e SWR1 também atua no reparo de DSBs de modo independente, controlando os níveis de γ-H2A nos sítios de

dano (Osley, Tsukuda, and Nickoloff, 2007). Em cepas de leveduras deficientes em Htz1p, o complexo SWR1 promove um acúmulo de danos recombinogênicos no DNA, por um mecanismo dependente de γ-H2A, sugerindo que o complexo SWR1 impede o reparo de danos espontâneos ao DNA, nesses mutantes (Morillo-Huesca *et al*., 2010).

Adicionalmente, a inativação de Swr1p elimina a incorporação de Htz1p no nucleossoma, e restaura a fosforilação de H2A e a adaptação de *checkpoint* (Papamichos-Chronakis, Krebs, and Peterson, 2006). A substituição de H2A por Htz1p é um mecanismo essencial para o controle da dinâmica da cromatina, e também previne as consequências deletérias do remodelamento incompleto do nucleossoma (Morillo-Huesca *et al*., 2010).

5.6. Outros remodeladores de cromatina que atuam na reparação de DNA

Além dos complexos de remodelamento de cromatina anteriormente mencionados, diversas proteínas vêm sendo investigadas com respeito ao seu papel nas alterações epigenéticas necessárias para os processos de transcrição, replicação e reparação de DNA. A metiltransferase de histona Dot1p é responsável pela metilação da histona H3 na lisina 79, em um processo dependente da ubiquitilação de H2B na lisina 123, por Rad6p–Bre1p (Nakanishi *et al*., 2009; Chandrasekharan, Huang, and Sun, 2009). É possível que essas reações ocorram juntas, ativando respostas de *checkpoint* de dano ao DNA (Attikum and Gasser, 2005a). A atividade de Dot1p também previne o reparo de DSB de células meióticas na ausência de Dmc1p, usando a cromátide irmã como molde (San-Segundo and Roeder, 2000). Dot1p também atua na resposta ao dano ao DNA em células vegetativas, pela ativação de Rad53p, mediada por Rad9p, durante o checkpoint de danos ao DNA nas fases G1/S e intra-S (Giannattasio *et al*., 2005; Wysocki *et al*., 2005). É possível, ainda, que a metilação de H3 e Rad9p atuem juntas, possibilitando a transmissão do sinal do dano às cinases de *checkpoint*, e mantendo a resecção do DNA danificado sob controle, influenciando cascatas de *checkpoint* e contribuindo para a resposta ao dano (Lazzaro *et al*., 2008). Mutantes deficientes em dot1p mostram resistência aumentada ao agente alquilante MMS, que pode ser atribuída a um aumento na atividade da via de reparação por TLS, na ausência de Dot1p (Conde and San-Segundo, 2008).

A acetyltransferase Gcn5p atua na acetilação de diversas proteínas histonas, como histona H2B (H2BK11ac e H2BK16ac), histona H3 (H3K9ac, H3K14ac, H3K18ac H3K23ac, H3K27ac e H3K36ac) e, com menor afinidade, histona H4 (H4K8ac e H4K16ac), e também contribui para a acetilação da histona H2AZ. A deleção do gene GCN5 reduz o tempo de vida replicativa de células, através da indução de resposta retrógrada, que sinaliza disfunção mitocondrial e promove alterações na expressão gênica. A proteína Gcn5p e o transdutor de sinal retrogrado Rtg2p conectam o metabolismo à resposta ao estresse, à regulação gênica e à estabilidade genômica dependente da cromatina, no envelhecimento de leveduras (Kim *et al*., 2004).

Um trabalho recente demonstra que Gcn5p atua em paralelo com Rtt109p, uma lisina acetiltransferase (KAT), que acetila H3K56. Juntas, essas proteínas promovem a montagem do nucleossoma RC, em parte por promover a associação de H3 com CAF-1, via acetilação da histona H3, e células deficientes nas duas proteínas, Rtt109p e Gcn5p, são extremamente sensíveis a agentes causadores de danos ao DNA (Burgess and Zhang, 2010), apontando para um possível papel de Gcn5p na reparação de danos ao DNA. De fato, o aumento na acetilação de H3 nas lisinas 9 e 14 em resposta a danos ao DNA induzidos por radiação UV correlaciona-se com mudanças na estrutura da cromatina, o que está relacionado com a eficiência do GG-NER em leveduras. Nesse contexto, alterações na acetilação de H3 promovem remodelamento de cromatina, possibilitando a reparação eficiente do DNA danificado (Yu *et al*., 2011).

A proteína Rad54p e sua homóloga, chamada Rdh54p (em *S. cerevisiae*), estão presentes em leveduras e eucariotos superiores, e pertencem à família Swi/Snf de ATPases de remodelamento de cromatina. *In vitro*, Rdh54p atua como Rad54p, promovendo a formação do D-loop Rad51p, através da indução do super-enrolamento negativo do dúplex de DNA. Rad54p também tem atividade de remodelador de cromatina, e participa do reparo pela via HR modificando os nucleossomas dos sítios de dano (Hinz, 2010). *In vivo*, Rad54p remodela um único nucleossoma posicionado que oclui o sítio de corte HO do locus doador HMLα, durante o reparo de DSB induzida por HO no MAT, consistindo em um exemplo de remodelamento de cromatina sem movimentos nucleossomais de larga escala (Osley *et al*., 2007; Kelley, 2008). O produto do gene RDH54 atua no controle de algumas vias de reparação de danos ao DNA, e a proteína Rdh54p está envolvida na regulação de muitas vias de reparação de DNA, como BER, NER ou vias de reparação recombinacionais e mutagênicas. Essa função é possivelmente mediada por alterações na estrutura da cromatina em locais de

danos ao DNA, provavelmente pelo relaxamento das pontes DNA-histona, tornando o DNA acessível às proteínas de reparação (Latypov *et al*., 2010).

A proteína Yaf9p é uma subunidade dos complexos NuA4 e SWR1, e contém um domínio YEATS, encontrado em proteínas associadas com muitas enzimas que modificam a cromatina, em complexos de transcrição presentes em eucariotos (Wang *et al*., 2009). Yaf9p também participa da resposta celular à segregação cromossômica, silenciamento telomérico e estresse do fuso, e é necessária para a acetilação da histona telomérica H4 e deposição de H2AZ na cromatina *in vivo* (Lu *et al*., 2009; Wang *et al*., 2009). A histona *linker* Hho1p inibe a reparação de DNA por HR em leveduras, bem como a manutenção telomérica mediada pela via de recombinação. Sua incorporação nos nucleossomas também inibe a união das terminações de DNA pela DNA ligase IV/XRCC4 (LX) *in vitro*, mas essa inibição é comprometida pela fosforilação da histona H1 pela DNA-PK, reduzindo sua afinidade por DNA e diminuindo sua capacidade de inibir *end-joining*, o que resulta na facilitação da reparação por NHEJ (Altaf *et al*., 2007).

6. O código de histonas na reparação de danos ao DNA

A hipótese do código de histonas pressupõe que (*i*) as diferentes modificações que ocorrem em regiões específicas das caudas das histonas podem ser detectadas por outras proteínas associadas à cromatina, servindo como plataforma para o recrutamento de fatores nucleares específicos; (*ii*) alterações na mesma cauda de histona ou em diferentes caudas podem ser interdependentes e gerar várias combinações de modificações distintas em nucleossomas específicos; e (*iii*) diferentes tipos de cromatina de ordem maior, como heterocromatina ou eucromatina, são dependentes da concentração local e da combinação de nucleossomas diferencialmente modificados, levando à criação de um código epigenético (Escargueil *et al*., 2008).

Os modelos inicialmente propostos postularam que a redução na carga, que ocorre seguindo a acetilação da histona, seria capaz de desconectar as interações histona-DNA, tanto no plano inter como intranucleossomal, favorecendo uma abertura na estrutura da cromatina (revisado em Escargueil *et al*., 2008). Entretanto, trabalhos recentes vêm demonstrando que esses fenômenos são mais complexos e, de fato, mais

de 40 diferentes resíduos de histonas são passíveis de modificações. Além da acetilação, da metilação e da fosforilação, as histonas também podem ser ubiquitinadas, sumoiladas, ADP-ribosiladas e estão sujeitas a isomerização de prolina (Chervona and Costa, 2012). Adicionalmente, resíduos de lisina podem ser mono, di ou trimetilados, enquanto resíduos de arginina podem ser mono ou dimetilados, gerando um amplo espectro de possibilidades (Nagy and Turecki, 2012). Por fim, metilação e acetilação podem atuar de modo diverso na transcrição, dependendo do resíduo modificado (Escargueil *et al*., 2008).

As enzimas acetiltransferases de histonas, ou HAT, transferem grupamentos acetil (oriundos de acetil-CoA) para resíduos de lisinas, nas caudas N-terminais das histonas. Essa atividade resulta em maior acessibilidade de proteínas à molécula de DNA, pela neutralização das cargas das lisinas acetiladas, levando também à ativação gênica (Icardi *et al*., 2012; Escargueil *et al*., 2008). A desacetilação das histonas, por sua vez, é efetuada por desacetilases de histonas, ou HDAC, e tem seu papel associado à repressão transcricional. A acetilação e desacetilação de histonas por meio da atividade de HAT e HDAC controlam, além da expressão gênica, os processos de apoptose e proliferação celular (Kristensen *et al*., 2009), progressão de ciclo celular, recombinação e reparação de DNA (Dinant *et al*., 2008; Luijsterburg and Attikum, 2011). A metilação de histonas é efetuada por enzimas chamadas DNA metiltransferases, ou DNMT. Essas enzimas catalisam a transferência de grupamentos metil da S-adenosilmetionina (ou AdoMet), para citosinas, argininas, e lisinas das caudas das histonas (Shilatifard, 2006; Szyf, 2009). A metilação da histona H3 nas lisinas 4, 36 e 79 parece estar relacionada à ativação da expressão gênica, enquanto a metilação da mesma histona H3 nas lisinas 9 e 27, bem como a metilação de H4 na lisina 20, levam à inativação da expressão gênica (Dinant *et al*., 2008). Por outro lado, a metilação de citosinas em regiões de início de transcrição conduz ao silenciamento gênico. A metilação pode interferir na ligação de fatores de transcrição, ou atrair proteínas metiladas que se ligam ao DNA, silenciando a expressão gênica (Szyf, 2009). A metilação também é importante no desenvolvimento embrionário, na repressão transcricional de regiões repetitivas e centroméricas, na inativação do cromossomo X em fêmeas e no *"imprinting"* genômico (Groth *et al*., 2007; Gal-Yam and Egger, 2008). Adicionalmente, padrões aberrantes de metilação do DNA podem atuar na tumorigênese. Estudos relatam a existência de células tumorais com quantidades reduzidas de 5-metilcitosina, além de hipometilação global de elementos repetitivos de DNA, contribuindo para a instabilidade genética e a super-

expressão de proteínas oncogênicas. A hipermetilação de regiões promotoras de supressores tumorais resulta em silenciamento gênico, contribuindo ainda mais para a tumorigênese. Além disso, a hipermetilação de promotores de supressores tumorais, que resulta em silenciamento, também atua no desenvolvimento tumoral, Esse fenômeno foi observado em retinoblastoma, no gene VHL (von Hippel-Lindau), além de tumores de rim, CDKN2 A/p16, de bexiga, e no gene MLH1 em tumor de cólon (Gal-Yam and Egger, 2008). No tocante à reparação de DNA, deficiências na metilação de H3 na lisina 79 por Dot1p causam hipersensibilidade à radiação UV, além de problemas de *checkpoint* na fase intra-S do ciclo celular. A metilação de H4 na lisina 20 também é importante para a sinalização de *checkpoint* após DSBs geradas por radiação UV (Dinant *et al*., 2008).

A fosforilação de H3 na serina 10 e na treonina 11 leva à ativação transcricional em resposta a dano ao DNA (Dinant *et al*., 2008). A histona H2B também é fosforilada, na sua serina 14, antes da formação de γH2AX, mas a função desta fosforilação ainda é desconhecida (Luijsterburg and Attikum, 2011).

A ubiquitinação de histonas consiste na conjugação de ubiquitina (um peptídeo de 8.5 kDa) a resíduos de lisina nas caudas das histonas. A função da ubiquitinação pode ser a de marcar proteínas para a degradação nos proteassomos, ou até a de alterar funções de proteínas. A ubiquitinação ocorre por meio da atuação de enzimas de ativação (E1), de conjugação (E2) e de ligação (E3) (Dinant *et al*., 2008; Méndez-Acuña *et al*., 2010), e 10-15 % das histonas H2A nucleares encontram-se ubiquitinadas na lisina 119 em células humanas, o que está relacionado à repressão transcricional. Por outro lado, somente cerca de 1% das histonas H2B são ubiquitinadas na sua lisina 120, causando a ativação transcricional, e as histonas H3 e H4 também podem ser ubiquitinadas, em níveis baixos (~ 0,1 %) (Luijsterburg and Attikum, 2011).

A reação de ubiquitinação parece ser importante na resposta à DSB, e as histonas H2A e H2AX são ubiquitinadas na sua lisina 119, pelas enzimas RNF8 e RNF168 (ambas ubiquitinas ligases), juntamente com a enzima de conjugação UBC13. A ubiquitinação de H2A atua na sinalização e recrutamento de BRCA1, 53BP1 em resposta a DSB, e depende de ATM/DNA-Pk, ATR e γ-H2AX (Luijsterburg and Attikum, 2011). As histonas H3 e H4 também podem ser temporariamente ubiquitinadas em resposta ao dano ao DNA, conforme o tipo de dano (Dinant *et al*., 2008). Em leveduras, o reparo de diferentes tipos de lesões, incluindo aquelas derivadas de exposição à radiação UV, requer a ubiquitinação de H2B, na lisina 123, pelo

complexo Rad6p/Bre1p E2/E3. Caso a ubiquitinação não ocorra, a ativação de Rad53p é afetada, e Rad9p é ativada (Game and Chernikova, 2009).

OBJETIVOS

1. Objetivo geral

O presente trabalho tem como principal objetivo avançar na compreensão dos efeitos de diferentes agentes antitumorais nos processos de remodelamento da cromatina e reparação de danos ao DNA, utilizando como modelo experimental a levedura *Saccharomyces cerevisiae*.

1.1. Objetivos específicos

1. Analisar as relações entre o remodelamento da cromatina e a reparação de danos ao DNA na levedura *Saccharomyces cerevisiae*, em resposta à fotoadição de psoralenos mono (3-CPs) e bifuncionais (8-MOP).

2. Avaliar os efeitos do tratamento com DTDF quanto à reparação dos danos ao DNA e a participação dos remodeladores de cromatina dependentes de ATP na citotoxicidade induzida por este composto, estabelecendo uma comparação com a resposta observada para a fotoadição de psoralenos mono (3-CPS) e bifuncionais (8-MOP).

3. Estabelecer a relação entre as vias de reparação de DNA e a citotoxicidade induzida pelo tratamento com a combinação de 5-FU e cisplatina, utilizando linhagens de *S. cerevisiae* proficientes e deficientes em diferentes vias de reparação de DNA e em remodeladores de cromatina dependentes de ATP.

CAPÍTULO I

Relationships between chromatin remodeling and DNA damage repair induced by 8-methoxypsoralen and UVA in yeast Saccharomyces cerevisiae

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Relationships between chromatin remodeling and DNA damage repair induced by 8-methoxypsoralen and UVA in yeast *Saccharomyces cerevisiae*

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Running title: Chromatin remodeling and DNA repair in yeast

Keywords: DNA repair, psoralen, chromatin remodeling, histones, DNA interstrand cross-links.

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Abstract

Eukaryotic cells have developed mechanisms to prevent genomic instability, such as DNA damage detection and repair, control of cell cycle progression and cell death induction. The bifunctional compound furocumarin 8-methoxypsoralen (8-MOP) is widely used in the treatment of various inflammatory skin diseases. In this review, we summarize recent data about the role of chromatin remodeling in the repair of DNA damage induced by treatment with 8-methoxypsoralen plus UVA (8-MOP+UVA), focusing on repair proteins in budding yeast *Saccharomyces cerevisiae*, an established model system for studying DNA repair pathways. The interstrand crosslinks (ICL) formed by the 8-MOP+UVA treatment are detrimental lesions that can block transcription and replication, leading to cell death if not repaired. Current data show the involvement of different pathways in ICL processing, such as nucleotide excision repair (NER), base excision repair (BER), translesion repair (TLS) and double-strand break repair. 8-MOP+UVA treatment in yeast enhances the expression of genes involved in the DNA damage response, double strand break repair by homologous replication, as well as genes related to cell cycle regulation. Moreover, alterations in the expression of subtelomeric genes and genes related to chromatin remodeling are consistent with structural modifications of chromatin relevant to DNA repair. Taken together, these findings indicate a specific profile in 8-MOP+UVA responses related to chromatin remodeling and DNA repair.

Introduction

Eukaryotic cells have developed mechanisms to prevent genomic instability, such as DNA damage detection and repair, control of cell cycle progression and cell death induction (Lobrich and Jeggo, 2007). Recent studies have revealed the importance of histone modification in the recruitment of proteins involved in the cellular response to DNA damage (Escargueil *et al*., 2008; van Attikum and Gasser, 2009; Lu *et al*., 2009). In this review, we compile recent data about the role of chromatin remodeling in the repair of DNA damage induced by treatment with 8-methoxypsoralen plus UVA (8-MOP+UVA), focusing on the respective repair proteins in budding yeast

Saccharomyces cerevisiae, a proven excellent model system for studying DNA repair pathways (Henriques *et al.*, 1997; Noll *et al.*, 2006).

8-methoxypsoralen: DNA damage and repair

The bifunctional furocumarin 8-methoxypsoralen (8-MOP) is widely used in the treatment of various inflammatory skin diseases (Dardalhon *et al*., 2007). The cellular effects of 8-MOP are due to its ability to bind to pyrimidine bases in DNA upon UVA irradiation. This binding occurs in a specific and covalent manner, resulting in the formation of both monoadducts and interstrand cross-links (ICLs) (Brendel and Henriques, 2001; Brendel *et al*., 2003; Noll *et al*., 2006). ICL caused by 8-MOP has specific structural characteristics which distinguish them from ICLs caused by other alkylating agents such as nitrogen mustard, mitomycin C, cisplatin, carmustine and malondialdehyde (reviewed by Guainazzi and Schärer, 2010). Moreover, the psoralen-induced ICL can block transcription and inhibit DNA replication, leading to cell death if not repaired (Lehoczký *et al*., 2007). Furthermore, due to the chemical stability of the cross-links and the facility of psoralen-DNA ICL synthesis, many studies have addressed DNA repair using psoralen ICL (Brendel *et al*., 2003; Noll *et al*., 2006 Vasquez, 2010; Le Breton *et al*., 2011).

The repair of ICL in *S. cerevisiae* is initiated by recognition of the lesion by the Nucleotide Excision Repair (NER, Rad4p/Rad23p) mechanism. Subsequently, Rad2p nicks one of the DNA strands at 3' to the ICL, while the Rad1p/Rad10p complex cleaves the same strand at 5' to the damage. The incised ICL is flipped out and the opposite strand serves as a template for the translesion polymerase machinery that has the ability to bypass the ICL. Next, cleavage occurs on the opposite strand to completely remove the ICL damage, leading to double strand breaks (DSBs), which then can be repaired by the homologous recombination machinery (reviewed by Wood, 2010). In this process, Pso3p/Rnr4p plays an important role in the regulation of translesion polymerase ζ in error-prone repair of psoralen ICL (Brendel *et al*., 2003). Curiously, genes involved in the NER pathway (*RAD2, RAD16* and *RAD23*) and the post-replication repair (PRR) pathway (*RAD18*), which participates in the repair of 8-MOP+UVA lesions (Henriques and Moustacchi, 1981; Brendel and Henriques, 2001;

Saffran *et al*., 2004), were not up-regulated by 8-MOP+UVA treatment in yeast (Dardalhon *et al*., 2007).

It has been shown that DSBs, formed as repair intermediates of 8-MOP photo-additions in DNA, are preferentially located in intergenic regions, where the TATA boxes and/or transcription termination consensus sequences are also situated (Dardalhon *et al*., 1998). Furthermore it is known that during the chromatin photoreaction with psoralen, ICLs are preferentially made in the linker DNA between adjacent nucleosomes (Lucchini and Wellinger, 2001). Recently, Mace-Aime *et al*. (2010) showed that the base excision repair (BER) pathway can provide an alternative pathway for the repair of psoralen-DNA photoadducts in human cells by the DNA glycosylase NEIL1, which directly participates in the removal of ICLs.

More than 30 years ago, Henriques and Moustacchi (1980) isolated a new class of *S. cerevisiae* mutants sensitive to photo-activated psoralens, so-called *pso* mutants. One of the mutant proteins, Pso2p, was shown to participate in the repair of DSBs induced by DNA inter-strand cross-linking agents such as cisplatin, nitrogen mustard or photo-activated bi-functional psoralens. The molecular function of Pso2p in DNA repair has only partially been characterized, but yeast and mammalian cell line mutants for *PSO2* show the same cellular responses as strains with defects in NHEJ, *viz.* sensitivity to ICLs and apoptosis. Pso2p/Snm1p is a member of the metallo-β-lactamase family of proteins, it has DNA 5'-exonuclease activity *in vitro*, and seems to have a specific role in repair of ICL (Magana-Schwencke *et al.*, 1982; Brendel and Henriques, 2001; Bonatto *et al*., 2005).

Pso2p/Snm1p can be directed to incised cross-links to resect repair intermediates, thus supplying substrates for downstream repair, such as translesion synthesis (TLS, by polymerases Rev1, Polζ, Polη, Polκ, Polι and Polν) in G_1 phase or homologous recombination (HR) in S- and G_2-phases (Figure 1) (Magana-Schwencke *et al*., 1982; Bonatto *et al*. 2005; Yan *et al*., 2010; Cattell *et al*., 2010). Pso2p/Snm1p also showed an overlapping role with the 5′–3′ mismatch repair exonuclease Exo1 during HR repair of collapsed replication forks (Sarkar *et al*., 2006; Yan *et al*., 2010). Hazrati *et al.* (2008) suggested that hSnm1A is a functional homologue of the yeast Pso2p/Snm1p. Although Pso2p/Snm1p binds to the 5'-phosphate moiety, present in both, DNA or RNA molecules, its activity on double-stranded substrates is very low (Hejna *et al*., 2007). Furthermore, Dudás *et al.* (2007) provided evidence that the

function of Pso2p/Snm1p in repair of ICL-associated DSB can be individual, without crosstalk with DSB repair machinery.

DSB damage signaling: The role of γ-H2A

One of the first responses triggered by the cells as a consequence of DSBs formed as intermediates during the repair of 8-MOP/UVA induced ICL is the fast induction of H2AX histone phosphorylation (in mammals at serine 139) surrounding the DSB site. This reaction is catalyzed by members of the phosphoinositide 3-kinase related protein kinase (PIKK) family (ATM, ATR and DNA-PK in mammalian cells or Tel1p and Mec1p in yeast, Figure 2) (Attikum and Gasser, 2005a). In response to DNA damage, H2A histone is also phosphorylated at serine 129, located four residues from the carboxy terminus. The phosphorylated species, referred to as γ-H2A (or γ-H2AX), spreads up to 50 kb around the DSB (Gangaraju and Bartholomew, 2007). In addition, H2A serine 122 (or threonine 119 in mammals) was also shown to be involved in mediating cell survival after many kinds of DNA damage, but its function in repair is independent from that of S129 (Ataian and Krebs, 2006).

The formation of γ-H2A results in the recruitment of several chromatin remodeling complexes at the site of DNA damage promoting changes in chromatin structure, so that the repair factors can gain access to the lesion. After 8-MOP+UVA treatment, the accumulation of DNA damage repair proteins occurs in sub-nuclear foci called ionizing-radiation-induced foci (IRIF), and studies in mice show that the histone variant H2AX is a core element in IRIF formation, although it is still unclear how proteins are actually recruited and retained as foci (Attikum and Gasser, 2009). In yeast, mutations in H2A (Ser129) increase cell sensitivity to DNA-damaging agents and impair DSB repair. In mice, the mutation or deletion of H2AX impairs or abolishes the accumulation of DNA damage response proteins into IRIF, suggesting a role of γ-H2AX as a key regulator of checkpoint signaling or repair itself (Celeste *et al*., 2002; Celeste *et al*., 2003). H2AX phosphorylation also recruits cohesin complexes to DSBs, which may explain the formation of a large domain of γ-H2AX-containing chromatin around DSBs. This prevent the loss of entire chromosome regions, as well as the progression of DNA breaks to chromosome breaks and translocations, keeping the DNA ends in close proximity for efficient repair (Altaf *et al*., 2007).

The DNA end-binding Mre11–Rad50–Nbs1 (MRN) complex, which is involved in recognition, signaling and repair of DSBs in mammalian cells, specifically binds to the γ-H2AX by the Nbs1 subunit. This interaction between Nbs1 and γ-H2AX is required for retention of the MRN complex at the damaged site, although recent work has shown that the MRN complex can initiate DNA end resection and HR repair in an H2AX-independent pathway, which also starts ATM activation and cell cycle checkpoints (Yuan and Chen, 2010). Moreover, 53BP1 and its yeast homologs *Saccharomyces cerevisiae* Rad9p and *Schizosaccharomyces pombe* Crb2p act as adaptor proteins between the sensing and effector kinases ATM and Chk1p, respectively, operating in the checkpoint response to DNA damage (Sweeney *et al*., 2005). 53BP1 also interacts with γ-H2AX and depends on it for the checkpoint function (Altaf *et al*., 2007). Several studies have demonstrated the recruitment of chromatin remodeling complexes to DSBs by γ-H2AX. Indeed, in yeast, the γ-H2A binder list includes the histone acetyltransferase (HAT) NuA4, as well as the chromatin remodeling complex proteins INO80 and SWR1 (Table 1).

Role of chromatin remodeling in DNA damage repair

The recruitment of chromatin remodeling complexes to the site of DNA damage is essential to allow changes in chromatin structure, thus enabling the access and retention of repair factors to the lesion. It is worthy of note that changes in chromatin structure induced by the lesion itself also play a role in their recognition. The chromatin structure can suffer post-translational modifications of histones, incorporation of histone variants and ATP-dependent remodeling (Wang *et al*., 2009). The emerging concept is "access-repair-restore", in which changes in chromatin structure modulate: a) the access of repair factors to the damaged site, b) their retention until the end of the repair process, and c) the restoration of chromatin to its initial state. 8-MOP/UVA treatment in yeast enhances the expression of genes involved in the DNA damage response (*RNR2, DUN1*), in DSB repair by HR (*RAD54, RAD51*) as well as genes of the Mec1p/Rad53p kinase signaling cascade (Dardalhon *et al*., 2007). Other induced genes are *NSE1* (encoding a protein component of the SMC5 SMC6-complex), *PAK1* (encoding an upstream kinase for the SNF1 complex), *YRF1-2* (a helicase that is highly expressed in mutants lacking the telomerase component *TLC1*), *CDC45* (encoding a DNA replication

initiation factor), *ESC8* (encoding a protein involved in telomeric and mating-type locus silencing) and genes related to cell cycle regulation, including *DUN1*, *SPO2*, *CTF19* and *CDC27* (Dardalhon *et al*., 2007). In this scenario, we proposed a model whereby Pso2p would act on DNA hairpin substrates induced by ICLs during DNA replication, where the potential endonucleolytic function of Pso2p would be activated via Pak1p-induced phosphorylation (Brendel *et al*., 2003, Bonatto *et al*., 2005).

Furthermore, the induction of *YRF1-2* and other subtelomeric genes, such as *YFL061W*, *YFL065C* and *YFL066C* is consistent with the need for changes in chromatin structure to allow repair of DNA damage after treatment with 8-MOP+UVA (Dardalhon *et al*., 2007). On the other hand, the repression of genes related to chromatin remodeling by treatment with 8-MOP+UVA, such as *RPD3* (encoding a histone deacetylase), *IOC4* (implicated in chromatin remodeling) and the histone genes *HTA1*, *HTA2* and *HTB2* may also result in structural modifications of chromatin, relevant to DNA repair.

In general terms, DSB repair is related to local changes in chromatin structure, such as histone modifications, chromatin remodeling and nucleosome disruption (Osley *et al*., 2007). The NuA4 complex, which interacts with γ-H2AX via its Arp4p subunit, is the first complex that appears at the break site (van Attikum and Gasser, 2009). The presence of NuA4 at the DSB site promotes the recruitment of DNA repair factors and of other chromatin modifying complexes, like SWR1-C and INO80 (Figure 3), perhaps by increasing accessibility to chromatin structure promoted by H4 acetylation (Altaf *et al*., 2007). In addition, the H2A.Z acetylation by NuA4 may promote the replacement of H2A with γ-H2A, in an SWR1-dependent manner, controlling the ratio of these two forms of H2A around the DSB site. This affects a phenomenon called checkpoint adaptation (Osley *et al*., 2007), in which cells can escape an extended checkpoint arrest and re-enter the cell cycle even with unrepaired DSBs (Papamichos-Chronakis *et al*., 2006; Osley *et al*., 2007).

A great deal of evidence connects INO80-dependent chromatin remodeling to DSB repair (reviewed by van Attikum and Gasser, 2009). The INO80 complex is recruited to DSB by interaction with γ-H2A, perhaps via its Nhp10p and Arp4p subunits, and the Arp8p subunit influences the rate of Rad51p loading at breaks, possibly promoting nucleosome displacement (Sarkar *et al*., 2010). On the other hand, the INO80 complex may act as a nucleosome acceptor during remodeling for repair, either by temporarily sequestering the displaced nucleosomes or by stabilizing

nucleosomes changed during repair and cooperating with other assembly factors to restore chromatin structure after the conclusion of repair (Sarkar *et al*., 2010).

Mutants of the Arp subunits in the *S. cerevisiae* INO80 complex have defects in NHEJ, as well as in the HR pathway (Morrison and Shen, 2009). Recently, Sarkar *et al* (2010) have shown that the INO80 complex operates in the same way as the NER pathway, also contributing to the removal of UV photoproducts in the regions of high incidence of nucleosomes. In addition, these authors also reported that the INO80 complex interacts with the Rad4p–Rad23p complex, a damage recognition complex of the NER pathway, after its recruitment to chromatin by Rad4p in a UV damage-dependent manner. After DNA damage response activation, the INO80 complex is phosphorylated at its Ies4p subunit, by ataxia-telangiectasia mutated (ATM) and A-T and RAD3-related (ATR) kinases, and it modulates the cellular response to DNA-damaging agents. The Ies4p subunit is also phosphorylated by the Tel1p and Mec1p kinases, modulating DNA replication checkpoint responses without altering DSB repair processes (Morrison and Shen, 2009).

Curiously, Czaja *et al*. (2010) reported that *ino80*Δ yeast mutants are hypersensitive to DNA lesions induced by ultraviolet (UV) radiation and methyl methanesulfonate (MMS), but are less sensitive to the DNA double-strand break (DSB)-inducing agents ionizing radiation and camptothecin. These data suggest that INO80 is involved in DNA damage tolerance through a role in the stabilization and recovery of broken replication forks, but not in the repair of lesions leading to such events. In addition, only 5% of the genes induced by 8-MOP+UVA treatment are also affected in *ino80*Δ mutants, showing that INO80 does not appear to regulate the HR pathway at the transcriptional level. Rather it may influence the NHEJ pathway, which is believed to play a modest role in the repair of the photo adducts and DSBs that indirectly result from 8-MOP+UVA treatment (Dardalhon *et al*., 2007). Furthermore, this study also found an unexpected correlation between the transcriptional profiles caused by 8-MOP+UVA treatment and histone H4 depletion, with about 62% of 8-MOP+UVA response genes also being affected by the lack of this histone.

About 15% of the genes affected after 8-MOP+UVA treatment in yeast are also modulated in *swr1*Δ, *htz1*Δ and *sir2*Δ cells, suggesting that the replacement of histone H2A with H2.Z in nucleosomes, as well as a Sir contribution to telomeric silencing, may influence the repair of 8-MOP+UVA induced damage (Dardalhon *et al*., 2007). The biology of SWR1 and Htz1 has been intensely studied for their roles in

transcriptional regulation. SWR1 also acts in DSB repair in an independent manner, controlling the γ-H2A levels at DSB sites (Osley *et al*., 2007; Escargueil *et al*., 2008). In yeast strains lacking Htz1, the SWR1 complex promotes an accumulation of recombinogenic DNA damage by a mechanism dependent on γ-H2A, suggesting that the SWR1 complex hinders the repair of spontaneous DNA damage in these mutants (Morillo-Huesca et al. 2010). In addition, the inactivation of Swr1p eliminates the incorporation of histone variant Htz1 and restores the phosphorylation of H2A and checkpoint adaptation (Papamichos-Chronakis *et al*., 2006). The replacement of H2A with Htz1 is an essential mechanism for the control of chromatin dynamics, and also prevents the deleterious consequences of incomplete nucleosome remodeling (Morillo-Huesca et al. 2010).

Mutations in the yeast RSC chromatin remodeling complex (Table 1) cause hypersensitivity to DNA break-inducing agents (van Attikum and Gasser, 2005a). RSC has homologs in other eukaryotes and acts in transcriptional activation and repression, maintenance of cell viability and in DSB repair (Osley *et al*., 2007). The RSC complex subunit Rsc8p and the catalytic subunit Sth1p are recruited to DSBs, where the latter rapidly binds to the lesion and accumulates in regions immediately adjacent to the damage (van Attikum and Gasser, 2005b). Defects in RSC complex activity cause hypersensitivity to DSBs, which can be attributed to defects in NHEJ and HR repair pathways. Furthermore, the RSC complex is recruited to the DSB break at the same time as the MRX (Mre11p/Rad50p/Xrs2p) complex (Figure 3), involved in NHEJ, HR and checkpoint signaling, both of which can lead to remodeling of chromatin near the break site and facilitate the access of NHEJ factors (Osley *et al*., 2007). The RSC complex can also load cohesin onto chromosome arms, maintaining the sister chromatids close, and during DSB repair it may promote the binding of proximal ends during NHEJ and of distal ends during HR (Osley *et al*., 2007). The importance of this cohesin loading reflects the fact that defects in the establishment of cohesion during S-phase or *de novo* loading of cohesin at DSBs in the G_2 phase reduce the efficiency of post-replication repair (PRR) (van Attikum and Gasser, 2005a).

The histone methyltransferase Dot1p is responsible for methylation of histone H3 on lysine 79, and this process is dependent on the ubiquitylation of H2B lysine 123 by Rad6p–Bre1p (Giannattasio *et al.*, 2005). It is possible that these reactions work together, activating checkpoint responses to DNA damage (van Attikum and Gasser, 2005b). The activity of Dot1p also prevents DSB repair of meiotic cells in the absence

of Dmc1p, using the sister chromatid as a template (San-Segundo and Roeder, 2000). Dot1p also acts in the DNA damage response by activating Rad53p. This is mediated by Rad9p during the G_1/S and intra-S DNA damage checkpoints (Conde *et al*., 2009). In this scenario, Cardone *et al*. (2006) proposed a model for yeast checkpoint activation after 8-MOP+UVA treatment in which the checkpoint is activated by means of Rad9p phosphorylation in a process dependent on the recruitment of the Ddc1p/Mec3p/Rad17p complex. The latter can regulate Chk1p and Rad53p, suggesting that *PSO9*/*MEC3* is crucial for sensing DNA lesions generated by photo-induced psoralens or other mutagens. A recent study suggests that histone H3 methylation and Rad9p act together, enabling transmission of the damage signal to checkpoint kinases and keeping resection of damaged DNA under control (Lazzaro et al. 2008). This would affect checkpoint cascades and contribute to the DNA damage response. Interestingly, the *dot1*Δ mutant shows increased resistance to the alkylating agent MMS. Accordingly, the reduced sensitivity of some mutants deficient in DNA repair has also been attributed to an increase in TLS repair pathway activity in the absence of Dot1p (Conde and San-Segundo, 2008).

A recent study by Faucher and Wellinger (2010) shows that monoubiquitylation of histone H2B and trimethylation of histone H3 at lysin 4 (H3K4me3) are important for both the response to DNA damaging agents and the passage through S-phase in budding yeast. The recruitment of the methyltransferase Set1p to the DSB is dependent on the presence of the RSC complex, thus contributing to DNA damage repair. In this context, yeast mutants lacking Set1p show impaired ability to recruit the Yku-proteins to DSB sites, leading to reduced efficiency of the NHEJ pathway. In addition, H2BK123ub is also required for H3K79 methylation by Dot1p. The loss of NHEJ-efficiency and the synthetic interactions with MRX are specific to a lack of H3K4me3 and not H3K79me3, showing that the roles of Set1p in genome stability are different from those of Dot1p methyltransferase.

The analysis of global transcriptional responses after treatment with 8-MOP+UVA in yeast shows that 25% of the genes modified by this treatment were also changed in *snf2* and/or *swi1* deletion mutants, indicating that the *S. cerevisiae* SWI/SNF complex is implicated in gene expression following 8-MOP+UVA exposure (Dardalhon *et al*., 2007). The yeast SWI/SNF complex acts in gene transcription and DSB repair (Osley *et al*., 2007). SWI/SNF increases accessibility to DNA in the nucleosome core, even though the NER repair proteins also increase their remodeling activity. This

suggests a cooperative relationship between DNA damage repair and chromatin remodeling. The chromatin remodeling activity of the SWI/SNF complex changes the histone-DNA contacts, slides or remodels nucleosomes or transfers histone dimers *in vitro* (Osley *et al.*, 2007). Yeast mutants lacking SWI/SNF activity are sensitive to DNA damage agents that cause DSBs, and there is strong evidence that suggests the role of this complex in the HR repair pathway.

The up-regulation of the genes *RAD51*, *RAD54*, *RNR2*, *RNR4*, *DIN7* and *RNR3* in response to treatment with 8-MOP+UVA, MMS and ionizing radiation (Dardalhon *et al*., 2007) is also consistent with the importance of recombination pathways in the repair of ICLs. Rad54 protein and its homolog Rdh54p in *S. cerevisiae* are present in yeast and higher eukaryotes. They belong to the Swi/Snf family of ATPase chromatin remodeling factors. *In vitro*, Rdh54p acts like Rad54p, promoting Rad51p D-loop formation through the induction of negative supercoiling of the DNA duplex. The product of the *RDH54* gene acts in the control of damage repair and Rdh54p is involved in the regulation of many DNA repair pathways like BER, NER or recombinational and mutagenic repair pathways. This function is possibly mediated by changes in chromatin structure in DNA damage sites, probably by alleviation of DNA-histone bonds, making the DNA accessible to the repair proteins (Latypov et al. 2010).

Final considerations

DNA repair is a critical cellular function that maintains genomic stability and prevents mutations. Current data support the idea that histone modifications are essential for cell survival in response to DNA damage through induction of cell cycle checkpoints and DNA repair. The interstrand crosslinks formed by 8-MOP+UVA are highly detrimental lesions that are too complex to be processed by a single repair pathway. The existing data show the involvement of NER, BER, TLS and double-strand break repair (HR and NHEJ) in ICL processing. As described above, the repair of 8-MOP+UVA lesions in *Saccharomyces cerevisiae* can result in the transient formation of DSBs, and both NER and a Pso2p/Msh2p/Exo1p-dependent pathway are required to process ICLs in S-phase cells prior to DSB repair. Moreover, 8-MOP+UVA treatment leads to up-regulation of genes related to cell cycle regulation and repression of genes related to chromatin remodeling, which is consistent with changes in chromatin

structure relevant to DNA repair. Taken together, these findings indicate a specific profile of 8-MOP+UVA responses related to chromatin remodeling and DNA repair. Further studies are certainly required to clarify the relationships between the DNA damage response to psoralen, cell cycle checkpoint induction and chromatin remodeling.

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Figure legends

Figure 1 - Main pathways involved in the repair of 8-MOP+UVA induced ICL in yeast *Saccharomyces cerevisiae.* After treatment, ICL is formed preferentially in linker DNA between adjacent nucleosomes. Initially, psoralen ICL is recognized by NER machinery and subsequently incised from one of the DNA strands. Pso2p can be directed to incised cross-links in order to resect repair intermediates, thus supplying substrates for downstream repair such as translesion synthesis (TLS) in G_1 phase or homologous recombination (HR) in S- and G_2-phases.

Figure 2 - DSB formation and γ-H2AX signaling following ICL formation.

Figure 3 - Main factors involved in chromatin remodeling after 8-MOP+UVA induced DNA damage. After ICL incision, MRX complex and Yku-proteins bind to DSB. RSC complex interacts with Mre11p, promoting loading of cohesin at DSB site. The kinases Tel1p and Mec1p are recruited and phosphorylate the histone H2AX. The γ-H2AX recruits NuA4 HAT complex that acetylates H2AX and H4 tails, promoting the binding of SWR1 complex that exchange γ-H2AX with H2AZ. The INO80 complex interacts with γ-H2AX and alters the position of nucleosomes, facilitating the MRX-dependent formation of single-stranded DNA. For more details, see the text.

Table 1 - Summary of the main activities of chromatin remodeling in yeast

Chromatin remodeler	Main activities
NuA4 complex	Histone acetyltransferase acetylates H4 and H2A histones, plays an important role in DNA repair and interacts with γ-H2AX.
SWR1 complex	Helicase modulates the replacement of histone H2A with HTZ (or H2AZ).
RSC complex	Member of SWI/SNF class of remodelers. Acts in transcriptional activation and repression, maintenance of cell viability and plays a role in DSB repair
SWI/SNF complex	Nucleosome remodeling complex with ATP-dependent activity, acts in gene transcription and DSB repair.
INO80 complex	ATPase with chromatin remodeling activity and 3' to 5' DNA helicase activity in vitro.
Sin3/Rpd3 complex	Histone deacetylase complex that regulates gene transcription and silencing.
Rdh54	DNA-dependent ATPase, stimulates strand exchange, involved in recombinational repair of DSBs.
Dot1	Nucleossomal histone H3K79 methylase

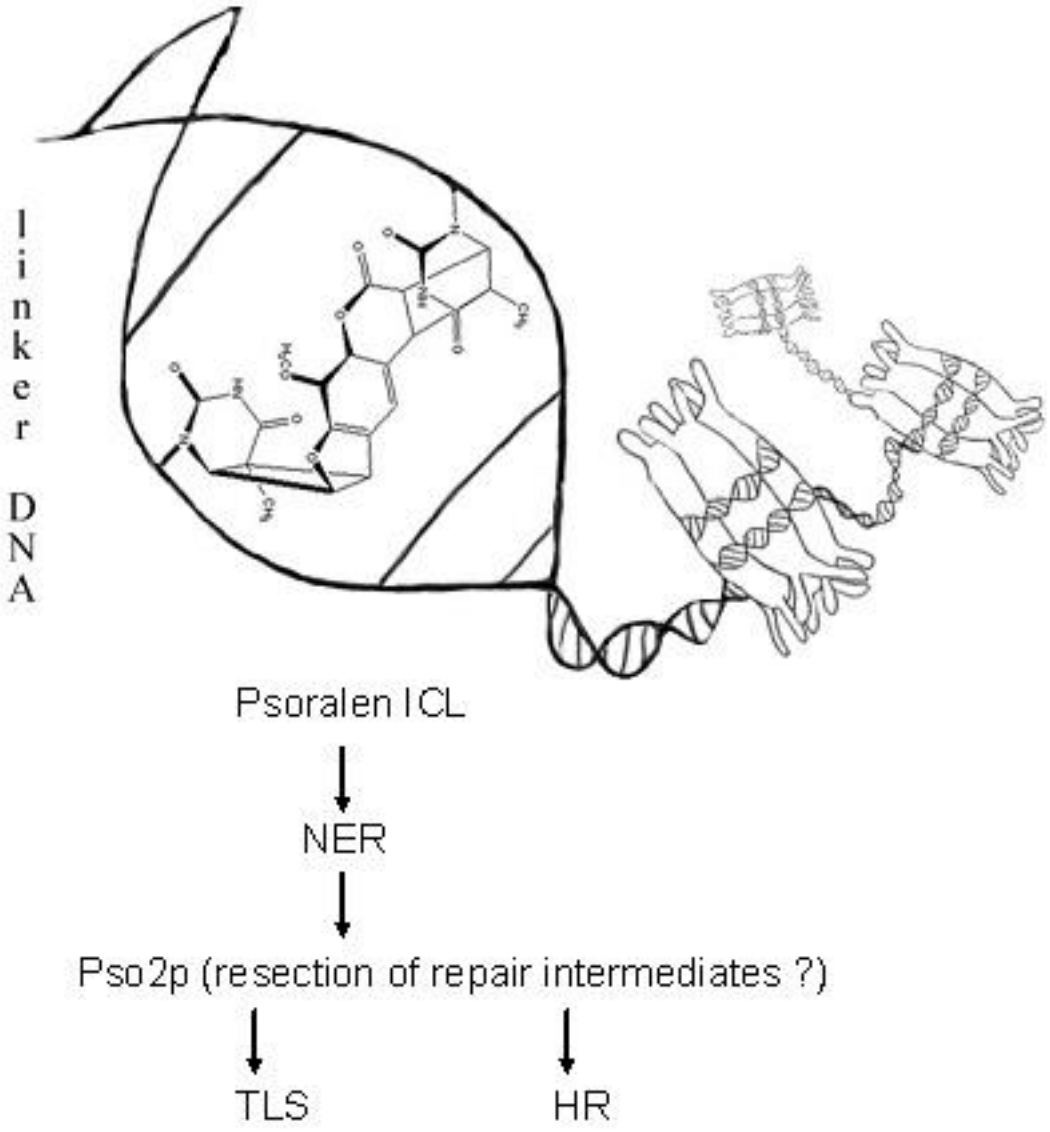
linker DNA
Psoralen ICL
NER
Pso2p (resection of repair intermediates ?)
TLS
HR

Figure 1.

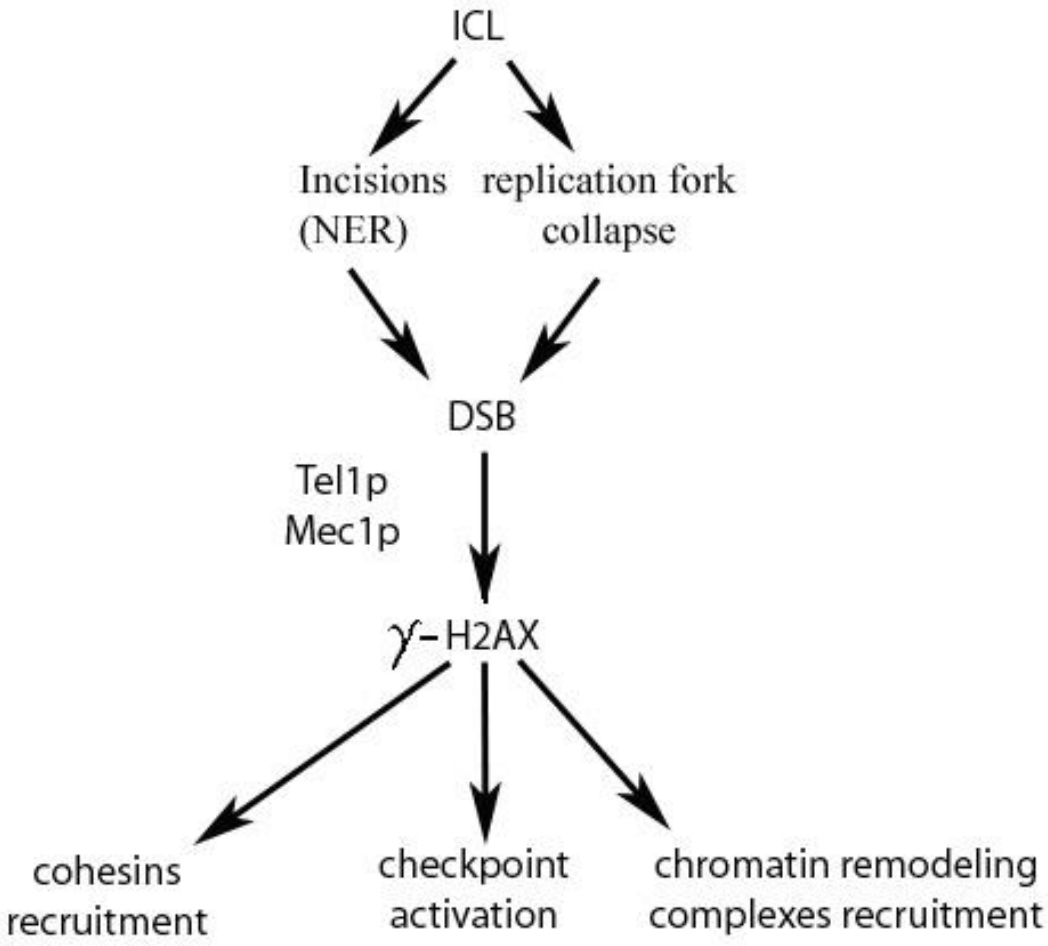
ICL
Incisions (NER)
replication fork collapse
DSB
Tel1p
Mec1p
γ-H2AX
cohesins recruitment
checkpoint activation
chromatin remodeling complexes recruitment

Figure 2.

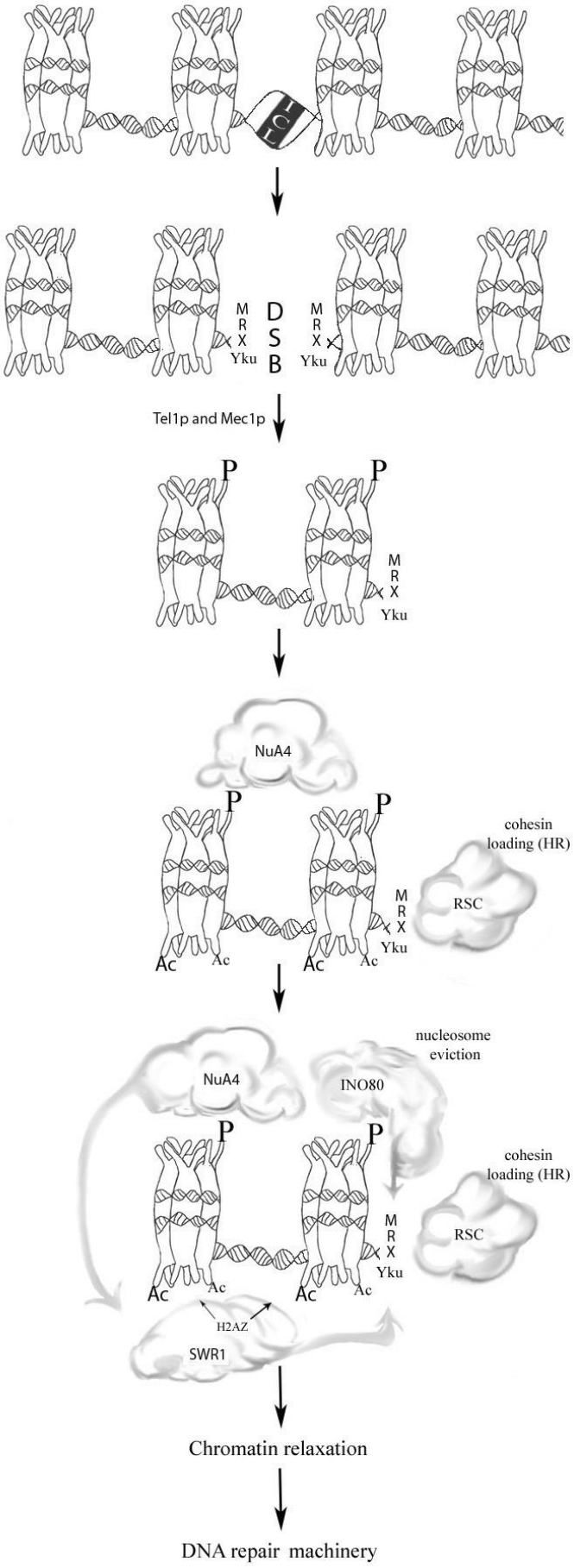
ICL
M
R
X
Yku
D
S
B
Tel1p and Mec1p
P
NuA4
cohesin
loading (HR)
RSC
Ac
nucleosome
eviction
INO80
H2AZ
SWR1
Chromatin relaxation
DNA repair machinery

Figure 3.

CAPÍTULO II

The role of Pso2p in the response to diphenyl ditelluride induced damage in Saccharomyces cerevisiae

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The role of Pso2p in the response to diphenyl ditelluride induced damage in *Saccharomyces cerevisiae*

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Abstract

Diphenyl ditelluride (DPDT) is a potential prototype for development of novel biologically active molecules. Thus, it is important to investigate the mechanism of genetic toxicity of this compound. In the present study, we evaluated the effect of DPDT treatment in chromatin remodeling and DNA damage repair, by the use of *Saccharomyces cerevisiae* yeast strains deficient in several proteins involved in these processes. Cell survival assays reveals that Pso2p, a member of the metallo-β-lactamase family of proteins, have an important role in repair of DPDT induced lesions. The set of our results suggest that DPDT treatment may cause hairpin-capped DNA double-strand breaks, or other lesion that blocks the replication fork. In this scenario, Pso2p could have a key role in lesion processing, providing substrate for the DNA double-strand break repair pathways.

Key words: Pso2p, DNA repair, interstrand crosslink, psoralen, Diphenyl ditelluride, chromatin

Introduction

Diphenyl ditelluride (DPDT) is a potential prototype for development of novel biologically active molecules. The employment of organotellurium compounds in anti-proliferative therapy has been suggested based on their cytotoxicity (Engman *et al*., 2000). One of the strategies that are currently employed in cancer treatment involves drugs able to cause irreversible damage to the genome of tumor cells. These damages, when detected by cell, can lead to cell cycle arrest and repair or induction of cell death. Many therapeutic protocols combine drugs that cause different kinds of DNA damage. These damages are recognized and trigger responses of proteins belonging to specific repair pathways, making the DNA repair machinery an important biological target in the fight against cancer (Damia and D'Incalci, 2007; Deans and West, 2011; Tang *et al*, 2011).

DPDT has shown to induce DNA double-strand breaks (DSBs) in mammalian cells (Degrandi *et al*. 2010). DNA DSBs present the most severe kind of DNA damage and risk for cell viability since their inaccurate repair can result in deleterious mutations, cancer or cell death (Wyman and Kanaar, 2006; Mladenov and Iliakis, 2011). Two evolutionary conserved pathways mediate DSB repair: homologous recombination (HR) and non-homologous end-joining (NHEJ). The canonical NHEJ is DNA-PK and Ku-dependent, whereas microhomology-mediated end joining (MMEJ), or error-prone NHEJ, requires MRX complex (Mladenov and Iliakis, 2011; Symington and Gautier, 2011).

The photo-activated bifunctional psoralens such as 8-methoxypsoralen (8-MOP) are antitumoral drugs able to induce DNA interstrand crosslinks (ICLs) (for review, see Ho and Schärer, 2010). The ICL repair requires involvement of different DNA repair pathways, as well as the interaction of these proteins with factors involved in chromatin remodeling. The collapsed replication fork at ICL can result in a DSB formation, considered a severe damage to the cell (Wyman and Kanaar, 2006; Mladenov and Iliakis, 2011). In *S. cerevisiae*, there are three pathways known for its role in ICL repair, defined by (1) homologous recombination repair (HRR, by RAD52); (2) translesion bypass synthesis (TLS, by REV3) and (3) an mechanism still incompletely understood, by PSO2, that is involved in processing of ICL-induced DSBs, (Henriques and Moustacchi, 1981; Henriques *et al*., 1997; Grossmann *et al*., 2001; Li and Moses, 2003; Matuo *et al*., 2012). In addition, many studies demonstrated that chromatin remodeling

can modulate the DNA repair mechanisms, and also regulates the transcriptional activation in eukaryotic cells (Czaja *et al.*, 2010; Lee *et al.*, 2010; Sarkar *et al.*, 2010; Palomera-Sanchez and Zurita, 2011). In budding yeast, after DSB induction the DNA broken ends are bound by yKu heterodimer (yKu70p–yKu80p) and the MRX complex (Mre11p, Rad50p, Xrs2p), with consequent recruitment of Tel1p checkpoint kinase. The SWR1 complex facilitates yKu80p binding to the DNA ends, and could affect yKu80p-dependent repair of the DSB by error-free NHEJ. Mutants deficient in SWR1 complex are hypersensitivity to a wide range of DNA damaging agents (van Attikum *et al.*, 2007) and defective in error-free NHEJ pathway (Morrison and Shen, 2009).

Many efforts aimed to improve existing therapeutic protocols consist of the development of new drugs, whose antitumoral activity is more specific and with less adverse effects for patients. The development of new therapeutic strategies based on relationships between chromatin remodeling and DNA repair pathways constitutes a promising possibility (Ellis *et al.*, 2009; Kwa *et al.*, 2011; Luijsterburg and van Attikum, 2011; Chervona and Costa, 2012). In order to advance the understanding of the relationship between chromatin remodeling and repair of DNA damage, this work investigates the role of different proteins involved in these processes in response to treatment with the organotellurium compound DPDT and the mono (3-carbethoxypsoralen, 3-CPS) and bifunctional (8-MOP) photo-activated psoralens.

Materials and methods

Chemicals

Diphenyl ditelluride (DPDT) (CAS 32294-60-3) was provided by Dr. Luis Antônio Braga from Federal University of Santa Catarina, Brazil. 8-Methoxypsoralen (8-MOP) was obtained from Sigma (St. Louis, MO, USA). 3-carbethoxypsoralen (3-CPS) was provided by Dr. E. Bisagni (Institute Curie, Orsay, France). Reagents for culture medium (yeast extract, bacto-peptone, bacto-agar and glucose) were acquired from Merck.

Yeast strains, media and growth conditions

Yeast strains deficient in chromatin remodeling factors and DNA repair proteins were kindly provided by Dr. Lisiane Meira (Biological Engineering Division, MIT, Cambridge, USA), acquired from Euroscarf (European *Saccharomyces cerevisiae* Archive for Functional Analysis) or constructed by gene replacement. The *pso2*Δ strain was constructed in our laboratory (Revers et al. 2002).The relevant genotypes of *S. cerevisiae* strains used in this study are listed in Table 1. Techniques in yeast genetics and standard molecular techniques were performed according to Sambrook *et al*. (Sambrook and Russell 2001) and Ausubel *et al*. (Ausubel *et al*. 1996). Media, solutions, and buffers were prepared as previously described by Burke (Burke *et al*., 2000). The complete medium (YPD) containing 0.5% yeast extract, 2% peptone and 2% glucose was routinely used for cultures growth.

Genotoxic treatments

Survival curves

The sensitivity to DPDT treatment was assayed by plating of 5 x 10^6 cells/mL, in exponential phase, in YPD medium plates containing DPDT 5 μM, 10 μM and 20 μM. Control group were plated in YPD plates without DPDT. For treatment with the furocumarins, 5 x 10^6 cells/mL in exponential growth phase were treated with 8-MOP or 3-CPS [5x10^{-5} M] during 20 minutes in the dark. After, the cels are irradiated with differents doses of UVA, submited to serial dilution and plated in YPD plates, according to Henriques and Moustacchi (1980). After all treatments, the cells were incubated during 3-7 days at 28°C in the dark, and the colonies were counted for determination of cells survivors. All experiments were independently repeated at least threefold, with triplicate samples for each treatment.

Drop tests

Logarithmic cultures were serially diluted by 1:10 steps and 6 μL aliquots per mL are spotted onto synthetic complete media (SC) plates with or without DPDT 5μM, 10μM and 20μM. Plates were incubated at 28°C for 2 days, and photographed with digital camera.

Double-mutants construction

Double mutants were obtained by disruption of *SWR1* gene by homologous recombination with their cassete. pGADT7 vector was the template for *swr1*::LEU2 cassete. The primers used in this study are described in Table 2. Cassete were amplified with Platinum´s Taq DNA polymerase High Fidelity (Invitrogen), purified with PureLinkTM gel extraction kit (Invitrogen) and used for transformation. Transformations were conduced using LiAc/PEG protocol (Gietz and Woods, 2002). Disruption was confirmed by PCR and restriction analysis performed with purified genomic DNA from yeast transformant colonies selected in synthetic media lacking leucine. The accuracy of gene replacements was verified by PCR analysis, using specific primers for SWR1 gene (data not shown).

Results

Chromatin remodeling

In order to investigate the role of the chromatin remodeling in response to DNA damage induced by treatment with mono- and bifunctional photoactivated furocumarins and DPDT, we used *S. cerevisiae* strains deficient in different proteins related to chromatin remodeling (Table 1). The results on Figure 1 showed that only the single mutants *swr1*Δ and *gcn5*Δ appear to be slightly more sensitive to DPDT treatment, when compared to the Wild Type strain. The similar results were obtained after treatment with 3-CPS and 8-MOP plus UVA (supplementary data, S1 and S-2).

However, when the drop test was performed on the plates containning DPDT at doses of 5 µM, 10 µM and 20 µM, the *gcn5*Δ single mutant shows a sensibility more pronounced as a function of the dose to the treatment of this agent (Fig. 2).

Relationships between chromatin remodeling and DNA damage repair

In order to evaluate the mode of interaction between different genes involved in processes of chromatin remodeling and DNA damage repair in response to DPDT treatment, single and double mutants of *S. cerevisiae* deficients in Swr1p (CR) (on

BY4742 WT background) and mutants involved in DSB repair pathways (*swr1*Δ*rad52*Δ, *swr1*Δ*yku80*Δ, *swr1*Δ*pso2*Δ) were constructed by gene disruption. The cytotoxic effects of treatment with DPDT were determined by cell survival assays (Figures 3, 4 and 5).

In the Figure 3 we can see that the *swr1*Δ mutants showed sensitivity similar to the WT for both, ICLs induced by furocoumarins photoaddition and DNA damage induced by DPDT treatment. The *rad52*Δ mutant showed a strong sensitivity to all three treatments performed, indicating a strong contribution of homologous recombination pathway in repairing the damage induced by the studied agents, as has been reported in previous studies from our laboratory (Henriques and Moustacchi, 1981; Henriques *et al*., 1997; Rosa *et al*., 2004; Degrandi *et al*., 2010).. Furthermore, the sensitivity of the double-mutant *swr1*Δ*rad52*Δ was similar to the single-mutant *rad52*Δ (Figure 3), showing no functional interactions between these proteins.

Interestingly, none of the genotoxic treatments used in this study increased cell death in mutants deficient in canonical NHEJ repair pathway, the single mutant *yku80*Δ, as well as the double-mutant *swr1*Δ*yku80*Δ, when compared to WT (Figure 4).

Curiously, the single- and double mutants deficient in Pso2p, known for its role in the repair of ICLs induced by psoralens photoaddition (Brendel *et al*., 2003; Cardone *et al*., 2006, 2008; Ho and Scharer, 2010), showed marked sensitivity to treatment with DPDT when compared to Wild Type (Figure 5).

Discussion

Previous study conducted by our research group evaluated the cytotoxic and mutagenic properties of DPDT, and demonstrated that treatment with this compound causes disturbances in cellular redox balance (by glutathione depletion), resulting in induction of oxidative stress and DNA damage in both, yeast and mammalian cells (Degrandi *et al*. 2010). The same study also found that, in mammalian cell culture, DPDT induces DSBs and micronucleus formation. The induction of frameshift mutations was also observed in yeast and *Salmonella typhimurium* TA97a and TA98 strains. These frameshift mutations cannot be related to direct oxidative damage as DPDT does not induce mutagenesis in the *S. typhimurium* strains detecting base pair substitution (TA100 and TA1535 strains). Similarly, diphenyl diselenide showed the

same behavior as DPDT in the Salmonella/microsome assay (Ames test) (Rosa *et al*., 2004). These results suggest that the lesion induced by these compounds could be rather specific and not detectable in the test system used.

Influence of chromatin remodeling in DNA damage induced by furocumarins and DPDT

Several studies demonstrated that chromatin remodeling can modulates the DNA repair mechanisms, and also regulates the transcriptional activation in eukaryotic cells. In budding yeast, after DSBs induction the DNA broken ends are bound by yKu heterodimer (yKu70p–yKu80p) and the MRX complex (Mre11p, Rad50p, Xrs2p), with consequent recruitment of Tel1p checkpoint kinase. Together, Tel1p and Mec1p phosphorylate H2A histone, which is referred as γ-H2A (or γ-H2AX). Following this pathway, γ-H2A recruits the chromatin remodeling complexes SWR1 and INO80 to DSB sites. Loss of Swr1p affects yKu80p binding but not Mre11p association or end-resection (van Attikum *et al*., 2007). Cells with failure in INO80 complex show defects in HR and NHEJ pathways, while mutants deficient in SWR1 complex are defective in error-free NHEJ pathway (Morrison and Shen, 2009).

The high sensitivity of yeast strains deficient in the MRX complex subunits (Pastwa and Blasiak, 2003, Degrandi *et al*., 2010), which in addition to its role in HR is also involved in the processing of DSBs by NHEJ error-prone pathway (or MMEJ), indicates a contribution of this complex on mutagenesis induced by DPDT treatment. Our results show that the double mutant *swr1*Δ*ino80*Δ, as well as the respective single mutants, exhibited only a moderate sensitivity to DPDT treatment, indicating that the loss of Ino80p did not significantly affect the DNA damage repair although HR seems to be the preferential pathway for repair of damage caused by this compound. Moreover, the absence of Swr1p, that confer deficiency in the canonical NHEJ, had no effect on the ability of cells to repair the damage induced by DPDT. Czaja *et al*. (2010) reported that *ino80*Δ yeast mutants are hypersensitive to DNA lesions induced by ultraviolet (UV) radiation and methyl methanesulfonate (MMS), but show low sensitivity to the DNA double-strand break (DSB)-inducing agents ionizing radiation and camptothecin. These data suggest that INO80 complex is involved in DNA damage tolerance through a role in stabilization and recovery of broken replication forks, but not in the repair of lesions leading to such events.

Interestingly, although the single-mutant *gcn5*Δ showed an increased sensitivity to treatment with DPDT, this effect was abrogated in the double mutant *swr1*Δ*gcn5*Δ, whose sensitivity was similar to that observed for swr1Δ. Gcn5p is a nuclear HAT that acetylates the H2B histone, in addition to the H3 histone, with low preference for the H4 histone. Kim and colleagues (2004) demonstrated that the deletion of *GCN5* gene decreases the replicative lifespan in yeast, by induction of retrograde response, which signalizes mitochondrial dysfunction and promote changes in gene expression (Kim *et al*., 2004). Furthermore, a recent work shows that the H3 histone acetylation by Gcn5p regulates the nucleosome assembly, by a mechanism which involves the association of the H3 histone with the CAF-1 chaperone histone (Falbo and Shen, 2010). In addition, more recently it has been demonstrated that GG-NER regulates the chromatin remodeling induced by UV light, through the modulation of H3 histone acetylation in chromatin (Yu *et al*., 2011). The authors concluded that this modulator activity is due to control of the accessibility of Gcn5p in chromatin, and the changes in H3 histone acetylation facilitate the DNA damage repair by promoting the chromatin remodeling. Furthermore, it seems that GCN5 play a role in NER, in human cells.

Role of \Pso2p in DNA damage induced by DPDT

In this study, the yeast mutant *pso2*Δ showed DPDT sensitivity pattern similar to that of mono- (3-CPs) and bifunctional (8-MOP) furocumarins photoaddition suggesting involvement of Pso2p in the repair of DPDT induced lesions. The cellular effects of 8-MOP is due to its ability to bind to pyrimidine bases in DNA upon UVA irradiation by specific and covalent manner, resulting in the formation of both monoadducts and interstrand cross-links (ICLs). Moreover, yeast and mammalian cell lines mutants for PSO2 show the same cellular responses as strains with defects in NHEJ, e.g., sensitivity to ICLs and apoptosis (Bonatto *et al*., 2005). The existing data show involvement of different pathways in ICL processing such as nucleotide excision repair (NER), base excision repair (BER), translesion repair (TLS) and double-strand break repair (Guainazzi and Scharer, 2010; Deans and West, 2011). ICLs can block transcription and inhibit DNA replication, leading to cell death if not repaired. The Pso2p is a member of the metallo-β-lactamase family of proteins with DNA 5'-exonuclease activity *in vitro*, that was shown to participate in the repair of DSBs induced by DNA inter-strand cross-

linking agents such as cisplatin, nitrogen mustard or photo-activated bi-functional psoralens (Bonatto *et al*., 2005).

A role of Pso2p outside ICL repair was suggested by Tiefenbach and Junop (2012) that reported a molecular function of this protein as a structure-specific DNA hairpin opening nuclease in budding yeast. Also, Pso2p/Snm1p showed an overlapping role with the 5′–3′ mismatch repair exonuclease Exo1p in the repair of DNA ICL during the S phase of cell cycle (Barber *et al*., 2005). ICL processing involves function of NER and Pso2p, or Exo1p and MutSp, prior to the repair of DSB formed during replication (Barber *et al*., 2005). The loss of both Pso2p and Exo1p/Msh2p reduces spontaneous HR involved in repair of DSBs that arise from collapsed replication forks (Barber *et al*., 2005). In order to clarify whether function of Pso2p in the repair of ICL-associated DSB could be mediated through protein-protein interactions, Dudas *et al* (2007) conducted a two-hybrid screen examining a possibility of interaction of Pso2p with Yku70p, Yku80p, Nej1p, Lif1p, Dnl4p, Rad50p, Mre11p, Xrs2p, Rad51p, Rad52p, Rad54p, Rad55p, Rad57p, Rad59p and Rdh54p. The authors showed that Pso2p associates with none of the above DSB repair proteins, suggesting that its function in DNA repair seems to be rather individual.

Our previous results demonstrated mutagenic properties of DPDT and involvement of proteins members of BER, HR and NHEJ pathways, but not of NER pathway, in the repair of the induced lesions (Degrandi *et al*., 2010). Furthermore, a possible induction of replication blocking lesions by DPDT was supported by the results of Sailer *et al* (2004) that reported cell cycle specific apoptosis induction by DPDT, from the S and G2/M phase, in the promyelocytic HL-60 cell line (Sailer *et al*., 2003). Restart of stalled forks can occur through a recombination-associated pathway or mutagenic TLS. The *rad6*Δ (ubiquitin-conjugating enzyme (E2), involved in TLS) and *rev3*Δ (deficient in the catalytic subunit of DNA polymerase zeta, which is involved in DNA repair and TLS) mutants did not present sensitivity to DPDT (Degrandi *et al*., 2010), suggesting that TLS alone is not important in repairing DPDT-induced DNA damage. On the other hand, the hypersensitivity of the *rad52*Δ mutant strain suggests that HR is critical for the processing of potentially lethal genetic lesions induced by this agent. These repair intermediates could stall the replication forks, resulting in DSBs that are substrates for the HR repair pathway.

The Mre11p and Pso2p nucleases function in homologous recombination and interstrand cross-link (ICL) repair pathways, respectively, while the Exo1p nuclease is involved in homologous recombination and mismatch repair. However, elimination of all three nucleases confers higher sensitivity to IR than any of the single or double mutant combinations indicating a high degree of redundancy and versatility in the response to DNA damage (Lam *et al*., 2008). In addition, Mre11p is essential for processing the chemically modified or damaged DNA ends by MMEJ which is active throughout the cell cycle (Mladenov and Iliakis, 2011). In addition, Mre11p is essential for processing the chemically modified or damaged DNA ends by MMEJ which is active throughout the cell cycle (Mladenov and Iliakis, 2011). Also, the idea that Mre11p/MRX could process ICL or capped hairpins independently of Pso2p is supported by the increased sensitivity of the double-mutant *pso2Δmre11Δ* to 8-MOP/UVA and nitrogen mustard treatment, when compared to the *pso2Δ* single-mutant (Munari *et al*., unpublished data). The set of our results and literature data showed that DPDT is highly toxic for dividing cells. If ICL or hairpin capped DSBs occur during cell replication, these lesions are mainly processed by Pso2p in yeast, or Exo1p-Msh2p or Mre11p/MRX(N) (reviewed by Cattell *et al*., 2010; Tiefenbach and Junop, 2012). Thus, Pso2p can provide the substrate for HR (during S-phase) or MMEJ (also referred as error-prone NHEJ, MRX-dependent), or canonical NHEJ (but this pathway is not active during S-phase of cell cycle). Furthermore both, Exo1p and MRX can provides substrate for HR, and in absence of this pathway, the substrate may be directed to MMEJ.

Our results showed that the double mutants *pso2Δswr1Δ* and *rad52Δswr1Δ* are not more sensitive to the tested DPDT, 3-CPS and 8-MOP agents than the more sensitive single mutant (*pso2Δ* and *rad52Δ,* respectively). The fact that *swr1Δ* (deficient in NHEJ) do not enhance the sensitivity of the double-mutants, in addition to the observed low sensitivity of the *yku80Δ* mutant to DPDT and psoralens photoaddition suggest that the induced lesions could be directed to HR or MMEJ pathways rather than to canonical NHEJ. It is worth to notice that Henriques and Moustacchi, (1981) and Henriques *et al*. (1985) showed that Rad52 and Pso2 are non-epistatic in repair of 3-CPS and 8-MOP photoinduced lesions. This suggests that Pso2 could provide substrate for repair pathway different from HR that could be the MMEJ. The error-prone nature of MMEJ could explain the fact that *pso2Δ* mutant is blocked in mutagenesis (Cassier *et al*., 1980; Brendel *et al*., 2003). On the other hand, the

involvement of MMEJ in the repair of DPDT induced lesions could explain the observed frameshift mutation induction by this agent (Degandi *et al*., 2010).

In conclusion, our results suggested that 8-MOP induces ICLs and hairpin capped DSBs, because mutants deficient in NER, TLS, HR and MMEJ pathways, besides *pso2Δ*, are sensitive to this agent. DPDT, at this time, probably can form hairpin capped DSBs, or other lesions able to block replication, once only mutants deficient in HR pathway, Pso2p and MRX are sensitive. The low sensitivity of *yku80Δ* to DPDT and psoralens + UVA treatment could be explained by the fact that the NHEJ pathway is not the preferential one during the S-phase of cell cycle. This could be also the case of *swr1Δ* mutant that is deficient in the canonical NHEJ (yKu80 loading), but not in Mre11 association and end-resection indispensable for the HR and MMEJ.

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Table 1. List of *S. cerevisiae* yeast strains used in this study.

Strains	Relevant genotypes	Pathway affected	Source
BY4741 (WT)	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	-	Euroscarf
arp4Δ	BY4741; with *arp4::kanMX4*	CR	L. Meira
ino80Δ	BY4741; with *ino80::kanMX4*	CR	L. Meira
swr1Δ	BY4741; with *swr1::kanMX4*	CR	L. Meira
nhp10	BY4741; with *nhp10::kanMX4*	HMG	L. Meira
elp3Δ	BY4741; with *elp3::kanMX4*	HAT	L. Meira
esa1Δ	BY4741; with *esa1::kanMX4*	HAT	L. Meira
gcn5Δ	BY4741; with *gcn5::kanMX4*	HAT	L. Meira
hat1Δ	BY4741; with *hat1::kanMX4*	HAT	L. Meira
hat2Δ	BY4741; with *hat2::kanMX4*	HAT	L. Meira
hpa2Δ	BY4741; with *hpa2::kanMX4*	HAT	L. Meira
hpa3Δ	BY4741; with *hpa3::kanMX4*	HAT	L. Meira
hta1Δ	BY4741; with *hta1::kanMX4*	HAT	Euroscarf
hta2Δ	BY4741; with *hta2::kanMX4*	HAT	L. Meira
sas2Δ	BY4741; with *sas2::kanMX4*	HAT	L. Meira
sas3Δ	BY4741; with *sas3::kanMX4*	HAT	L. Meira
hda1Δ	BY4741; with *hda1::kanMX4*	HDAC	L. Meira
sin3Δ	BY4741; with *sin3::kanMX4*	HDAC	L. Meira
BY4742 (WT)	*MATα his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*	-	Euroscarf
pso2Δ	BY4742; *pso2::URA3*	NER	Revers
rad52Δ	BY4742; with *rad52::kanMX4*	HR	Euroscarf
yku80Δ	BY4742; with *yku80::kanMX4*	NHEJ	Euroscarf
swr1Δ	BY4742; with *swr1::LEU2*	CR	This study
swr1Δpso2Δ	BY4742; with *swr1::LEU2, pso2::URA3*	CR/NER	This study
swr1Δrad52Δ	BY4742; with *swr1::LEU2, rad52::kanMX4*	CR/NER	This study
swr1Δyku80Δ	BY4742; with *swr1::LEU2, ; yku80::kanMX4*	CR/NHEJ	This study

CR: Chromatin Remodeling; HMG: High-Mobility Group; HAT: Histone Acetyltransferase; HDAC: Histone Deacetylase; NER: Nucleotide Excision Repair; NHEJ: Non-homologous end joining.

Table 2. Primers used in this study

Primers for disruption
5’- ATGACCACATCTCGTAAATCGCATGCGAAAGACAAAAAGGGGTCAGGTTGCTTTCTCAGG-3’ 3’- TCAATAATAATAACCGTTGGCAATAAACCTGATCATGTACTTTATGCTTCCGGCTCGTAT-5’
Specific primers for the *SWR1* gene
5’-GACGATTTAACGCTGGAAGA-3’ 3’-CATATCATCGTCACTTTCAC-5’

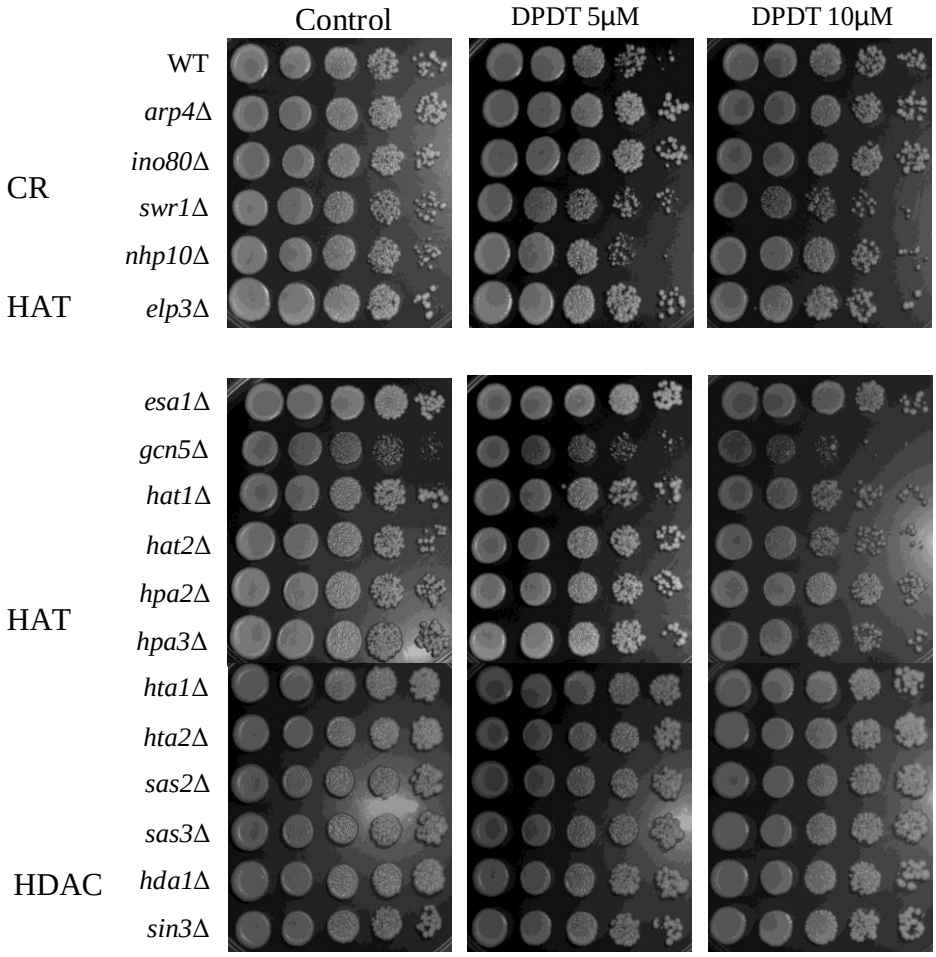

Control
DPDT 5μM
DPDT 10μM
WT
arp4Δ
ino80Δ
swr1Δ
nhp10Δ
elp3Δ
esa1Δ
gcn5Δ
hat1Δ
hat2Δ
hpa2Δ
hpa3Δ
hta1Δ
hta2Δ
sas2Δ
sas3Δ
hda1Δ
sin3Δ
CR
HAT
HAT
HDAC

Figure 1.

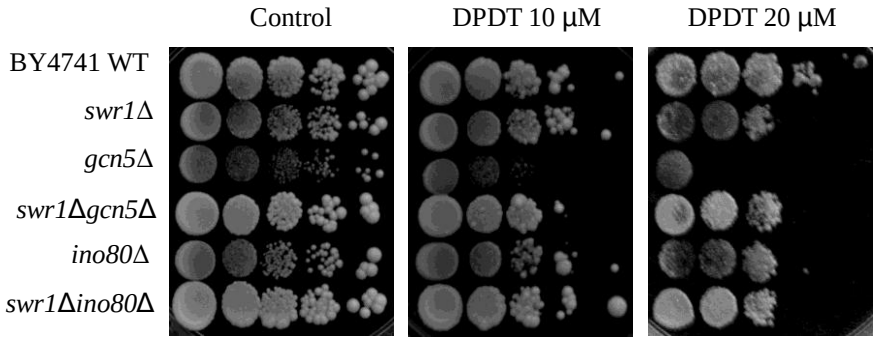

Control
DPDT 10 µM
DPDT 20 µM
BY4741 WT
swr1Δ
gcn5Δ
swr1Δgcn5Δ
ino80Δ
swr1Δino80Δ

Figure 2.

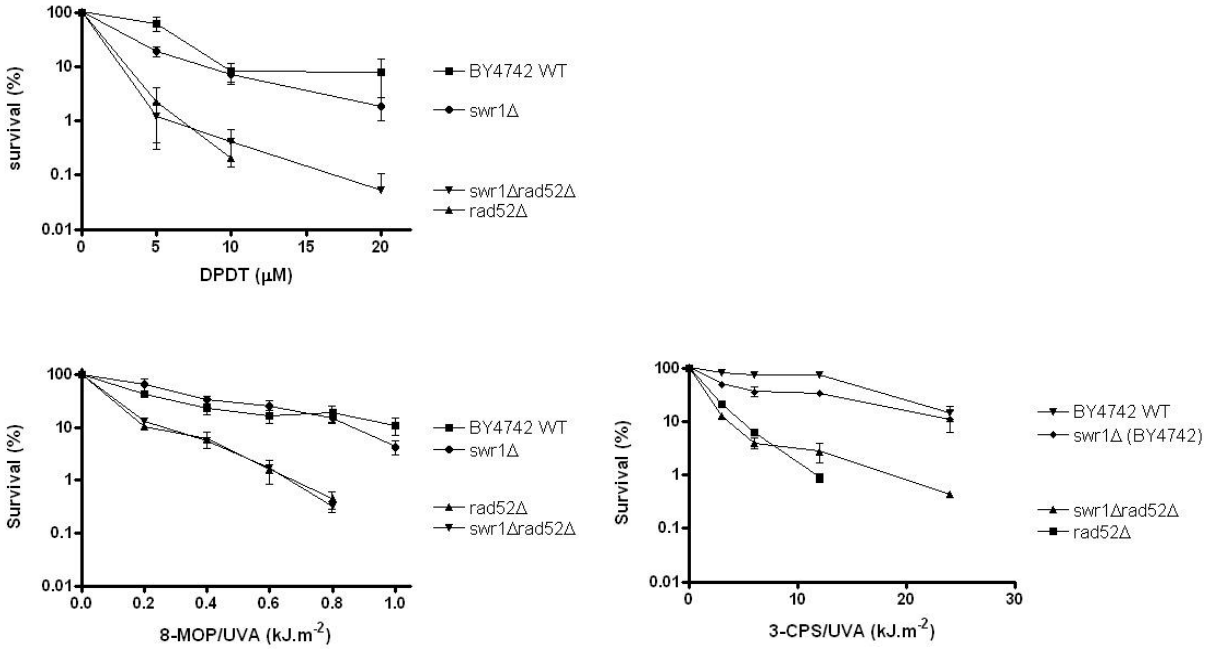

survival (%)
100
10
1
0.1
0.01
0
5
10
15
20
DPDT (μM)
BY4742 WT
swr1Δ
swr1Δrad52Δ
rad52Δ
Survival (%)
0.0
0.2
0.4
0.6
0.8
1.0
8-MOP/UVA (kJ.m-2)
BY4742 WT
swr1Δ
rad52Δ
swr1Δrad52Δ
30
3-CPS/UVA (kJ.m-2)
swr1Δ (BY4742)

Figure 3.

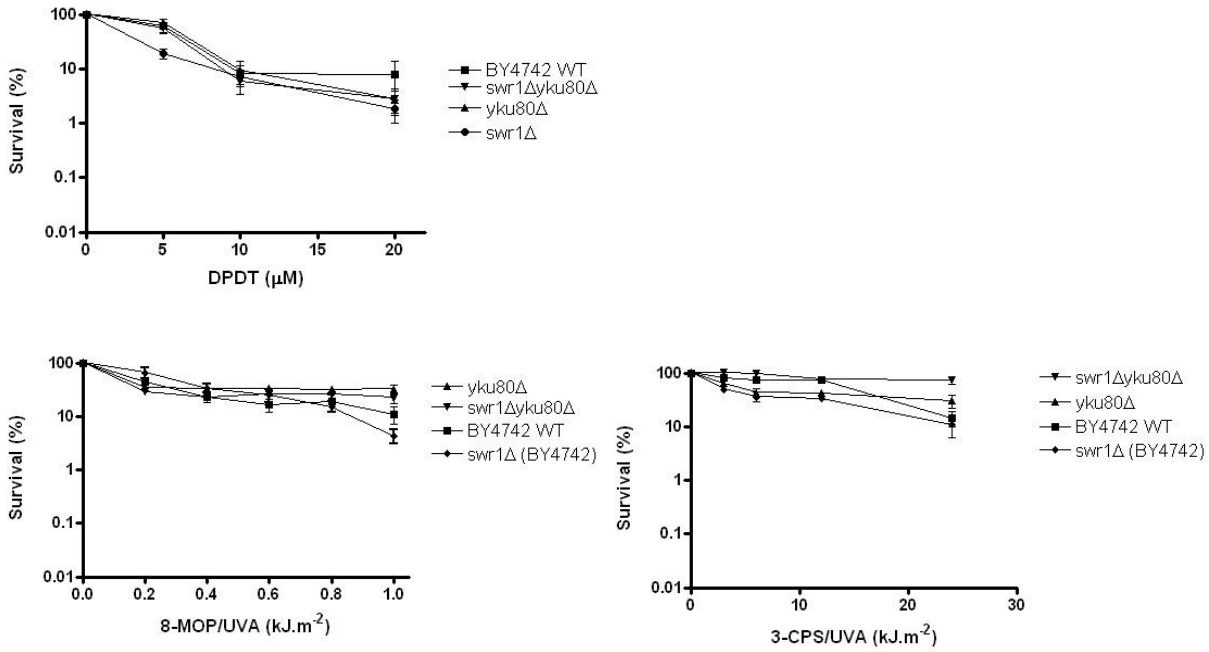

Survival (%)
100
10
1
0.1
0.01
0
5
10
15
20
DPDT (μM)
BY4742 WT
swr1Δyku80Δ
yku80Δ
swr1Δ
Survival (%)
0.0
0.2
0.4
0.6
0.8
1.0
8-MOP/UVA (kJ.m-2)
yku80Δ
swr1Δyku80Δ
BY4742 WT
swr1Δ (BY4742)
Survival (%)
0
10
20
30
3-CPS/UVA (kJ.m-2)
swr1Δyku80Δ
yku80Δ
BY4742 WT
swr1Δ (BY4742)

Figure 4.

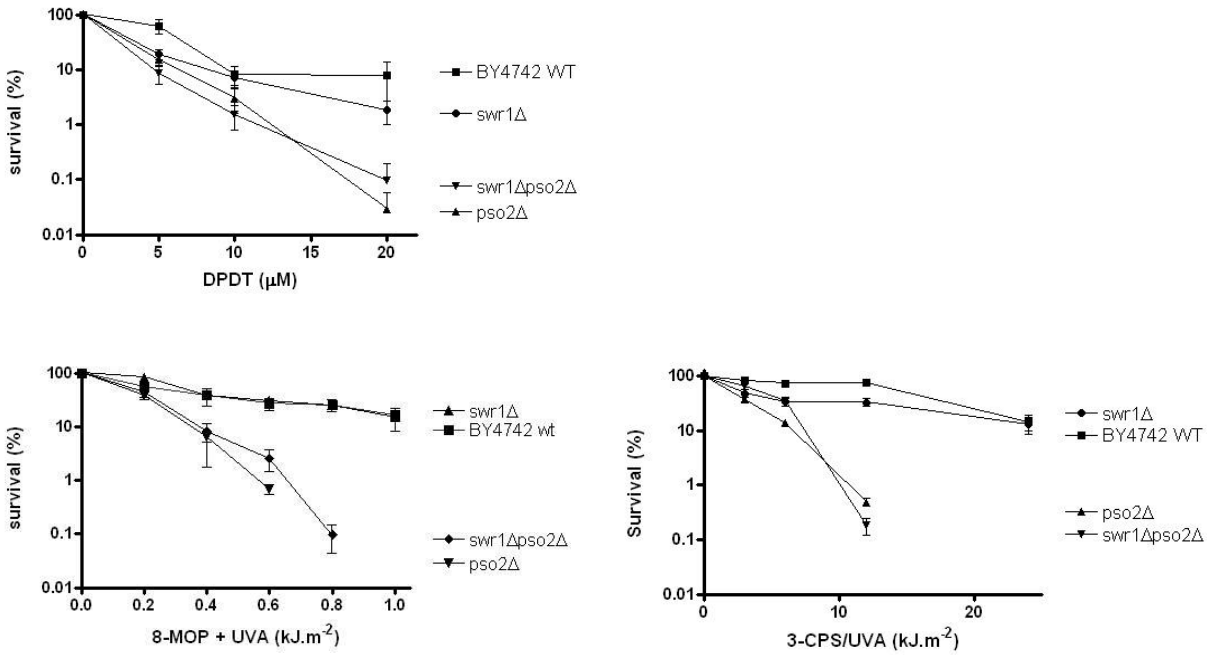
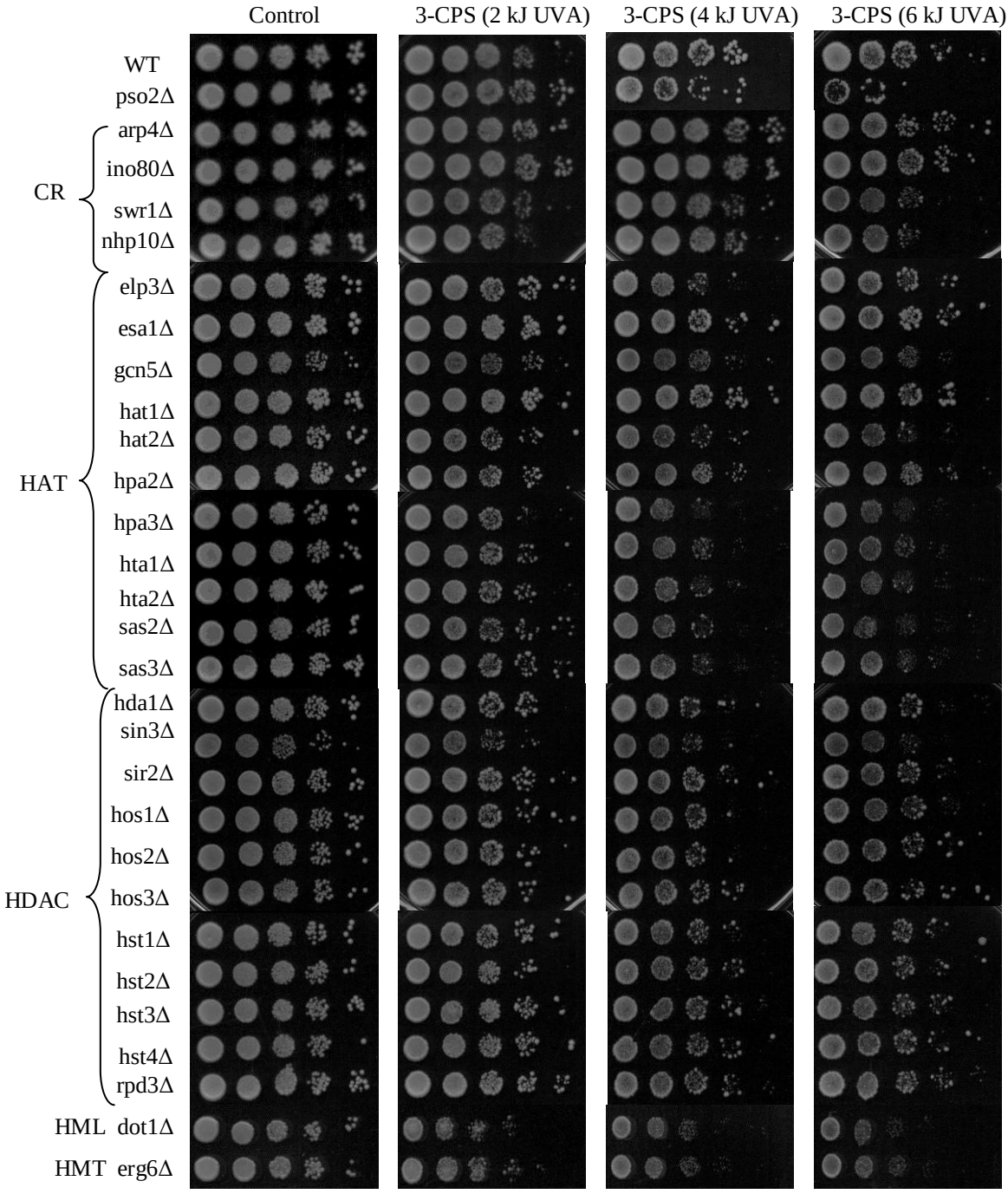
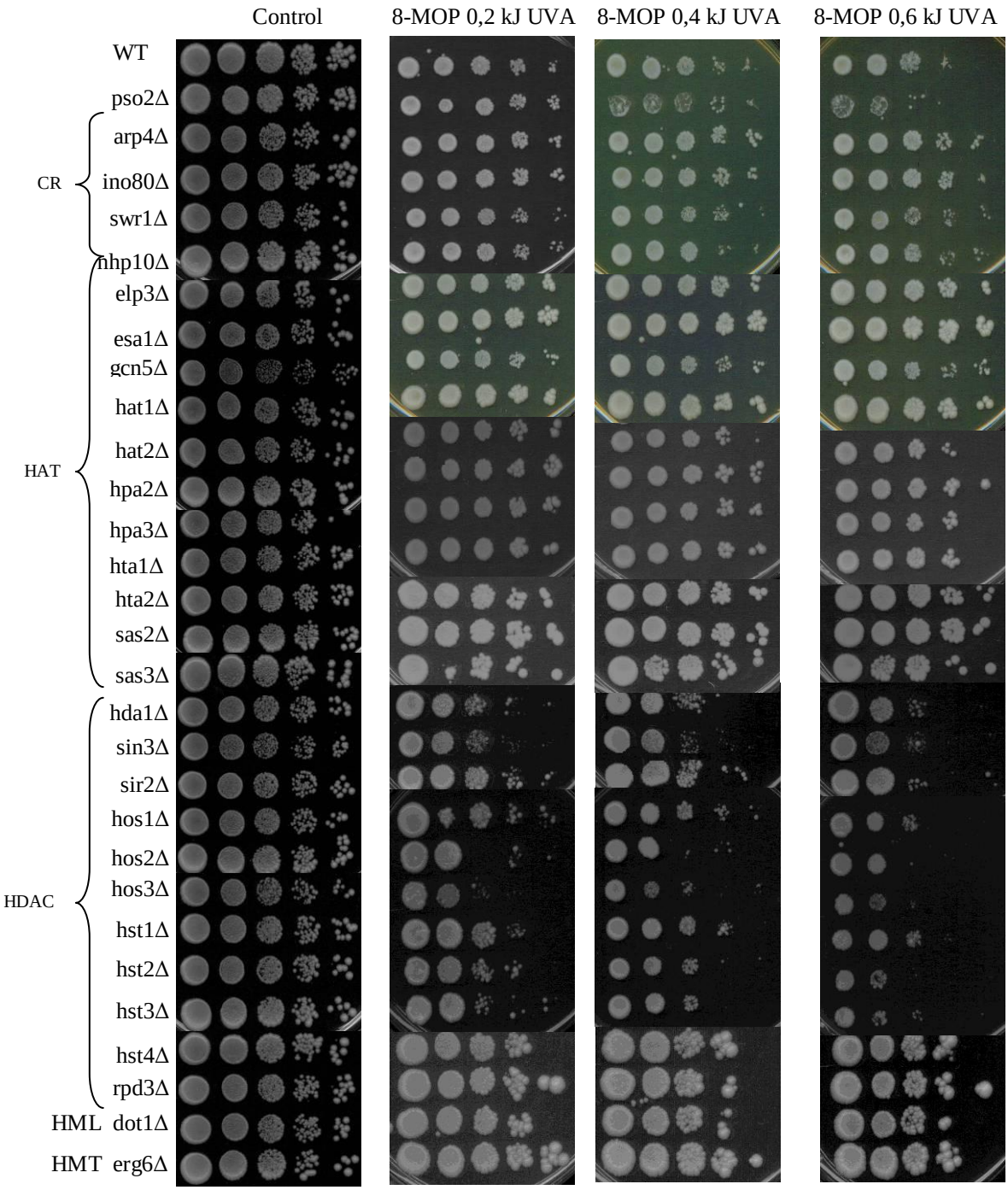

survival (%)
100
10
1
0.1
0.01
0
5
10
15
20
DPDT (µM)
BY4742 WT
swr1Δ
swr1Δpso2Δ
pso2Δ
survival (%)
0.0
0.2
0.4
0.6
0.8
1.0
8-MOP + UVA (kJ.m-2)
swr1Δ
BY4742 wt
swr1Δpso2Δ
pso2Δ
Survival (%)
0
10
20
3-CPS/UVA (kJ.m-2)
swr1Δ
BY4742 WT
pso2Δ
swr1Δpso2Δ

Figure 5.



Supplementary data S-1.



Supplementary data S-2.

Figure 1. Drop tests of *S. cerevisiae* strains deficient in proteins involved in chromatin remodeling. Cells in exponential growth phase were incubated in SynCo medium with (or without) DPDT at concentrations of 10μM and 5μM, during 20:00 h at 28 °C, under shaking. After serial dilutions of cell suspensions, the cells were plated on YPD plates.

Figure 2. Drop tests of *S. cerevisiae* strains deficient in proteins involved in chromatin remodeling. Cells in exponential growth phase were serially diluted and plated on SynCo medium plates with (or without) DPDT, at concentrations of 10 μM and 20 μM.

Figure 3. Effect of different cytotoxic treatments in *S. cerevisiae* strains deficient in CR and HR protein.

Figure 4. Effect of different cytotoxic treatments in *S. cerevisiae* strains deficient in CR and NHEJ protein.

Figure 5. Effect of different cytotoxic treatments in *S. cerevisiae* strains deficient in CR and Pso2p.

Supplementary data S-1. Drop tests of *S. cerevisiae* yeast strains deficient in proteins related to chromatin remodeling. Cells in exponential growth phase were treated with 3-CPS + UVA at different doses, according to Henriques and Moustacchi, 1980. After serial dilution, cells were plated on YPD medium plates.

Supplementary data S-2. Drop tests of *S. cerevisiae* yeast strains deficient in proteins related to chromatin remodeling. Cells in exponential growth phase were treated with 8-MOP + UVA at different doses, according to Henriques and Moustacchi, 1980. After serial dilution, cells were plated YPD plates.

CAPÍTULO III

Effects of cisplatin and 5-FU chemotherapeutic protocol in chromatin remodeling and DNA damage repair pathways in *Saccharomyces cerevisiae:* ***insights in cancer therapy***

Artigo a ser submetido ao periódico *Genetics and Molecular Research*

Effects of cisplatin and 5-FU chemotherapeutic protocol in chromatin remodeling and DNA damage repair pathways in *S. cerevisiae*: insights in cancer therapy

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Introduction

The high incidence of cancer in human populations has motivated researchers around the world in search for potential therapeutic targets for the treatment of this disease. Due to the great variability in the kinds of existing tumors whose morphologic, genotypic and physiological characteristics are distinct, it is necessary to treat a multidirectional approach, using cytotoxic chemotherapeutic drugs with different action mechanisms (Ohtsu, 2008; Rosell *et al*., 2008; Luchenko *et al*., 2011; Takara *et al*., 2012). Understand and exploit the genetic reasons why some tumors respond to certain treatments and not others, is the central role of biological and medical research. Since the conventional antitumoral agents are not selective for cancer cells, patients treated with these drugs generally have a range of undesirable effects. In this sense, elucidate the action mechanisms of chemotherapeutic drugs used in the treatment of various cancers is extremely important to support the development of new therapeutic protocols, whose drug combinations showing up increasingly effective in preventing the progression of tumor growth, while reducing the side effects (Goekkurt and Al-Batran 2009). For this, it is necessary a more detailed understanding of the action mechanism of these drugs, so they are more efficiently administered to the patient, thus contributing effectively as a therapy against cancer

The chemotherapeutic regimen which combines 5-fluorouracil (5-FU) and cisplatin has been widely used in the treatment of gastric cancer (Goekkurt and Al-Batran 2009). However, both treatment efficacy as their toxicity have a high individual variability (Takara *et al.* 2012). 5-FU is a pyrimidine analog antimetabolite which has been prescribed in the treatment of adenocarcinomas of the breast, ovary and the gastrointestinal tract (Matuo *et al.* 2010). The cytotoxicity of the drug is assigned to three different mechanisms: fluoronucleotideos incorporation of the DNA, the RNA, and inhibition of enzyme thymidylate synthase (Matuo *et al.* 2009). Cisplatin (cis-diamminedichloroplatinum (II), CDDP), in turn, is used to treat various kinds of tumors, although their efficiency is affected by mutations in genes involved in DNA repair machinery. The cisplatin cytotoxicity is due mainly to its ability to form adducts with DNA, resulting mainly in 1,2-intrastrand cross-links between guanines or between guanine and adenine, and to a lesser extent, DNA interstrand crosslink (reviewed in Tang *et al*., 2011). Such changes in the structure of duplex DNA can block the progression of DNA replication, and activate cell cycle checkpoint.

Several studies have shown that chromatin remodeling (CR) can modulate the mechanisms of DNA repair, and regulate transcriptional activation in eukaryotic cells (Matuo *et al*. 2012; Czaja *et al*. 2010; Lee *et al*. 2010; Palomera-Sanchez and Zurita 2011; Yu *et al*. 2011). Moreover, arise every day new evidences that point to the use of histone code in the search for new therapeutic targets that may be useful in cancer combat (Witt *et al*., 2009; Dekker & Haisma, 2009; Espinosa *et al*., 2010; Faucher & Wellinger, 2010; Chervona & Costa, 2012). In this context, the epigenetic modifications can lead to oncogenes activation, silencing of tumor suppressors and to the uncontrolled tumor cells proliferation (Parthun, 2012). Currently, drugs that inhibit DNA methylation and histone deacetylases inhibitors are used to treat myelodysplastic syndrome, and acute and chronic myelogenous leukemia (Kristensen *et al*., 2009). Moreover, changes in the activity of histone acetyl transferases (HATs) are observed in solid and hematological tumors either by mutations that cause the inactivation of HATs or by the action of viral oncoproteins (Ellis *et al*., 2009). Then it seemed interesting to use *S.cerevisae* yeast as a model to test the response of strains proficient and deficient in chromatin remodeling factors and DNA repair proteins to antitumoral drugs, once this microorganism provide a better understanding of the interplay between chromatin remodeling and other intracellular processes, and their DNA repair systems are well understood and similar to the human (Matuo *et al*., 2012). Thus, this study aims to broaden the understanding of epigenetic modulation and DNA damage repair machinery involved in the biological response to chemotherapeutic protocol combining cisplatin and 5-FU, focused in the *S. cerevisiae* yeast biology.

Materials and methods

Yeast strains and growth conditions

Yeast strains deficient in chromatin remodeling factors and DNA repair proteins were kindly provided by Dr. Lisiane Meira (Biological Engineering Division, MIT, Cambridge, USA) and acquired from Euroscarf (European *Saccharomyces cerevisiae* Archive for Functional Analysis) or constructed by gene replacement. The *htz1Δ* strain was constructed in our laboratory, by Dr. Renata Matuo. The relevant genotypes of *S. cerevisiae* strains used in this study are listed in Table 1.

The yeast *Saccharomyces cerevisiae* strains growing to exponential phase (LOG) by inoculation of $5x10^5$ cells/mL of YPD culture in stationary phase (STAT) into 5 mL of YPD medium. After 14 hours incubation, at 30°C with aeration, the cultures contained (1-2) x 10^7 cells/mL with 20-30 % budding cells. The number of cells was determined by counting in Neubauer chamber.

Survival assays

Survival after treatment with 5-FU and cisplatin was measured by preparing cell suspension containing 1 x 10^7 LOG cells/mL. The cells were incubated in culture medium at 30 °C for 4h (for yeast strains deficient in DNA repair proteins) or 20:00h (for yeast strains deficient in chromatin remodeling), with agitation. After incubation, samples were diluted in saline solution, plated onto YPD agar, and incubated at 30 °C for 2-3 days. 5-FU concentrations employed were 75 µM; and cisplatin concentrations employed were 200 and 500 µM with or without 5-FU. Assays were performed twice.

Results

Aiming to establish the relationship between DNA repair pathways and cytotoxicity induced by treatment with CDDP, 5-FU and CDDP + 5-FU, we used yeast strains proficient and deficient in proteins involved in different DNA repair pathways. The results showed that the strains deficient in Rad1p (NER) Rad50p (NHEJ), Rad52p (HR) Rev3p (TLS) and Rad6p (PRR) were markedly sensitive to the antitumoral agent cisplatin, which was dose-dependent (Figure 1), confirming the results already existing in literature, in which the DNA interstrand crosslinks induced by cisplatin can be removed by NER, and also requires HR pathway, which is supported by the fact that this drug increases the rate of recombination, possibly in response to DSB formation (reviewed by Tompkins *et al*., 2012). Furthermore, the single-strand breaks resulting of the processing of the lesion by NER machinery can also be converted into DSBs, so that the repair of DNA platinum damage involves NER, MMR, TLS and HR factors (Boulikas *et al*. 2007; Lehoczký *et al.*, 2007; Ho and Schärer 2010). The high sensitivity of the mutant *rad6Δ* to cisplatin shows the importance of the TLS pathway for the repair of these adducts, although the *rad18Δ* mutant did not show increased sensitivity after

the treatment (Figure 1). The *rad30Δ* mutant, deficient in DNA polymerase η, showed low cell survival rate when compared to Wild Type, independently of the treatment employed, indicating the importance of this polymerase to the survival of cells under standard conditions. Moreover, strains deficient in Ogg1p (BER) and Mlh1p (MMR) exhibited similar sensitivity to that observed in Wild Type cells (Figure 1).

In relation to treatment with 5-FU, an earlier study from our laboratory has investigated the DNA repair pathways involved in repair of lesions generated by 5-FU or its major metabolite, FdUMP, in yeast *S. cerevisiae* (Matuo *et al*. 2010). The results showed that mutants deficient in BER pathway (*ntg1Δ, ntg2Δ, apn1Δ, apn2Δ*) showed greater sensitivity to both, 5-FU and FdUMP, as well as MMR deficient mutants (*mlh1Δ, pms1Δ*). The same study also reveals that the lesions induced by 5-FU were repaired by BER, MMR, HR and PRR pathways, while the lesions induced by FdUMP were repaired by BER and MMR pathways. Curiously, all the yeast strains deficient in DNA repair used in this study showed sensitivity similar to that observed in Wild Type cells, when treated with 75 μM of 5-FU (Figure 1). This response may be due to the dose employed, once the previous study conducted by Matuo *et al*. (2010) has used 5-FU at a concentration of 150 μM and, in this study, we used 75 μM of 5-FU. The choice of doses was based on the fact that 150 μM of 5-FU caused a very high cell mortality in the combined treatment of CDDP + 5-FU, rendering it impossible to interpret the results (data not shown).

In order to investigate the role of histone code in response to DNA damage induced by treatments with CDDP, 5-FU and their combination, *S. cerevisiae* yeast strains deficient in proteins related to chromatin remodeling were used. The screening presented in Figure 2 indicates a higher sensitivity of the mutants deficient in CR (*ino80Δ*), HATs (*elp3Δ, gcn5Δ, and hat2Δ hpa2Δ*), HDACs (*sin3Δ, sir2Δ, hos2Δ, hst1Δ, hst2Δ, hst3Δ*), HML (*dot1Δ*) and HMT (*erg6Δ*) to the combined treatment (CDDP + 5-FU), when compared to Wild Type. However, the same mutants treated only with 5-FU and CDDP exhibited similar sensitivity to that observed in wild type, except *sin3Δ*, which was also sensitive to treatment with 5-FU. Interestingly, the mutant *hos3Δ*, sensitive to 5-FU and CDDP, showed a similar response to that seen in Wild Type, when treated with CDDP + 5-FU. Furthermore, the combined treatment (5-FU + CDDP) showed marked toxicity to all strains studied, including the Wild Type (Figure 2).

Discussion

The effect of combined treatment of CDDP + 5-FU reflects the action of each drug alone, concerning to DNA damage repair machinery from yeast S. cerevisiae.

The alkylating agent cisplatin and the pyrimidine analog 5-FU are drugs widely used to treat various types of cancer, and treatment combining the pharmacological properties of these drugs is effective against gastric, testicular, lung and head and neck tumors. The biological effects of cisplatin are attributed to the drug's ability to form adducts with the DNA molecule, mainly 1,2-intrastrand crosslinks between adjacent guanines or between guanine and adenine, causing structural changes in DNA. Such distortions interrupt replication and transcription processes, with activation of cell cycle checkpoint and, if not repaired, can lead to cell death (Tompkins *et al.*, 2012). The mechanism of cytotoxic action of 5-FU, at this time, involves its conversion to fluoronucleotides (which are erroneously incorporated into DNA and RNA). In mammalian cells, 5-FU is converted to the active metabolite FdUMP, which inhibits the enzyme thymidylate synthase, resulting in imbalance of the levels of dTMP (decrease) and dUMP (increase) (Seiple *et al.* 2006; Berger *et al.*, 2008; Matuo *et al.* 2009, 2010). The yeast *S. cerevisiae*, our experimental model, does not possess the enzyme thymidine kinase (responsible for the conversion of 5-FU into FdUMP), which enabled the analysis of drug effects and its metabolite individually by Matuo *et al.* (2010). The authors found differences in the machinery involved in resolving repair of damage induced by 5-FU and FdUMP, so that injuries caused by the direct action of 5-FU were repaired by BER, MMR, HR and PRR pathways, while its metabolite FdUMP recruited BER and MMR pathways for the repair of DNA damage. Matuo *et al.* (2009) showed that, in SW620 colon adenocarcinoma cell line, 5-FU induces SSB, DSB and apoptosis more quickly than FdUMP, and treatment with 5-FU leads to arrest in S-phase, while FdUMP causes arrest in G2-phase of cell cycle. The authors suggested that this effect results from the induction of different types of primary lesions, which in turn, trigger different checkpoints, with consequent recruitment of DNA repair pathways which also differ. The combined treatment of CDDP and 5-FU showed synergistic clinical effects in the treatment of gastrointestinal cancer, when compared to the individual effects of the drugs (Takara *et al.*, 2012). Although our results showed that the combination of the two drugs (cisplatin + 5-FU) appears to be slightly more efficient in inducing cell death,

it appears to reflect the sensitivity of the specific strains to each individual drug used (Figure 1). This finding suggests that the enhanced effect of the combined treatment in cancer therapy could be due to the action of each agent alone on different clones from the heterogeneous population of transformed cells.

The Chromatin remodeling factors are differently affected by CDDP, 5-FU and CDDP + 5-FU cytotoxic treatments

An important recent study suggest that chromatin remodeling complex SWI/SNF is involved in modulation of cisplatin cytotoxicity by relaxing the chromatin structure, facilitating the formation of platinum-DNA adducts (Kothandapani and Gopalakrishnan, 2012). In addition, the 1,2-d(GpG) cross-link, the more abundant cross-link formed by cisplatin treatment, causes changes in the rotational setting of the DNA on the histone octamer core, and also influences the conformation of the DNA within the nucleosome depending on the position of the adduct in the DNA sequence (Ober and Lippard, 2008). Our results concerning the chromatin remodeling proteins participation in the cell response induced by the treatments used in this study showed that the drugs combination demonstrates a higher specificity in the cell response to the treatments, with greater sensitivity observed in mutants deficient in HATs, in addition to the HDAC mutant *hos3Δ*, for cisplatin treatment (Figure 2). A similar result was found by Matuo *et al.* (2012), in which the treatment with 200 μM CDDP directly added on YPD plates caused high cytotoxicity in several HAT deficient strains, except for *elp3Δ* and *esa1Δ* mutants. The same response was also observed for HDAC mutants, in which all studied strains were very sensitive to treatment with 200 μM CDDP, except *sir2Δ* (Matuo *et al.*, 2012). Collectively, the HATs regulate transcription, and some HATs are over expressed in human cancer cells resistant to cisplatin, as Clock and Tip60, that regulates glutathione biosynthesis and modulates the expression of DNA repair genes, respectively (Hirano *et al.*, 2010). The acetylation of lysine ε-amino groups of the chromosomal core of histones controls the accessibility to DNA molecule, and the enzymes responsible for histone acetylation and deacetylation constitutes important targets for the development of new anti-cancer drugs (Bandyopadhyay and Banères, 2009). The HAT inhibition promotes a transient arrest of DNA synthesis, as well as a transient delay in S- phase progression, and also inhibits the NER pathway and the DSB repair, increasing the cellular sensitivity to the chemotherapeutic drugs cisplatin

(Platinol™), 5-fluorouracil, camptothecin and also to UV-C irradiation (Bandyopadhyay and Banères, 2009).

Our results also demonstrates that the treatment with 75 μM of 5-FU alone induced marked sensitivity in strains deficient in HAT (*gcn5Δ*) and HDACs (*sin3Δ* and *hos3Δ*, the latter is also very sensitive to CDDP treatment) (Figure 2). Gcn5p is a lysine acetyltransferase (KAT) which acetylates H2B and H3 histones (H2BK11ac, H2BK16ac, H3K9ac, H3K14ac, H3K23ac, H3K27ac and H3K36ac), and have less preference for H4 histone (H4K8ac and H4K16ac). In yeast cells, the *GCN5* gene is involved in the control of lifespan (by modulation of retrograde response and genome stability) and gene expression (Kim *et al*., 2004). Furthermore, the H3 histone acetylation by Gcn5p controls the nucleosome assembly by a mechanism that involves the association of H3 histone with CAF-1 (Dekker and Haisma, 2009; Eitoku *et al*., 2008), and GCN5 deficient cells, as well as those that express mutations in the N-terminus of H3 histone, are defective in the deposition of new H3 molecules on replicative DNA, and also exhibit a reduction in binding of the H3 to CAF-1 (Xu and Zhu, 2010). A recent study also demonstrates that changes in H3 histone acetilation by Gcn5p facilitates the DNA damage repair, by promoting chromatin remodeling, and it is known that the GCN5 gene plays a role in NER pathway in human cells (Yu *et al*., 2011). Sin3p, in turn, is a component of the Sin3p-Rpd3p histone deacetylase complex, which participates in activation of several processes, like meiosis and mating-type switching, besides transcriptional repression. It has been reported that the absence of deacetylation of H4K16 by Sin3/Rpb3 impairs NHEJ, although the deacetylation of the same residue is also required for HR pathway (reviewed by Escargueil *et al*., 2008). A study recently published show that in the absence of the histone deacetylases Hos3 and Hos2, Gcn5p associates throughout intron-containing genes, enhancing histone H3 acetylation (Gunderson and Merkhoferb, 2011).

Our results with the combined treatment (5-FU + CDDP) showed high toxicity for all strains studied, including the Wild Type, with greater sensitivity to CR (*ino80Δ*), HATs (*elp3Δ, gcn5Δ, hat2Δ* and *hpa2Δ*), HDACs (*sin3Δ, sir2Δ, hos2Δ , hst1Δ, hst2Δ,* and *hst3Δ*), HML (*dot1Δ*) and HMT (*erg6Δ*). The Ino80p, a member of INO80 complex, operates in the same way that the NER pathway and contributes to the removal of UV photoproducts in the regions of high incidence of nucleosomes (reviewed by Cruz *et al*., 2012). INO80 complex also interacts with Rad4p–Rad23p complex, a damage recognition complex of NER, and may influence NHEJ pathway

(reviewed by Cruz *et al*., 2012). Concerning to the HAT mutants affected by treatment with 5-FU + CDDP, its know that Elp3p is a subunit of elongator complex, which promotes RNAPII transcript elongation through histone acetylation in the nucleus and tRNA modification in the cytoplasm, and also controls several biological processes, including exocytosis and heat shock resistance in yeast, besides cell migration and neuronal differentiation in higher eukaryotes, and also regulates cell migration, invasion and tumorigenicity of melanoma cells (Close *et al*., 2012). Hat2p is subunit of two different complexes, Hat1p-Hat2p complex (cytoplasmatic complex that acetylates H4 histone newly synthesized, similar to human RbAp46 and 48, with a role in telomeric silencing), and the NuB4 complex (a nuclear complex that also contains Hat1p and Hif1p, the H3/H4-specific histone chaperone), and both complexes have diverse effects on the repair-linked chromatin reassembly process, and *S. cerevisiae hat1*Δ mutants showed defects in recombinational repair (reviewed in Ge *et al*., 2011). Hpa2 is an tetrameric HAT that have similarity to Gcn5p, Hat1p, Elp3p and Hpa3p, exhibiting autoacetylation activity and also acetylating the H3 and H4 histones, *in vitro* (Vernarecci *et al*., 2010).

Many therapeutic protocols currently used against cancer make use of HDAC inhibitors in addition to the combined treatment of CDDP + 5-FU, besides other therapeutic regimens using drugs with different action mechanisms (Ohtsu, 2008; Batty *et al*., 2009; Johanne *et al*., 2011; Luchenko *et al*., 2011). The HDACs inhibition makes DNA less compacted and thus more sensitive to DNA damage induced by cisplatin, by facilitating the establishment of cisplatin-DNA adducts. This fact was confirmed by the increase in acetylation of H3 and H4 histones, used as markers of relaxation of the chromatin structure (Tesei and Brigliadori, 2012). As mentioned above, the HDAC yeast mutants *sin3Δ, sir2Δ, hos2Δ, hst1Δ, hst2Δ,* and *hst3Δ* showed high sensitivity to CDDP + 5-FU treatment. Cells lacking Sin3p, a component of Sin3p-Rpd3p HDAC complex, shows defects in NHEJ repair pathway, and the H4 histone acetyl group turnover (dependent of NuA4 complex, for acetylation, and Sin3p, for deacetylation) is important for efficient DSB repair, and also cooperate to promote transcription (Van Attikum and Gasser, 2005). The yeast HDAC Sir2p has homology to the human members of the HDACs class III, also called sirtuins, whose activity is related to the neurological and metabolical diseases, in addition to their association with HIV and cancer pathogenesis (Lawson *et al*., 2010). Furthermore, yeast Sir2p also affects the polarisome function, in retrograde transport of the damaged proteins from bud to mother

cell, and thus ensuring the youth of the daughter cells (Gan and Tang, 2010). Human sirtuins participates in several cellular processes, as in the control of cell metabolism, transcriptional repression, aging, DNA damage responses and apoptosis (Greiss and Gartner, 2009). Hos2p is a subunit of Set3 (a meiotic-specific repressor of sporulation) and Rpd3L HDAC complexes, and deacetylates H3 and H4 histone tails on their lysine residues, and co-localizes with Cmr1p in nuclear foci in response to DNA damage by the alkylating agent methyl methanesulfonate (MMS) (Tkach *et al*., 2012). Hst1p is a subunit of Sum1p/Rfm1p/Hst1p HDAC complex, necessary for ORC-dependent silencing and mitotic repression, and is also involved in telomere maintenance (Askree *et al*., 2004). Hst2p modulates the nucleolar and telomeric silencing, and Hst3p, at this time, is involved in the short-chain fatty acid metabolism, in addition to the cell cycle progression, genomic stability, telomeric silencing, and radiation resistance (Starai and Takahashi, 2003).

Histone methylation and demethylation occurred within CpG islands are the best-studied epigenetic alterations in cancer, plays an important role in transcriptional regulation, and it is normally altered during malignant cell transformation (Dawson and Kouzarides, 2012). Additionally, the methylation is also observed in inactive X chromosomes, centromeres, telomeres and repeat sequences, and hypomethylation is commonly found in cancer cells (You and Jones, 2012). Our results showed that the combined treatment of CDDP + 5-FU showed high toxicity for *dot1Δ* and *erg6Δ* mutants, which did not occur during the treatment of the cells with CDDP or 5-FU alone. Dot1p is responsible by methylation of H3 histone at their lysine 79 residue, and is required for G1/S and intra-S DNA damage checkpoints, in vegetative cells, and also for the meiotic recombination checkpoint, as well as for chromatin silencing (reviewed in Conde and San-Segundo, 2008). Dot1p has been proposed as capable to inhibits the TLS pathway by Polz/Rev1, and H3K79 methylation by Dot1p allows the transmission of the damage signal to checkpoint kinases, in a mechanism dependent of Rad9p, controlling the resection of damaged DNA and also influencing checkpoint cascades (Conde and San-Segundo, 2008). Bostelman *et al*. (2007) found that Dot1p deletion or point mutation at lysine 79 of H3 histone causes increased sensitivity to UV radiation, and epistasis analysis between dot and genes involved in repair of UV damage indicates that H3K79 methylation plays roles overlapped with NER, PRR and HR pathways, besides their checkpoint function mediated Rad9p. Furthermore, the DOT1L inhibitor (EPZ004777) prevents transcription of genes that are involved in leukemogenesis, and

kills cancer cells bearing MLL translocations (Daigle *et al*., 2011). Erg6p is an important bottleneck enzyme for ergosterol biosynthesis in *S. cerevisiae* by methylation of C-24 (Zahedi *et al*., 2006). The sterols are the major structural lipids of the plasmatic membrane of eukaryotes, and play an important role in maintaining the stability of the lipid bilayer during physicochemical disorders (Zahedi *et al*., 2006). The *erg6Δ* mutants show increased cation uptake (Welihinda *et al.*, 1994), and sensibility to drug brefeldin A, which disrupts secretion (Graham *et al*., 1993), and our results demonstrates that this mutant was also sensitive to CDDP + 5-FU treatment, and the hypersensitivity of the erg6Δ mutant may be related to changes in membrane properties.

Concluding remarks

The expression of different HDAC families in tumor tissues is up-regulated, and it is related to clinical response to chemotherapy (Witt *et al*., 2009), so that HDACs are currently considered the most important enzymes as targets of anticancer drugs (Tesei and Brigliadori, 2012). Thus, the differences in the sensitivity to treatments with CDDP, 5-FU and CDDP + 5-FU in mutants deficient in different HDACs may explain the clinical synergism existing in the combined treatment, since the pathways involved in DNA damage repair after treatment with the drugs individually did not show this effect. Our results points to involvement of chromatin remodeling proteins as important therapeutic targets in response to genotoxic agents, and indicates a possible interaction of these drugs not only with the DNA molecule, but also with the ATP-dependent chromatin remodeling. Therefore, these findings provide new insights for understanding the mechanisms involved in the response to treatment with CDDP + 5-FU, as well as in the choice of therapeutic protocols that consider the effect of antitumoral drugs on chromatin, although many studies are still needed to understanding these interactions.

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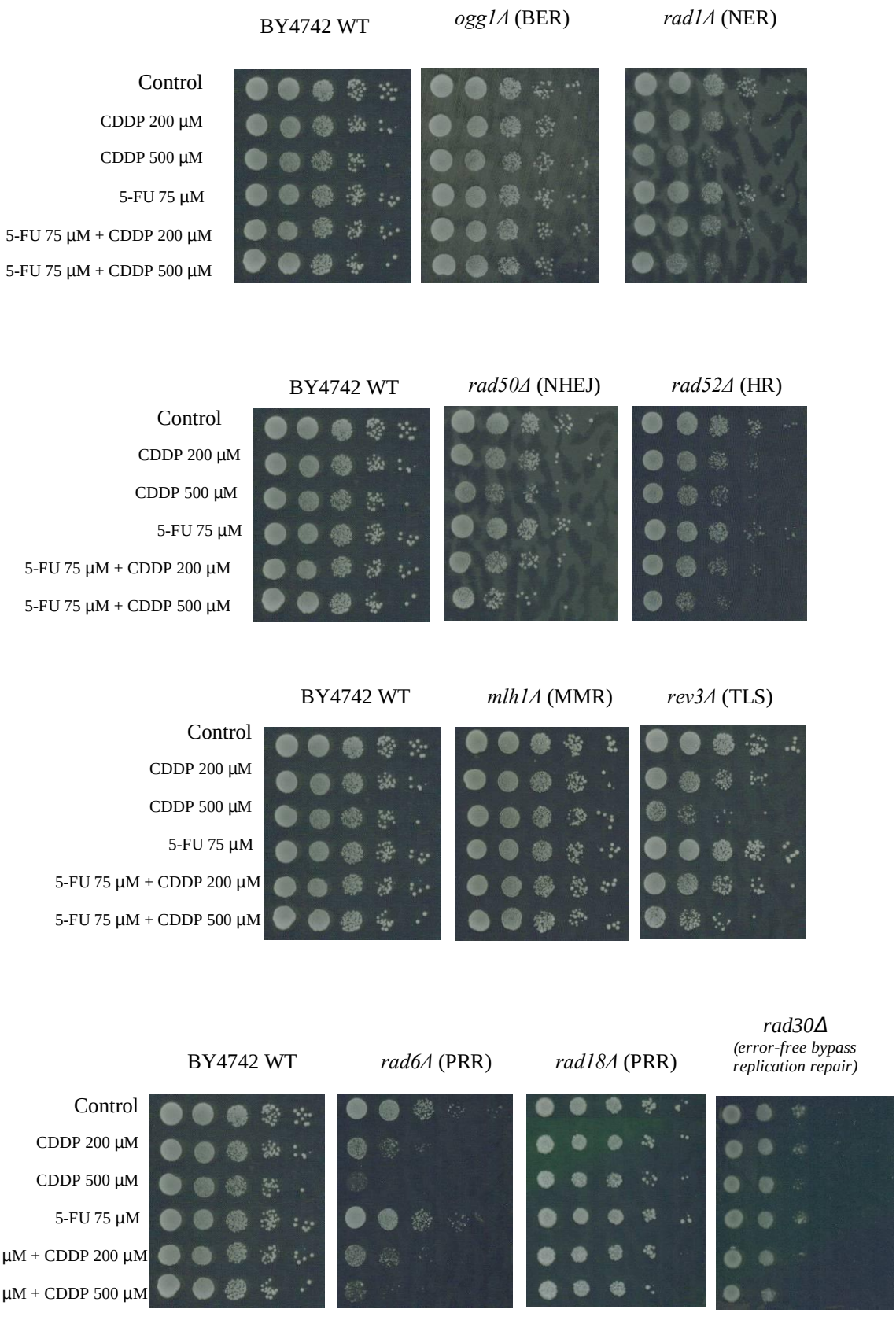
BY4742 WT
ogg1Δ (BER)
rad1Δ (NER)
Control
CDDP 200 µM
CDDP 500 µM
5-FU 75 µM
5-FU 75 µM + CDDP 200 µM
5-FU 75 µM + CDDP 500 µM
BY4742 WT
rad50Δ (NHEJ)
rad52Δ (HR)
Control
CDDP 200 µM
CDDP 500 µM
5-FU 75 µM
5-FU 75 µM + CDDP 200 µM
5-FU 75 µM + CDDP 500 µM
BY4742 WT
mlh1Δ (MMR)
rev3Δ (TLS)
Control
CDDP 200 µM
CDDP 500 µM
5-FU 75 µM
5-FU 75 µM + CDDP 200 µM
5-FU 75 µM + CDDP 500 µM
BY4742 WT
rad6Δ (PRR)
rad18Δ (PRR)
rad30Δ
(error-free bypass replication repair)
Control
CDDP 200 µM
CDDP 500 µM
5-FU 75 µM
5-FU 75 µM + CDDP 200 µM
5-FU 75 µM + CDDP 500 µM

Figure 1.

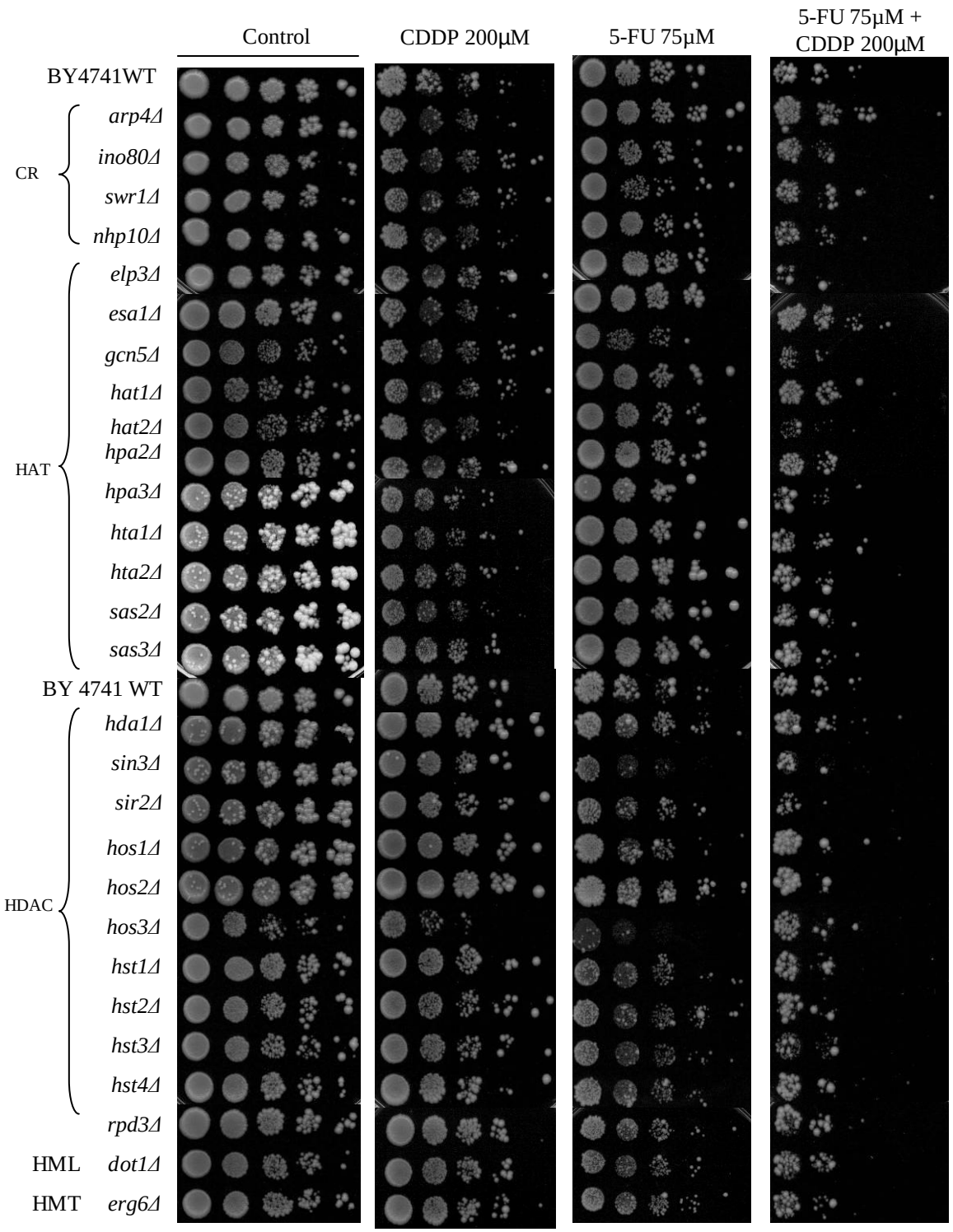
Control
CDDP 200μM
5-FU 75μM
5-FU 75μM +
CDDP 200μM
BY4741WT
CR
arp4Δ
ino80Δ
swr1Δ
nhp10Δ
HAT
elp3Δ
esa1Δ
gcn5Δ
hat1Δ
hat2Δ
hpa2Δ
hpa3Δ
hta1Δ
hta2Δ
sas2Δ
sas3Δ
BY 4741 WT
HDAC
hda1Δ
sin3Δ
sir2Δ
hos1Δ
hos2Δ
hos3Δ
hst1Δ
hst2Δ
hst3Δ
hst4Δ
rpd3Δ
HML
dot1Δ
HMT
erg6Δ

Figure 2.

Table 1. List of *S. cerevisiae* yeast strains used in this study.

Strains	Relevant genotypes	Pathway affected	Source
BY4741 (WT)	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	-	Euroscarf
arp4Δ	BY4741; with *arp4::kanMX4*	CR	L. Meira
ino80Δ	BY4741; with *ino80::kanMX4*	CR	L. Meira
swr1Δ	BY4741; with *swr1::kanMX4*	CR	L. Meira
nhp10	BY4741; with *nhp10::kanMX4*	HMG	L. Meira
elp3Δ	BY4741; with *elp3::kanMX4*	HAT	L. Meira
esa1Δ	BY4741; with *esa1::kanMX4*	HAT	L. Meira
gcn5Δ	BY4741; with *gcn5::kanMX4*	HAT	L. Meira
hat1Δ	BY4741; with *hat1::kanMX4*	HAT	L. Meira
hat2Δ	BY4741; with *hat2::kanMX4*	HAT	L. Meira
hpa2Δ	BY4741; with *hpa2::kanMX4*	HAT	L. Meira
hpa3Δ	BY4741; with *hpa3::kanMX4*	HAT	L. Meira
hta1Δ	BY4741; with *hta1::kanMX4*	HAT	Euroscarf
hta2Δ	BY4741; with *hta2::kanMX4*	HAT	L. Meira
sas2Δ	BY4741; with *sas2::kanMX4*	HAT	L. Meira
sas3Δ	BY4741; with *sas3::kanMX4*	HAT	L. Meira
hda1Δ	BY4741; with *hda1::kanMX4*	HDAC	L. Meira
sin3Δ	BY4741; with *sin3::kanMX4*	HDAC	L. Meira
sir2Δ	BY4741; with *sir2::kanMX4*	HDAC	L. Meira
hos1Δ	BY4741; with *hos1::kanMX4*	HDAC	L. Meira
hos2Δ	BY4741; with *hos2::kanMX4*	HDAC	L. Meira
hos3Δ	BY4741; with *hos3::kanMX4*	HDAC	L. Meira
hst1Δ	BY4741; with *hst1::kanMX4*	HDAC	L. Meira
hst2Δ	BY4741; with *hst2::kanMX4*	HDAC	L. Meira
hst3Δ	BY4741; with *hst3::kanMX4*	HDAC	L. Meira
hst4Δ	BY4741; with *hst4::kanMX4*	HDAC	L. Meira
rpd3Δ	BY4741; with *rpd3::kanMX4*	HDAC	L. Meira
dot1Δ	BY4741; with *dot1::kanMX4*	HML	L. Meira
erg6Δ	BY4741; with *erg6::kanMX4*	HMT	L. Meira
htz1Δ	BY4741; with *htz1::kanMX4*	HTZ histone	R. Matuo
BY4742 (WT)	*MATα his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*	-	Euroscarf
rad1Δ	BY4742; *rad1::kanMX4*	NER	Euroscarf
rad6Δ	BY4742; *rad6::kanMX4*	PRR	Euroscarf
rad18Δ	BY4742; *rad18::kanMX4*	PRR	Euroscarf
rad30Δ	BY4742; *rad30::kanMX4*	error-free bypass replication repair	Euroscarf
rad50Δ	BY4742; *rad50::kanMX4*	NHEJ	Euroscarf
rad52Δ	BY4742; with *rad52::kanMX4*	HR	Euroscarf
mlh1Δ	BY4742; *mlh1::kanMX4*	MMR	Euroscarf
rev3Δ	BY4742; with *rev3::kanMX4*	TLS	Euroscarf
ogg1Δ	BY4742; *ogg1::kanMX4*	BER	Euroscarf

CR: Chromatin remodeling; HMG: High-mobility group; HAT: Histone acetyltransferase; HDAC: Histone deacetylase; HML: Histone methylase; HMT: Histone methyltransferase; NER: Nucleotide excision repair; PRR: Post-replication repair; NHEJ: Non-homologous end joining; HR: Homologous recombination; MMR: Mismatch repair; TLS: Translesion sinthesis repair; BER: Base excision repair

Figure 1. Drop tests with *S. cerevisiae* yeast strains deficient in proteins from different DNA repair pathways. Cells in exponential growth phase were treated with CDDP, 5-FU and CDDP + 5-FU, during 4:00 h. After serial dilution, cells were seemed on YPD plates.

Figure 2. Drop tests with *S. cerevisiae* yeast strains deficient in proteins involved in chromatin remodeling. Cells in exponential growth phase were treated with CDDP, 5-FU and CDDP + 5-FU, during 20:00 h. After serial dilution, cells were seemed on YPD plates.

DISCUSSÃO GERAL

Muitas drogas atualmente empregadas em quimioterapia, embora de eficácia clínica comprovada, não tem seus mecanismos de ação completamente compreendidos. Além disso, o uso de combinações de antineoplásicos com diferentes efeitos biológicos é uma prática já bem estabelecida, de modo que a elucidação dos processos desencadeados por essas drogas é imprescindível, tanto para o aperfeiçoamento dos protocolos terapêuticos já existentes, como para o desenvolvimento de novos fármacos. De fato, a resistência celular a agentes genotóxicos tem sido associada com o aumento da atividade de enzimas que atuam em vias de reparação de DNA, enquanto defeitos nestas vias induzem à hipersensibilidade a estas drogas (Damia and D'Incalci, 2007).

Diversos estudos demonstraram que o remodelamento da cromatina pode modular os mecanismos de reparação de DNA, além de regular a ativação transcricional em células eucarióticas (Dinant *et al*., 2008; Kouzarides, 2007; Sarkar *et al*., 2010; Morrison and Shen, 2009; Czaja *et al*., 2010; Luijsterburg and Van Attikum, 2011; Parthun, 2012; Thomas and Stott, 2012). Até o momento, acredita-se que um determinado conjunto de modificações de histonas pode determinar a ativação de uma via de reparação de DSB ao invés de outra. Contudo, o modo como as células escolhem determinadas vias ainda não está claro, e parece que ao invés da criação de um código específico de histonas nas proximidades do DNA danificado, a escolha da via de reparação depende dos fatores envolvidos na própria maquinaria de reparação (Escargueil *et al*., 2008; You and Bailis, 2010; Symington and Gautier, 2011). Atualmente persiste a idéia de que as modificações de histonas são imprescindíveis para a sobrevivência celular em resposta ao dano ao DNA, por meio da indução de paradas de ciclo celular (*checkpoints*) e reparação de DNA. Contudo, muitos aspectos da cascata de eventos que ocorre em resposta a tipos específicos de dano ao DNA ainda permanecem obscuros.

Desse modo, buscando avaliar a interação direta entre genes envolvidos nos processos de remodelamento de cromatina e reparação de DNA em resposta a diferentes tratamentos genotóxicos, foram construídos duplos mutantes de *S. cerevisiae* deficientes em Swr1p (CR) e outras proteínas relacionadas ao remodelamento da cromatina (*swr1Δgcn5Δ, swr1Δdot1Δ, swr1Δhtz1Δ*), além de proteínas envolvidas em vias distintas de reparação de DNA (*swr1Δrad52Δ, swr1Δyku80Δ, swr1Δpso2Δ*) por disrupção gênica. Os resultados obtidos são apresentados no Capítulo II desta Tese, e mostraram que os mutantes *pso2Δ* e *swr1Δpso2Δ* apresentaram elevada sensibilidade ao

tratamento com DTDF, e esta sensibilidade foi similar à observada após o tratamento destes mutantes com 8-MOP + UVA.

A proteína Pso2p faz parte da família metallo-β-lactamase de proteínas, com atividade de 5'-exonuclease e participa da reparação de DSBs induzidas por agentes como cisplatina, mustarda nitrogenada ou psoralenos fotoativados (Cattell *et al*., 2010). Pso2p é conhecida por seu papel na reparação de ICLs induzids por psoralenos fotoativados, e atualmente também vem sendo referido por sua atuação na resolução de estruturas de DNA do tipo *hairpins* (Tiefenbach and Junop, 2012).

O capítulo I desta Tese apresenta uma revisão dos principais mecanismos que controlam as relações entre o remodelamento da cromatina dependente de ATP e proteínas envolvidas na reparação de danos ao DNA, mediante lesões genotóxicas induzidas pela fotoadição com 8-MOP em leveduras. Assim, durante o reparo dos ICLs resultantes do tratamento com 8-MOP + UVA, DSBs são transitoriamente formadas, induzindo a fosforilação da histona H2AX, pela ação de ATM, ATR e DNA-PK, em células de mamíferos, ou Tel1p e Mec1p, em leveduras (revisado em Cruz *et al*., 2012) nos arredores das DSBs. Este processo resulta no recrutamento de diversos complexos de remodelamento da cromatina ao local da lesão, com acúmulo de proteínas de reparação de danos ao DNA em *foci* subnuclear. Além disso, o tratamento com 8-MOP + UVA induz a repressão de alguns genes relacionados ao remodelamento da cromatina, acompanhado pela indução da expressão de genes relacionados ao controle do ciclo celular e reparação de DNA. Este perfil transcricional é compatível com a necessidade de alterações na estrutura da cromatina, possibilitando a reparação do dano (revisado em Cruz *et al*., 2012).

Conforme mencionado anteriormente, a reparação das lesões genotóxicas causadas pelo tratamento com 8-MOP + UVA em *S. cerevisiae* implica na formação transitória de DSBs, e tanto a via NER como a via dependente de Pso2p/Msh2p/Exo1p são necessárias para o processamento de ICLs na fase S do ciclo celular, antes da reparação da DSBs (revisado em Cruz *et al*., 2012). Pso2p pode ser direcionada para os ICLs incisados, a fim de efetuar a ressecção de estruturas intermediárias do processo de reparação de DNA, fornecendo substratos para a reparação por TLS, na fase G1 do ciclo celular ou por HR. Nas fases S e G2, a Pso2p apresenta um papel de sobreposição com a exonuclease Exo1p durante a reparação de forquilhas de replicação colapsadas pela via HR. Desse modo, a ICL formada por 8-MOP + UVA consiste em uma lesão complexa, e os dados existentes mostram a participação das vias NER e TLS, além de vias de

reparação de DSBs, como HR and NHEJ, no processamento e reparação destas ICLs (revisado em Cruz *et al*., 2012).

Um estudo anterior do nosso laboratório avaliou a citotoxicidade e as propriedades mutagênicas do DTDF, um potencial protótipo para o desenvolvimento de novas drogas antitumorais, e mostrou que o tratamento com este composto causa alterações no balanço redox celular (pela depleção de glutationa), resultando na indução de estresse oxidativo e dano ao DNA, em leveduras e células de mamíferos (Degrandi *et al*., 2010). O mesmo trabalho também revelou que o tratamento com DTDF induz a formação de DSBs e micronúcleos em células de mamíferos, bem como a formação de mutações *frameshift* em leveduras e nas cepas TA97 e TA98 de *Salmonella typhimurium*. Os autores demonstraram as propriedades mutagênicas do DTDF, e o envolvimento de proteínas pertencentes às vias BER, HR e NHEJ, mas não a via NER, na reparação das lesões induzidas por este composto. Além disso, Sailer *et al*. (2004) relataram a indução de apoptose (especificamente nas fases S e G2/M do ciclo celular) por DTDF, em células pró-mielocíticas HL60. Essa observação dá suporte à idéia de que o DTDF talvez possa causar lesões genotóxicas que bloqueiam a replicação. Nesse caso, a restauração da forquilha de replicação colapsada pode ocorrer por uma via associada à recombinação, ou por TLS mutagênica.

Degrandi *et al*. (2010) demonstraram que as cepas mutantes de *S. cerevisiae rad6*Δ e *rev3*Δ (deficientes na reparação pós-replicativa e na via TLS, respectivamente), não apresentam sensibilidade a DTDF, sugerindo que estas vias, isoladamente, não sejam importantes para a reparação dos danos induzidos por esta substância. Por outro lado, a grande sensibilidade do mutante *rad52*Δ após o tratamento com DTDF evidencia o papel crítico da via HR no processamento dessas lesões (Degrandi *et al*., 2010). Estruturas intermediárias do processo de reparação podem bloquear as forquilhas de replicação, resultando na formação de DSBs, que são substratos para a via HR. As nucleases Mre11p and Pso2p atuam na via HR e em vias de reparação de ICLs, respectivamente, enquanto a nuclease Exo1p está envolvida nas vias HR e MMR. Contudo, a eliminação das três nucleases confere ao triplo mutante uma sensibilidade a radiação ionizante (IR) superior à observada nos simples ou duplos mutantes, indicando o elevado grau de redundância e versatilidade em resposta a danos ao DNA (Lam *et al*., 2008).

A análise do conjunto dos resultados apresentados no Capítulo II desta tese, associada aos dados da literatura, leva à criação de um modelo (Figura 15) no qual, caso

o dano genotóxico ocorra durante a replicação celular, as ICLs/ DSBs *hairpin capped* são processadas, principalmente, por Pso2p em leveduras, ou por Exo1p-Msh2p or Mre11p/MRX(N) (revisado por Cattell *et al*., 2010; Tiefenbach and Junop, 2012). Desse modo, Pso2p pode fornecer substrato para a via NHEJ canônica (dependente de Yku70/Yku80), mas esta via não está ativa durante a fase S do ciclo celular (Wu *et al*., 2008; Mladenov and Iliakis, 2011). Assim, o substrato gerado por Pso2p também pode ser direcionado para a via HR (principalmente durante a fase S) ou MMEJ (também conhecida como NHEJ alternativa, independente de Yku70/Yku80). Além disso, tanto Exo1p como MRX podem fornecer substrato para HR, e na ausência desta via, o substrato pode ser direcionado para MMEJ. Esta idéia encontra embasamento no fato de que o duplo mutante *pso2Δmre11Δ* apresenta acentuada sensibilidade ao tratamento com 8-MOP/UVA e mustarda nitrogenada, quando comparado ao tipo selvagem (Munari *et al*., dados não publicados). Por outro lado, as ICLs formadas durante a fase G1 do ciclo celular são processadas pela via NER, a qual fornece substrato para Pso2p e para a via TLS. Pso2p, por sua vez, processa a lesão e fornece substrato para as vias NHEJ canônica, MMEJ e TLS, uma vez que HR encontra-se inativa durante a fase G1 do ciclo celular. A maquinaria da via NER também pode direcionar estruturas intermediárias para MRX, que fornece substrato para a via MMEJ, durante a fase G1 do ciclo celular.

Os psoralenos fotoativados são capazes de formar ICLs e DSBs *hairpin capped*, sendo que os mutantes *pso2Δ*, além dos mutantes deficientes em MRX e nas vias NER, TLS e HR, exibem grande sensibilidade ao tratamento com psoralenos fotoativados. Nós acreditamos que o tratamento com DTDF, por sua vez, possivelmente forma apenas DSBs *hairpin capped*, ou outra lesão capaz de bloquear a forquilha de replicação, pois além de *pso2Δ*, mutantes deficientes na via HR e em MRX são sensíveis a este composto. Assim, o fato de o mutante *yku80Δ* mostrar resistência ao tratamento com DTDF pode estar relacionado com o fato que o composto induz lesões citotóxicas durante a fase S do ciclo celular, quando a via NHEJ canônica é menos utilizada pela célula. Nesse contexto, o mutante *swr1Δ* também se mostrou pouco sensível aos tratamentos empregados, uma vez que esta cepa é deficiente em NHEJ canônica, livre de erros, embora nesses mutantes a associação com Mre11p (necessária para as vias HR e MMEJ) não seja afetada (Van Attikum *et al.*, 2007).

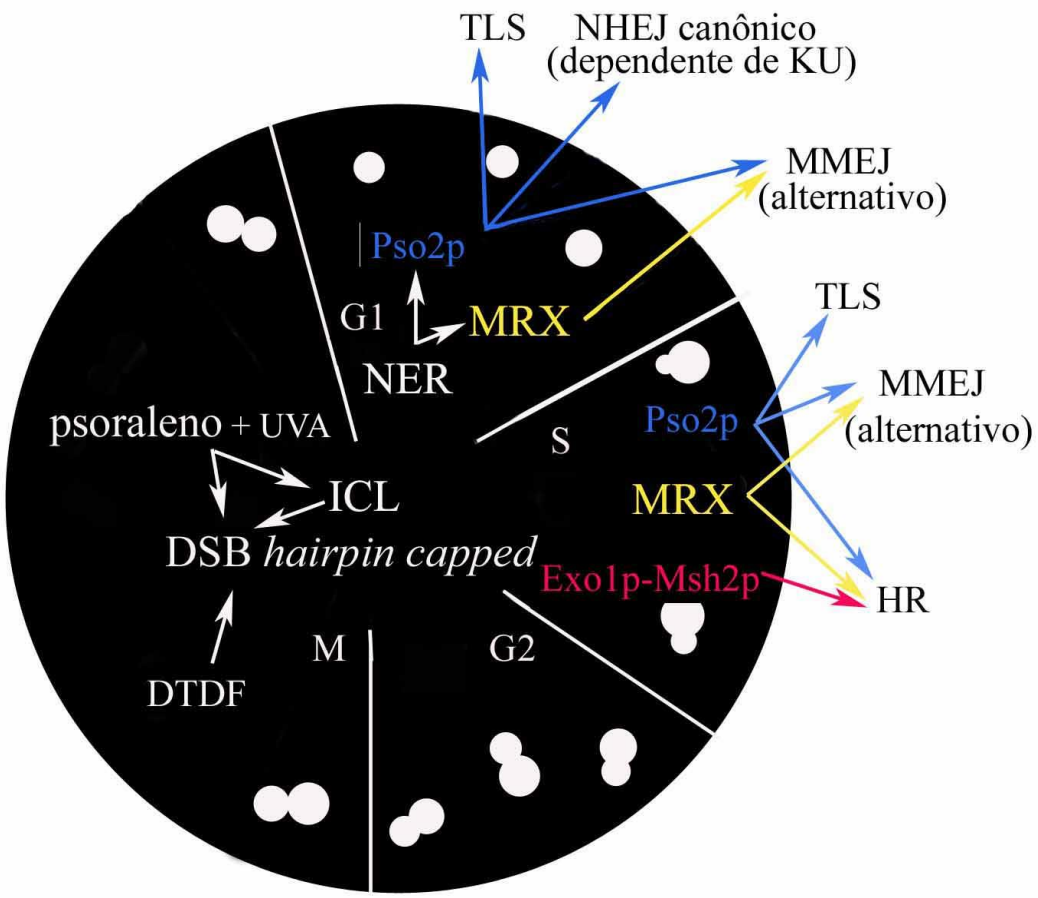

Figura 15. Modelo proposto para o papel de Pso2p na reparação de danos ao DNA induzidos por psoralenos fotoativados e DTDF . Psoralenos fotoativados formam ICLs e DSBs *hairpin capped*, e DTDF possivelmente forma DSBs *hairpin capped* ou outra lesão capaz de bloquear a forquilha de replicação. Durante a fase G1 do ciclo celular, as ICLs são resolvidas pela via NER, que fornece substrato para Pso2p e TLS. Pso2p, por sua vez, processa a lesão e fornece substrato para NHEJ canônica, MMEJ e TLS. Durante a replicação, ICLs e DSBs *hairpin capped* são resolvidas por Pso2p, Exo1p-Msh2p ou Mre11/MRX(N). Destes, Pso2p é o único que pode fornecer substrato para NHEJ canônico, ou este pode também ser direcionado para TLS, HR ou MMEJ.

O Capítulo III desta tese avalia os efeitos do tratamento combinado de dois quimioterápicos clássicos, o 5-fluorouracil (5-FU) e a cisplatina (CDDP), utilizados no tratamento de diversos tipos de câncer (ver Introdução, itens 1.2.1 e 1.2.2), quanto às vias de reparação de DNA e remodelamento de cromatina, em *S. cerevisiae*. O

tratamento com 5-FU ou CDDP isoladamente mostra efeitos significativos no tratamento de tumores gástricos e câncer de cólon, além de tumores testiculares, câncer de pulmão e tumores de cabeça e pescoço (Takara *et al*., 2012; Ohtsu 2008; Hata *et al*., 2008). A terapia utilizando a combinação das duas drogas produz efeitos clínicos sinergísticos no tratamento de câncer gastrointestinal, quando comparado aos efeitos individuais das drogas (Takara *et al*., 2012), sendo também empregada no tratamento do carcinoma metastático peniano (Lorenzo *et al*., 2012).

Os efeitos biológicos da cisplatina são atribuídos à sua capacidade de formar adutos com a molécula de DNA, principalmente pontes intercadeias, além de ligações cruzadas entre guaninas adjacentes ou entre guanina e adenina, provocando alterações estruturais no dúplex de DNA. Estas alterações são capazes de bloquear a replicação e a transcrição, e desencadeiam a ativação de *checkpoint* de ciclo celular. Caso não reparadas, podem levar à morte da célula (Tompkins, Wu, and Her, 2012). No caso do 5-FU, a citotoxicidade da droga requer a sua conversão a fluoronucleotídeos, os quais são erroneamente incorporados ao DNA e ao RNA. Além disso, o 5-FU também pode ser convertido ao seu metabólito ativo, o FdUMP (por ação da enzima timidina-cinase, ausente na levedura *S. cerevisiae*, nosso modelo experimental), que inibe a enzima timidilato sintetase, resultando em redução dos níveis de dTMP e aumento de dUMP (Seiple *et al*., 2006; Berger, Pittman, and Wyatt, 2008; Matuo *et al*., 2009; Matuo *et al*., 2010).

De fato, a combinação das duas drogas (5-FU + cisplatina) parece ser mais eficiente na indução de morte celular em comparação com a sua aplicação individual. Os nossos resultados indicam a inexistência de interações entre as vias envolvidas na reparação dos danos genotóxicos induzidos por estas drogas, dentro de cada linhagem específica, uma vez que a sensibilidade das linhagens aos fármacos utilizados parece refletir apenas seus efeitos individuais, sem aditividade ou sinergismo. Este achado sugere que o melhor efeito antitumoral do tratamento combinado é devido à ação de cada um dos agentes sobre clones diferentes das populações heterogêneas de células transformadas.

No que se refere ao remodelamento da cromatina, a investigação da participação do código de histonas na resposta ao dano ao DNA induzido pelos tratamentos com CDDP, 5-FU e sua combinação demonstraram que as células respondem de forma bastante específica aos diferentes tipos de danos induzidos pelos tratamentos. Sendo assim, a maior sensibilidade foi observada em mutantes deficientes em HAT, além do

mutante *hos3Δ* (HDAC), para o tratamento com cisplatina, enquanto o tratamento com 5-FU induziu acentuada sensibilidade em linhagens deficientes em Gcn5p (HAT), além de Sin3p e Hos3p (HDACs). O tratamento combinado (5-FU + CDDP) mostrou elevada toxicidade para todas as linhagens estudadas, inclusive a selvagem, com maior sensibilidade para CR (*ino80Δ*), HATs (*elp3Δ, gcn5Δ, e hat2Δ hpa2Δ*), HDACs (*sin3Δ, sir2Δ, hos2Δ, hst1Δ, hst2Δ, hst3Δ*), HML (*dot1Δ*) and HMT (*erg6Δ*). Estes resultados demonstram que o tratamento combinado de 5-FU + CDDP de fato amplia o painel de remodeladores de cromatina envolvidos na citotoxicidade das drogas, quando estas são empregadas em associação.

Recentemente, a citotoxicidade da cisplatina vem sendo relacionada ao complexo SWI / SNF, que facilitaria a formação de adutos platina-DNA por meio do relaxamento da estrutura da cromatina (Kothandapani and Gopalakrishnan, 2012). Nossos resultados demonstraram que as linhagens de *S. cerevisiae* deficientes em HATs e na HDAC Hos3p foram mais sensíveis ao tratamento com CDDP. Coletivamente, as HATs regulam a transcrição, e algumas HATs são super expressadas em células tumorais humanas resistentes a cisplatina, como Clock (que regula a biossíntese de glutationa) e Tip60 (que modula a expressão de genes de reparação de DNA) (Hirano *et al*., 2010), e a inibição de HATs promove paradas na síntese de DNA, atraso na progressão da fase S do ciclo celular e inibe vias de reparação de DSBs e também a via NER (Bandyopadhyay and Banères, 2009).

O tratamento com 75 μM de 5-FU, por sua vez, induziu grande sensibilidade em linhagens deficientes em HAT (*gcn5Δ*) e HDACs (*sin3Δ* e *hos3Δ*, este último também sensível a CDDP). Sabe-se que o gene *GCN5* está relacionado à regulação da expressão gênica e ao controle da longevidade em leveduras (Kim *et al*., 2004), e a acetilação de H3 por Gcn5p regula a montagem dos nucleossomas por um mecanismo que envolve a associação de H3 a CAF-1 (Dekker and Haisma, 2009; Eitoku *et al*., 2008). Além disso, a acetilação de H3 por Gcn5p facilita a reparação de danos ao DNA por promover o remodelamento da cromatina, e o gene GCN5 atua na via NER, em células humanas (S. Yu *et al*., 2011). Sin3p (membro do complexo Sin3p/rpd3p), também sensível ao tratamento com 5-FU, atua na ativação da meiose, troca de *mating-type* e repressão transcricional, e sua ausência causa defeitos nas vias NHEJ e HR, uma vez que a desacetilação de H4K16ac por Sin3p/Rpd3p é importante para a reparação de DNA por essas vias (revisado por Escargueil *et al*., 2008). Adicionalmente, na ausência das

HDACs Hos3p e Hos2p, a HAT Gcn5p aumenta a acetilação de H3 (Gunderson and Merkhoferb, 2011).

O tratamento combinado de CDDP + 5-FU mostrou-se extremamente citotóxico para todas as linhagens estudadas, incluindo a selvagem, com destaque para CR (*ino80Δ*), HATs (*elp3Δ, gcn5Δ, hat2Δ* e *hpa2Δ*), HDACs (*sin3Δ, sir2Δ, hos2Δ , hst1Δ, hst2Δ,* e *hst3Δ*), HML (*dot1Δ*) e HMT (*erg6Δ*). Ino80p, membro do complexo INO80, opera na mesma via do NER, e contribui para a remoção de fotoprodutos UV em regiões de alta incidência de nucleossomas, podendo também influenciar a via NHEJ (revisado por Cruz *et al*., 2012). Elp3p, subunidade do complexo elongador, o qual controla diversos processos biológicos, como exocitose e resistência a choque térmico (em leveduras), além de migração celular e diferenciação neuronal (em eucariontes superiores), também controla migração celular, invasão tecidual e tumorigênese de células de melanoma (Close *et al*., 2012). Hat2p (subunidade do complexo citoplasmático Hat1p-Hat2p, que atua no silenciamento telomérico, e também do complexo nuclear NuB4). Mutantes *hat1*Δ mostram defeitos no reparo recombinacional (revisado em Ge *et al*., 2011). Hpa2p é uma HAT tetramérica com similaridade com Gcn5p, Hat1p, Elp3p e Hpa3p, com atividade de autoacetilação, também acetila as histonas H3 e H4, *in vitro* (Vernarecci *et al*., 2010).

Muitos protocolos terapêuticos atualmente empregados no combate ao câncer fazem uso de inibidores de HDACs em adição ao tratamento combinado de 5-FU + CDDP, bem como outros protocolos de associação de drogas com diferentes mecanismos de ação (Johanne *et al*., 2011; Ohtsu, 2008; Batty *et al*., 2009; Luchenko *et al*., 2011). A inibição de HDACs torna o DNA menos compactado e portanto, mais sensível aos danos induzidos pela cisplatina, por facilitar o estabelecimento dos adutos cisplatina-DNA. Este fato se confirma pelo aumento na acetilação das histonas H3 e H4, utilizadas como marcadores de relaxamento da estrutura da cromatina (Tesei and Brigliadori, 2012). Além disso, a expressão de diferentes famílias de HDACs em tecidos tumorais é *up*-regulada, e está relacionada com a resposta clínica ao tratamento quimioterápico (Witt *et al*., 2009), de modo que as HDACs são atualmente consideradas as enzimas mais importantes como alvos de drogas anticancer (Tesei and Brigliadori, 2012).

Conforme anteriormente mencionado, os mutantes deficientes em HDACs *sin3Δ, sir2Δ, hos2Δ, hst1Δ, hst2Δ,* e *hst3Δ* mostraram-se sensíveis ao tratamento com CDDP + 5-FU. Sin3p é componente do complexo Sin3p-Rpd3p, e células deficientes

em Sin3p apresentam defeitos na via NHEJ e no *turnover* do grupo acetil de H4, e é importante para a eficiência da reparação de DSBs, também cooperando na promoção da transcrição (van Attikum and Gasser, 2005). Sir2p tem homologia com membros de HDACs de classe III (humanas), também conhecidas como sirtuinas, cuja atividade está relacionada a doenças neurológicas e metabólicas, além da sua associação com a patologia do HIV e câncer (Lawson *et al*., 2010). Sir2p de leveduras também afetam a função do polarissoma, no transporte retrógrado de proteínas danificadas da célula-filha (broto) para a célula-mãe (Gan and Tang, 2010). Sirtuinas humanas atuam no controle do metabolismo celular, repressão transcricional, envelhecimento, respostas de dano ao DNA e apoptose (Greiss and Gartner, 2009). Hos2p, subunidade dos complexos Set3 e Rpd3L, desacetila H3 e H4, e co-localiza-se com Cmr1p no foci nuclear em resposta a danos ao DNA por metil metanosulfonato (MMS) (Tkach *et al*., 2012). Hst1p (subunidade do complexo Sum1p/Rfm1p/Hst1p) atua no silenciamento transcricional dependente de ORC e na repressão da mitose, estando também envolvido na manutenção telomérica (Askree *et al*., 2004). Hst2p modula o silenciamento nuclear e telomérico, e Hst3p atua no metabolismo de ácidos graxos de cadeia curta, na progressão do ciclo cellular, na estabilidade genômica, no silenciamento telomérico e na resistência a radiação (Starai and Takahashi, 2003).

Os mutantes *dot1Δ* e *erg6Δ*, envolvidos na metilação e desmetilação de histonas, respectivamente, também foram muito sensíveis ao tratamento com CDDP + 5-FU, o que não ocorreu no tratamento com as drogas individualmente. Dot1p atua na metilação de H3 na lisina 79, necessária para os *checkpoints* de dano ao DNA nas fases G1/S e intra-S, em células vegetativas, e também para o *checkpoint* de recombinação meiótica, além do silenciamento da cromatina (revisado em Conde and San-Segundo, 2008).Foi proposto que Dot1p seria capaz de inibir a via TLS por Polz/Rev1, e a metilação de H3K79 por Dot1p permite a transmissão do sinal de dano a cinases de *checkpoint*, por um mecanismo dependente de Rad9p, controlando a resecção do DNA danificado (Conde and San-Segundo, 2008). Além disso, a deleção de Dot1p causa aumento na sensibilidade a radiação UV, e a metilação de H3K79 por Dot1p tem papel nas vias NER, PRR and HR (Bostelman *et al*., 2007). Erg6p, por sua vez, é uma importante enzima envolvida na biossíntese de ergosterol em *S. cerevisiae*, atuando na manutenção da estabilidade das membranas (Zahedi *et al*., 2006).

Assim, as diferenças observadas na sensibilidade aos tratamentos com CDDP, 5-FU e CDDP + 5-FU nos mutantes deficientes em diferentes HDACs talvez explique o

sinergismo clínico existente no tratamento combinado de CDDP + 5-FU, uma vez que as vias envolvidas na reparação dos danos promovidos pelo emprego individual das drogas estudadas não demonstra este efeito. Desse modo, os nossos resultados apontam para os remodeladores de cromatina envolvidos nos processos de acetilação e desacetilação de histonas como alvos terapêuticos importantes na resposta a agentes genotóxicos, e indica uma possível interação destas drogas não apenas com a molécula de DNA, mas também com os remodeladores de cromatina dependentes de ATP. Estes achados, portanto, fornecem novos *insights* para a compreensão dos mecanismos envolvidos na resposta ao tratamento com CDDP + 5-FU, bem como na escolha de protocolos terapêuticos que considerem o efeito de drogas antitumorais na cromatina, embora muitos estudos ainda sejam necessários para a compreensão destas interações.

CONCLUSÕES

1. Conclusão geral

A presente Tese avaliou as relações entre reparação de danos ao DNA e remodelamento de cromatina dependente de ATP, em resposta a agentes antineoplásicos com diferentes mecanismos de ação, em *Saccharomyces cerevisiae*. Inicialmente, investigamos os efeitos do Ditelureto de difenila (DTDF), e os nossos resultados revelaram que a via de reparação de ICLs mediada por Pso2p é de extrema importância no reparo dos danos genotóxicos causados por esse agente. Além disso, os efeitos do tratamento combinado de cisplatina + 5-fluorouracil também foram avaliados, e os resultados demonstraram que a elevada citotoxicidade observada nessa associação de drogas está mais estritamente relacionada a modificações epigenéticas do que a maquinaria de reparação de DNA *per se*.

1.1. Conclusões específicas

- As lesões genotóxicas causadas pelo tratamento com DTDF resultam em extrema sensibilidade por parte de linhagens de *S. cerevisiae* deficientes em Pso2p.

- O tratamento com DTDF parece causar DSBs *hairpin capped*, ou outras lesões capazes de bloquear a forquilha de replicação;

- Pso2p é o único fator capaz de fornecer substrato para a via NHEJ canônica, o qual também podendo ser direcionado para TLS, HR ou MMEJ, durante o processamento dos danos por DTDF.

- O tratamento de *S. cerevisiae* com CDDP + 5-FU causa uma resposta mais efetiva na indução de morte celular, em comparação ao uso individual de cada droga;

- A maior citotoxicidade do tratamento combinado de CDDP + 5-FU não está relacionada à maquinaria de reparação de DNA;

- Enzimas envolvidas em modificações pós-traducionais das caudas das histonas são diferencialmente afetadas pelos tratamentos com CDDP, 5-FU e a combinação das duas drogas em *S. cerevisiae;*

- O tratamento com CDDP causa sensibilidade em mutantes deficientes em diferentes HATs, além do mutante deficiente na HDAC Hos3p;

- 5-FU, na concentração de 75 µM, causa maior sensibilidade em HAT (*gcn5Δ*) e HDACs (*sin3Δ* e *hos3Δ*);

- O tratamento combinado de CDDP + 5-FU, causa elevada citotoxicidade em todos os mutantes estudados, incluindo o tipo selvagem, com destaque para mutantes deficientes em CR (*ino80Δ*), HATs (*elp3Δ, gcn5Δ, hat2Δ* e *hpa2Δ*), HDACs (*sin3Δ, sir2Δ, hos2Δ , hst1Δ, hst2Δ,* e *hst3Δ*), HML (*dot1Δ*) e HMT (*erg6Δ*).

PERSPECTIVAS

Visando ampliar os conhecimentos acerca das relações entre remodelamento de cromatina e reparação de danos ao DNA em resposta a agentes genotóxicos antineoplásicos clássicos, bem como DTDF, os resultados obtidos nesse estudo apresentam como perspectivas:

- Avançar no esclarecimento do papel da proteína Pso2p na reparação de danos genotóxicos, por meio da construção de duplos e triplos mutantes de leveduras deficientes em Pso2p e proteínas de reparação de DNA e remodelamento da cromatina;

- Avaliar a citotoxicidade do tratamento combinado de CDDP + 5-FU, quanto ao remodelamento da cromatina e reparação de danos ao DNA, utilizando as linhagens de duplos mutantes de *S. cerevisiae swr1Δrad52Δ, swr1Δyku80Δ, swr1Δhtz1Δ, swr1Δpso2Δ, swr1Δino80Δ, swr1Δerg6Δ, swr1Δdot1Δ, swr1Δhat1Δ*.

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