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Funções da proteína Pso9/Mec3 no controle do ciclo celular e reparação de DNA em *Saccharomyces cerevisiae*

Tese de Doutorado

Jacqueline Moraes Cardone

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Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do grau de Doutor em Ciências.

Orientador: Prof. Dr. João Antonio Pêgas Henriques

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(...)

Sonharei com o poente azul e com seus astros,
Repuxos no jardim chorando entre alabastros,
Beijos como canções desde a manhã à tarde,
Tudo o que de infantil o Idílio ainda guarde.
O Alvoroço lá fora em tempestade cresça
Mas eu nunca erguerei da carteira a cabeça;
Mergulhado serei nesta sensualidade
Do mês de abril chamar só com minha vontade,
De um sol todo extrair de minha alma que espera
Mudar meu peito ardente em tépida atmosfera.

(*Paysage, Charles Baudelaire*)

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SUMÁRIO

LISTA DE ABREVIATURAS

3-CPS	3-carbetoxipsoraleno
4-NQO	4-hidroxiquinoleína
8-MOP	8-metoxipsoraleno
8oxoG	8- oxoguanina
ATP	adenosina trifosfato
BER	*base excision repair* (reparação por excisão de bases)
CPD	*cyclobutane pyrimidine dimer* (dímero de pirimidina)
DSBs	*double strand breaks* (quebras duplas da fita de DNA)
DSBR	*double strand break repair* (reparação de quebras duplas da fita de DNA)
EMS	etil-metanosulfonato
EROs	espécies reativas de oxigênio
HN2	mostarda nitrogenada bifuncional
HR	*homologous recombination* (recombinação homóloga)
ICLs	*interstrand cross-links* (pontes intercadeias)
MMR	*mismatch repair* (reparação de erros de emparelhamento de bases)
MNNG	*N*-metil-*N'*-nitro-*N*-nitrosoguanidina
NER	*nucleotide excision repair* (reparação por excisão de nucleotídeos)
NHEJ	*non-homologous end joining* (recombinação não-homóloga)
PCNA	*proliferating cell nuclear antigen* (antígeno nuclear de

	proliferação celular)
PHR	*photoreactivation repair* (reparação por fotorreativação)
Pol	*DNA polymerase* (DNA polimerase)
PRR	*post-replication repair* (reparação pós-replicativa)
PUVA	psoralenos + UVA
RFC	*replication factor C* (fator de replicação C)
RPA	*replication protein A* (proteína de replicação A)
SDSA	*synthesis-dependent strand annealing* (síntese dependente de anelamento de fita de DNA)
SSBs	*single strand breaks* (quebras simples da fita de DNA)
SUMO	*small ubiquitin-like modifier* (conjugador de proteínas similares a ubiquitina)
Tg	timina glicol
TLS	*translesion synthesis* (síntese translesão)
UV	luz ultravioleta
UVA	luz ultravioleta de 365 nm
UVC	luz ultravioleta de 254 nm

RESUMO

A manutenção da estabilidade do material hereditário das células requer fidelidade na replicação do DNA, precisão na segregação dos cromossomos e a capacidade de evitar mutações herdáveis, causadas por injúrias espontâneas e induzidas no genoma. Para enfrentar estes desafios, as células possuem processos evolutivamente conservados, incluindo reparação do DNA e mecanismos de *checkpoint*. Falhas no cumprimento destes mecanismos podem resultar em morte celular, no acúmulo de mutações herdáveis e na indução de instabilidade genômica, a qual é uma característica predominante de células tumorais.

Em *Saccharomyces cerevisiae*, a visão mais recente dos estágios iniciais da resposta de *checkpoint* indica que dois complexos de proteínas, Mec1-Ddc2 e Rad17-Mec3-Ddc1, são recrutados à região de lesão no DNA. Rad17p, Mec3p, e Ddc1p (os homólogos humanos são Rad1, Hus1, e Rad9, respectivamente) formam um complexo heterotrimérico estruturalmente relacionado ao antígeno nuclear de proliferação celular (PCNA), o fator de processividade das DNA polimerases δ e ε. Recentemente, identificamos um alelo mutante de *MEC3 – pso9-1* – o qual é fortemente sensível a vários agentes indutores de danos no DNA e apresenta baixa mutabilidade induzida.

Para prosseguir nas investigações dos efeitos da proteína mutante Pso9-1, a seqüência completa do alelo mutante foi determinada. A comparação das seqüências obtidas com a seqüência tipo-selvagem depositada nos bancos de dados revelou a deleção de um único nucleotídeo, na posição 802 (802delA), a qual leva a uma forma truncada de Pso9p. O alelo mutante *pso9-1*[802delA] codifica, um polipeptídeo de 276 aminoácidos, no qual os últimos nove aminoácidos estão fora da fase leitura apropriada e seu domínio carboxi-terminal, drasticamente diminuído. Esta mutação afetou as

propriedades de ligação de Pso9-1p, impedindo as interações com seus parceiros de complexo Rad17p e Ddc1p. Ensaios adicionais de interação empregando construções de *mec3* que não continham os últimos 25 e 75 aminoácidos do domínio carboxi-terminal também não foram capazes de manter interações estáveis. Além disso, a linhagem mutante *pso9-1* perdeu a capacidade de detectar danos no DNA, uma vez que dava continuidade no ciclo-celular, após tratamento com 8-metoxipsoraleno fotoativado.

Uma vez analisada a reposta a danos no DNA de mutantes deficientes no componente de PCNA-*like*, Pso9p/Mec3p, em combinação à inativação de subunidades de DNA polimerases replicativas (Pols α, δ and ε), foi encontrada uma interação aditiva entre *PSO9/MEC3* e *POL32*, o qual codifica a terceira subunidade da DNA polimerase δ. A hipersensibilidade de *pol32*Δ a 8-MOP fotoativado, revelada por estas análises genéticas, sugeriu fortemente a participação ainda não descrita de uma polimerase replicativa na reparação de pontes intercadeias. Esta hipótese foi confirmada, já que o mutante *pol32*Δ também se mostrou extremamente deficiente em ligar extremidades de DNA não-coesivas, particularmente em casos de extremidades protuberantes 5', sugerindo um papel para a DNA polimerase δ em recombinação não-homóloga (NHEJ).

A coleção de dados apresentada neste trabalho reafirma a importância de proteínas como Pso9p/Mec3p que iniciam a sinalização de anormalidades no DNA. Além disso, amplia a rede de proteínas que responde a danos do tipo pontes intercadeias, mostrando que uma proteína em particular, como visto para Pol32p, pode ter tarefas adicionais como mecanismo de segurança, a fim de garantir a estabilidade genômica.

ABSTRACT

Maintenance of stability of hereditary material of cells requires fidelity in DNA replication, precision in chromosome segregation, and the ability to avoid heritable mutations caused by spontaneous and induced genomic insults. To cope with these challenges cells have evolved evolutionarily conserved processes, including DNA repair and checkpoint mechanisms. Failure to enforce these mechanisms can result in cell death or in accumulation of heritable mutations and the induction of genome instability, which is a predominant characteristic of cancer cells.

In *Saccharomyces cerevisiae*, it is the current understanding of the initial stages of the checkpoint response that two protein complexes, Mec1-Ddc2 and Rad17-Mec3-Ddc1, are recruited to sites near a DNA lesion. Rad17p, Mec3p, and Ddc1p (the human homologues are Rad1, Hus1, and Rad9, respectively) form a heterotrimeric complex structurally related to the proliferating cell nuclear antigen (PCNA), the processivity factor of DNA polymerases δ and ε. We have recently identified a yeast mutant allele of *MEC3 – pso9-1 –* which is strongly sensitive to DNA damaging agents and confers low induced mutability.

To further investigate the effects of the Pso9-1 mutant protein, the complete sequence of the mutant allele was determined. The comparison of obtained sequences with the databank-deposited wild-type *MEC3* sequence revealed a single deletion of nucleotide position 802 (802delA) that leads to a truncated Pso9p. The 802delA frameshift mutant allele thus encodes a 276 amino acid polypeptide in which the last nine amino acids are out of proper reading frame and that has its carboxyl terminus dramatically shortened. This mutation affected the binding properties of Pso9-1p, abolishing its interactions with both Rad17p and Ddc1p. Further interaction assays

employing *mec3* constructions lacking the last 25 and 75 amino acid carboxyl termini were also not able to maintain stable interactions. Moreover, the *pso9-1* mutant strain could no longer sense DNA damage since it continued in the cell cycle after photoactivated 8-methoxypsoralen treatment.

When analyzing the response to DNA damage of yeast mutants lacking the yeast PCNA-like component Pso9p/Mec3p in combination with defective subunits of three replicative DNA polymerases (Pols α, δ and ε), we found an additive interaction between *PSO9/MEC3* and *POL32*, which codifies the third subunit of DNA polymerase δ. The hypersensitivity of *pol32*Δ to photoactivated 8-MOP revealed by these genetic analyses strongly suggested the hitherto unknown participation of a replicative polymerase in the repair of interstrand cross-links. This assumption was confirmed since we also found the *pol32*Δ mutant extremely deficient in joining non-cohesive DNA ends, particularly in cases of mismatched 5' overhangs, suggesting a role for DNA polymerase δ in non-homologous end-joining (NHEJ).

The collection of data presented in this work reasserts the relevance of sensor proteins as Pso9p/Mec3p in initiating the signaling of structural abnormalities in DNA. Moreover, it amplifies the network of interstrand cross-links responsive proteins, showing that a particular protein, as seen for Pol32p, may have additional tasks as a safety mechanism in ensuring genomic stability.

CAPÍTULO I

INTRODUÇÃO

1. Introdução geral

A estética fascinante da dupla-hélice do DNA, tida como uma entidade macromolecular altamente estável, inicialmente retardou considerações sobre mutação e reparação de DNA, o que necessariamente interferiria no seu estado imaculado. Mas os trabalhos subseqüentes em metabolismo de DNA – replicação, transcrição e recombinação – acabaram por revelar o estado dinâmico desta molécula. Tornou-se claro que o DNA de todos os organismos vivos sofre continuamente uma miríade de tipos de danos e que mecanismos engenhosos foram criados pelas células para tolerar e reparar estes danos.

Conforme a percepção de que o DNA poderia sofrer alterações foi tornando-se mais difundida, os cientistas passaram a esperar que a identificação de qualquer novo tipo de dano que viesse a ocorrer naturalmente, direcionaria, ao descobrimento de um ou mais mecanismos para sua reparação ou tolerância (Lindahl, 1993; Friedberg, 1997). De fato, este tem sido o caso. A reparação de DNA agora abrange não só a reversão direta de alguns tipos de dano (como a fotorreativação de dímeros de timina), como também múltiplos mecanismos distintos para excisar bases com danos ou corrigir erros durante a replicação. Além de lidar com este tipo de injúria, as células também sofrem quebras em uma ou ambas as fitas do DNA e conseguem repará-las, fazendo trocas de regiões de DNA entre cromossomos homólogos (recombinação homóloga). Alternativamente, estas quebras podem ser reparadas pela ligação de extremidades de DNA de uma maneira independente de homologia de seqüência (recombinação não-homóloga). Existe ainda a possibilidade de que um dano persista no genoma, sendo tolerado por mecanismos nos quais ou a maquinaria de replicação é capaz de utilizar o DNA lesado como molde (síntese translesão), ou é efetuada uma recombinação pós-

replicativa que pode ser acompanhada ou não de regressão da forquilha, permitindo que a síntese de DNA e os processos posteriores tenham continuidade (para revisão, ver Friedberg, 2003; 2005).

Atualmente, sabe-se que a resposta às agressões ao material genético abrange mais do que reparação e tolerância aos danos. Adicionalmente, as células têm evoluído complexas vias de sinalização para parar o ciclo celular na presença de danos no DNA (*checkpoint*), proporcionando assim, um maior tempo para que a reparação e os demais mecanismos atuem (Zhou e Elledge, 2000). Finalmente, quando a agressão ao genoma é simplesmente muito grande para ser efetivamente solucionada pelas respostas referidas anteriormente, as células são capazes de iniciar sua morte de uma forma programada (apoptose), eliminando-se assim da população, a qual de outra forma poderia sofrer sérias conseqüências patológicas, como o câncer (Cory e Adams, 2002). A magnitude das respostas celulares às injúrias ao genoma esta sumarizada na Figura 1.

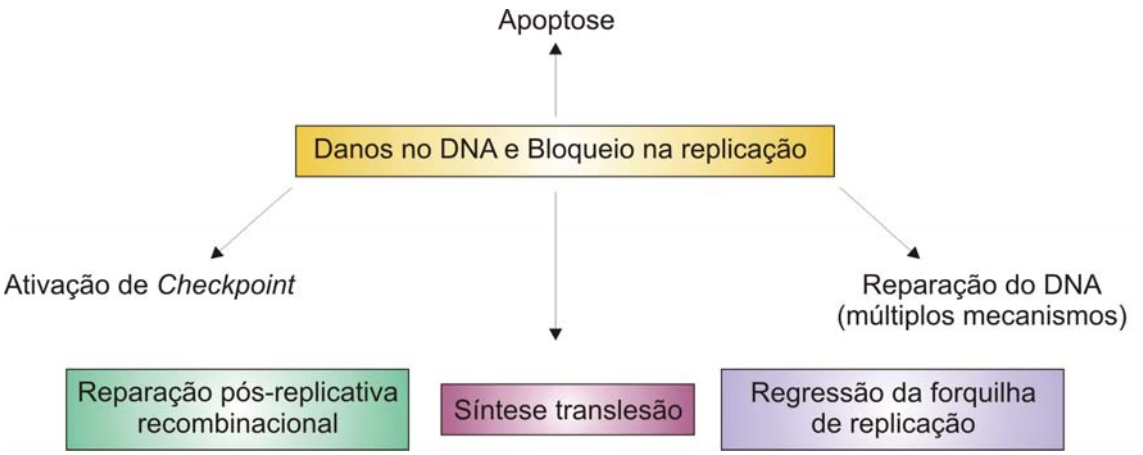

Figura 1. Respostas celulares aos danos no DNA. As respostas podem resultar na reparação ou tolerância às lesões, contando ainda com estratégias como *checkpoint* e apoptose. Os mecanismos de tolerância conhecidos estão indicados nas caixas verde, rosa e azul (adaptado de Friedberg, 2005).

Progressos na compreensão dos mecanismos de reparação de DNA estão sendo alcançados em diversos organismos, especialmente com células de mamíferos, e leveduras continuam sendo um excelente modelo para o estudo da reparação em humanos. Quase todas as vias de reparação são bioquimicamente muito similares de leveduras a mamíferos (para revisão, ver Taylor e Lehmann, 1998; Resnick e Cox, 2000; Costa *et al.*, 2003; Aylon e Kupiec, 2004; Lisby e Rothstein, 2005).

A levedura *Saccharomyces cerevisiae* é o microrganismo eucariótico mais estudado e de melhor aplicação para o entendimento de diversas funções biológicas (Hieter, 1998; Resnick e Cox, 2000; Dolinski e Botstein, 2005). Algumas das propriedades que fazem da levedura particularmente apropriada para estudos biológicos incluem seu rápido crescimento, o fato de ser unicelular, possuir um sistema genético bem-definido e mais significantemente, o conhecimento da seqüência completa de seu genoma, que foi concluído em 1996 (Goffeau, 1996; Dujon, 1996), mudando a forma com que geneticistas e biólogos moleculares planejavam e conduziam seus estudos. Ao contrário da maioria dos microrganismos, linhagens de *S. cerevisiae* são estáveis tanto em estado haplóide quanto diplóide. Assim, mutações recessivas podem ser convenientemente isoladas e manifestadas em linhagens haplóides, e testes de complementação podem ser conduzidos em linhagens diplóides. A levedura também possui um sistema de transformação de DNA altamente versátil, sendo viável com numerosos marcadores genéticos; plasmídeos podem ser introduzidos nas células tanto como moléculas replicativas, quanto para integração no genoma. Diferente do que ocorre em outros organismos, a integração de uma seqüência de DNA procede exclusivamente via recombinação homóloga. Igualmente, a recombinação homóloga, combinada ao alto grau de conversão gênica das leveduras, possibilitou o desenvolvimento de técnicas para a substituição direta de seqüências de DNA

geneticamente modificadas, no lugar de sua cópia normal no cromossomo. Deste modo, genes intactos, mesmo aqueles dos quais não se conhecem mutações, podem ser convenientemente substituídos por alelos modificados. Também exclusivo à levedura, transformações podem ser conduzidas com oligonucleotídeos sintéticos, permitindo a produção conveniente de numerosas formas alteradas de proteínas. Estas técnicas têm sido extensivamente exploradas em análises que vão desde regulação gênica até relação estrutura-função de proteínas, incluindo mutagênese e reparação de DNA (para revisão, ver Pringle *et al.*, 1997; Brown e Tuite, 1998; Burke *et al.*, 2000).

1.1 Tipos de lesões mais comuns ao DNA

Os danos na molécula de DNA compreendem modificações químicas que ocorrem nas bases nitrogenadas (purinas e pirimidinas), na desoxirribose e na ponte fosfodiéster (Figuras 2 e 3). Estas modificações, na sua totalidade, são provenientes da radiação solar (na forma de luz UV e radiação ionizante), da geração de espécies reativas de oxigênio (EROs) através de diferentes processos metabólicos ou mesmo de substâncias químicas de origem natural e/ou sintética (Figura 2).

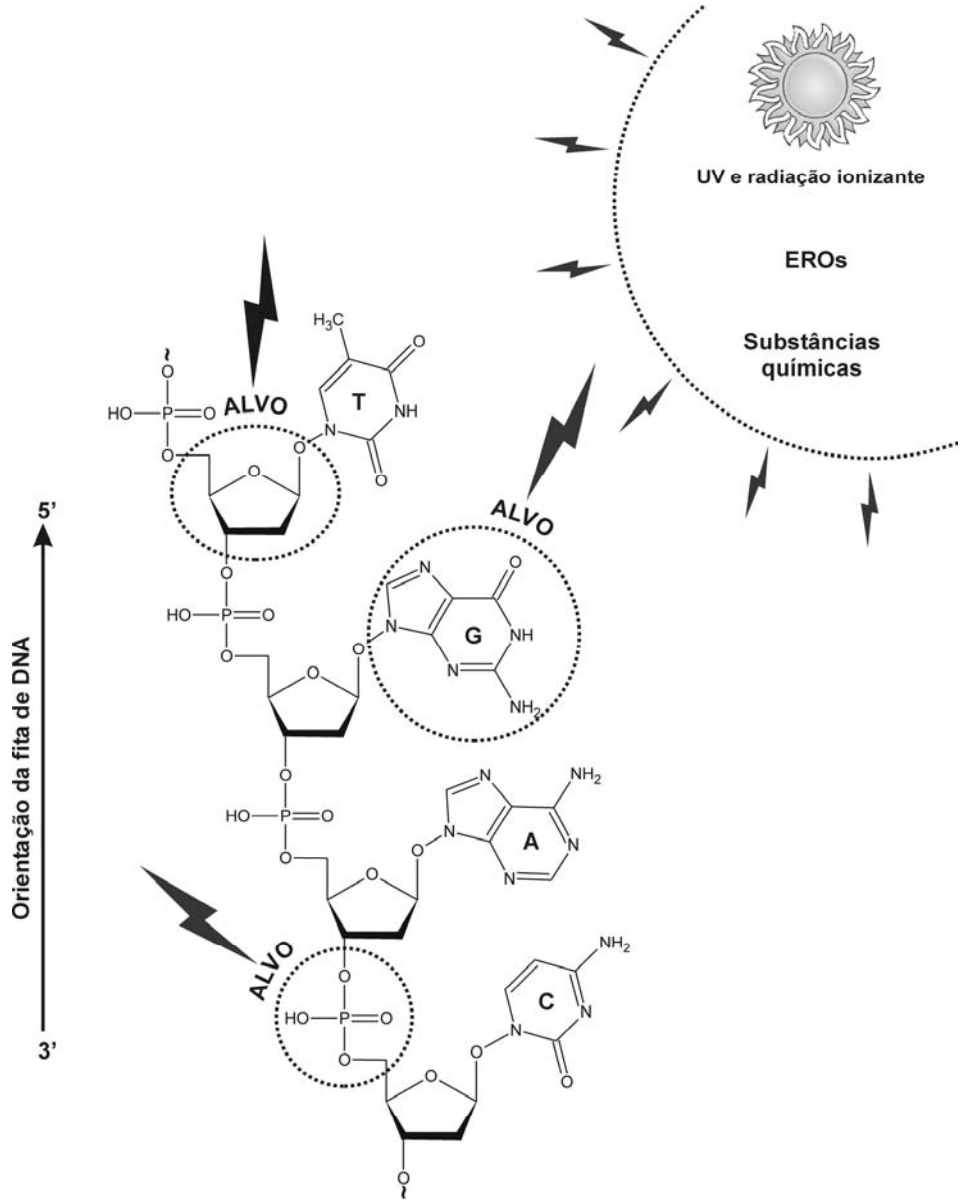

Figura 2. Principais alvos para danos na molécula de DNA. Os danos, que podem acontecer nas bases, na desoxirribose ou na ponte fosfodiéster (indicados pelos círculos pontilhados) são originados a partir da radiação solar (na forma de radiação UV e ionizante), de EROs e de substâncias químicas. A orientação da fita de DNA é mostrada na figura. Siglas: T, G, A e C - timina, guanina, adenina e citosina respectivamente (cedido por Bonatto, 2005).

1.1.1 Bases modificadas

De todas as alterações químicas que podem ocorrer no DNA, as bases modificadas são as mais comuns e também as mais perigosas, desde que não reparadas corretamente (Figura 3; Slupphaug *et al.,* 2003; Evans *et al.,* 2004). Os processos químicos que originam as modificações nas bases nitrogenadas são complexos em sua natureza (Slupphaug *et al.*, 2003) e resultam de uma maior ou menor interação dos agentes indutores de danos *per se* com moléculas de importância biológica, tanto de natureza enzimática (superóxido dismutases, tiorredoxinas, citocromo P450, entre outros), quanto de natureza não-enzimática (por exemplo, glutationa, aminoácidos sulfurados, água ou O_2) (Slupphaug *et al.,* 2003; Evans *et al.,* 2004).

As bases modificadas que mais prevalecem no DNA são a 8-oxoguanina (8oxoG) e a timina glicol (Tg) (Slupphaug *et al.,* 2003). A 8oxoG é uma base fracamente mutagênica (freqüência de mutação de 2,5% a 4,8% em células de mamíferos; Leadon e Avrutskaya*,* 1997) e resulta, em grande parte, em substituições do tipo G $\rightarrow$ T. Alternativamente, a 8oxoG pode emparelhar erroneamente com uma adenina, produzindo uma substituição do tipo A $\rightarrow$ T (Cheng *et al.,* 1992). Por outro lado, a Tg é considerada como uma base muito pouco mutagênica (freqüência de mutação de 0,3% em células de mamíferos), causando substituições do tipo T $\rightarrow$ C (Basu *et al.,* 1989). Contudo, a principal alteração causada pela Tg é a modificação da estrutura do DNA, o que leva a um bloqueio do processo de replicação (McNulty *et al.,* 1998). Este mesmo fenômeno é observado em outra alteração de base: os dímeros de pirimidinas ou CPDs (*ciclobutane pyrimidine dimers*) (Figura 3). Diversos tipos de CPDs podem ser gerados *in vivo* na presença de luz solar, tais como os dímeros de timina-timina, a pirimidina-pirimidona (6,4)-fotoprodutos e os isômeros de 'Dewar'

(Vink e Roza, 2000). Os CPDs podem provocar modificações na estrutura do DNA, causando bloqueios nos processos de replicação e transcrição. Além disso, grande parte da atividade mutagênica observada em células de mamíferos deve-se à formação de CPDs (Ravanat *et al.,* 2001). Interessantemente, os CPDs foram as primeiras lesões de DNA estudadas (Beukers *et al.,* 1960) e ainda possuem um papel de destaque nas pesquisas relacionadas com as modificações de bases provocadas por agentes químicos e físicos, em especial a luz solar (Vink e Roza, 2001; Douki *et al.,* 2003). Recentemente, a atuação da luz UVA na formação de CPDs ganhou importância ao ser mostrado que esta radiação é a principal fonte de indução de CPDs em células de mamíferos, o que explicaria a alta incidência de tumores de pele após a exposição prolongada à radiação solar em seres humanos (Douki *et al.,* 2003).

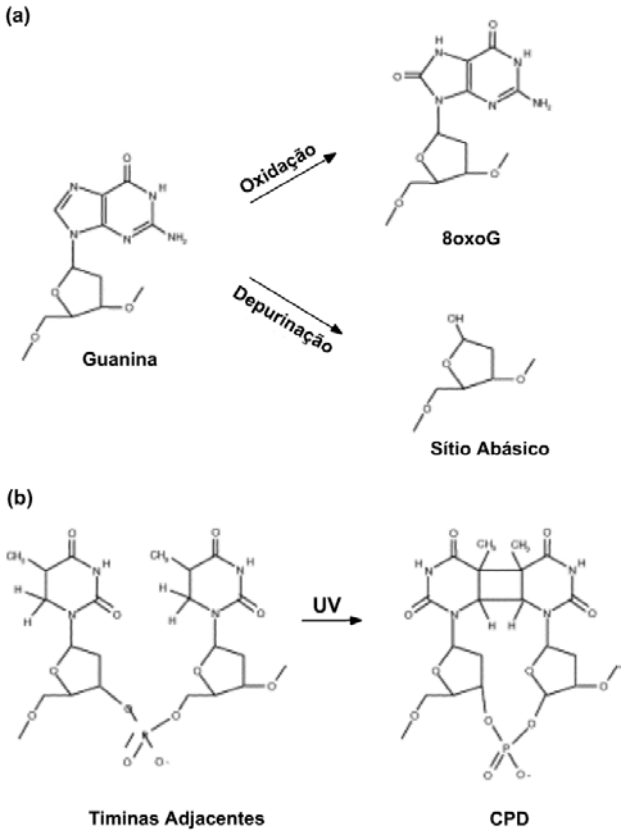

Figura 3. Diagramas exemplificando a formação de bases modificadas: **(A)** 8oxoG e sítio abásico; **(B)** CPD (adaptado de Hogg *et al.,*2005).

1.1.2 Adutos mono- e bifuncionais

As ligações covalentes que se formam entre determinadas classes de substâncias químicas e as bases nitrogenadas do DNA são conhecidas como 'adutos' (Shärer, 2005). Quimicamente, os compostos ou agentes formadores de adutos podem ser separados em dois grandes grupos: (i) agentes monofuncionais, quando induzem a formação de ligação covalente entre um composto químico e apenas uma base nitrogenada (adutos monofuncionais) e (ii) agentes bi- ou polifuncionais, quando a substância química possui a habilidade de se ligar covalentemente a duas bases nitrogenadas (adutos bifuncionais), estejam estas situadas na mesma fita de DNA (pontes intracadeia) ou em fitas separadas (pontes intercadeias, ou *interstrand cross-links* - ICLs) (Shärer, 2005).

Os agentes monofuncionais compreendem várias classes de substâncias químicas dos quais destacam-se os agentes alquilantes. Estes agentes possuem a propriedade de se ligarem às bases nitrogenadas do DNA por meio de grupos metila ou etila (Sanderson e Shield, 1996). Neste grupo predominam vários compostos com finalidades terapêuticas, especialmente para o tratamento de tumores (Izbicka e Tolcher, 2004).

Por outro lado, os agentes bi- ou polifuncionais podem atuar tanto na indução de adutos monofuncionais quanto de pontes intracadeias e ICLs (Dronkert e Kanaar, 2001). Uma ampla variedade de compostos químicos é considerada como agentes bifuncionais, sendo que muitos destes possuem aplicações diretas na terapia clínica para tratamento de tumores ou patologias de pele (Dronkert e Kanaar, 2001). Dentre os agentes bifuncionais com importância médica estão os psoralenos, a mitomicina C, a cisplatina e as mostardas nitrogenadas (Figura 4a). Os psoralenos são representados por moléculas pertencentes à classe das furocumarinas, que consistem em metabólitos secundários isolados de plantas das famílias *Umbelliferae, Rutaceae, Moraceae e*

Leguminosae (Guo e Yamazoe, 2004). Vários psoralenos sintéticos estão hoje disponíveis para o uso clínico, dos quais destaca-se o 8-metoxipsoraleno (8-MOP, Figura 4a), especialmente para o tratamento de psoríase, de vitiligo e de mais 30 tipos diferentes de patologias de pele (Morison, 2004). O modo como os psoralenos, em especial o 8-MOP, formam ICLs no DNA é bastante conhecido (Bethea *et al.,* 1999) e consiste de duas etapas (Figura 4b). Na primeira etapa, o 8-MOP intercala-se entre as bases pirimídicas (com alta afinidade por timinas) sem formar ligações covalentes (Figura 4b). Na segunda etapa, os adutos são formados por uma fotocicloadição entre a dupla ligação 4,5 do grupo furano ou 3,4 do grupo cumarínico com a ligação dupla 5,6 da timina. Um segundo fóton de luz UVA induz a formação de ICLs (Figura 4b; Bethea *et al.,* 1999).

A mitomicina C (Figura 4a) por sua vez, é um antibiótico natural que possui a propriedade de formar monoadutos nas posições N-7 e N-2 da guanina, pontes intracadeias e ICLs (Tomasz, 1995; Kumar *et al.,* 1997). Neste último caso, a formação de ICLs dá-se entre as posições N-2 da guanina em seqüências do tipo CpG, e constituem cerca de 5% a 13% dos adutos totais em células de mamíferos (Warren *et al.,* 1998). A mitomicina C possui uma ampla aplicabilidade médica, sendo bastante utilizada em combinação com outras drogas para o tratamento de tumores de mama, pulmão, próstata e bexiga (Cummings *et al.,* 1998).

A cisplatina (Figura 4a) é um dos compostos mais utilizados para o tratamento de diferentes tipos de tumores (Gupta *et al.,* 2005). As chances de cura para pacientes com tumores de testículos e de ovários pode chegar a 90% quando a cisplatina é administrada em conjunto com outros quimioterápicos (Bosl e Motzer, 1997). A ação genotóxica da cisplatina deve-se à formação de adutos bifuncionais de DNA do tipo pontes intracadeia (65% GpG e 25% ApG) e ICLs entre guaninas situadas em regiões

do DNA ricas em GpC (5% a 8% do total de adutos) (Dronkert e Kanaar, 2001). Em comparação aos psoralenos e à mitomicina C, a cisplatina provoca grandes distorções na estrutura do DNA, as quais podem resultar em quebras da fita e bloqueios nos processos de replicação e transcrição de DNA (Malinge *et al.,* 1999).

As mostardas nitrogenadas, em especial as bifuncionais (HN2; Figura 4a), constituem na principal classe de drogas antitumorais hoje disponíveis (Souliotis *et al.,* 2003). Seu mecanismo de ação baseia-se na alta reatividade que possui com macromoléculas biologicamente importantes, tais como DNA, RNA e proteínas, induzindo múltiplos tipos de lesões (Souliotis *et al.,* 2003). Entretanto, o seu principal alvo é o DNA, alquilando a posição N-7 da guanina ou a posição N-3 da adenina (Osborne *et al.,* 1995). Além disso, as mostardas nitrogenadas bifuncionais podem induzir a formação de ICLs que modificam radicalmente a estrutura do DNA (Rink e Hopkins, 1995) e que constituem cerca de 5% de todos os danos gerados (Dronkert e Kanaar, 2001).

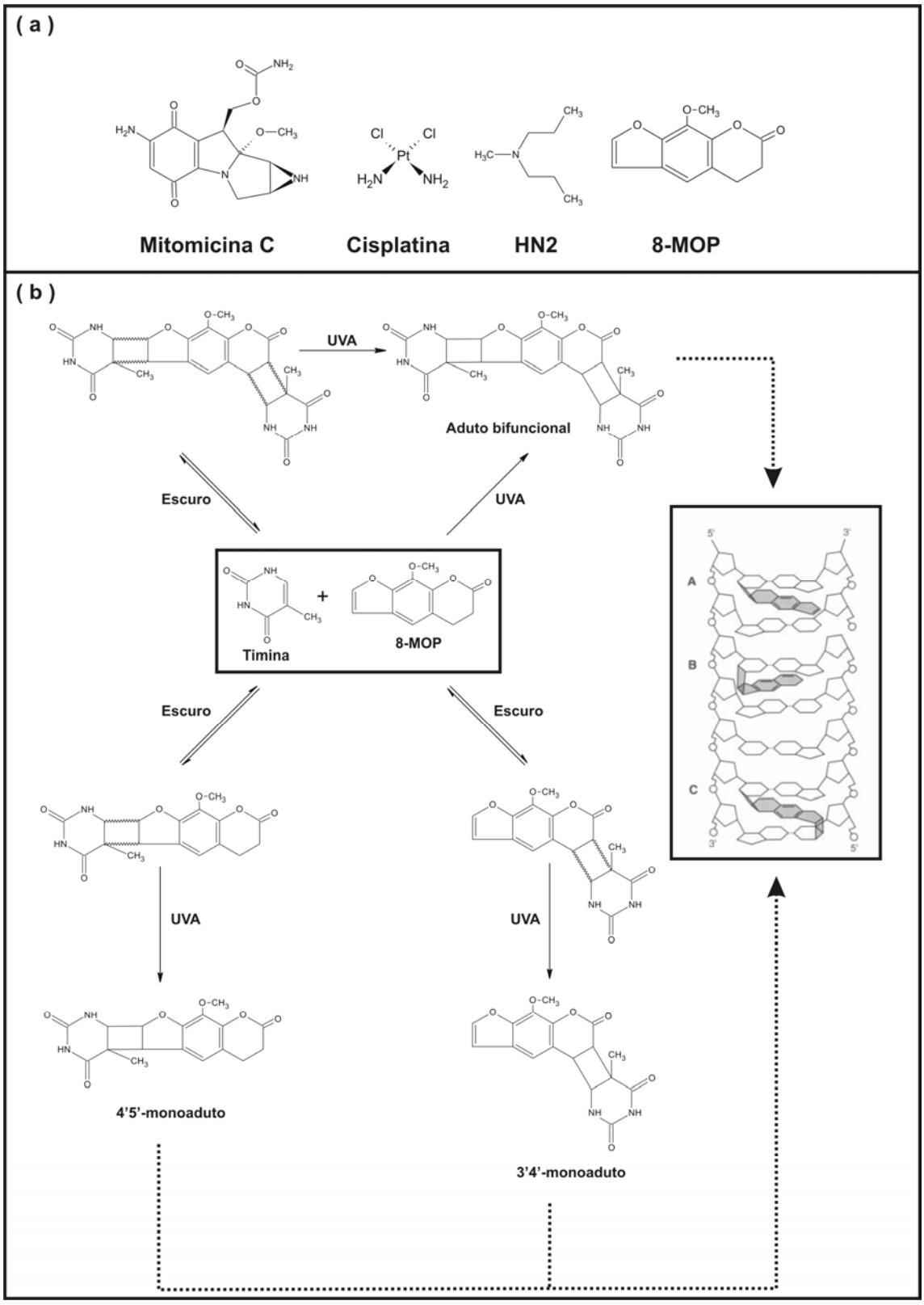

Figura 4. Em (a), exemplos de agentes mono- e/ou bifuncionais. Em (b), esquema da indução de adutos mono- e bifuncionais com 8-MOP e timina, bem como sua disposição espacial na molécula de DNA (quadro interno – A, B, C). Neste caso, quando a reação é feita na ausência de luz UVA, a molécula de 8-MOP tem a

propriedade de interagir não covalentemente com a timina. Na presença de UVA, formam-se as ligações covalentes do tipo 4'5'- e 3'4'-monoadutos (A e B) ou um aduto bifuncional quando duas timinas estão espacialmente próximas (C) (modificado de Bonatto, 2005).

1.1.3 Quebras simples e duplas de DNA

As quebras de DNA podem acontecer como resultado (i) do ataque de um agente químico ou físico à ponte fosfodiéster (Caldecott, 2001; Dudáš e Chovanec, 2004; Purdy e Su, 2004), (ii) de um bloqueio no processo de replicação do DNA (Michel *et al.,* 2004) ou (iii) da ação de enzimas específicas com funções de reparação ou que atuam no metabolismo geral do DNA (Haber, 1999; Pastink e Lohman, 1999; Flores-Rozas e Kolodner, 2000; Pastink *et al.,* 2001; van den Bosch *et al.,* 2002).

As lesões do tipo quebras de DNA podem ser separadas em dois grupos: simples (*single strand breaks* - SSBs) e duplas (*double strand breaks* - DSBs) (Caldecott, 2001; Dudáš e Chovanec, 2004; Purdy e Su, 2004). As quebras simples são consideradas as menos tóxicas para a célula e milhares de SSBs são geradas por dia em células de mamíferos como resultado das atividades de reparação ou metabólicas do DNA (Caldecott, 2001). Contudo, esta falta de toxicidade é devida à rápida reparação de SSBs, as quais, de outra forma, poderiam converter-se em DSBs (Caldecott, 2001). A reparação de SSBs é realizada por uma via bioquímica especializada, denominada de SSBR (SSB *repair;* Caldecott, 2001). Interessantemente, a ausência de reparação de SSBs está relacionada a um conjunto de patologias, tais como a ataxia espinocerebelar (Caldecott, 2003) e outros processos neurodegenerativos (Caldecott, 2004).

As lesões do tipo DSBs são consideradas as mais tóxicas para a célula (Dudáš e Chovanec, 2004). Quando não reparadas corretamente, as DSBs podem originar mutações e rearranjos cromossômicos (Jeggo, 1998; Kanaar *et al.,* 1998; Haber, 1999),

podendo levar à perda de cromossomos ou à morte celular (Kaina, 2003; Dudáš e Chovanec, 2004).

1.2 Mecanismos de reparação de DNA

1.2.1 Vias de reparação de DNA

Como visto na seção anterior, a enorme complexidade dos diferentes tipos de lesões que se acumulam no DNA podem induzir instabilidades genômicas que, se não reparadas corretamente, levam à morte celular ou a processos tumorais. Desta maneira, não é surpreendente que a reparação de todas estas lesões requeira a atuação de várias proteínas que, juntas, compõem vias ou mecanismos discretos, mas que se mostram espacialmente e temporalmente interconectados (Eisen e Hanawalt, 1999). Esta diversidade de vias de reparação de DNA pode ser observada quando as mesmas são comparadas entre si. Por exemplo, algumas vias são usadas para reparar apenas um tipo de dano, enquanto que outras são mais abrangentes em suas atuações enzimáticas. Da mesma forma, algumas vias de reparação são mecanisticamente simples, requerendo apenas algumas enzimas, enquanto que outras são bastante complexas, envolvendo muitos passos enzimáticos e diferentes tipos de complexos protéicos atuando, não apenas no processo de reparação em si, mas também em outros processos celulares (Eisen e Hanawalt, 1999). Esta diversidade de vias pode ser melhor compreendida quando as mesmas são agrupadas levando-se em conta os mecanismos de ação. Assim, três grandes vias são atualmente conhecidas: a reparação direta, a reparação por excisão e a reparação recombinacional (Figura 5; Eisen e Hanawalt, 1999).

A reparação direta envolve dois mecanismos principais: (i) a fotorreativação, catalisada por enzimas pertencentes à família das fotoliases/criptocromos (Figura 5;

Thompson e Sancar, 2002) e (ii) a reversão de bases alquiladas, catalisadas pelas DNA metiltransferases (Christmann *et al.,* 2003). Por sua vez, a reparação por excisão é formada por três mecanismos principais: a excisão de nucleotídeos (*nucleotide excision repair* - NER, para revisão, ver Prakash e Prakash, 2000; Christmann *et al.,* 2003 e Costa *et al.,* 2003), a excisão de bases (*base excision repair* - BER, para revisão, ver Boiteux e Guillet, 2004) e a reparação de erros de emparelhamento de bases (*mismatch repair* - MMR, para revisão, ver Christmann *et al.,* 2003). Tanto as vias de reparação direta quanto a de excisão são essenciais para a remoção de bases modificadas e adutos, de forma que respondem por grande parte da atividade de reparação de DNA na célula (Huang *et al.,* 1994; Reardon *et al.,* 1997; Memisoglu e Samson, 2000). A reparação recombinacional (abordada em maiores detalhes no item 1.2.2) é recrutada em caso de lesões do tipo quebras de DNA, geradas endogenamente, ou por agentes exógenos físicos e químicos.

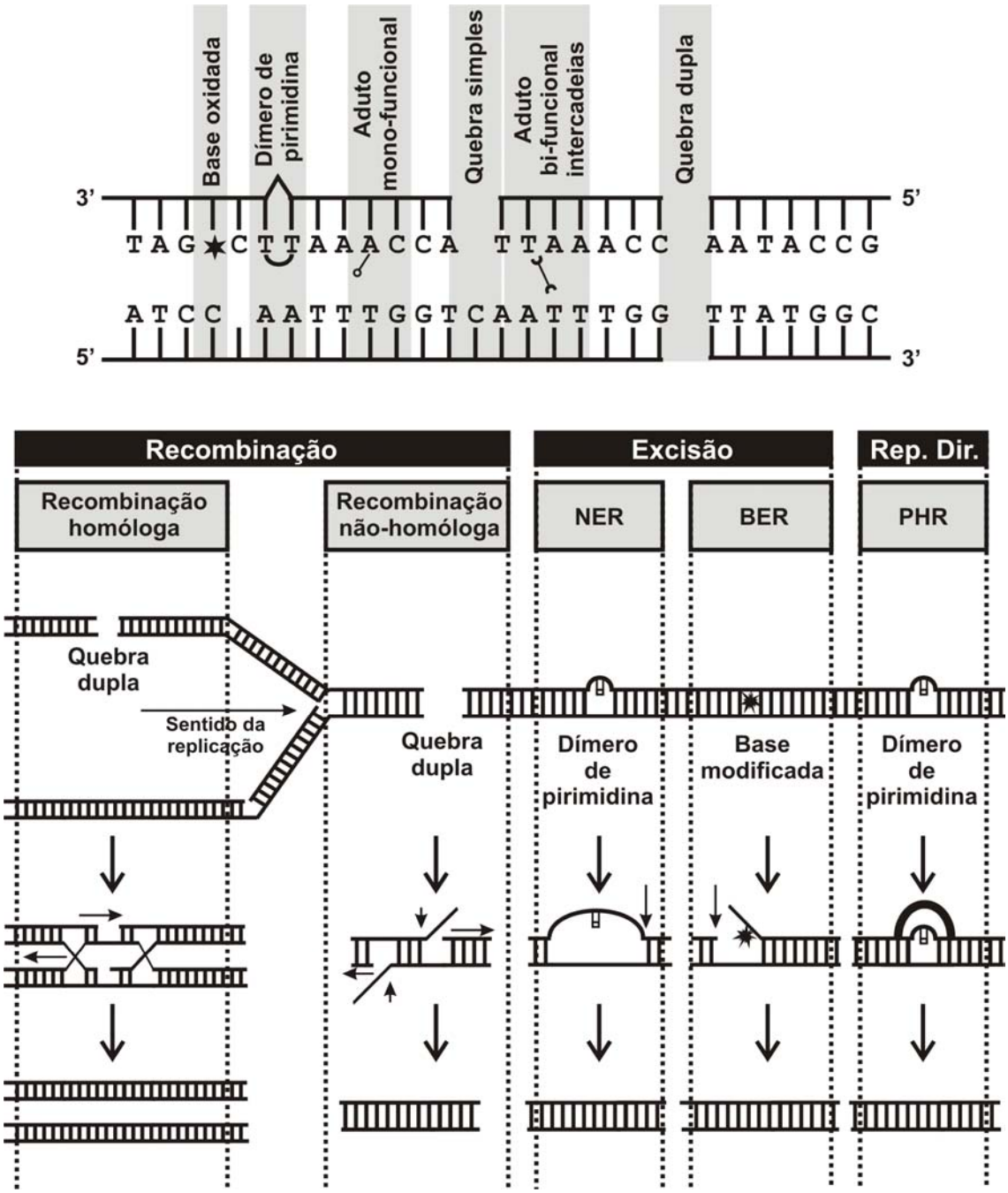

Figura 5. Diagrama esquemático dos três mecanismos principais de reparação de DNA (recombinacional, excisão e reparação direta), bem como de algumas subvias de reparação. A presença de várias lesões em uma fita de DNA que está sendo replicada é reconhecida por subvias diferentes, por proteínas sensoras. Estas, por sua vez, recrutam as proteínas responsáveis pela reparação do DNA, conforme cada situação (NER – *nucleotide excision repair*; BER - *base excision repair*; PHR – *photoreactivation repair*; modificado de Bonatto, 2005).

1.2.2 Reparação recombinacional

Como visto previamente, a geração de bases modificadas no DNA bem como adutos mono- e bifuncionais podem resultar em quebras altamente genotóxicas que, se não forem reparadas, podem ser letais (Kaina, 2003; Dudáš e Chovanec, 2004). Estas quebras são reparadas por um mecanismo especializado denominado de reparação recombinacional.

A reparação recombinacional possui dois mecanismos principais: a recombinação homóloga (*homologous recombination* - HR, não mutagênica) e a recombinação não-homóloga (*non-homologous end joining* - NHEJ, mutagênica). Cada uma destas vias possui requerimentos enzimáticos únicos, sendo que o recrutamento de uma ou de outra via é dependente de uma série de fatores fisiológicos celulares (Lieber, 1999). Interessantemente, as vias HR e NHEJ podem ter funções sobrepostas para a manutenção da integridade cromossomal em eucariotos (Takata *et al.,* 1998).

1.2.2.1 Recombinação homóloga

A recombinação homóloga em eucariotos é uma via evolutivamente conservada, sendo que os homólogos protéicos, necessários para realizar esta função, estão presentes tanto em eucariotos unicelulares quanto multicelulares (Jackson, 2002). Os eventos relacionados à via HR são complexos em sua natureza, sendo que dois modelos são conhecidos atualmente: a reparação de quebras duplas (*double strand breaks repair* - DSBR) e a síntese dependente de anelamento de fita (*synthesis-dependent strand annealing* - SDSA) (Krogh e Symington, 2004). O modelo descrito para esta seção representa a via DSBR, pois esta é a principal via HR na célula (Krogh e Symington, 2004; Fig. 6).

A função primária da HR em células mitóticas, independente do modelo proposto, é a reparação de DSBs ou SSBs que se formam em conseqüência do colapso

da forquilha de replicação de DNA e que é resultante da ação de diferentes mecanismos de reparação de bases modificadas ou adutos (Krogh e Symington, 2004). A HR também é requerida para a manutenção telomérica e para a meiose, sendo essencial para estabelecer uma conexão física entre cromossomos homólogos a fim de assegurar a correta disjunção dos mesmos na primeira divisão meiótica (Krogh e Symington, 2004). Adicionalmente, a alta freqüência de recombinação meiótica promovida pela HR contribui para a geração de diversidade genética observada nos gametas (Krogh e Symington, 2004).

A via HR envolve um conjunto de proteínas pertencentes ao grupo de epistasia Rad52 (Wood *et al.,* 2001; Krogh e Symington, 2004). O primeiro evento relacionado à HR é a ressecção nucleolítica da fita 5'-3', promovida por um complexo protéico que contém Rad50, Mre11p e Xrs2p (Complexo MRX; Jackson, 2002; Krogh e Symington, 2004). O objetivo desta ressecção é a geração de extremidades invasivas do tipo 3' fita simples que resultarão no processo recombinacional (Figura 6). O repertório nucleolítico da Mre11p inclui: (i) atividade exonucleásica 3'$\rightarrow$5' em extremidades cegas e rescindidas, (ii) atividade endonucleásica em fitas simples de DNA, tanto circulares quanto lineares e (iii) atividade endonucleásica em estruturas secundárias de DNA, como grampos (*hairpins*). Uma vez que as extremidades 3' fita simples estejam disponíveis, a proteína Rad51 (em um filamento nucleoprotéico) liga-se a estas e inicia o evento de invasão a uma fita homóloga (Figura 6). Esta invasão é consideravelmente facilitada pelas proteínas mediadoras Rad54 e RPA, as quais são responsáveis pela eliminação de estruturas secundárias da região 3' terminal. Desta maneira, a região 3' terminal da fita danificada é restaurada por uma DNA polimerase, que copia as informação da fita intacta. Terminada a extensão, as extremidades livres são novamente reunidas pela DNA ligase I (Figura 6). Entretanto, este processo gera junções do tipo

Holliday, que necessitam ser resolvidas a fim de gerarem duas moléculas intactas de DNA (Figura 6; Krogh e Symington, 2004). A resolução das junções de Holliday é realizada pelas enzimas Mus81 e Mms4 (Eme1), as quais pertencem à família XPF de endonucleases estrutura-específicas (Heyer, 2004). O resultado final da resolução das junções de Holliday pode ser ou não a permuta de fitas (*crossover*), especialmente durante o processo de recombinação meiótica (Krogh e Symington, 2004).

Em seres humanos são conhecidas duas patologias como resultado de deficiências na via HR: a anemia de Fanconi, cujos pacientes apresentam uma alta instabilidade cromossômica e propensão a leucemias de origem mielóide (Faivre *et al.,* 2000) e a síndrome de Bloom, cujos pacientes apresentam uma alta propensão a desenvolver diferentes tipos de tumores, além da instabilidade cromossômica associada (Ellis e German, 1996).

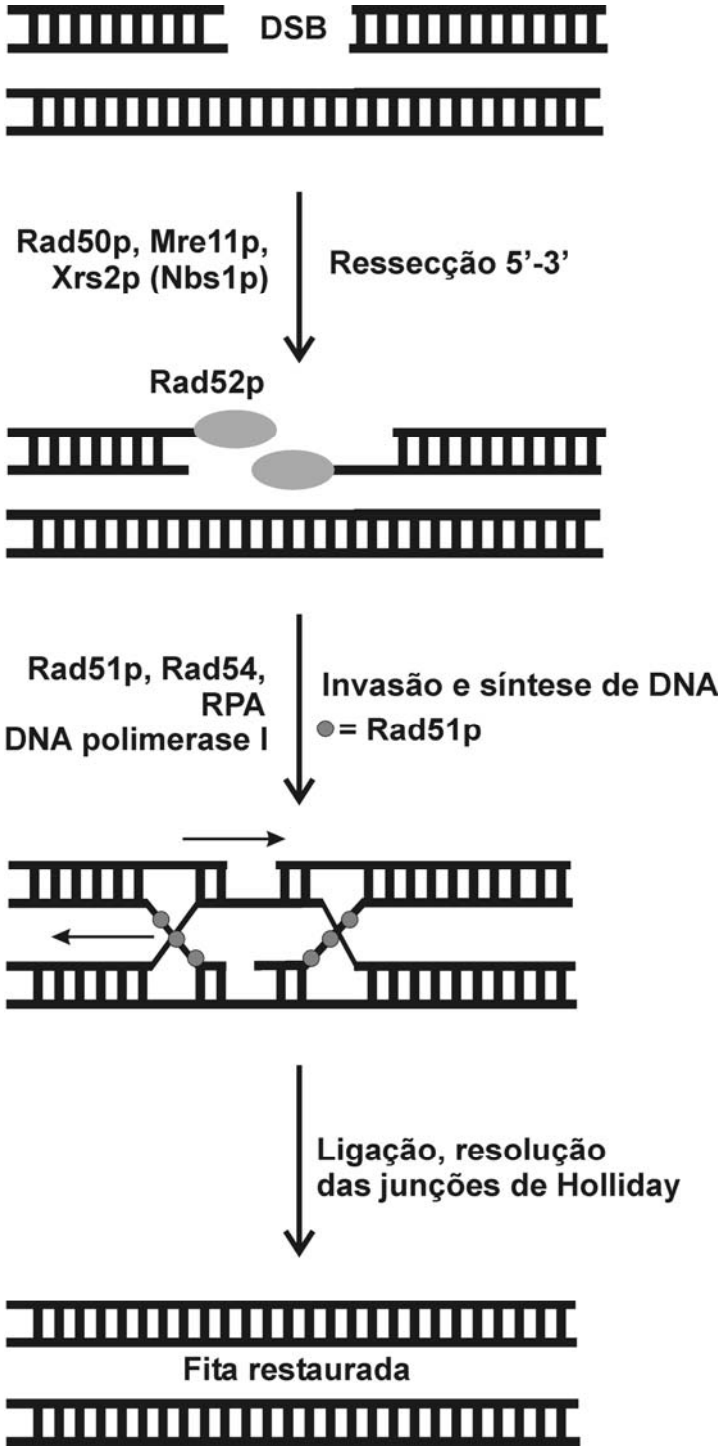

Figura 6. Modelo simplificado da via HR. A quebra dupla é reconhecida pelo complexo Rad50p/Mre11p/Xrs2p, o qual induz a ressecção da fita 5'→3'. Com a ajuda das proteínas Rad51, Rad54 e RPA ocorre a invasão das extremidades 3' danificadas na fita homóloga não danificada, a qual é utilizada como molde pela DNA polimerase. Após a ligação dos fragmentos, as junções de Holliday resultantes são resolvidas e ambas as fitas restauradas (cedido por Bonatto, 2005).

1.2.2.2 Recombinação não-homóloga

A recombinação não homóloga ou NHEJ representa um dos ramos principais da via recombinacional. Presente em todos os eucariotos, a via NHEJ é recrutada quando há a necessidade de religar duas extremidades de DNA, resultantes de uma quebra, e que não possuem homologia entre si. Além disso, pequenas regiões de homologia (ou microhomologias, quando abrangem apenas alguns nucleotídeos) existentes entre duas fitas não homólogas de DNA podem ser utilizadas pela via NHEJ para a reparação (Labhart, 1999). Assim, diversas subvias de reparação pertencentes à via NHEJ são conhecidas, cada qual com características próprias, mas cujo resultado final é a reparação de DSBs de uma forma sujeita a erros.

Inúmeras patologias associadas a deficiências na via NHEJ já foram descritas em seres humanos e outros mamíferos. Estas deficiências, em grande parte, resultam no aumento da incidência de tumores malignos, envelhecimento precoce, inviabilidade embrionária e em instabilidades cromossômicas de diferentes naturezas (Iliakis *et al.,* 2004). Em *S. cerevisiae*, embora os mecanismos moleculares da via NHEJ ainda não sejam conhecidos em detalhes, a maquinaria básica da via NHEJ consiste nos complexos Ku70/80, Mre11/Rad50/Xrs2, Dnl4/Lif1 e, em alguns casos, conta com a participação das proteínas Rad27 e Pol4. A junção de duas extremidades de DNA requer, pelo menos, quatro passos: (1) a detecção da DSB; (2) a formação de uma ponte molecular que mantenha as duas extremidades próximas uma da outra; (3) o processamento das extremidades, de forma a torná-las compatíveis para a ligação e (4) a ligação das extremidades (para revisão, ver Dudášová *et al.*, 2004; Weterings e van Gent, 2004; Hefferin e Tomkinson, 2005). O primeiro componente a se ligar na DSB é o heterodímero Ku70/80, o qual presumivelmente protege as extremidades do DNA contra degradação e sinaliza a região de dano para os demais componentes da via

(Milne *et al.*, 1996; Siede *et al.*, 1996). Subseqüentemente, o complexo Mre11/Rad50/Xrs2 é recrutado à DSB, mediando a sinapse em conjunto com Ku70/80 (Moore *et al.*, 1996; Tsukamoto *et al.*, 1996; Chen *et al.*, 2001). O processo é finalizado com o processamento – onde atuam Rad27p e Pol4p (Wu *et al.*, 1999; Daley *et al.*, 2005) e ligação das extremidades por Dnl4p/Lif1p (Figura 7; Wilson *et al.*, 1997; Herrmann *et al.*, 1998; Teo e Jackson, 2000).

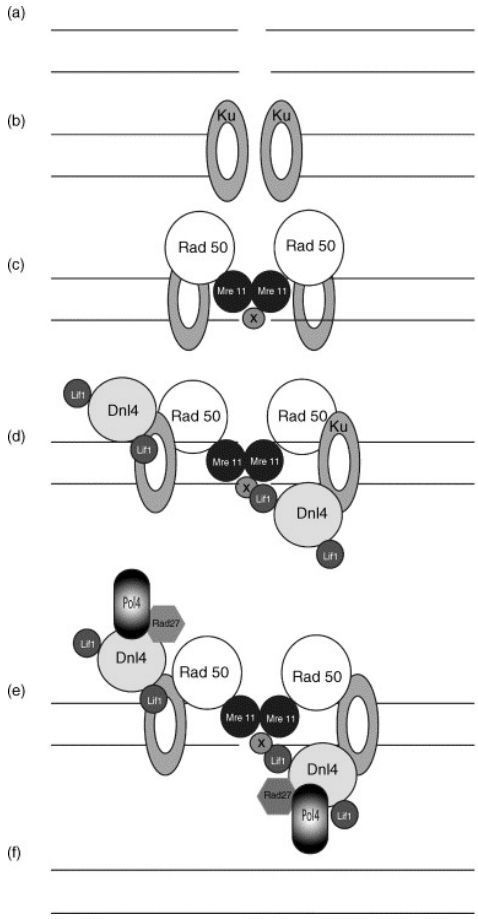

Figura 7. Modelo da via NHEJ em *S. cerevisiae*. Seguindo a formação de uma quebra dupla **(a)**, o passo inicial é a ligação das extremidades por Ku70/80 **(b)**. O complexo Mre11/Rad50 /Xrs2 associa-se ao heterodímero Ku, já ligado ao DNA, formando uma ponte entre as extremidades **(c)**. A DNA ligase Dnl4, específica da via NHEJ, e seu fator associado Lif1 são recrutados ao sítio da quebra **(d)**, ocorrendo processamento pela endonuclease Rad27 e preenchimento da lacuna pela DNA polimerase Pol4 **(e)**, antes das extremidades serem religadas por Dnl4 **(f)** (extraído de Hefferin e Tomkinson, 2005).

1.2.3 Processos de tolerância a danos ou reparação pós-replicativa

Como visto nos itens anteriores, uma variedade de processos existe para que as células reparem os danos no DNA, a fim de garantir que a fidelidade do genoma seja mantida. No entanto, na ausência de um sistema que resolva uma forquilha de replicação bloqueada, a decorrente parada no ciclo-celular pode levar, em última análise, à morte celular. Por este motivo, mecanismos que permitem o desvio (*bypass*) de lesões e a conclusão da replicação, sem a real remoção dos danos, são essenciais para a sobrevivência das células (Lehmann, 2002; McGlynn e Lloyd, 2002). Estes mecanismos são coletivamente chamados de processos de tolerância a danos ou reparação pós-replicativa (*post-replication repair* – PRR; para revisão, ver Friedberg, 2005).

A PRR compreende tanto processos livres de erro, quanto sujeito a erro. A via sujeita a erro envolve síntese translesão (*translesion synthesis* - TLS), onde a DNA polimerase replicativa é substituída por uma DNA polimerase com fidelidade reduzida. A natureza das vias livres de erro é menos clara, mas deve envolver recombinação e/ou eventos de reversão da forquilha de replicação. Até recentemente, havia pouca informação sobre como ocorreria esta canalização e como seria escolhida uma via em detrimento da outra. No entanto, nos últimos anos tem sido mostrado que o antígeno nuclear de proliferação celular (*proliferating cell nuclear antigen* – PCNA) – o fator de processividade de DNA polimerases, envolvido em síntese e reparação de DNA (para revisão, ver Maga e Hubscher, 2003) - tem papel fundamental na determinação das conseqüências do encontro do dano pela forquilha de replicação (Hoege *et al.*, 2002; Stelter e Ulrich, 2003; Pfander *et al.*, 2005; Papouli *et al.*, 2005).

Em *S. cerevisiae*, a via PRR canaliza as lesões para diferentes vias de desvio do dano, onde atuam proteínas do grupo de epistasia Rad6 (Lawrence e Hinkle, 1996). A

proteína Rad6 é uma ubiquitina-conjugante (Jentsch *et al.*, 1987), a qual, em conjunto com Rad18p (Bailly *et al.*, 1994), mono-ubiquitina a lisina 164 do PCNA (Hoege *et al.*, 2002), uma modificação que promove TLS (Stelter e Ulrich, 2003). O processo seguinte emprega a DNA polimerase ζ, cujas duas subunidades são codificadas pelos genes *REV3* e *REV7* (Nelson *et al.*, 1996a; Lawrence, 2004), a DNA polimerase η, codificada por *RAD30* (Johnson *et al.*, 1999) e Rev1p, uma desoxicitidil-transferase (Nelson *et al.*, 1996b) que também participa na TLS (Figura 8a) (Nelson *et al.*, 2000).

Por outro lado, a poli-ubiquitinação da lisina 164, efetuada pelo complexo Rad5p/Ubc13p/Mms2p (Ulrich e Jentsch, 2000), promove o processo de tolerância ao dano, de uma forma livre de erro (Hoege *et al.*, 2002). Este processo evita o uso da região lesada como molde para síntese de DNA, utilizando a fita recém sintetizada da cromátide irmã para restaurar a seqüência (Cox, 2002; McGlynn e Lloyd, 2002). Apesar de não se conhecer detalhes sobre este mecanismo em eucariotos, acredita-se que este envolva uma reversão temporária da forquilha de replicação, o que permitiria o pareamento das duas fitas recém sintetizadas, em uma estrutura chamada "pé-de-galinha" (*chicken-foot*; Figura 8b) (Ulrich, 2005).

Este mesmo resíduo de lisina pode ainda sofrer modificação por SUMO (*small ubiquitin-like modifier*), já tendo sido demonstrado que a substituição desta lisina por uma arginina (resíduo incapaz de atuar como aceptor tanto de ubiquitina, quanto de SUMO) resulta em sensibilidade a agentes indutores de danos no DNA (Hoege *et al.*, 2002; Stelter e Ulrich, 2003). Quando modificado por SUMO, PCNA recruta a helicase Srs2, originalmente identificada como supressora de mutantes *rad6* e *rad18* (Lawrence e Christensen, 1979). Esta proteína é capaz de inibir recombinação através de sua capacidade de dissociar filamentos nucleoprotéicos de Rad51 (Krejci *et al.*, 2003; Veaute *et al.*, 2003). Logo, o papel de PCNA modificado por SUMO seria prevenir a

associação de Rad51p à forquilha bloqueada, caso contrário seria desencadeado um evento de recombinação homóloga não vantajoso (Figura 8c; Watts, 2006).

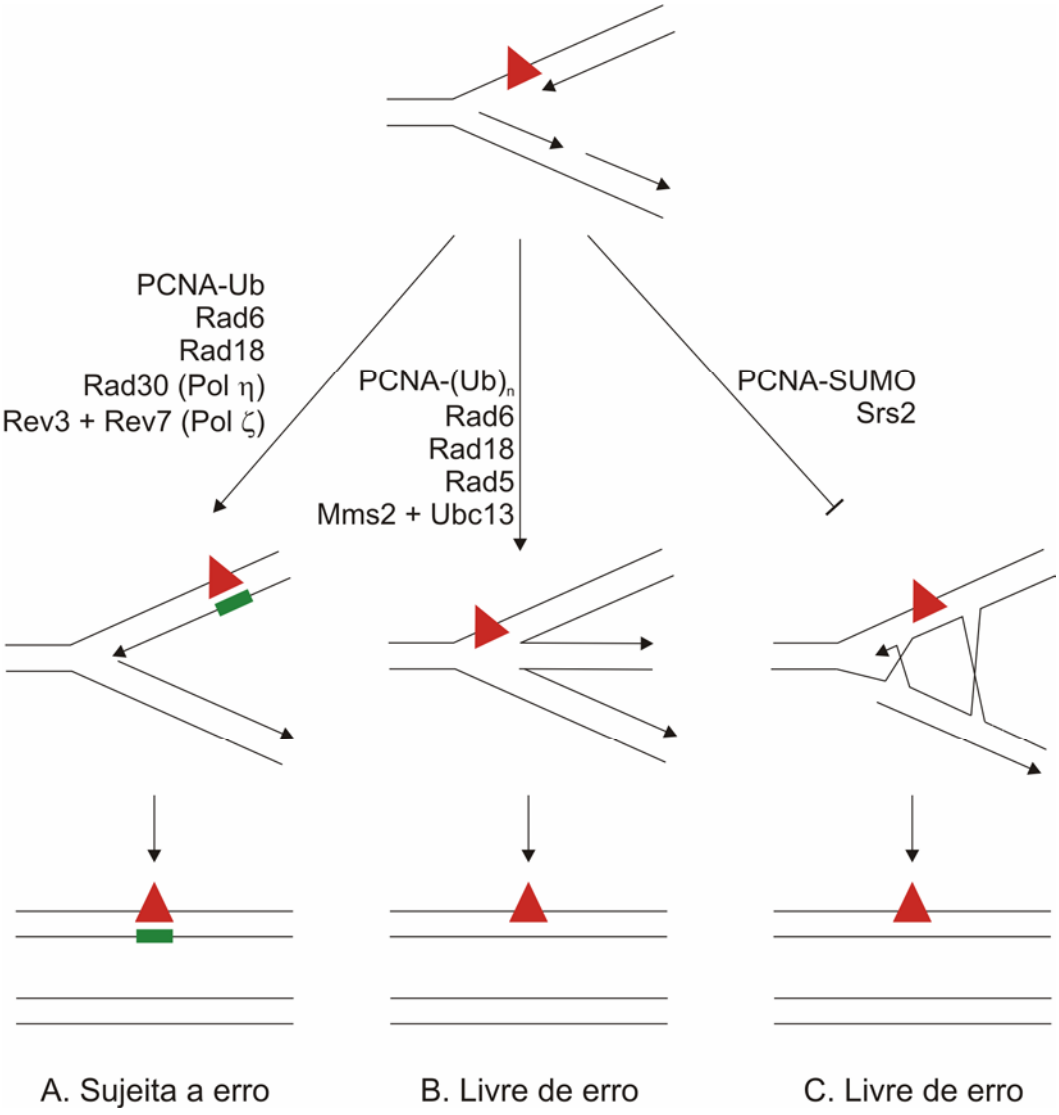

Figura 8. Possíveis conseqüências que sucedem um bloqueio na forquilha de replicação, devido a danos no DNA. **(A)** A via sujeita a erro resulta na incorporação de uma base incorreta (■), por uma polimerase de síntese translesão (Pol η ou Pol ζ) em face à lesão (▲). **(B)** Uma via livre de erro poderia envolver troca de molde, sem a participação de Rad51p, podendo estar associada à regressão temporária da forquilha **(C)** Outro processo livre de erro dependente de recombinação poderia envolver a invasão da fita descontínua pela contínua para fazer o desvio da lesão. Esta via pode requerer Rad51p e ser inibida por Srs2p (adaptado de Watts, 2006).

1.2.4 DNA polimerases replicativas na reparação do DNA

Em eucariotos, a replicação cromossomal é garantida por pelo menos três DNA polimerases essenciais: polimerase α (Pol α), polimerase δ (Pol δ) e polimerase ε (Pol ε). A Pol α, em conjunto com a primase, está envolvida na iniciação dos fragmentos de Okazaki e é composta de quatro polipeptídeos (Lucchini *et al.*, 1985), cada um codificado por um gene essencial (Campbell e Newlon, 1991; Campbell, 1993). A Pol δ - que possui três unidades, em *S. cerevisiae* - é a polimerase da fita descontínua e evoluiu para lidar eficientemente com a maturação dos fragmentos de Okazaki (Gerik *et al.*, 1998). Finalmente, a Pol ε consiste em um heterotetrâmero (Chilkova *et al.*, 2003) e é requerida para a síntese da fita contínua, sendo provida de atividade de 'leitura de prova' (*proofreading*) (Wintersberger e Wintersberger, 1970; Morrison *et al.*, 1990). Informações sobre a identidade e função das subunidades estão resumidas na Tabela 1 (para revisão, ver Hubscher *et al.*, 2002; Garg e Burgers 2005).

Tabela 1. Estrutura das DNA polimerases replicativas de *S. cerevisiae*

DNA polimerase	Massa molecular da subunidade (kDa)	Gene	Essencial?	Função principal
α	165	*POL1*	Sim	Subunidade catalítica
	86	*POL12*	Sim	Estrutural; interações proteína-proteína
	58	*PRI2*	Sim	Primase
	49	*PRI1*	Sim	Primase
δ	125	*POL3*	Sim	Subunidade catalítica
	58	*POL31*	Sim	Estrutural
	55	*POL32*	Não[a]	Multimerização; interação com PCNA
ε	256	*POL2*	Sim	Subunidade catalítica
	80	*DPB2*	Sim	Multimerização
	23	*DPB3*	Não	Estrutural; interações proteína-proteína
	22	*DPB4*	Não	Estrutural; interações proteína-proteína

[a] Essencial em *Schizosaccharomyces pombe*

Há uma diversidade de substratos de DNA que se apresentam para as DNA polimerases, indo desde estruturas com lacunas relativamente simples até forquilhas de replicação complexas, nas quais ambas as fitas precisam ser replicadas simultaneamente. Sendo assim, seria esperado que as células desenvolvessem um conjunto bem definido de DNA polimerases, com cada uma unicamente adaptada para uma via específica. E até determinado grau, este parece ser o caso, como abordado na seção anterior. Mas ocorre que uma DNA polimerase em particular pode ter mais de

uma tarefa funcional na célula e determinados substratos de DNA podem requerer mais de uma polimerase, o que sugere que a evolução tenha providenciado que polimerases replicativas tivessem tarefas adicionais, além de duplicar o genoma. De fato, estudos genéticos e bioquímicos têm demonstrado o envolvimento das polimerases α, δ e ε na reparação de DNA.

Mutantes defectivos na subunidade catalítica da Pol δ mostram alta sensibilidade à metilação de bases por MMS, o que foi ligado diretamente a sua função em BER (Blank *et al.* 1994; Jin *et al.*, 2003; Wang *et al.*, 1993), e também apresentam bloqueio parcial na mutagênese e recombinação induzidas (Giot *et al.*, 1997). A ausência da subunidade Pol32 também altera a mutagênese induzida e aumenta a freqüência de deleções em levedura, associando Pol δ à via mutagênica (Huang, 2000; 2002). Esta mesma subunidade mostrou-se ser a mediadora da interação entre Pol δ e WRN (a proteína responsável pela síndrome de Werner), sugerindo que esta seria requerida para processar o dano no DNA e Pol δ, efetuar a síntese de reparação (Kamath-Loeb *et al.*, 2000). Recentemente, foi evidenciado que Pol32 é requerida para o desvio de sítios abásicos e (6,4)-fotoprodutos (Gibbs *et al.*, 2005). Em células humanas, foi demonstrado que Pol δ também participa em MMR (Longley *et al.*, 1997) e a alta incidência de tumores em camundongos deficientes na atividade de *proofreading* desta polimerase corrobora sua participação nesta via (Goldsby *et al.* 2001; 2002).

De forma semelhante à Pol δ, a Pol ε também mostrou atuação em BER (Wang *et al.*, 1993), e adicionalmente em NER (Aboussekhra *et al.*, 1995; Stucki *et al.*, 1998). Recentemente, McCulloch *et al.* (2004) mostraram a importância desta polimerase no desvio de dímeros de timina.

Finalmente, a Pol α é a que mostra atuação mais limitada na reparação do DNA, mas é sugerida sua participação na reparação de DSBs, por um mecanismo que envolve

a geração de uma forquilha de replicação modificada (Holmes e Haber, 1999) e também na remoção de CPDs, em extratos de oócitos de *Xenopus* (Oda *et al.*, 1996).

A soma destas evidências aponta a Pol δ como a principal DNA polimerase replicativa para a maioria das vias de reparação de DNA, enquanto a Pol ε seria capaz de substituí-la em BER e NER. Uma função mais clara na reparação ainda está por ser encontrada para a Pol α, em leveduras e células de mamíferos.

1.3 Os genes *PSO*

A fotoquimioterapia empregando psoralenos e luz UVA (PUVA terapia) é largamente utilizada para tratar desordens dermatológicas (ver item 1.1.2). Os produtos formados pela fotoativação de psoralenos nos fibroblastos da pele e em células tumorais são responsáveis pela eficácia deste tratamento. Contudo, embora a PUVA terapia seja efetiva na terapia clínica, os fotoprodutos resultantes são altamente citotóxicos, gerando espécies reativas de oxigênio e inibindo a replicação e a transcrição do DNA genômico e mitocondrial. Neste sentido, as alterações promovidas pela PUVA terapia no metabolismo de DNA podem resultar em diferentes tipos de mutações, em quebras simples e duplas e danos ao nível cromossomal. Como conseqüências gerais, a PUVA terapia pode induzir apoptose e elevar os riscos de surgimento de tumores, de um modo dose-dependente (Dronkert e Kanaar, 2001; Greenberg *et al.,* 2001).

Com o objetivo de estudar as conseqüências fotoquímicas, biológicas e genéticas da PUVA terapia em células vivas, Henriques e Moustacchi (1980) isolaram, a partir de uma população de células mutagenizadas com etil-metanossulfonato (EMS), uma nova classe de mutantes (*pso*) de *S. cerevisiae,* sensíveis à fotoadição de psoralenos mono- (3-CPs) e bi-funcionais (8-MOP). A análise molecular desses genes e a caracterização

fenotípica de seus alelos mutantes têm progredido consideravelmente (para revisão ver, Henriques *et al.,* 1997; Brendel e Henriques, 2001; Brendel *et al.,* 2003). Atualmente, dez genes *PSO* estão caracterizados fenotipicamente, sendo que nove foram identificados molecularmente (Tabela 2; para revisão ver, Brendel *et al.,* 2003). A clonagem e a caracterização molecular permitiu o agrupamento desses genes em três classes funcionais. O maior grupo compreende sete genes PSO que estão ou amplamente, ou especificamente envolvidos em reparação de DNA sujeita a erro e logo afetam mutabilidade e recombinação induzidas; um gene PSO (*PSO5*) que representa a via de excisão livre de erro (Paesi-Toresan *et al.*, 1995); e dois genes PSO (*PSO6* e *PSO7*) codificando proteínas que não estão relacionadas a ácidos nucléicos, mas participam na manutenção do equilíbrio redox intracelular (Schmidt *et al.*, 1999; Pungartnik *et al.*, 1999). Entre os sete genes de reparação envolvidos em mutagênese induzida, três locos (*PSO1/REV3*, *PSO8/RAD6*, *PSO9/MEC3*) mostraram-se alélicos a genes de reparação já conhecidos (Cassier-Chauvat e Moustacchi, 1998; Rolla *et al.*, 2002; Cardone, 2002), enquanto três, *PSO2/SNM1*, *PSO3/RNR4*, e *PSO4/PRP19* representam novos genes envolvidos em reparação e metabolismo de ácidos nucléicos em *S. cerevisiae*. O gene *PSO2* codifica uma proteína indispensável para a reparação de ICLs e parece ser importante para uma função de reparação similar em humanos (Bonatto *et al.*, 2005; Li *et al*., 2005; Revers *et al.*, artigo submetido [Anexo 1]). A redução acentuada da mutação induzida em mutantes *pso3/rnr4* indica um importante papel desta subunidade da ribonucleotídeo redutase (RNR) na regulação da DNA polimerase ζ, na reparação sujeita a erro (Cassier *et al.*, 1980; Moustacchi *et al.*, 1983; Brendel *et al.,* 2003). A proteína Pso4/Prp19 influencia a eficiência de reparação via processamento de mRNAs de genes de reparação interrompidos, mas sua atuação na estabilidade da matriz nuclear também pode estar associada à capacidade de reparação

(Revers *et al.*, 2002). O gene *PSO10* controla um passo ainda desconhecido na mutagênese induzida, já que permanece por ser clonado (para revisão, ver Brendel *et al.,* 2003).

Tabela 2. Propriedades das proteínas codificadas pelos genes *PSO* e os fenótipos dos seus alelos mutantes

Gene/Alelo	**Proteína (kDa)/Função**	**Fenótipo do mutante**
PSO1/REV3	173; DNA polimerase translesão	Sensível à radiação e a agentes mutagênicos químicos; baixa mutabilidade
PSO2/SNM1	72; endo/exonuclease	Sensível ao tratamento com todos os agentes indutores de ICLs
PSO3/RNR4	40; ribonucleotídeo redutase	Redução na mutabilidade induzida e recombinação; as células não atingem a fase estacionária de crescimento
PSO4/PRP19	57; proteína associada ao spliceossomo	Sensível a agentes mutagênicos, não recombina em mitose, baixa muta-bilidade e defectivo em esporulação
PSO5/RAD16	91; DNA helicase	Sensível à UVC e lesões oxidativas
PSO6/ERG3	43; ergosterol dessaturase	Sensível a danos oxidativos
PSO7/COX11	28; associa-se ao complexo da citocromo oxidase	Células sensíveis a 4-NQO
PSO8/RAD6	19; ubiquitina-conjugante	Sensível à UVC e a vários agentes mutagênicos; mutagênese reduzida
PSO9/MEC3	53; controle da parada de ciclo celular, em resposta a danos no DNA	Sensível a vários agentes mutagênicos; mutagênese reduzida
PSO10	Desconhecida	Sensível a vários agentes mutagênicos; mutagênese reduzida; bloqueado em esporulação

1.3.1 O gene *PSO9*

Continuando a análise dos mutantes isolados por Henriques e Moustacchi (1980) um novo mutante *pso*, denominado *pso9-1*, foi caracterizado fenotipicamente (Machado, 2000). Este mutante mostra sensibilidade a diversos agentes mutagênicos, incluindo UVC, 3-CPs e 8-MOP fotoativados, e MNNG (metil-nitro-nitrosoguanidina). Adicionalmente, apresenta redução na mutagênese induzida, quando tratado com psoralenos fotoativados e UVC. Essa mutação não afeta a esporulação, e quando submetido a agentes oxidativos como H_2O_2 e paraquat, o mutante *pso9-1* mostra resistência semelhante à linhagem selvagem (Machado, 2000).

A clonagem molecular do gene *PSO9* revelou que este é alelo do gene *MEC3* (Cardone, 2002). Mutantes *mec3* já haviam sido descritos por sua sensibilidade à UVC, MMS e hidroxiuréia (Longhese *et al.*, 1996) e parecem reduzir a mutabilidade induzida após tratamento com UVC (Paulovich *et al.*, 1998). A proteína codificada por este gene está envolvida em *checkpoint*, uma respeitável estratégia que coopera para o sucesso da manutenção da integridade/estabilidade genômica.

1.3.1.1 Pso9p/Mec3p e *checkpoint* em *S. cerevisiae*

A sobrevivência dos organismos é dependente de uma acurada transmissão da informação genética de uma célula para suas filhas. Para alcançar esta fidelidade, as células têm evoluído mecanismos de sobrevivência que monitoram a estrutura dos cromossomos e coordenam reparação de DNA e progressão do ciclo celular, chamados *checkpoints* (para revisão, ver Elledge, 1996; Paulovich *et al.*, 1997; Weinert, 1998; Melo e Toczyski, 2002; Longhese *et al.* 2003; Kai e Wang, 2003). Estes mecanismos foram inicialmente definidos como vias regulatórias não-essenciais que controlam a capacidade da célula de parar o ciclo-celular em resposta a danos no DNA, concedendo

tempo para a reparação. No entanto, recentes evidências sugerem que a definição clássica de *checkpoint* é inadequada para explicar a função deste mecanismo por completo. Além de parar o ciclo-celular, tem sido demonstrado que estas vias controlam a ativação das vias de reparação de DNA (Cortez *et al*., 1999; Mercier *et al.*, 2001; Smirnova *et al.*, 2005; DeMase *et al.*, 2005), composição da cromatina telomérica e extensão dos telômeros (Martin *et al.*, 1999; Naito *et al.*, 1998; Ritchie *et al.*, 1999, Enomoto *et al.*, 2002; Bi *et al.*, 2005; Viscardi *et al.*, 2005), direcionamento das proteínas de reparação de DNA aos sítios de dano (Mills *et al.*, 1999; Lukas *et al.*, 2004), ativação de programas transcricionais (Elledge, 1996; Ishii *et al.*, 2005; Zaim *et al.*, 2005; Yin *et al.*, 2004) e, em alguns tipos de células, indução de morte celular por apoptose (Lowe *et al.*, 1993; Hirao *et al.*, 2000; Jiang *et al.*, 2005; Chen *et al.*, 2005; Bhonde *et al.*, 2006). Desta forma, fica claro que o *checkpoint* faz parte de uma complexa rede que responde a danos no DNA, a qual regula uma resposta multifacetada, como ilustra a Figura 9.

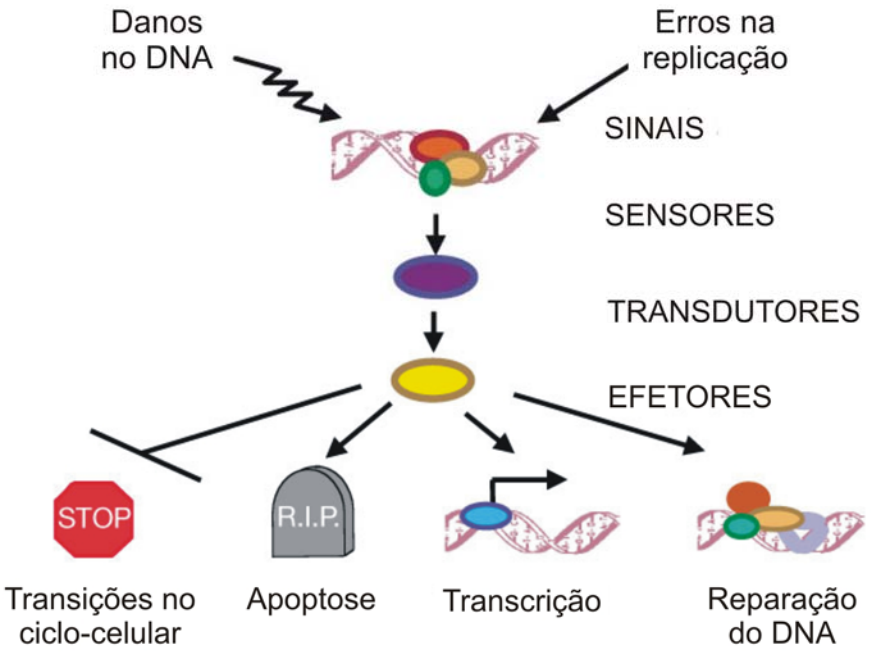

Figura 9. Esquema geral da via de transdução de sinais em *checkpoint*. Flechas representam eventos de ativação e finais perpendiculares representam eventos inibitórios. A hélice de DNA com uma flecha representa transcrição induzida por dano, enquanto a hélice de DNA com várias subunidades circulares representa reparação induzida por dano (adaptado de Zhou e Elledge, 2000).

Como todas as vias de transdução de sinais, o *checkpoint* envolve moléculas que atuam como sensores, transdutores e efetores. Os sensores monitoram o DNA para anormalidades estruturais e iniciam o sinal de *checkpoint*. Mais adiante, os transdutores transmitem e amplificam este sinal. Finalmente, as moléculas efetoras controlam as conseqüências biológicas desencadeadas pela via.

Uma das questões mais cruciais neste campo é definir os sinais que ativam o *checkpoint*. Agentes genotóxicos causam muitos tipos de lesões primárias que podem ser convertidas em lesões secundárias durante a replicação de um molde danificado. Todas estas lesões podem ser diretamente reconhecidas por determinadas proteínas de *checkpoint*, sozinhas ou em complexos especializados, ou ainda podem ser processadas

a intermediários comuns que por sua vez irão desencadear a ativação do *checkpoint*. Como esta resposta igualmente pode ser influenciada pelo estágio do ciclo-celular no qual o dano ocorre, múltiplos sensores podem reconhecer os sinais, em fases específicas do ciclo-celular (para revisão, ver Melo e Toczyski, 2002; Longhese *et al.*, 2003; Kai e Wang, 2003).

Dois complexos distintos localizam-se independentemente aos sítios de dano, em *S. cerevisiae*, mas a presença de ambos os complexos é necessária para uma função adequada de *checkpoint* (Kondo *et al.*, 2001; Melo *et al.*, 2001; Rouse e Jackson, 2002). A proteína-cinase Mec1 forma um complexo com Ddc2p e pode atuar tanto no reconhecimento de danos, quanto na transdução destes sinais (Rouse e Jackson, 2002; Sun *et al.*, 1996). O outro grupo compreende as proteínas Rad24, Rad17, Mec3 e Ddc1, o qual atua principalmente no reconhecimento e processamento dos danos. A proteína Rad24 apresenta similaridade de seqüência com a subunidade Rfc1 do fator de replicação C (RFC) e interage com as subunidades menores de RFC (Griffths *et al.* 1995; Lydall e Weinert, 1997; Shimomura *et al.*, 1998). O RFC é um complexo heteropentamérico, consistindo de uma subunidade maior, Rfc1, e as quatro subunidades menores Rfc2-5, o qual adiciona PCNA ao redor das junções *primer*-molde de DNA, em um processo dependente de ATP (Ellison e Stillman, 2001). Por sua vez, o complexo formado por Rad17p, Mec3p e Ddc1p (Paciotti *et al.*, 1998) compartilha similaridade de seqüência com PCNA (Caspari *et al.*, 2000; Venclovas e Thelen, 2000). Como abordado na seção 1.2.3, PCNA é o fator de processividade das DNA polimerases δ e ε, e atua como plataforma para integrar múltiplos fatores envolvidos em replicação, reparação de DNA, montagem da cromatina e regulação do ciclo-celular (para revisão, ver Maga e Hubscher, 2003).

Algoritmos para predição de dobramento de proteínas já haviam sugerido uma estrutura PCNA-*like* para Rad17/Mec3/Ddc1 e que este complexo seria adicionado ao DNA por uma versão modificada de RF-C, mediada por Rad24p (RFC-Rad24). Este complexo atuaria como um grampo específico de detecção de danos que, uma vez ligado ao DNA, tornaria-se competente a recrutar fatores adicionais de *checkpoint*, responsáveis pela transdução de sinais (Figura 10) (Venclovas e Thelen, 2000; Melo *et al.*, 2001; Melo e Toczyski, 2002; Venclovas *et al.*, 2002; Majka e Burgers, 2004). Esta analogia de estrutura-função foi confirmada recentemente, mostrando que após a hidrólise de ATP, o grampo Rad17/Mec3/Ddc1 desprende-se de RFC-Rad24 e consegue percorrer mais de 1 Kb de dúplex de DNA, o que se torna muito adequado para desempenhar sua função de monitoramento (Majka e Burgers, 2003).

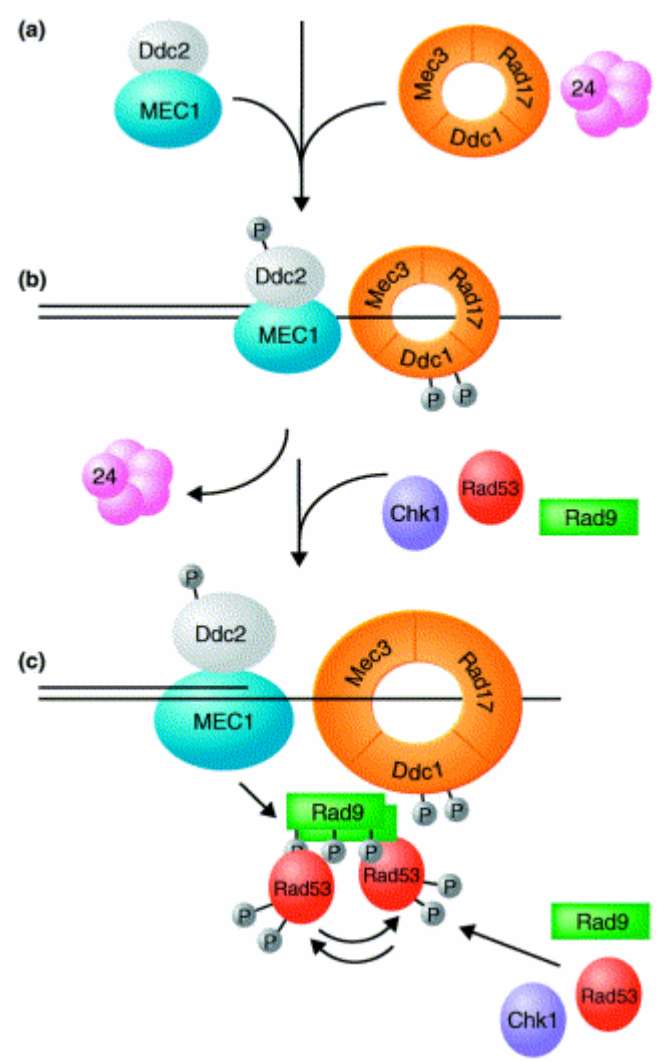

Figura 10. A) Modelo para ativação de *checkpoint* que responde a danos no DNA. **(a)** Os complexos Mec1/Ddc2 (azul) e Rad17/Mec3/Ddc1 (laranja) são independentemente recrutados aos sítios de dano no DNA, **(b)** promovendo sua interação por um efeito dependente da concentração de dano. **(c)** múltiplos complexos Mec1/Ddc2 e Rad17/Mec3/Ddc1 são recrutados ao sítio de dano, promovendo uma rápida ativação da via de *checkpoint*, onde o recrutamento de Rad17/Mec3/Ddc1 é catalisado por RFC-Rad24 (rosa) (extraído de Melo e Toczyski, 2002).

As proteínas de *checkpoint* são bem conservadas de leveduras a células humanas (para revisão, ver Venclovas e Thelen, 2000; Kai e Wang, 2003), indicando que a organização básica destas vias tem sido preservada ao longo da evolução. Alguns dos componentes destas vias encontram-se mutados em síndromes humanas raras, estando associados à predisposição ao câncer (para revisão, ver Hartwell e Kastan, 1994; McDonald e El-Deiry, 2001; Baker *et al.*, 2005). Portanto, torna-se relevante investigar os papéis destes componentes em organismos modelos, podendo-se assim utilizar estes dados para o esclarecimento dos mecanismos de controle da divisão celular e reparação de DNA em eucariotos superiores.

CAPÍTULO II

OBJETIVOS

1. Objetivo geral

Com a finalidade de buscar novas informações que possam auxiliar na compreensão dos mecanismos implicados na reparação de lesões induzidas por psoralenos fotoativados, este trabalho foi conduzido com o objetivo principal de ampliar a caracterização molecular e bioquímica da forma modificada de Pso9p/Mec3p, Pso9-1p, avaliando sua atuação com seus parceiros moleculares na reparação do DNA, em *Saccharomyces cerevisiae*.

2. Objetivos específicos

- Identificar o local e o tipo de mutação do alelo mutante *pso9-1*, possibilitando avaliar os seus efeitos na função da proteína Pso9/Mec3;

- Definir os domínios de Pso9p/Mec3p requeridos para as interações com seus parceiros de complexo Rad17p e Ddc1p, através da expressão de construções truncadas do gene *PSO9/MEC3*, utilizando o 'Sistema Dois-Híbridos';

- Avaliar o perfil de ciclo-celular do mutante *pso9-1* diante tratamento com 8-MOP+UVA;

- Analisar os efeitos da combinação da mutação *pso9*Δ*/mec3*Δ com mutações em genes envolvidos na replicação do DNA, por meio de genética clássica;

- Avaliar o papel de DNA polimerases replicativas na reparação de danos induzidos por psoralenos fotoativados.

CAPÍTULO III

Psoralen-sensitive mutant *pso9-1* of *Saccharomyces cerevisiae* contains a mutant allele of the DNA damage checkpoint gene *MEC3*

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Psoralen-sensitive mutant *pso9-1* of *Saccharomyces cerevisiae* contains a mutant allele of the DNA damage checkpoint gene *MEC3*

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Abstract

Complementation analysis of the *pso9-1* yeast mutant strain sensitive to photoactivated mono- and bifunctional psoralens, UV-light 254 nm, and nitrosoguanidine, with *pso1* to *pso8* mutants, confirmed that it contains a novel *pso* mutation. Molecular cloning via the reverse genetics complementation approach using a yeast genomic library suggested *pso9-1* to be a mutant allele of the DNA damage checkpoint control gene *MEC3*. Non-complementation of several sensitivity phenotypes in *pso9-1/mec3*Δ diploids confirmed allelism. The *pso9-1* mutant allele contains a −1 frameshift mutation (deletion of one A) at nucleotide position 802 (802delA), resulting in nine different amino acid residues from that point and a premature termination. This mutation affected the binding properties of Pso9-1p, abolishing its interactions with both Rad17p and Ddc1p. Further interaction assays employing *mec3* constructions lacking the last 25 and 75 amino acid carboxyl termini were also not able to maintain stable interactions. Moreover, the *pso9-1* mutant strain could no longer sense DNA damage since it continued in the cell cycle after 8-MOP + UVA treatment. Taken together, these observations allowed us to propose a model for checkpoint activation generated by photo-induced adducts.

Keywords: *Saccharomyces cerevisiae*; Psoralen sensitivity; *MEC3*; *PSO9*; DNA repair; Checkpoint

1. Introduction

The isolation of yeast mutants specifically sensitive to photoactivated mono- and bifunctional psoralens, so-called *pso* mutants, was initiated by Henriques and Moustacchi (1980) in order to study possible genotoxic effects of these medically important furocoumarins that are applied in the photo-therapy of dermatological disorders like psoriasis and vitiligo [1]. Depending on their molecular structure photoactivated psoralens, upon their photo-activation with UVA (UV-light of 360 nm), may form mono- and di-adducts with thymine in DNA [2–6]. The di-adduct DNA lesion results from two consecutive reactions of, for example, 8-MOP with two thymine residues of opposite DNA strands and leads to DNA inter-strand cross-links (ICL). Psoralen-sensitive mutants, therefore, are putatively DNA repair-deficient. Psoralen + UVA induced ICL are very stable and may constitute one of the most toxic types of DNA damage encountered by yeast as one to two ICL per chromosome, i.e. 20 ICL contained in 16 chromosomes of a haploid *Saccharomyces cerevisiae* wild-type strain, are lethal [7–9]. Studies of *pso* mutants have contributed to our knowledge on the different interactions of photoactivated psoralen-induced DNA damage with repair functions that, when impaired or absent, may alter viability and mutagenic responses.

Phenotypic, genetical and molecular analysis of the hitherto known eight *pso* mutants have allowed their separation into three groups; five which either directly or indirectly play a role in the mechanisms of error-prone DNA repair, two that lack protection against reactive oxygen species (ROS) or had an altered energy metabolism, and one that represents error-free excision repair. Of the five DNA repair genes involved in induced mutagenesis two PSO loci [*PSO1/REV3* and *PSO8/RAD6*] were allelic to already known repair genes, whereas three, *PSO2/SNM1*, *PSO3/RNR4*, and *PSO4/PRP19* represent new genes involved in

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DNA repair and nucleic acid metabolism of *S. cerevisiae*. Gene *PSO2* encodes a protein indispensable for repair of ICL and may have a role in non-homologous end joining [10]. The low induced mutability of *pso3/rnr4* mutants indicates an important role of this subunit of ribonucleotide reductase (RNR) in regulation or function of translesion polymerase zeta in error-prone repair (reviewed in [11]). Prp19p/Pso4p influences efficiency of DNA repair via splicing of pre-mRNAs of intron-containing repair genes but also may function in the stability of the nuclear scaffold that might influence DNA repair capacity [12]. Mutations in genes *PSO6* (allelic to *ERG3*) and *PSO7* (allelic to *COX11*) alter the respective mutants' sensitivities by interfering with protective mechanisms [13] or by modulating the metabolism of certain mutagens [14], respectively.

Maintaining genome integrity is the main challenge for living cells. To achieve this cells have, therefore, evolved surveillance mechanisms, the so-called checkpoints, that monitor the structure of chromosomes and coordinate DNA repair and cell-cycle progression. Such mechanisms refer to the biochemical pathways which are activated in response to internal and external aggressions (DNA damage), responsible for the induction of transcriptional programs for the inhibition of cell-cycle progression. This would allow DNA repair before completion of cell division (for reviews, see [15–18]).

To extend our understanding of the genetical and biochemical basis of UVA-activated psoralen-induced DNA repair we have identified and cloned a further *pso* mutant from the original stock of mutagenized yeast cells [19]. In this report, we describe the phenotypic characteristics and the molecular cloning of this mutant which complemented all hitherto described *pso* mutant strains (*pso1* to *pso8*). By classical and molecular genetics methods we show that the mutagen sensitivity of the yeast strain containing mutant allele *pso9-1* is due to a deficiency in an important checkpoint protein, encoded by the yeast *MEC3* gene.

2. Materials and methods

2.1. Strains, plasmids and media

The genetic constitution of the yeast strains used in this study is given in Table 1. The *Escherichia coli* strain TOP10 (Invitrogen) was employed for plasmid manipulation and propagation. A yeast genomic library contained in the centromeric vector pRS200 was used for molecular cloning of *PSO9*. pRS200 is a modification of pRS314 by replacing *Sma*I with *Bgl*II linkers [20]. DNA fragments of interest were sub-cloned either into the centromeric vector pRS314 [20] or into pCR® Blunt vector (Invitrogen). Yeast media and bacterial genetic procedures were as described by Burke et al. [23] and Sambrook et al. [24], respectively. Yeast was routinely grown and stored on YPD. Auxotrophy markers were controlled on synthetic medium—SynCo (1.7 g yeast nitrogen base w/o amino acids and w/o ammonium sulfate/L, 5 g ammonium sulfate/L, 20 g glucose/L) supplemented with the appropriate amino acids and bases (40 μg/mL).

2.2. Mutagen treatments

Stationary phase cells were washed in 0.9 % NaCl and resuspended in the same buffer to a titer of 10^8/mL. Treatment with the furocoumarins 8-methoxypsoralen (8-MOP) and 3-carbethoxypsoralen (3-CPs) was as previously described [19]. Sensitivity to $UV_{254\,nm}$ (UVC) was assayed by irradiating (Stratalinker, Stratagene) cells plated on solid medium. Cells were treated with *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG [SIGMA] CAS No. 70-25-7) at exposure doses ranging from 0 to 200 μM, for 18 h in a shaker at 30 °C. For drop tests, stationary cultures were serially diluted in 1:10 steps and 5 μL aliquots were then spotted onto SynCo medium and treated as above.

2.3. Mutation induction

Reversion was detected by measuring frequencies of Lys revertants (*lys1* → *LYS1*) on SynCo-Arg-Lys media and forward mutation was measured with the canavanine resistance assay (CAN1 → $can1^R$) after induction with UVC, 3-CPs + UVA and 8-MOP + UVA. Concentration of canavanine in SynCo-Arg medium was 40 mg/mL [25]. Mutant frequencies were scored per surviving cells.

2.4. Molecular cloning

Yeast *pso9-1* mutant strain JC003-8C was transformed with a yeast genomic library contained in the centromeric pRS200 vector [20]. Transformants were selected in synthetic medium lacking tryptophan (SynCo-Trp) and then screened for sensitivity to UVC (50 J/m^2 UVC dose) and, afterwards to 8-MOP + UVA (0.5 kJ/m^2 UVA dose). In cases of wild-type-like growth, transformants were subjected to a plasmid loss experiment and assayed for concomitant loss of tryptophan prototrophy, UVC and 8-MOP + UVA resistance. The resistant isolate had its plasmid extracted and transformed into *E. coli* for propagation. Sequencing was performed using T3 and T7 primers (Amersham Biosciences) in an automated sequencer (ABI PRISM® 3100—Applied Biosystems), according to the manufacturer's instructions.

2.5. Identification of pso9-1 mutant

PCRs of genomic DNA, using specific primers [MEC3F (5′-ATCATGCATTCGTTCAAA-3′) and MEC3R (5′-GCTTTGGTGTACCATTCC-3′)] and a high fidelity thermostable DNA polymerase (Platinum® *Pfx*, Invitrogen)

Table 1
Yeast strains used in this study

Strain	Genotype	Source
N123	*MAT***a** *his1-1 gsh1-L PSO9*	[19]
$N123_{35(11)}$	*MAT***a** *his1-1 gsh1-L pso9-1*	[19]
MKPo	*MATα can1-100 ade2-1 lys2-1 ura3-52 leu2-3 his3Δ200 trp1Δ901*	[20]
RMH2001-1B	*MAT***a** *his3Δ200 ade2-1 lys1-1 ura3-52 GSH1 pso9-1*	This work
RMH2001-1C	*MAT***a** *his3Δ200 ade2-1 lys1-1 ura3-52 GSH1 PSO9*	This work
W303	*MATα his3-11ade2-1 ura3-1 leu2-3 trp1Δ*	[21]
JC003-8C	*MAT***a** *his3Δ200 ade2-1 ura3-52 trp1Δ GSH1 pso9-1*	This work
JC003-8D	*MATα his3Δ200 ade2-1 ura3-52 trp1Δ GSH1 PSO9*	This work
Y10'000	*MATα his3Δ1leu2Δ0 lys2Δ0 ura3Δ0*	EUROSCARF
Y00'000	*MAT***a** *his3Δ1leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
Y15198	*MATα his3Δ1leu2Δ0 lys2Δ0 ura3Δ0 mec3::kanMX4*	EUROSCARF
Y187	*MAT***a** *ura3-52 his3-200 ade2-101 trp1-901 leu2-3112 gal4Δ met⁻ gal80Δ URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*	[22]

were performed to amplify the sequence of the mutant allele. The amplified fragments were ligated into the pCR-Blunt vector (Invitrogen) and three clones from independent PCR reactions (both for the mutant and the wild-type allele) were sequenced, according to standard procedures.

2.6. Yeast two-hybrid protein–protein interaction assay

Protein interaction experiments were carried out employing the Matchmaker Gal4 Yeast Two-Hybrid System-3 (Clontech). Briefly, *S. cerevisiae* Y187 cells were co-transformed with either pGBKT7-*PSO9* or pGBKT7-*pso9-1* and with the activation domain constructs pGADT7-*DDC1* and pGADT7-*RAD17*. To clone the genes in frame with the Gal4BD or GAl4AD, PCRs of genomic DNA were performed using gene specific primers flanked by *Nco*I and *Bam*HI restriction sites [MEC3N (5′-CCATGGCAATGAAATTAAAATTGATAGT-3′), MEC3C (5′-GGATCCGATGTGATAGTCTGCAACTC-3′), DDC1N (5′- CCATGGCAATGTCATTTAAGGCAACTAT-3′), DDC1C (5′-GGATCCCAGCGATCGATATTATCATG-3′), RAD17N (5′-CCATGGCAATGCGAATCAACAGTGAGCT-3′) and RAD17C (5′-GGATCCAAGTTCTGCGTTTTCTGCGA-3′)] and a high fidelity thermostable DNA polymerase (Platinum® *Pfx* DNA Polymerase, Invitrogen). Deletions in the C-terminal region of *MEC3* gene used in THS were obtained by PCR using the primer MEC3-1350C (5′-CGGGATCCTTCCAAGCTACCACG-3′) to delete the last C-terminal 25 amino acids (aa), yielding pGBK*PSO9*-ΔC75 and the primer MEC3-1200C (5′-CGGGATCCCTGTTCAGCCTTTTC-3′) to delete the last c-terminal 75 aa, yielding pGBK*PSO9*-ΔC225). The C-terminal portions to be deleted were defined by hydrophobic cluster analysis (HCA) [26], comparing fungal Mec3p sequences. The in-frame and accurate sequence of baits and preys were confirmed by sequencing. Transformants were seeded on appropriate selective media and subsequently assayed for activation of the reporter gene in X-Gal plates. The interactions were quantitatively tested for β-galactosidase activity according to Burke et al. [23].

2.7. Cell cycle analysis

Overnight cultures were re-inoculated in YPD at a titer of $\sim 5 \times 10^6$ cells/mL and aliquots were submitted to 8-MOP + UVA as previously described [19], applying 0.2 kJ of UVA radiation. Control and treated cultures were shaken vigorously at 30 °C. Distribution of cells in G1, S and G2/M was analyzed by monitoring the fraction of cells that were unbudded, small-budded, or large-budded. About 200–300 cells were counted at each time point, with a hemacytometer after sonication. Large-budded cells were defined as those in which the bud was >50% of the size of the mother cell.

3. Results

3.1. Phenotype of pso9-1 mutant

The yeast mutant strain *pso9-1* can be phenotypically characterized as sensitive to photoactivated 3-CPs and 8-MOP as well as to UVC and MMNG. Survival assays showed a sensitivity phenotype about three times greater than WT, for all tested mutagens (Fig. 1). The reverse mutation rate was significantly lower than in WT after photo-activated 3-CPs, 8-MOP and UVC treatments, but not as much restricted as that of forward mutation in the *CAN1* locus for the same mutagens (Fig. 2). Sporulation in diploids either homoallelic for *pso9-1* or heteroallelic *pso9-1/mec3*Δ were not affected and *pso9-1* did not confer a sensitivity phenotype to oxidative damage-inducing agents H_2O_2 and paraquat (data not shown).

3.2. Molecular cloning of PSO9

The original *pso9-1* mutant $N123_{35(11)}$ was crossed to MKPo in order to introduce appropriate markers for molecular cloning

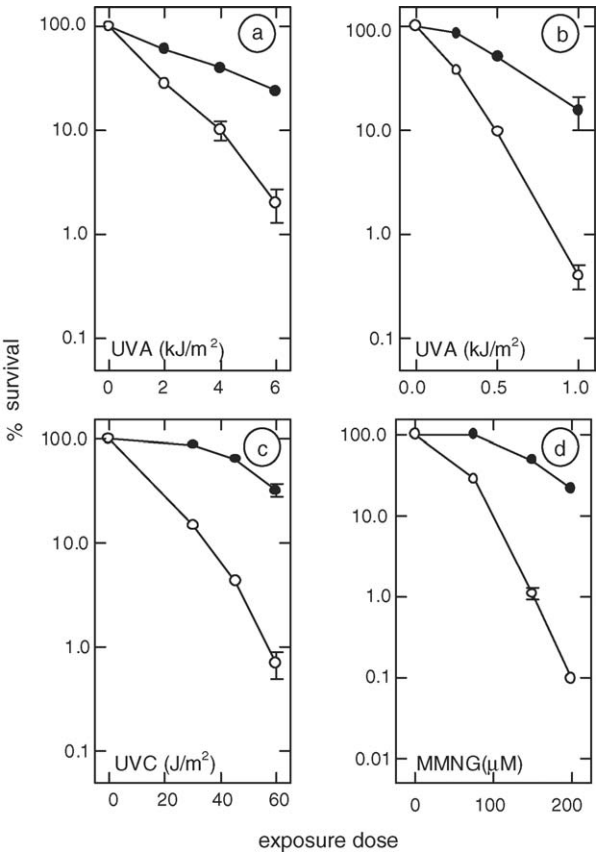

Fig. 1. Sensitivity of *pso9-1* (open circles) and WT (filled circles) to treatment by: (a) 3-CPS + UVA; (b) 8-MOP + UVA; (c) UVC; (d) MMNG.

and also to replace the *gsh1-L* mutant allele (leaky allele) of N123 with *GSH1*, thereby avoiding a strongly reduced glutathione pool in that strain and its derivatives [27] which in turn might alter mutagen-sensitivity phenotypes, e.g., to MNNG [27,28]. The new *pso9-1* haploid isolated, called RMH3001-1C, had a very poor transformability. Therefore, it was crossed to W303 and after two additional backcrosses, one WT (JC003-8D) and a *pso9-1* mutant (JC003-8C) were selected from the same tetrad.

Molecular cloning of *PSO9* gene was achieved by complementing UVC and photoactivated 8-MOP sensitivities of the *pso9-1* mutant strain JC003-8C, using a single-copy vector pRS200-based yeast genomic DNA library. Analysis of 9120 transformants yielded one isolate showing WT resistance to both mutagens. Complementation of UVC and photoactivated 8-MOP sensitivities were plasmid-linked, since the strains which had lost the plasmid were sensitive to the aforementioned agents and once more resistant when re-transformed with the plasmid. The complementing plasmid, pJC9, was isolated from yeast and sequence analysis revealed a 8611 bp DNA fragment from the left arm of chromosome XII, containing two unknown ORFs (YLR287c and YLR290c), and the known genes *RPS30A*, *MEC3* and *GUF1*.

Analysis of mutant phenotypes and physiological functions indicated *MEC3* as the most likely candidate for complementation of *pso9-1*. Two types of experiments confirmed this

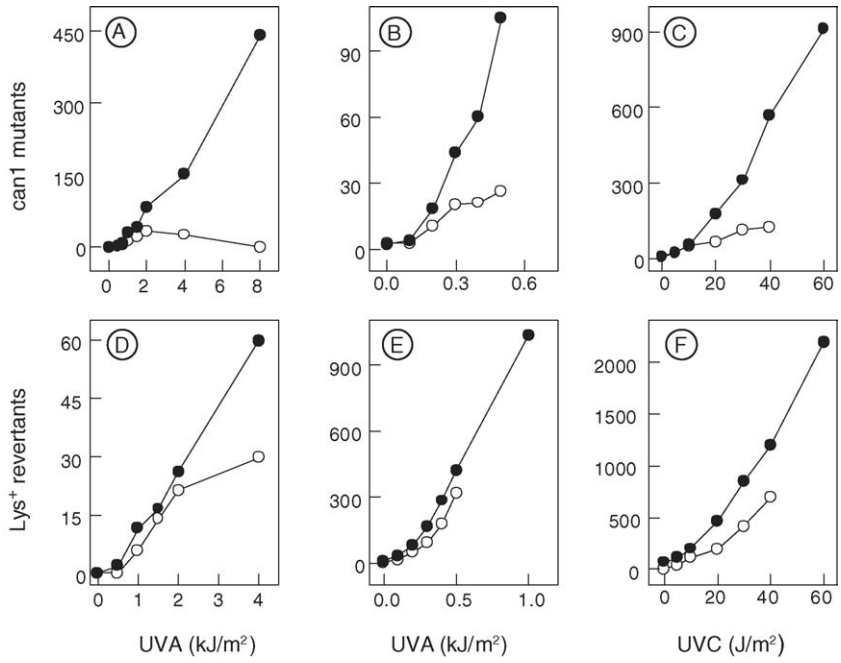

Fig. 2. Mutability by 3-CPS + UVA (A, C), 8-MOP + UVA (B, D), and by UVC (C, E) of *pso9-1* (open circles) and WT (filled circles). Induced reversion in the *lys1* locus (per 10^8 survivors) and induced forward mutation in the *CAN1* locus (per 10^6 survivors).

assumption: (1) sub-cloning of the 8611 bp complementing fragment followed by re-transformation into *pso9-1* and (2) crosses of *pso9-1* to already established *mec3* mutants. Therefore, a 2461 bp *Pst*I–*Sna*BI DNA fragment containing the *MEC3* gene was sub-cloned into pRS314, originating pJC91. This plasmid was able to complement UVC and photoactivated 8-MOP sensitivities of *pso9-1* (Fig. 3a), revealing that the *MEC3* gene alone was sufficient to restore the WT-like phenotype, indicating that *PSO9* might be allelic to *MEC3*. Final evidence for the allelism of the cloned ORF with *PSO9* was obtained by demonstrating that the *mec3*Δ allele was unable to complement any known phenotype of the *pso9-1* mutant in a heteroallelic diploid (Fig. 3b), constructed by crossing *pso9-1* with a haploid *mec3*Δ of opposite mating type. Since the heterozygous diploid *PSO9/mec3*Δ displayed the WT phenotype of *PSO9/MEC3* and a *mec3*Δ allele was unable to complement the *pso9-1* mutant phenotype, we have good evidence that both genes are allelic. Furthermore, we observed that *pso9-1* and *mec3*Δ had similar sensitivities to UVC and 8-MOP + UVA: considering UVC treatment, the dose reduction factor (DRF) for *pso9-1* was 2.3 and for *mec3*Δ was 2.0 at 37% survival. After 8-MOP + UVA, DRF numbers were 1.7 and 1.6, respectively.

Further information about the mutation present in the *MEC3* locus was obtained from the complete sequence of the *pso9-1* mutant allele. Comparing the three identical sequences from three different clones with the databank-deposited WT sequence revealed a single nucleotide deletion at position 802 (802delA), which led to an altered and prematurely ended Pso9-1 protein. The 802delA frameshift mutation results in a 276 aa truncated Pso9-1p, in which the last nine aa are out of proper reading frame and that has its carboxyl terminus dramatically shortened (GenBank accession number AY957606). Since *pso9-1* mutant allele does not encode a full-length protein it was not surprising that

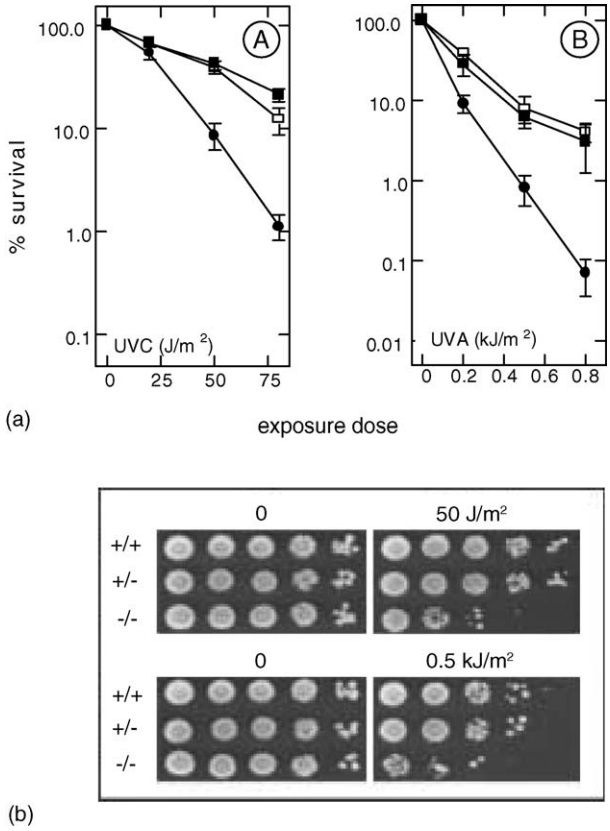

Fig. 3. (a) Complementation of *pso9-1* strain by the pJC91 plasmid harboring a WT *MEC3* gene. (A) UVC and (B) 8-MOP + UVA treatment. Open squares: *pso9-1* transformed with *MEC3*-containing pJC91, filled squares and circles: wild-type and *pso9-1* transformed with pRS314, respectively. (b) Non-complementation assay in the heteroallelic *pso9-1/mec3*Δ diploid for UVC (50 J/m^2) and 8-MOP + UVA (0.5 kJ/m^2) sensitivity. (+/+) *PSO9/MEC3*; (+/−) *PSO9/mec3*Δ; (−/−) *pso9-1/mec3*Δ.

Table 2
Effect of the 802delA, 75Δc-term and 225Δc-term mutations on the Mec3p/Pso9p interactions with Ddc1p and Rad17p[a]

DNA-binding domain plasmid (pGBKT7)	Activation domain plasmid (pGADT7)	β-Galactosidase activity[b]
pGBK*PSO9*	pGAD*DDC1*	76.4 ± 11.3
pGBK*PSO9*	pGAD*RAD17*	132.3 ± 34.6
pGBK*pso9-1*	pGAD*DDC1*	0.39 ± 0.08
pGBK*pso9-1*	pGAD*RAD17*	0.37 ± 0.10
pGBK*PSO9*-ΔC225	pGAD*DDC1*	0.51 ± 0.11
pGBK*PSO9*-ΔC225	pGAD*RAD17*	0.38 ± 0.09
pGBK*PSO9*-ΔC75	pGAD*DDC1*	0.47 ± 0.17
pGBK*PSO9*-ΔC75	pGAD*RAD17*	0.56 ± .010
pGBK	pGAD*DDC1*	0.56 ± 0.09
pGBK	pGAD*RAD17*	2.9 ± 0.63

[a] Plasmids that directed the synthesis of the DNA-binding domain (pGBKT7) and activation domain (pGADT7) fusion proteins were introduced into Y187. All constructions expressed full-length fusion proteins.
[b] Expressed in Miller units. The values are averages from three to four independent transformants each assayed in duplicate.

8-MOP + UVA and UVC sensitivities were not restored when *pso9-1* allele was over-expressed in a *mec3*Δ background (data not shown).

3.3. Ddc1/Pso9-1/Rad17 interactions in the yeast two-hybrid system

In order to investigate whether Pso9-[802delA] could be compromising the Ddc1p/Pso9-1p/Rad17p complex assembly, the yeast two-hybrid system was employed to evaluate the effect of the 802Adel mutation on the interactions of Pso9-1p with Ddc1p and Rad17p. Both Gal4Pso9-1 mutant and Gal4Pso9 WT baits were individually co-expressed with the activation domain fusion plasmids pGADT7*DDC1* or pGADT7*RAD17*, expressing Ddc1p and Rad17p fusion proteins, respectively. Table 2 shows that the Pso9-[802delA] mutation abolished the interaction of Pso9-1p with both Ddc1p and Rad17p, suggesting that the deletion in the *pso9-1* mutant allele did not only lead to a truncated protein but that this also strongly compromised the functional heterotrimeric structure of the Ddc1p/Mec3p/Rad17p complex. In order to clarify which domains in the C-terminal region could be responsible for an efficient complex assembly we further constructed baits containing *PSO9* C-terminal deletions lacking the last 25 aa (pGBK*PSO9*-ΔC75) and 75 aa (pGBK*PSO9*-ΔC225). No significant β-galactosidase activity was detected when the mutated baits were used (Table 2), indicating that the integrity of the C-terminal region of Mec3p is crucial for a proper Ddc1p/Mec3p/Rad17p complex assembly.

3.4. pso9-1 cell-cycle profile

To further examine the consequences of the *pso9-1*[802delA] mutation on the DNA damage checkpoint we monitored the proportion of non-budded (G1 phase), small-budded (an approximation of cells undergoing S phase), and large-budded (G2/M phase) cells 2, 4 and 6 h after 8-MOP + UVA treatment. Fig. 4 shows the cell-cycle progression of untreated and 8-MOP treated WT and *pso9-1* cells. Treated WT cells had their cell cycle delayed and resumed normal cycling after 6 h. This delay was not observed in *pso9-1* cells which continued their cell cycle in both normal and damage-induced situations, suggesting that this mutant is not proficient in sensing DNA damage, thus not triggering the DNA damage checkpoint pathways.

4. Discussion

Photo-chemotherapy employing 8-MOP and long wave ultraviolet radiation (PUVA) is widely used in the treatment of skin disorders. Unfortunately, the efficacy of PUVA is linked to the formation of DNA photo-adducts in the target cells. High doses of 8-MOP + UVA lead to G2 delay and G1 arrest in budding yeast [29] and a similar phenomenon occurs after cisplatin treatment where a G2 arrest is triggered [30]. In the fission yeast *S. pombe*, nitrogen mustard and mitomicyn C activate a G2/M checkpoint [31]. Our results are consistent with these findings since we could show that an 8-MOP + UVA treated *pso9-1* mutant fails to arrest its cell cycle confirming its role in responding to ICLs.

The screening of a mutagenized yeast culture for sensitivity to photoactivated psoralens performed by Henriques and Moustacchi [19] yielded mutants with impaired functions related to this kind of damage, all of them lacking a protein with direct or indirect function in DNA repair or protection from DNA damage [11]. In this work, we were able to show that the *PSO9/MEC3* allelism includes DNA damage checkpoint to the Psop functions. *MEC3* is required for the DNA damage checkpoint response in mitosis and is probably also required for the meiotic recombination checkpoint [32–34]. Mec3p exists in a complex with Rad17p and Ddc1p [35] and is required for both localization [36] and phosphorylation of Ddc1p [37]. The Ddc1/Mec3/Rad17 complex shares sequence similarity with PCNA (Proliferating Cellular Nuclear Antigen), a DNA sliding clamp (also known as DNA polymerase processivity factor), suggesting that this factor acts by sensing DNA damage directly [38]. The *mec3* mutants were known to be sensitive to UVC, methyl methanesulfonate and hydroxyurea [39], and to reduce UVC-induced mutability in a *rad1*Δ background [40]. These phenotypes match the *pso9-1* phenotypic characterization shown in our work, and have been extended for sensitivities to photoactivated mono- and bifunctional psoralens and to MNNG (Figs. 1 and 2).

Sequencing of the *pso9-1* mutant allele revealed it to contain an 802delA frameshift mutation leading to an altered and truncated Pso9p carboxyl terminus. The 802delA mutation resulted in the mutagen-sensitivity and low-induced mutability phenotypes, which hinted at an altered tertiary structure of the Pso9-[802delA] protein, thus not allowing its interaction with either Ddc1p or Rad17p [36], a pre-requisite for the DNA damage checkpoint response. This hypothesis was supported since over-expression of *pso9-1* allele in a *mec3*Δ background did not show any residual function regarding 8-MOP + UVA and UVC sensitivities (data not shown), suggesting that lack of the 207 carboxy terminal aa results in severe distortion of the tertiary structure of Pso9-1p.

Molecular modeling studies predict that Rad1, Hus1 and Rad9 (Rad17p, Mec3p and Ddc1p, in budding yeast, respec-

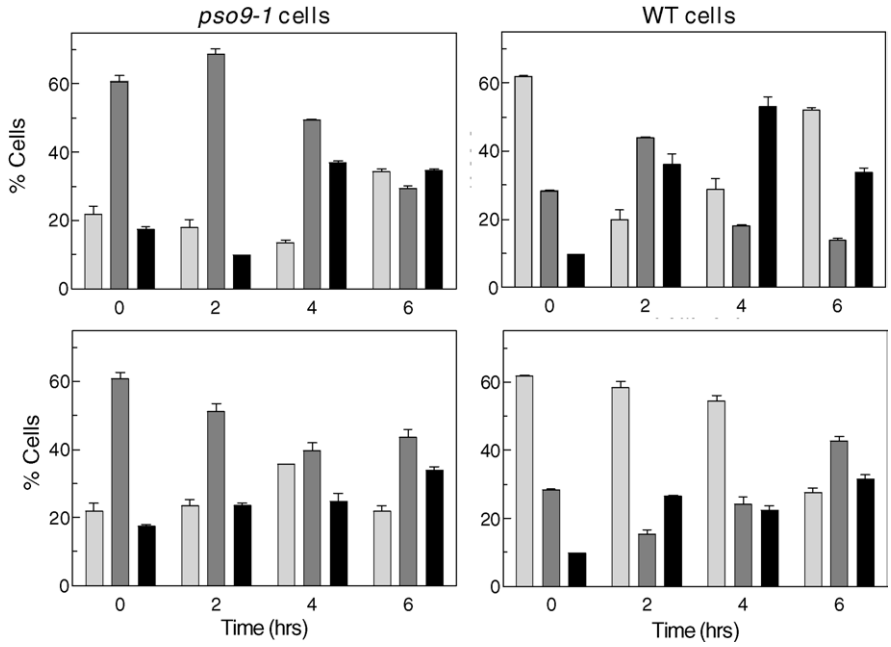

Fig. 4. Distribution of G1 (grey), S (dark grey), and G2/M (black) cells in wild-type and *pso9-1* strains: upper panel, untreated control; lower panel, cells with DNA damage (8-MOP + UVA).

tively) fold into PCNA-like structures [38,41] and each member of the complex has been shown to interact in a pair-wise fashion where the amino terminus of hHus1 interacts with hRad9 [42,43]. According to this interaction manner (amino terminus with the carboxyl terminus of the binding partner), the Pso9-[802delA] protein could be defective in its ability to interact with Rad17p, thus compromising the complex assembly and/or its functional heterotrimeric structure. Using the yeast two-hybrid system we could show that Pso9[802delA]p loses ability to interact with Rad17p and with Ddc1p as well (Table 2). When the pGBK*PSO9*-ΔC225 and pGBK*PSO9*-ΔC75 constructions were used as baits we could observe that not only the last 75 aa but the last 25 aa of Pso9p/Mec3p are essential for maintaining the complex interactions. This would indicate that both Mec3p–Rad17p and Mec3p–Ddc1p interactions require the carboxyl-terminal region of Pso9p/Mec3p and may be crucial for a functional DNA damage checkpoint. Since *MEC3*, *RAD17* and *DDC1* belong to the same epistasis group and were shown to be sensitive to the same damaging agents tested (UV, MMS, HU) [44,37,45] and that they all fail to arrest cell cycle after DNA damage as well [32,39,37,45] we infer that deletion of the other two components of the PCNA-like complex would lead to a similar response regarding photoactivated psoralen sensitivity and checkpoint response. The fact that stability of the Ddc1p/Mec3p/Rad17p complex depends on the presence of all three proteins [35,46,43] (this last concerning *Schizosaccharomyces* data) suggests an equivalent phenotypic response in mutants lacking any of the three components.

We must also take into account that sliding clamps interact with a large number of cellular factors (including proteins needed for nucleic acid metabolism and cell-cycle regulation) and regulate their activity (reviewed in [47]). Therefore, Pso9-1p could be affecting interactions of the Ddc1p/Mec3p/Rad17p complex with other proteins, i.e. DNA repair and recombination enzymes, a fact that could also explain the *pso9-1* mutant's high sensitivity to DNA damaging agents. This is also supported by the low induced mutability in the *pso9-1* mutant (Fig. 2), a fact probably derived from the deficiency in recruiting error-prone repair proteins, e.g. Polζ. It is known that Mec3p, among other checkpoint proteins, is required for promotion of error-prone repair of UV lesions by Rev3p/Rev7p [40] and recent observations have indeed indicated that PCNA plays a key role in promoting the access of translesion synthesis DNA polymerases to the replication fork (reviewed in [48]).

Although the response of cells to DNA damaging agents like UVC and ionizing radiation is well established, little is known about cell cycle arrest after treatment with photoactivated psoralens. As a pioneering step of an investigation aiming to better understand the molecular events involved in the repair of photo-induced adducts, Henriques and Moustacchi [49] showed that, among the *rad50* mutant group, *rad53-1* was the only to show a WT phenotype with respect to 8-MOP and 3-CPS photoaddition in stationary as well as in exponential phase of growth. In contrast, the *rad9-4* mutant was twice as sensitive after equal treatment [50,51]. At the time of these findings the intricate network of proteins involved in a process called DNA damage checkpoint was far to be discovered. With today's knowledge it is possible to fit those early evidences with recent findings and propose a model for checkpoint activation generated by photo-induced adducts (Fig. 5). Psoralen-induced adducts do not cause major changes in the architecture of the DNA helix [5] and it is, therefore, unlikely that these lesions are recognized directly; thus, recognition would occur when a replication fork stalls at an ICL. A mechanism by which a functional uncoupling of the MCM (minichromosome maintenance) helicase and DNA polymerases leads to checkpoint activation after DNA damage

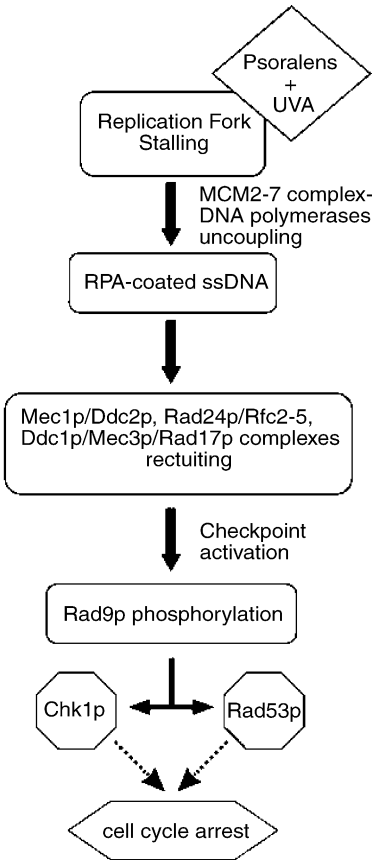

Fig. 5. Model for checkpoint activation in yeast cells exposed to photoactivated psoralens. The photo-induced lesions result in the slowing of DNA replication, leading to a functional uncoupling of the MCM2-7 complex from DNA polymerases. The ssDNA generated in the lagging strand is bound by RPA that recruits Ddc2p/Mec1p and Rad24p/Rfc2-5 which in turn loads the Ddc1p/Mec3p/Rad17p complex. Next, checkpoint is activated by means of Rad9p phosphorylation, that now can regulate Chk1p and Rad53p. By targeting their effectors these proteins regulate cell cycle arrest (cf. text for details).

has been proposed [52]. Considering this mechanism, a slowing of replication, followed by uncoupling of the yeast MCM2-7 complex [53] from the replicative polymerases would generate a single-strand DNA (ssDNA) region. The ssDNA in turn would be bound by the replication protein A (RPA), thus generating a structure that is commonly found after DNA damage (reviewed in [54]). This RPA-coated ssDNA would then recruit the Mec1p/Ddc2p complex [55] and the Rad24p/Rfc2-5 complex [56] which would load the Ddc1p/Mec3p/Rad17p complex onto DNA [57]. The proximity of the Ddc1p/Mec3p/Rad17p and Mec1p/Ddc2p complexes leads to Rad9p phosphorylation in a Mec1p-dependent manner [58], becoming responsible for regulation of Chk1p and Rad53p [59,60]. The functional overlapping between Chk1p and Rad53p in targeting the effectors to arrest the cell cycle [59] explain the non-sensitivity to photoactivated psoralens of *rad53-1* [50]. On the other hand, *rad9-4* with its increased sensitivity to these agents [51,52], suggests a central role for the upstream adaptor Rad9p. Similarly, we could demonstrate that Pso9p/Mec3p integrity is also crucial for proper signal response to photoactivated psoralen-generated DNA lesions, emphasizing the role of this protein in initiating the biochemical cascade of the DNA damage checkpoint.

In summary, *PSO9/MEC3* is crucial for sensing DNA lesions generated by photo-induced psoralens or by other mutagens, allowing an optimal repair either by regulating the progression of cell cycle or bringing specific DNA repair factors to the substrate. Future investigation of Pso9-1p might contribute to further elucidate the connections between checkpoint pathways and DNA repair in lower eukaryotes.

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CAPÍTULO IV

DNA repair by polymerase δ in *Saccharomyces cerevisiae* is not controlled by the PCNA-like Rad17/Mec3/Ddc1 complex

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DNA Repair by Polymerase δ in *Saccharomyces cerevisiae* is not Controlled by the PCNA-like Rad17/Mec3/Ddc1 Complex

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Running title: Genetic interactions between Rad17/Mec3/Ddc1 and Pol δ

Key words checkpoint, DNA replication, DNA repair, PCNA, Rad17/Mec3/Ddc1, *Saccharomyces cerevisiae*

Abstract DNA damage activates several mechanisms such as DNA repair and cell cycle checkpoints. The *Saccharomyces cerevisiae* heterotrimeric checkpoint clamp consisting of the Rad17, Mec3, and Ddc1 sub-units is an early response factor to DNA damage and activates checkpoint. This complex is structurally similar to the proliferating cell nuclear antigen (PCNA), which serves as a sliding clamp platform for DNA replication. Growing evidence suggests that PCNA-like complexes play a major role in DNA repair as they have been shown to interact with and stimulate several proteins, including specialized DNA polymerases. With the aim to extend knowledge concerning the link between checkpoint activation and DNA repair, we tested the possibility of a functional interaction between the Rad17/Mec3/Ddc1 complex and the replicative DNA polymerases α, δ and ε. The analysis of sensitivity response of single and double mutants to UVC and 8-MOP+UVA-induced DNA damage suggests that the PCNA-like component Mec3p of *S. cerevisiae* neither relies on nor competes with the third subunit of DNA polymerase δ, Pol32p, for lesion removal. No enhanced sensitivity was observed when inactivating components of DNA polymerases α and ε in the absence of Mec3p. The hypersensitivity of *pol32*Δ to photoactivated 8-MOP suggests that the replicative DNA polymerase δ also participates in the repair of mono- and bi-functional DNA adducts. Repair of UVC and 8-MOP+UVA-induced DNA damage via Pol δ thus occurs independent of the Rad17/Mec3/Ddc1 checkpoint clamp.

Cells ensure the stability of their genomes by activating a range of cellular responses following DNA damage, including DNA repair, apoptosis, damage tolerance mechanisms and complex signaling networks that arrest cell cycle at appropriate points. These so-called checkpoints allow recovery of the integrity of DNA before re-entering the cell cycle (1). Checkpoint activation requires the action of DNA damage sensors and transducers. While two distinct complexes independently bind to sites of DNA damage in *Saccharomyces cerevisiae*, the presence of both is required for proper checkpoint function. The first complex, the Mec1/Ddc2 heterodimeric protein kinase functions in DNA damage recognition and signal transduction (2). The second set of proteins is homologous to the replication clamp PCNA [proliferating cell nuclear antigen] and its clamp loader, replication factor C (RFC). The functional homolog of PCNA (PCNA–like) is a heterotrimeric clamp consisting of the *S. cerevisiae* Ddc1, Rad17, and Mec3 protein subunits, which are orthologous to the human and *S. pombe* Rad9, Rad1, and Hus1 subunits, respectively, the 9-1-1 complex. Protein threading algorithms have predicted a PCNA-like fold for these subunits, while biochemical studies show their heterotrimeric structure (3, 4). The five subunit Rad24-RFC clamp loader, consisting of Rad24 (Rad17 in human and *S. pombe*) and the four small subunits of RFC (Rfc2 to Rcf5) is a specific loader of the checkpoint clamp (reviewed in 5). Once loaded, the clamp has the ability to slide across double-stranded DNA similarly to PCNA, and search for DNA damage (5, 6).

Although the link between checkpoint engagement and recruitment of repair machinery to DNA lesions is far from being understood, recent studies have shown interaction or co localization of PCNA-like complexes with several DNA repair factors, including the Rad14 nucleotide excision repair (NER) protein of *S. cerevisiae* (7), the

human FEN1 nuclease (8), DNA ligase I (9), and the MYH glycosylase of *S. pombe* (10).

Central to these processes are DNA polymerases (Pols) that have also been found to interact with PCNA-like sliding clamps. It is known that the human 9-1-1 complex interacts with Pol β, pointing to a possible function in base excision repair (11). In *S. pombe* the 9-1-1 complex was found to co-immunoprecipitate with Pol κ which is important for translesion synthesis (12). The potential role of PCNA-like complex in regulating the access of translesion polymerases to DNA was recently confirmed by Sabbioneda *et al.* (13), who showed that the Rad17/Mec3/Ddc1 complex interacts with the Rev7 subunit of Pol ζ and regulates Pol ζ-dependent spontaneous mutagenesis. In order to address further the link between checkpoint proteins and DNA repair, we aimed to investigate whether the yeast PCNA-like sliding clamp could interact with other DNA polymerases than those engaged in translesion synthesis, particularly replicative DNA polymerases. Therefore, we analyzed the response to DNA damage (UVC and photoactivated 8-methoxypsoralen [8-MOP+UVA]) of yeast double mutants that lack the yeast PCNA-like component Mec3p and either Pol32p (third subunit of Pol δ), Dpb3p (subunit C of Pol ε), or Ctf4p (Pol α–binding protein) (reviewed in 14).

The genotypes of strains used in this study are listed in Table 1. Strains JC010, JC011, e JC012 were derived from Y16841, Y15550 e Y15726 respectively, by one-step gene replacement, using a *mec3::HIS3* disruption cassette. The deletion cassette was amplified by PCR with the primers MECHISF (5'*CAATGGTTGCGGCTACAAATATAGGCGAGTTATACTTGCC*CTGTGCGGTATTT CACACCG3') and MECHISR (5'*AGCCCTTCGATCTTGCTATATAATATATGATTTGTCCTCT*AGATTGTACTGAGA

GTGCAC3') where the first 40 bases are homologous to *MEC3* sequence just inside of the start and stop sites, respectively, of the *MEC3* coding region and the remaining bases are homologous to 20 bases of the 5' and 3' ends of *HIS3* in the plasmid pRS313 (15) used as template. When gene deletions were to be created the high efficiency lithium acetate method was used for transformation (16). Accuracy of all gene replacements was verified by PCR analysis using specific primers for the *MEC3* gene [MEC3F (5'-TCAGCATTTTTATGTGCAACTAGTTT-3') and MEC3R (5'-GTAGCAAAGAAATGTACCGCTGTAG-3')]. Yeast strains were routinely grown and stored on YPD medium (1% yeast extract, 2% peptone, 2% glucose). For mutagen treatments, stationary phase cells were washed and resuspended in 0.9% NaCl to a titer of 10^8/mL. Treatment with photoactivated 8-MOP was according to Henriques and Moustacchi (17) and sensitivity to UV_{254nm} (UVC) was assayed by irradiating (Stratalinker, Stratagene) cells plated on solid medium. Plates were incubated for 3-4 d at 30ºC. Survival data represent the average of at least three experiments.

Yeast strains were exposed to mutagen treatments and cell inactivation was analyzed. Figure 1 shows that the *dpb3*Δ *and ctf4*Δ single mutants have WT-like sensitivity to treatment with these agents, i.e, are not involved in repair of DNA lesions. In contrast, *pol32*Δ is clearly hypersensitive as it is inactivated like a *mec3*Δ mutant (18). The *mec3*Δ*pol32*Δ double mutant displayed the highest sensitivity of the three double mutants after both UVC and 8-MOP+UVA treatments. Since the sensitivity shown by *mec3*Δ*pol32*Δ is about the sum of the sensitivities of the single mutants, we can assume an additive interaction between *MEC3* and *POL32*.

The identification of the biochemical function of the subunits of PCNA-like complex and their roles in checkpoint activation have led to a model in which genotoxins create DNA structures that attract the loading of PCNA-like complexes via

the RFC clamp-loading complex (5, 6). Once bound to DNA, the PCNA-like complex serves as a sliding clamp that functions as a central regulator of checkpoint activation and DNA repair by tethering specific proteins to the sites of DNA damage. While experimental data point to its role in low fidelity DNA polymerase-utilizing translesion synthesis (12, 13, 14), little effort has been made in searching for interactions of PCNA-like complexes with replicative DNA polymerases.

In this work, we were able to show that the *S. cerevisiae* Rad17/Mec3/Ddc1 complex is not necessary for the repair function of the replicative DNA polymerases Pol δ. By analyzing the relative sensitivities of single and double mutants lacking Mec3p and components of the DNA polymerases α, δ e ε (Ctf4, Pol32 and Dpb3, respectively) to DNA damaging agents, we found an additive interaction between *MEC3* and *POL32*, for both UVC and 8-MOP+UVA treatments. This phenotype was exclusive to Pol δ, since the inactivation of Ctf4 and Dpb3 components did not lead to any sensitivity increase over the WT, and since the double mutants only showed an apparent epistatic interaction. Further characterization of conditional mutants of other polymerase subunits remains to be done.

Pol δ has been implicated in NER, BER and also in MMR (reviewed in 14) and the UVC and 8-MOP+UVA sensitivity phenotype of *pol32*Δ (Fig.1) confirms this. So far, only translesion polymerases were found to participate in cellular recovery from 8-MOP+UVA-induced DNA lesions (19). The high sensitivity of *pol32*Δ to 8-MOP+UVA suggests a role of Pol δ in the gap-filling prior to the re-ligation of DNA ends. Removal of ICL proceeds via DNA double strand breaks that are thought to be repaired either by homologous recombination or non-homologous end joining (NHEJ) (20). These findings together with our results, suggest that Pol32p might be playing a role in NHEJ.

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Table 1. Yeast strains used in this study

Strain	Genotype	Source
Y10'000	*MATα his3Δ1leu2Δ0 1ys2Δ0 ura3Δ0*	EUROSCARF
Y15198	*MATα his3Δ1leu2Δ0 lys2Δ0 ura3Δ0 mec3::kanMX4*	EUROSCARF
Y16841	*MATα his3Δ1leu2Δ0 lys2Δ0 ura3Δ0 pol32::kanMX4*	EUROSCARF
Y15550	*MATα his3Δ1leu2Δ0 lys2Δ0 ura3Δ0 ctf4::kanMX4*	EUROSCARF
Y15726	*MATα his3Δ1leu2Δ0 lys2Δ0 ura3Δ0 dpb3::kanMX4*	EUROSCARF
JC010	*MATα his3Δ1leu2Δ0 lys2Δ0 ura3Δ0 pol32::kanMX4mec3::HIS3*	This work
JC011	*MATα his3Δ1leu2Δ0 lys2Δ0 ura3Δ0 ctf4::kanMX4 mec3::HIS3*	This work
JC012	*MATα his3Δ1leu2Δ0 lys2Δ0 ura3Δ0 dpb3::kanMX4 mec3::HIS3*	This work

Figure legends

Figure 1. Sensitivity of DNA polymerase mutants in a *mec3*Δ background. UVC (upper panel); 8-MOP+UVA (lower panel). WT (■); *mec3*Δ (□); *ctf4*Δ (●); *ctf4*Δ *mec3*Δ (○); *dpb3* (▲); *dpb3*Δ *mec3*Δ (△); *pol32*Δ (▽); *pol32*Δ *mec3*Δ (▼).Where no error bar is seen, it is smaller than the symbol.

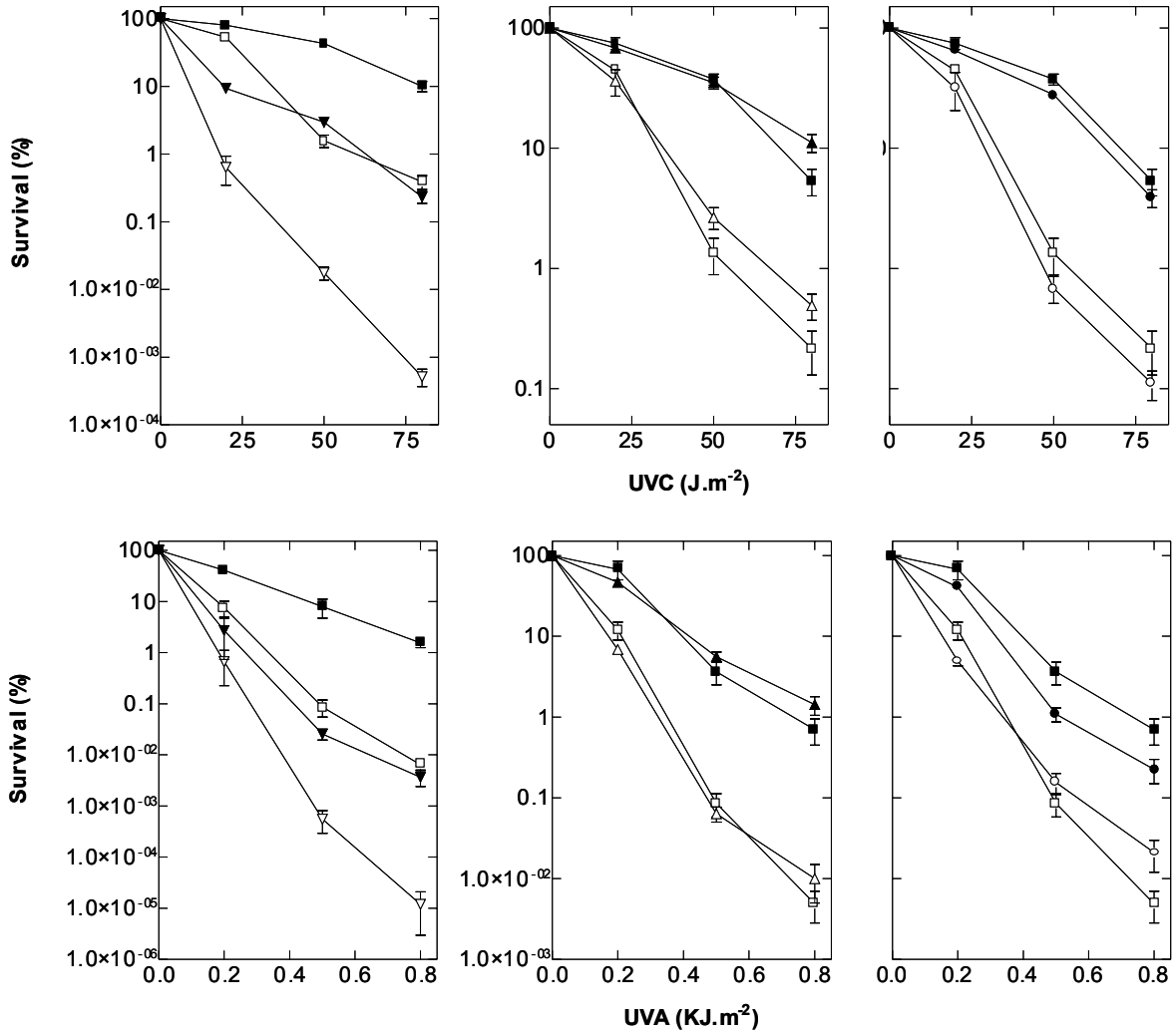
Survival (%)
100
10
1
0.1
1.0×10-02
1.0×10-03
1.0×10-04
0
25
50
75
UVC (J.m-2)
Survival (%)
1.0×10-05
1.0×10-06
0.0
0.2
0.4
0.6
0.8
UVA (KJ.m-2)

Fig.1

CAPÍTULO V

Role of DNA polymerase δ in the repair of psoralen-photoinduced DNA interstrand cross-links in *Saccharomyces cerevisiae*

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Role of DNA polymerase δ in the repair of psoralen-photoinduced DNA interstrand cross-links in *Saccharomyces cerevisiae*

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Abstract Characterization of DNA interstrand cross-link (ICL) repair in *Saccharomyces cerevisiae* has allowed us to determine that genes from all three epistasis groups of DNA repair [nucleotide excision repair, homologous recombination and post-replication/translesion repair] seem to encode proteins involved in this complex task. Although several of these genes are known, there has been little progress in clarifying the final steps of ICL removal from DNA. A yeast strain lacking subunit Pol32 of Pol δ, is about 3-fold more sensitive to 8-MOP+UVA whereas deletion of neither *CTF4* nor of *DPB3* genes resulted in increase of sensitivity over that of the isogenic wild type. Using a plasmid transformation assay, we found that *pol32*Δ mutant is extremely deficient in joining non-cohesive DNA ends, particularly in cases of mismatched 5' overhangs. Sequencing of the recovered plasmids showed that both WT and *pol32* mutant strains are able to generate NHEJ products, but the structures of junctions from the *pol32* strain were diverse with some of the joining events without obvious filling. Based on these findings we propose a model for removal of ICL from yeast DNA where the DSB, resulting from early steps in ICL processing, is subsequently repaired by DNA polymerase δ-mediated NHEJ.

Key words *Saccharomyces cerevisiae*, interstrand cross-links, psoralens, DNA polymerase, non-homologous end joining

Introduction

DNA interstrand cross-links (ICLs) represent a very toxic class of lesion since they prevent normal transcription and replication and are highly mutagenic and recombinogenic. ICLs are produced by many commonly used anticancer agents, including cisplatin, mitomycin C, nitrogen mustards, nitrosoureas and psoralens (Brendel and Ruhland 1984; Cassier *et al.* 1984; Averbeck *et al.* 1988).

Bi-functional psoralens are photoreactive DNA cross-linking agents that react with pyrimidine bases on opposite DNA strands in the presence of UVA (Cimino *et al.* 1985; Tessman *et al.* 1985). They belong to the group of furocoumarins, present in plants and cosmetics and are used in the treatment of several skin disorders like psoriasis and vitiligo (reviewed in Morison, 2004). The stability of an ICL may greatly influence efficiency of its removal via DNA repair enzymes and may be directly correlated to its genotoxicity (Henriques *et al.* 1997). Among all ICL, those induced by 8-MOP+UVA are the most chemically stable, and about 20 of these ICL suffice for a lethal hit in a haploid yeast WT cell (Bankmann and Brendel 1989). Moreover, up to 40% of the adducts formed after its photoactivation can be converted to ICLs, in contrast to the other agents where ICL constitute not more than 20% of total adducts (Brendel and Ruhland 1984).

The exact mechanism of ICL repair in eukaryotes is still not understood, though it seems clear that the enzymes of nucleotide excision repair (NER) start processing ICL-containing DNA (Jachymczyk *et al.* 1981; Magaña-Schwencke *et al.* 1982; Wilborn and Brendel 1989), resulting in double-strand breaks (DSBs) as repair intermediates (Jachymczyk *et al.* 1981; Magaña-Schwencke *et al.* 1982; Miller *et al.* 1982) which would

then require homologous recombination (HR) in an error-free process, in order to regenerate intact chromosomal DNA (Averbeck *et al.* 1992; Averbeck and Averbeck 1998; McHugh *et al.* 2000). An error-prone process dependent upon lesion bypass is also known to provide an additional route for repair of ICLs (Henriques and Moustacchi 1981; Miller *et al.* 1982; Meniel *et al.* 1995). While the participation of error-prone DNA polymerases in the repair of ICLs has already been described (Chanet *et al.* 1985; Lawrence and Hinkle 1996; Wang *et al*. 2001), little is known on the role of replicative DNA polymerases in this respect.

A processive and accurate DNA replication in *S. cerevisiae* requires at least the participation of DNA polymerases α, δ and ε. Pol α/primase associates with the initiation complex and starts to synthesize an oligonucleotide which is then utilized by Pol δ or Pol ε for processive elongation on the lagging and the leading strand (for reviews, see Hübscher 2002; Johnson and O'Donnell 2005). However, these polymerases have additional tasks besides genome duplication. Genetic and biochemical studies have demonstrated the involvement of Pol δ in a number of DNA repair pathways such as base excision repair (BER) (Wang *et al.* 1993; Blank *et al.* 1994; Jin *et al.* 2003), mismatch repair (Longley *et al*. 1997), in the mutagenic bypass pathway (Huang 2000; 2002). Recently, the Pol δ subunit Pol32 was shown important for the bypass of abasic sites and T-T (6-4) photoadducts (Gibbs *et al.* 2005). Similarly, Pol ε was shown to be involved in NER and BER (Wang *et al.* 1993; Aboussekhra *et al.* 1995; Stucki *et al.* 1998), besides bypassing T-T dimers (McCulloch 2004). Finally, Pol α/primase was suggested to participate in the repair of DSB, by generation of a modified replication fork (Holmes and Haber 1999).

Given the ample participation of replicative DNA polymerases in DNA repair processes and the paucity of knowledge regarding post-incision steps in repair of ICLs, we asked whether Pol δ, Pol α and Pol ε could also be implicated in the repair of psoralen-photoinduced ICLs in *S. cerevisiae*. Furthermore, in order to elucidate the mechanism involved, we investigated their capabilities in joining incompatible DNA ends. The present study strongly suggests that the late steps of ICL repair might go through NHEJ and require the function of DNA polymerase δ.

Material and Methods

Strains and media

The genetic constitution of the *S. cerevisiae* strains used in this study is given in Table 1. Yeast media were as described by Burke *et al.* (2000). Yeast was routinely grown and stored on YPD. Auxotrophy markers were controlled on synthetic medium - SynCo (1.7 g yeast nitrogen base w/o amino acids and w/o ammonium sulfate/L, 5 g ammonium sulfate/L, 20 g glucose/L) supplemented with the appropriate amino acids and bases (40 μg/mL).

8-MOP + UVA treatment

Stationary phase cells were washed in 0.9% NaCl and resuspended in the same solution to a titer of 10^8/mL. Treatment with the furocoumarin 8-methoxypsoralen was as previously described by Henriques and Moustacchi (1980). After dilution and plating all plates were incubated for 3-5 days at 28ºC. Survival data represent the average of at least three experiments.

Plasmid transformation assay

We have used the centromeric YCpLac33 (Gietz, 1988) appropriately digested as substrate for evaluating NHEJ. In order to generate 5' and 3' incompatible ends we have digested the plasmid with *Eco*RI/ *Hind*III and *Pst*I/ *Sac*I (Invitrogen), respectively. Single digestion with *Hind*III was used as a positive control (compatible ends). Restricted molecules were verified regarding linearity and integrity by agarose gel eletrophoresis, followed by a purification using a gel extraction kit (Invitrogen). Yeast strains were transformed with the substrate plasmids using a high efficiency lithium acetate method previously described (Gietz, 2002). Cells were transformed with 200 ng of digested plasmid and 50 ng of intact plasmid for the transformation efficiency control. The relative repair efficiency is expressed as the ratio of the colonies yielded from cut plasmids, to colonies yielded from uncut YCpLac33, considering the difference in the amount of DNA transformed. Repair efficiency data represent the average of at least three experiments. Yeast colonies had their plasmid extracted (Burke 2000) and transformed into *E. coli* for propagation (Sambrook *et al.*1989). Sequencing was performed using universal M13-40 primer (Amersham Biosciences) in an automated sequencer (ABI PRISM® 3100—Applied Biosystems), according to the manufacturer's instructions.

Results

To investigate the role of the replicative DNA polymerases Pol δ, Pol α and Pol ε in the repair of ICLs, we first measured cell survival of yeast strains lacking the third subunit of Pol δ- Pol32, the subunit C of Pol ε- Dpb3 and the Polα – binding protein Ctf4, after

subjecting cells to 8-MOP+UVA. In Figure 1, it can be noticed that the inactivation of *POL32* confers a hypersensitivity of yeast cells to killing by this agent. It becomes ~ 3 fold more sensitive comparing to its isogenic WT at a dose of 0.5 kJ UVA. In contrast, the absence of Dpb3p and Ctf4p does not increase sensitivity of the mutants to 8-MOP+UVA over that of the WT. Survival for a Pso2p-defective strain, known to be extremely sensitive to 8-MOP+UVA (Henriques and Moustacchi 1980; Cassier and Moustacchi 1981), is given as a reference (Fig.1).

Being aware of the high sensitivity of *pol32*Δ to 8-MOP+UVA, we needed to test the importance of Pol δ at the final steps of ICL repair. Given the role of Pol δ in BER (Wang *et al.* 1993; Blank *et al.* 1994; Jin *et al.* 2003), we hypothesized that it might be required for a gap-filling process prior the re-joining of DNA ends. Therefore, we used a plasmid transformation assay similar to previous studies of NHEJ in *S. cerevisiae* (Wilson and Lieber 1999, Zhang and Paul 2005, Daley *et al.* 2005). In this assay, test plasmids are linearized by restriction *in vitro* and transformed into yeast, where re-circularization is required for plasmid maintenance. The substrate plasmids were digested to yield incompatible ends of 3' (*Pst*I/ *Sac*I) and 5' (*Eco*RI/ *Hind*III) polarities, and as a positive control, *Hind*III compatible ends. Transformation efficiency was calculated by dividing the number of transformants obtained with the linearized plasmid by the number of transformants obtained with an equivalent amount of uncut DNA. Figure 2A shows that transformation efficiency of *Hind*III compatible ends was neither affected in WT nor in the mutants. Sequencing of the junctions from these plasmids showed that the ends were joined precisely with no nucleotides lost or gained (Fig.2B). This result was expected since DSBs with fully compatible overhangs can be joined by simple re-ligation, *i.e.* requiring

only DNA ligase (Wilson and Lieber 1999). No significant reduction in the transformation efficiency was observed with the *Pst*I/ *Sac*I cut plasmid (Fig.2A) and the sequences of the junctions generated showed that both WT and *pol32* had repaired this DSB in the same way (Fig.2B). In contrast, when transforming the strains with plasmids containing 5' incompatible ends (*Eco*RI/ *Hind*III), the number of yeast transformants recovered in the *pol32*Δ mutant was significantly lower, as compared to that obtained in the WT and to both *Hind*III and *Pst*I/*Sac*I linearized plasmids (Fig.2A). Sequencing of junctions from WT and *pol32* mutant, in this case, revealed that both strains are able to generate NHEJ products, but in a very different pattern. The structures of junctions from the *pol32* mutant strain were diverse with some of the joining events without obvious filling (Fig.2B). Even though both *ctf4*Δ and *dpb3*Δ strains were not sensitive to photoactivated 8-MOP we asked whether they could be implicated in joining incompatible DNA ends, but no impairment was noticed when the strains were transformed with the identical set of restricted plasmids (Fig.2A).

Discussion

DNA interstrand cross-linking chemicals are used widely in cancer chemotherapy because of their high cytotoxicity in replicating cells (Erickson *et al.* 1980; Lawley and Phillips 1996; Kohn 1996). The formed DNA lesions are complex, and their repair involves several different DNA repair pathways. As with other forms of chemical damage, excision repair systems incise the damaged DNA strands; however, there is no undamaged strand to act as a template, and full repair requires the participation of additional repair

enzymes (organized in other pathways of DNA repair). Recombinational repair pathways are involved in restoring the intact duplex structure after excision (Jachymczyk *et al.* 1981; Magaña-Schwencke *et al.* 1982; Li *et al.* 1999; McHugh *et al.* 2000). Additionally, cross-links efficiently induce mutations, implicating error-prone pathways in their repair (Averbeck 1989; Wang *et al*. 2001). However, late steps of ICL repair are still poorly understood. Considering that many DNA polymerases are known to serve a specific function apart from basic genome duplication (for reviews, see Hübscher 2002; Johnson and O'Donnell 2005) we asked whether replicative DNA polymerases could be involved in ICL repair.

To begin this investigation, we tested the sensitivity to photoactivated 8-MOP of yeast strains defective in subunits of polymerases Pol δ, Pol α and Pol ε (Fig.1). Lack of functional Pol δ, resulted in high sensitivity (Fig.1; Cardone *et al.* submitted) of the same magnitude as the one seen in absence of the Pso2p. This suggested involvement of Pol δ in the repair of 8-MOP+UVA-induced ICLs and the *pol32* mutant's sensitivity might thus be explained by failure of either of two functions: (1) lack of DNA synthesis required in the formation of Holliday junctions in HR (Heyer 2004; Kawamoto *et al.* 2005; McIlwraith *et al.* 2005) or (2) absence of gap filling in NHEJ (Mahajan *et al.* 2002; Lee *et al.* 2004). Since 8-MOP+UVA treatment was performed with stationary phase haploid cells, we considered NHEJ to be the most likely event.

By analyzing the transformation efficiency of WT and *pol32* mutant strains with plasmids containing 3' (*Pst*I/ *Sac*I) and 5' (*Eco*RI/ *Hind*III) incompatible ends, we found *pol32*Δ strain extremely deficient in joining incompatible DNA ends, particularly in cases of mismatched 5' overhangs (Fig.2B). Transformation efficiency of mutants lacking either

Ctf4p or Dpb3p was not significantly affected (Fig.2A), but it would be necessary to test conditional mutations of the remaining Pol subunits to affirm that Polε and Polα are not involved.

The different pattern of the 5'/5' mismatched ends resolution from WT and *pol32* mutant (Fig.2B) showed that inactivation of Pol32p still allows NHEJ to occur, but in a very inefficient way. When analyzing the role of Pol4p in NHEJ, Daley *et al.* (2005) showed the 3' end joining products of *pol4* mutant to contain gaps. Surprisingly, no gaps were detected in the sequences recovered from *pol32*Δ. Instead, they had in most of the cases a more extensive gap filling than WT (Fig.2B). Two hypotheses might explain these differences: the simplest is that Pol32p interacts, either directly or indirectly, with the critical terminal nuclease(s) at the NHEJ active site – which explains the base removal in the WT – and then Pol δ itself fills the remaining gap. Alternatively, another Pol is recruited in the absence of a functional Pol δ, resulting in a repair product different from that made in the WT. It is difficult to suggest which Pol could be involved, since Pol 4, Pol η and Pol ζ are known not to be required for the gap-filling of 5'-overhangs and we must, therefore, also consider the possibility of an involvement of an unknown polymerase. Although recent work suggests that NHEJ may only play a minor role in ICL repair (de Silva *et al.*, 2000; Wang *et al.*, 2001; Nojima *et al.*, 2005) our results strongly suggest that the DSB, resulting from early steps in ICL processing, are subsequently repaired by a Pol δ driven NHEJ. The fact that Pol32p interacts with the DNA helicase Srs2 (Huang *et al.* 2000) and this in required for NHEJ (Hegde and Klein 2000) indeed supports our findings. Interestingly, Srs2p has been shown to inhibit Rad52-dependent recombinational repair

(Krejci *et al.* 2003; Veaute *et al.* 2003) in what might represent a communication between NHEJ and HR pathways.

Considering existing data, multiple repair pathways are used to deal with ICLs and they depend on the type of ICL, ploidy, the cell cycle phase, and the proteins available for ICL repair. Evidently NER starts processing ICL-containing DNA (Magaña-Schwencke *et al.* 1982; Wilborn and Brendel 1989), resulting in double-strand breaks (DSBs) as repair intermediates. In haploid stationary cells most of these DSBs are different from X-ray or bleomycin-induced ones as only a minority of them is repaired by enzymes of the Rad52 pathway (Grossman *et al.* 2001). Not clear, however, are the later events, except for the well-established HR. Our data allow us to propose a model that depicts a Pol δ- mediated NHEJ (Fig.3). In stationary haploid cells, the DSB generated after ICL processing by NER and further processed by Pso2p, cannot be repaired by HR, being then bound by Ku70/Ku80 which presumably protects the broken DNA ends from nucleolytic degradation. This might mark the damaged site to be recognized by further NHEJ components (Milne *et al.* 1996; Siede *et al.* 1996). Next, the Mre11/Rad50/Xrs2 complex is recruited to the site of DSB (Moore *et al.* 1996; Tsukamoto *et al.* 1996; Chen *et al.* 2001) followed by the joining of the Lig4/Lif1/Nej1 complex to the Ku70/Ku80-bound DNA ends (Wilson *et al.* 1997; Herrmann *et al.* 1998; Teo and Jackson 2000). As the DNA ends are not fully complementary they undergo nucleolytic end-processing mediated by Rad27 (Wu *et al.* 1999) and the remaining gap would then be filled by Pol δ prior to ligation. The putative organization of Pso2p and Pol δ in the same pathway of ICL repair (via NHEJ) may be indicated by the fact that both *pso2* and *pol32* mutants have the same sensitivity to

8-MOP+UVA (Fig.1). A *pso2 pol32* double mutant with the same sensitivity as each of the single mutants would, by this epistatic interaction, strongly support this suggestion.

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Table 1. Yeast strains used in this study

Strain	Genotype	Source
Y10'000	*MATα his3Δ1leu2Δ0 1ys2Δ0 ura3Δ0*	EUROSCARF
Y15550	*MATα his3Δ1leu2Δ0 lys2Δ0 ura3Δ0 ctf4::kanMX4*	EUROSCARF
Y15726	*MATα his3Δ1leu2Δ0 lys2Δ0 ura3Δ0 dpb3::kanMX4*	EUROSCARF
Y16841	*MATα his3Δ1leu2Δ0 lys2Δ0 ura3Δ0 pol32::kanMX4*	EUROSCARF
Y16743	*MATα his3Δ1leu2Δ0 lys2Δ0 ura3Δ0 pso2::kanMX4*	EUROSCARF

Figure legends

Fig. 1 Viability of wild-type, *pso2*Δ, *ctf4*Δ, *dpb3*Δ and *pol32*Δ cells after 8-MOP +UVA treatment. WT (■); *pso2*Δ (□); *dpb3*Δ (●); *ctf4*Δ (▼); *pol32* (▲).

Fig. 2 The effect of *pol32*Δ, *ctf4*Δ and *dpb3*Δ on NHEJ involving incompatible ends.**(A)** Yeast strains transformation efficiency with YCpLac33 digested with *Hind*III (black), *Pst*I/*Sac*I (dark grey) and *Eco*RI/*Sac*I (grey). Each bar represents the mean S.D. from at least three independent transformations. **(B)** Sequences of the digested ends generated by *Hind*III, *Pst*I/*Sac*I and *Eco*RI/*Sac*I cleavages of YCpLac33. Bases in the joint are shown in bold. For *Hind*III and *Pst*I/*Sac*I five junctions were sequenced from each strain.

Fig. 3 Putative ICL repair pathway by Pol δ- mediated NHEJ, in stationary haploid yeast cells. **(A)** DSBs are formed on either side of the ICL, requiring NER and Pso2p participation, removing the lesion and yielding a gap. Next, DNA ends are bound by the Ku70/Ku80 heterodimer and the Mre11/Rad50/Xrs2 complex is recruited. Finally the

Lig4/Lif1/Nej1 complex is target to the DNA ends which are nucleolytically processed by Rad27 and gap-filled by Pol δ prior to end joining. **(B)** Schematic representation of the 5' overhangs of the digested plasmid used as substrate for the NHEJ efficiency evaluation.

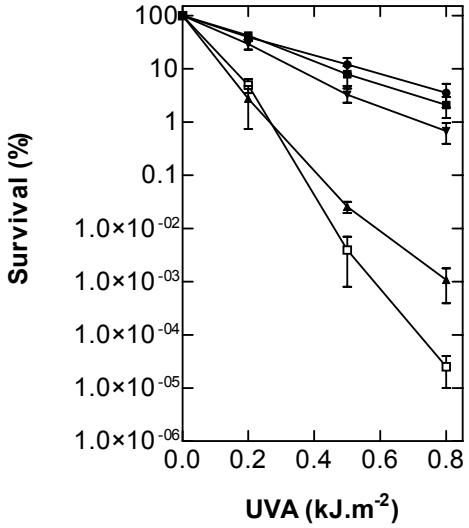
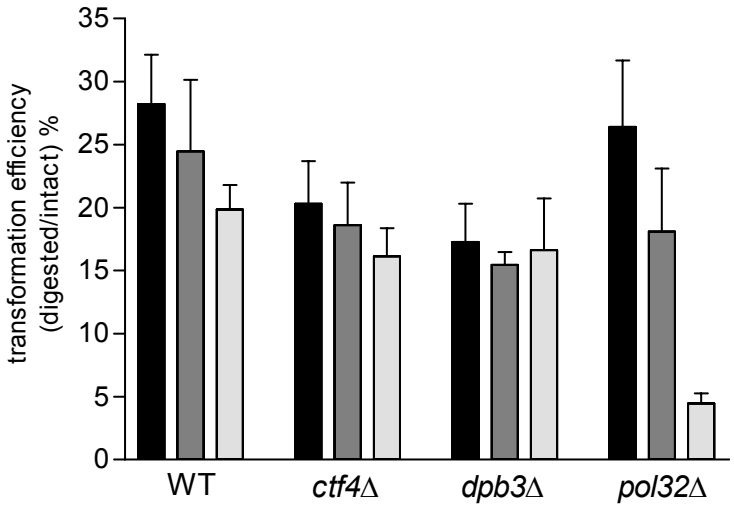

Survival (%)
100
10
1
0.1
1.0×10-02
1.0×10-03
1.0×10-04
1.0×10-05
1.0×10-06
0.0
0.2
0.4
0.6
0.8
UVA (kJ.m-2)

Fig. 1

(A)



(B)

*Hind*III (cohesive 5')

```
---AGGCATGCA       AGCTTGGCGTAATCA---
---TCCGTACGTTCGA       ACCGCATTAGT---
```

Junctions from all strains:

```
---AGGCATGCAAGCTTGGCGTAATCA---
---TCCGTACGTTCGAACCGCATTAGT---
```

*Pst*I/*Sac*I (3'/3')

```
---GCTTGCATGCCTGCA       CGAATTCACTG---
---CGAACGTACGG       TCGAGCTTAAGTGAC---
```

Junctions from all strains:

```
---GCTTGCATGCCTGCTCGAATTCACTG---
---CGAACGTAGCGACGAGCTTAAGTGAC---
```

*Eco*RI/*Hind*III (5'/5')

```
---GACGGCCAGTG       AGCTTGGCGTAATCA---
---CTGCCGGTCACTTAA       ACCGCATTAGT---
```

Junctions from wild-type:

```
5X ---GACGGCCAGTGATTGGCGTAATCA---
   ---CTGCCGGTCACTAACCGCATTAGT---
```

Junctions from *pol32*Δ:

```
2X ---GACGGCCAGTGATGCAGCTTGGCGTAATCA---
   ---CTGCCGGTCACTACGTCGAACCGCATTAGT---

   ---GACGGCCAGTGAATTAGCTTGGCGTAATCA---
   ---CTGCCGGTCACTTAATCGAACCGCATTAGT---

   ---GACGGCCAGTGAGCTTGGCGTAATCA---
   ---CTGCCGGTCACTCGAACCGCATTAGT---
```

Fig. 2

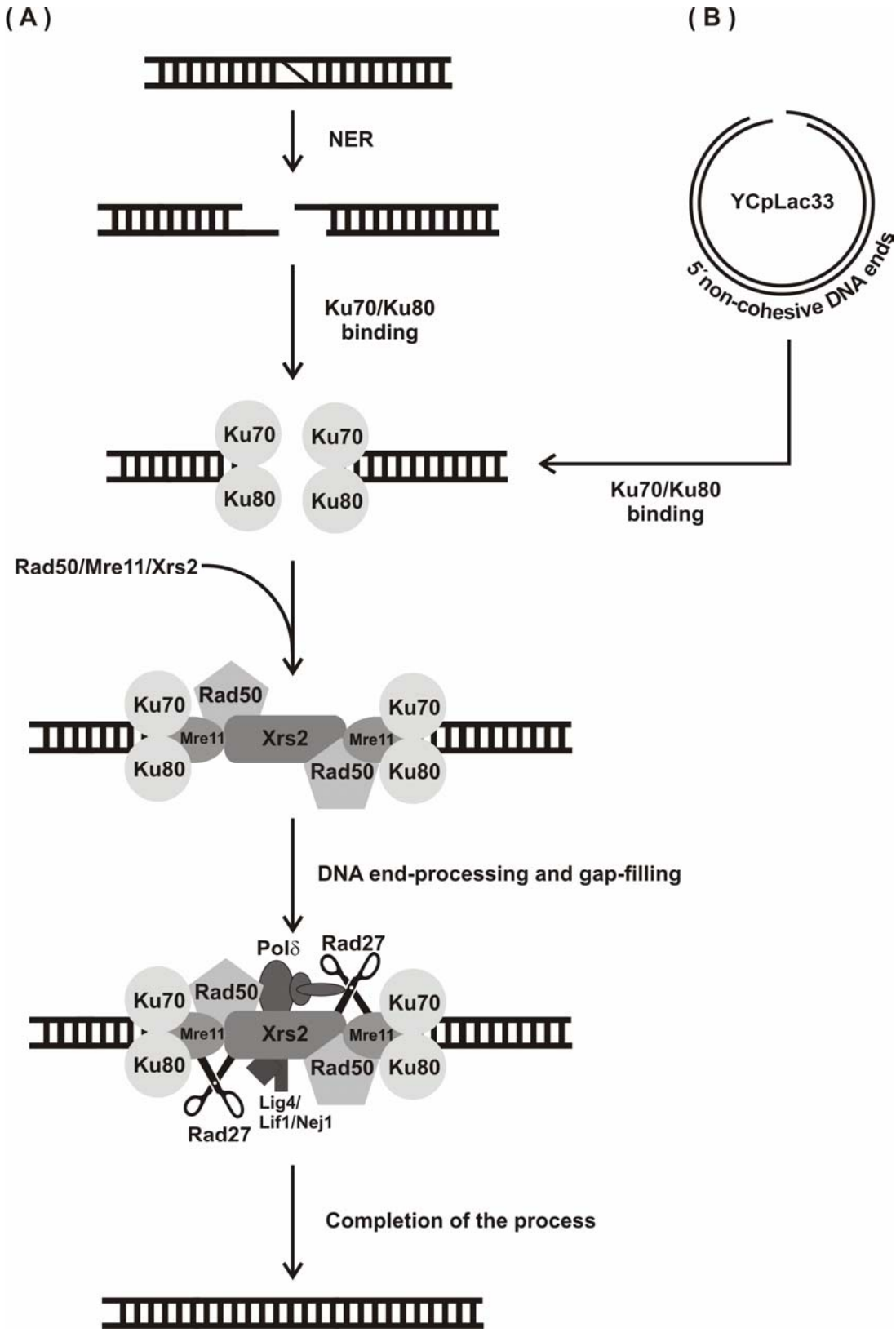

(A)
(B)
NER
YCpLac33
5' non-cohesive DNA ends
Ku70/Ku80
binding
Ku70
Ku70
Ku80
Ku80
Ku70/Ku80
binding
Rad50/Mre11/Xrs2
Ku70
Rad50
Mre11
Xrs2
Mre11
Ku70
Ku80
Rad50
Ku80
DNA end-processing and gap-filling
Polδ
Rad27
Ku70
Rad50
Mre11
Xrs2
Mre11
Ku70
Ku80
Rad50
Ku80
Lig4/
Lif1/Nej1
Rad27
Completion of the process

Fig. 3

CAPÍTULO VI

DISCUSSÃO GERAL

6. Discussão geral

O crescimento e a divisão de uma célula para gerar duas células filhas requerem a coordenação de numerosos eventos, em particular, fidelidade na replicação e na distribuição do material genético para cada célula filha. Em situações extremas, erros nestes processos podem significar a morte de um organismo unicelular e, para organismos multicelulares, culminar em defeitos no desenvolvimento ou oncogênese. Considerando que as células estão constantemente sob o ataque de formas endógenas e exógenas de mutágenos, manter um genoma completo e sem danos é um desafio contínuo e de vital importância para uma célula e suas futuras gerações. Para garantir esta fidelidade, as células evoluíram mecanismos que monitoram o sucesso de cada evento do ciclo-celular, a fim de que estes sejam completados corretamente e na ordem apropriada (Hartwell e Weinert, 1989).

Nos últimos anos, estudos abrangendo leveduras, fungos filamentosos (*Aspergillus*), nematódeos, *Drosophila* e mamíferos, têm contribuído para aumentar o conhecimento das vias de *checkpoint* que primam pela manutenção do DNA. O tema recorrente é a importância destes mecanismos na manutenção da estabilidade genética e a correlação da perda desta função com a etiologia do câncer. Células sem *checkpoints* funcionais mostram instabilidade genômica devido a falhas em responder apropriadamente aos danos no DNA, à replicação defeituosa, ou a uma segregação cromossômica aberrante, resultando em um fenótipo mutador acelerado (Loeb, 1991).

Os passos envolvidos no mecanismo de *checkpoint* que resultam na inibição do ciclo celular são relativamente bem entendidos (para revisão, ver Melo e Toczyski, 2002;

Longhese *et al.* 2003; Kai e Wang, 2003). No entanto, faltam ainda detalhes moleculares a respeito das etapas de reconhecimento do dano que dão início à via de *checkpoint*. A visão mais recente dos estágios iniciais desta via indica que dois complexos de proteínas, Mec1/Ddc2 e Rad17/Mec3/Ddc1, são recrutados a regiões de lesão no DNA, em *S. cerevisiae*, como já detalhado na Introdução (seção 1.3.1.1). Recentemente, identificamos um alelo mutante de *MEC3* – *pso9-1* – o qual é fortemente sensível a vários agentes indutores de danos no DNA e confere baixa mutabilidade induzida (Cardone, 2002).

Considerando a complexidade dos mecanismos implicados no início da via de transdução de sinais de *checkpoint* e o fato de se ter uma nova mutação do gene *MEC3* ainda por ser explorada, buscou-se estender a caracterização molecular e bioquímica da forma modificada de Pso9p/Mec3p, Pso9-1p, avaliando sua atuação com seus parceiros de complexo já conhecidos, bem como buscando novas interações envolvidas na reparação do DNA.

6.1 A influência de Pso9-1p nas interações no complexo Rad17/Mec3/Ddc1 e suas implicações na detecção de danos no DNA

Como abordado anteriormente, o primeiro passo para desencadear uma resposta a danos no DNA eficiente é o reconhecimento dos danos. Estudos em leveduras e mamíferos têm demonstrado que os complexos heterotriméricos Rad17/Mec3/Ddc1 e Rad9/Rad1/Hus1, respectivamente, são cruciais para o sucesso do início da via de *checkpoint* (Melo *et al.*, 2001; Parrilla-Castellar *et al.*, 2004). Para que fosse possível avaliar os efeitos da mutação presente em *pso9*-1 na função de Pso9p/Mec3p na formação deste complexo, o primeiro passo foi determinar a seqüência completa do alelo mutante. A

análise das seqüências revelou uma única deleção, de uma adenina, na posição 802 (802delA), resultando em uma forma truncada de Pso9p, com 276 aminoácidos (Capítulo 3). Várias mutações já haviam sido descritas para o gene *MEC3*, principalmente para seu ortólogo *Hus1*, em *S. pombe*. Empregando mutações sítio-dirigidas, Kaur *et al*. (2001) definiram as seguintes substituições para SpHus1: (1) que não perturba nenhuma das interações do complexo (R2S), (2) que diminuem a eficiência de interação com SpRad9 (ScRad17), mas não com SpRad1 (ScDdc1) (N80I, N121D e I244M) e (3) que enfraquecem a interação com SpRad1 e a eliminam com SpRad9 (W50R, H125R). Mais informações sobre estas interações foram obtidas a partir da reconstituição do complexo 9-1-1 de humanos, a qual revelou que cada uma das proteínas possui um sítio de ligação para os outros dois parceiros, com a região N-terminal de hRad9 (ScRad17) interagindo com hRad1 (ScDdc1), a região N-terminal de hRad1 interagindo com hHus1 (ScMec3) e com a região N-terminal de hHus1 interagindo com a região carboxi-terminal de hRad9 (Burtelow *et al*., 2001). Seguindo este padrão de interação, a mutação *pso9-1*[802delA] estaria comprometendo apenas a interação com Rad17p. Entretanto, as conseqüências para Pso9-1p mostraram-se muito mais drásticas, abolindo as interações com ambos os parceiros de complexo (Capítulo 3). Como esta mutação resulta na eliminação de quase todo o domínio carboxi-terminal de Pso9p/Mec3p, este não foi um resultado tão surpreendente. Por outro lado, o fato dos últimos 25 aminoácidos mostrarem-se essenciais para as interações Pso9p/Mec3p-Ddc1p e Pso9p/Mec3p-Rad17p (Capítulo 3) sugere fortemente o papel estrutural desta região na montagem do complexo heterotrimérico. Ao encontro destes dados, Majka e Burgers (2005) recentemente comprovaram que nenhum dos complexos parciais possíveis de Rad17/Mec3/Ddc1 forma um grampo capaz de ser adicionado ao DNA.

A natureza das estruturas de DNA que são reconhecidas por proteínas sensoras para ativar respostas de *checkpoint* permanece obscura. Mesmo o bem definido sistema SOS de bactérias - que leva à ativação da proteína RecA, definindo o sinal de dano no DNA - tem sido uma complexa tarefa, embora se tenha revelado o papel de DNA simples fita neste processo (Eder *et al*., 2001). Em células eucarióticas, mesmo a hipótese mais simples torna-se complexa quando se considera o tipo de dano, a quantidade de lesões, a fase do ciclo celular e a complexa rede envolvida na mediação destes sinais. Neste trabalho, foi possível constatar a importância da integridade do complexo Rad17/Mec3/Ddc1 na detecção de lesões induzidas por 8-MOP+UVA, uma vez que o mutante *pso9-1* continua interruptamente o ciclo celular, mesmo na presença de danos (Capítulo 3). Este é um aspecto interessante a ser investigado não só para a compreensão da instabilidade genômica, mas principalmente com a finalidade de sugerir estratégias para o desenvolvimento de terapêutica anti-câncer. Neste sentido, muitos esforços têm sido concentrados no estudo da proteína BRCA1 de humanos, também classificada como sensora na via de *checkpoint*, cujas alterações predispõem mulheres aos cânceres de mama e ovários (para revisão ver, Turner *et al.*, 2004). O conhecimento das funções de BRCA1 na sinalização para parada no ciclo-celular, integridade genômica, resposta a danos no DNA e tumorogênese facilitam a triagem de drogas e um melhor planejamento de abordagens terapêuticas. Reagentes que levam à reativação do ciclo-celular, morte celular, reparação defectiva de danos no DNA e/ou promovem a conclusão de uma mitose letal em células mutantes devem ser favoráveis no tratamento de cânceres associados à BRCA1. Trabalhos recentes explorando as debilidades de tumores BRCA1 têm mostrado resultados promissores a este respeito (Simeone *et al.*, 2005; Farmer *et al.*, 2005; Bryant *et al.*, 2005).

6.2 Interações de *PSO9*/*MEC3* com genes de DNA polimerases: definindo um novo papel para DNA polimerases replicativas

Até que se obtivessem maiores esclarecimentos a respeito das respostas geradas pela via de *checkpoint*, acreditava-se que sua função restringia-se à parada do ciclo-celular, apenas para garantir o tempo necessário para a reparação dos danos no DNA. Atualmente, sabe-se que a função desta via não é simplesmente acionar uma parada de ciclo (ver Introdução, seção 1.3.1.1), mas uma série de outras respostas, entre elas recrutar proteínas de reparação ou até mesmo atuar diretamente na reparação do DNA (para revisão, ver Helt *et al.*, 2005).

As primeiras evidências que suportaram este papel vieram do fato que células deficientes em *checkpoint* não eram tão viáveis quanto células tipo-selvagem, após tratamento com agentes indutores de danos ao DNA (para revisão, ver Rotman e Shiloh, 1999). Este fenômeno foi tornando-se mais evidente com o progresso do estudo das proteínas de *checkpoint*. A proteína ATM de mamíferos mostrou-se envolvida na reparação do DNA por recombinação homóloga (Morrison *et al.*, 2000), enquanto BRCA1 somou a este papel, participação na reparação acoplada à transcrição e também em MMR (Moynahan *et al.*, 1999; Gowen *et al.*, 1998). Em *S. cerevisiae*, a fosforilação específica de Rad55p – envolvida em reparação recombinacional - por Mec1p, foi uma evidência conclusiva para interligar diretamente a resposta de *checkpoint* à reparação do DNA (Bashkirov *et al.*, 2000). Ultimamente, acumulam-se dados a respeito de interações físicas e funcionais entre complexos PCNA-*like* e proteínas de reparação (ver Introdução do Capítulo 4). Recentemente, Sabbioneda e colaboradores (2005) mostraram a interação de Rad17/Mec3/Ddc1 com a subunidade Rev7 da DNA polimerase ζ. Neste trabalho, ao

buscar por novos interatores do complexo Rad17/Mec3/Ddc1, encontramos que mutantes *mec3*Δ em combinação à inativação da subunidade Pol32 da DNA polimerase δ, tornam-se muito mais sensíveis a danos causados por UVC e 8-MOP+UVA (Capítulo 4), mas não o suficiente para indicar interação física entre estas proteínas. Apesar de ser sugerido fortemente que este complexo possa desempenhar o papel de uma plataforma para proteínas de reparação, incluindo DNA polimerases, esta situação não foi observada para nenhuma das DNA polimerases replicativas essenciais (Capítulo 4), pelo menos quanto às subunidades testadas. Contudo, a aditividade encontrada para 8-MOP fotoativado indica que as tarefas executadas por Pol δ e Rad17/Mec3/Ddc1 são complementares, ou seja, Pol δ atua na reparação independentemente do complexo Rad17/Mec3/Ddc1, mas este potencializa a reparação das lesões, ao transmitir o sinal a outras proteínas efetoras. Esta situação exemplifica a complexa rede envolvida na reparação destas lesões, principalmente quando se considera DSBs como intermediários de reparação de ICLs (Tabela 3).

Tabela 3. Classes funcionais de proteínas que respondem a DSBs [a]

Classe funcional	***Saccharomyces cerevisiae***	***Homo sapiens***
Sensores de danos no DNA	Mre11/Rad50/Xrs2	Mre11/Rad50/Nbs1
	Rfa1/Rfa2/Rfa3	RPA1/RPA2/RPA3
Proteínas de *checkpoint*	Rad24/Rfc2-5	Rad17/Rfc2-5
	Ddc1/Mec3/Rad17	Rad9/Hus1/Rad1
	Mec1/Ddc2	ATR/ATRIP
	Tel1	ATM

continuação

Adaptadores/Transdutores	Mrc1	Claspina
	Rad9	BRCA1, MDC1, 53BP1
	Dpb11	TopBP1
Cinases efetoras	Rad53	Chk2
	Chk1	Chk1
Proteínas regulatórias	Sae2	?
	?	FancACEFGL
	?	FancD2
	?	c-AbI
Proteínas de recombinação homóloga	?	BRCA2/DSS1
	Rad52	Rad52
	Rad51, Rad55/Rad57	Rad51, Rad51B, Rad51C, Rad51D, Xrcc2 e Xrcc3
	Rad54	Rad54
	Rad59	Rad52B
	Rdh54	Rad54B
Proteínas de recombinação não-homóloga (NHEJ)	Ku70/Ku80	Ku70/Ku80
	Dnl4/Lif1	LIG4/XRCC4
	?	DNA-PKcs
	Pso2	Artemis

[a] Adaptado de Lisby e Rothstein, 2005

A hipersensibilidade do mutante *pol32*Δ a 8-MOP+UVA levou-nos a investigar a participação ainda não descrita de uma DNA polimerase replicativa na reparação deste tipo de dano. Sabe-se que tanto processos livre de erro quanto processos sujeito a erro estão envolvidos na reparação de ICLs, e que estes englobam proteínas envolvidas em excisão de nucleotídeos, recombinação homóloga e síntese translesão (para revisão, ver Dronkert e Kanaar, 2001; Noll *et al.*, 2006). No entanto, pouco se sabe a respeito dos passos posteriores a incisão do ICL. Ainda que alguns trabalhos apontem a via NHEJ como não essencial para a reparação de ICLs (De Silva *et al.*, 2000; Wang *et al.*, 2001; Nojima *et al.*, 2005), a sensibilidade à 8-MOP+UVA resultante da inativação de Pol32p poderia ser conseqüência de uma via NHEJ não funcional. A baixa eficiência na geração de produtos de NHEJ na linhagem *pol32*Δ confirmou a participação de Pol δ nesta via (Capítulo 5), enfatizando a importância da sobreposição de funções entre DNA polimerases. Considerando que alguns tipos de tumores desenvolvem resistência a agentes bi-funcionais, principalmente porque passam a ser capazes de reparar ICLs (Bramson *et al*., 1995; Chaney e Sancar, 1996; McHugh *et al.*, 2001; Panasci *et al.*, 2002), desvendar as vias alternativas para reparação destas lesões pode levar a estratégias mais efetivas para o tratamento do câncer ou ainda ao planejamento de novas drogas que produzam ICLs não reparáveis.

Como abordado, o estudo das respostas biológicas a danos no DNA compreende reparação do DNA, mutagênese, tolerância a danos, mecanismos de *checkpoint*, morte celular programada, entre outras repostas aos insultos no genoma. As vias regulatórias deste cenário integrado têm sido decifradas e com isto, conquistas na área de terapia gênica e na intervenção terapêutica irão oferecer novas estratégias para evitar conseqüências não

desejáveis dos danos ao DNA, especialmente o câncer. No entanto, embora as mutações possam ter conseqüências catastróficas, elas são fundamentais para a diversidade genética, a qual é favorecida por um balanço entre estabilidade e instabilidade genômica – entre mutação e reparação do DNA.

CAPÍTULO VII

CONCLUSÕES

7. Conclusões

A maioria dos processos celulares nucleares envolvendo a manutenção da integridade/estabilidade genômica é desempenhada por complexos multiprotéicos que interagem entre si e atuam de forma coordenada/regulada para garantir a fidelidade do fluxo da informação genética. O estudo da atuação da proteína Pso9/Mec3, bem como de sua forma modificada Pso9-1 na reparação do DNA nos permitiu formular as seguintes conclusões:

- A mutação 802delA encontrada na linhagem *pso9-1* codifica um polipeptídeo de 276 aminoácidos, no qual os últimos nove aminoácidos estão fora da fase de leitura apropriada e seu domínio carboxi-terminal encontra-se drasticamente diminuído;

- A forma modificada da proteína Pso9/Mec3, Pso9-1p, não é capaz de manter as interações com seus parceiros de complexo Rad17p e Ddc1p;

- O domínio carboxi-terminal de Pso9p/Mec3p é essencial para a montagem do complexo Ddc1/Mec3/Rad17, sendo que a perda dos 25 aminoácidos finais já anula suas interações;

- As células que portam a mutação *pso9-1* não são capazes de detectar danos no DNA e prosseguem no ciclo-celular, mesmo após tratamento com 8-MOP+UVA;

- *MEC3* interage geneticamente com o gene codificante da subunidade Pol32p da DNA polimerase δ, de forma aditiva, após tratamento com 8-MOP+UVA e UVC;

- O mutante *pol32*Δ é extremamente deficiente em religar extremidades de DNA 5' não coesivas, evidenciando a participação de Pol δ na reparação de pontes intercadeias induzidas por 8-MOP+UVA e sua atuação em NHEJ.

CAPÍTULO VIII

PERSPECTIVAS

8. Perspectivas

O conjunto de dados obtidos neste trabalho amplia a complexa rede de proteínas que responde a danos no DNA, especialmente pontes intercadeias. Contudo, detalhes importantes dos mecanismos que atuam na reparação de ICLs, em eucariotos, ainda são escassos. Assim, as perspectivas deste estudo podem ser resumidas nas seguintes abordagens:

- Reconstituir o complexo Ddc1/Mec3/Rad17 *in vitro* e avaliar uma possível especificidade de reconhecimento de danos, utilizando como substratos, plasmídeos contendo ICL ou quebras duplas, empregando a técnica de "gel shifting";

- Definir o tipo de interação genética entre *POL32* e *PSO2* na reposta ao tratamento com 8-MOP+UVA, tendo em vista a sensibilidade específica do mutante *pso2* a ICLs;

- Avaliar a eficiência de transformação, bem como analisar os produtos originados por NHEJ, de mutantes condicionais das demais subunidades de Pol α (Pol12 e Pol1) e Pol ε (Pol2, Dpb2 e Dpb3);

- Ampliar a caracterização dos padrões de ligação de extremidades não-homólogas nos mutantes de DNA polimerases replicativas, avaliando extremidades com diferentes tipos de microhomologia e também extremidades incompatíveis.

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ANEXO

Pak1p, a putative partner of Pso2p/Snm1p in repair of DNA interstrand cross-links in *Saccharomyces cerevisiae*

Manuscrito submetido ao periódico Molecular and General Genomics

Pak1p, a Putative Partner of Pso2p/Snm1p in Repair of DNA Interstrand Cross-Links in *Saccharomyces cerevisiae*

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Abstract

By isolating putative binding partners through the two-hybrid system (THS) we further extended the characterization of the specific ICL repair gene *PSO2*. Isolation of potential binding partners of Pso2p using the THS identified nine different fusion protein products, among them part of the protein kinase Pak1p, a temperature-sensitive suppressor of DNA Polymerase α mutations. Pak1p interacted with the C-terminal β-CASP domain of Pso2p, a region conserved among Pso2p-like orthologues. Integrity of the β-CASP domain was shown to be essential for WT-like DNA ICL repair in *pso2Δ* complementation assays. Further, comparison of mutagen-sensitivity phenotypes of *pso2Δ* and *pak1Δ* single as well as the *pso2Δ pak1Δ* double mutant revealed that *PAK1* is necessary for complete WT-like repair. In addition, *in vitro* phosphorylation assays demonstrated that Pso2p is a phosphorylation substrate (or target) of protein kinase Pak1p. The epistatic interaction of both mutant alleles in the double mutant suggests that Pak1p has a role in the same pathway of controling sensitivity to DNA damaging agents as Pso2p. Based on these facts, we propose a model for the function of Pso2p and Pak1p in removal of DNA-ICL from yeast DNA.

Key words: DNA repair, Interstrand cross-link, Two hybrid system, *Saccharomyces cerevisiae, PAK1, PSO2.*

Introduction

DNA interstrand cross-links (ICL) covalently link the two complementary DNA single strands, thereby preventing strand separation. This results in stalled transcription and DNA replication, and ultimately prevents segregation of DNA and chromosomes. In bacterial and yeast cells, the presence of one non-repaired ICL can be lethal (Brendel and Henriques 2001). Due to their extreme genotoxicity, agents that induce ICL in DNA are widely used in anti-tumor and in photo-therapy. Examples include cisplatin, mitomycin C, sulphur and nitrogen mustards and its derivatives, such as melphalan and cyclophosphamide, and photo-activated bi-functional psoralens. Most of these agents cause a number of different lesions in DNA, including monoadducts, DNA-protein cross-links, and DNA intra- and inter-strand strands cross-links (ICL). The latter constitute the most toxic DNA lesion and, therefore, the main cause of cell death, although they sometimes amount to only a small fraction of the total number of DNA adducts formed by a bi- or poly-functional chemical (c.f. reviews Brendel and Henriques 2001 and Brendel and Ruhland 1984).

The mechanism of ICL repair in yeast was partially elucidated by isolating genes specifically involved in ICL repair (Henriques and Moustacchi 1980, Ruhland et al. 1981, Siede and Brendel 1981). A large number of repair genes influence sensitivity of *S. cerevisiae* to ICL-forming treatments, amongst them many genes of the RAD3 and RAD6 epistasis groups of DNA repair (Henriques and Moustacchi 1981, Grossmann et al. 2000) but only the mutant alleles of the yeast gene *PSO2* (allelic to *SNM1* (Cassier-Chauvat and Moustacchi 1988) have been found to be specifically sensitive to all ICL-inducing DNA treatments (Kircher and Brendel 1983). Many other *snm* and *pso* mutants have been isolated after screening for strains with increased sensitivity to nitrogen mustard (HN2) and photo-activated 8-methoxypsoralen (8MOP+UVA), respectively (Henriques and Moustacchi 1980, Ruhland et al. 1981, Siede and Brendel 1981) but none of them exhibits such specific sensitivity to ICL-generating treatments as mutant alleles of *PSO2/SNM1*

(Ruhland et al. 1981, Kircher and Brendel 1983, Magaña-Schwencke et al. 1982). Following a suggestion of the Stanford yeast group (to avoid confusion with other *snm1* mutants in the yeast *S. cerevisiae)* we have recently introduced a new *PSO2* nomenclature that unifies *snm1* and *pso2* mutant alleles (Brendel and Henriques 2001).

Gene *PSO2* encodes a 76 kDa nuclear protein (Richter et al. 1992, Niegemann and Brendel 1994). In contrast to their extremely high sensitivity to ICL generating treatments *pso2* mutants are only mildly sensitive to mono-functional alkylating agents and to UV-light of 254nm (UVC) and are not hypersensitive to gamma rays (2, 3). In exponentially growing cells, expression of *PSO2* can be induced by ICL-forming agents or by UVC but not by the mono-functional alkylating agent MMS and by the UVC mimeticum 4NQO (Wolter et al. 1996) and induction is controlled by the *DUN1*-encoded kinase (Zhou and Elledge 1993). Over-expression of *PSO2* does not lead to an increased resistance to nitrogen mustard and *cis*platin but, to the contrary, results in slightly increased sensitivity of the transformants to these mutagens (Martin Strauss, personal communication). Analysis of DNA repair in 8-MOP+UVA, HN2 and *cis*platin-treated *pso2-1* and *pso2-11* mutants showed that incision near ICL and excision of this DNA damage proceeds WT-like, but a late step in ICL repair, necessary to reconstitute high m.w. DNA from low m.w. DNA generated by earlier incision/excision events (Magaña-Schwencke et al. 1982, Wilborn and Brendel 1989), is failing. The blocking of 3 modes of DNA repair (recombinational, non-homologous end joining, error-prone) each inhibits full repair of HN2- and *cis*platin-induced ICL, indicating that all 3 modes of repair are involved in removal of this DNA lesion (McHugh et al. 2000, Grossmann et al. 2001). However, contribution to repair of a particular pathway is cell cycle-dependent (McHugh et al. 2000).

Many DNA damage repair genes are conserved from bacteria via yeast to higher eukaryotes (Eisen and Hanawalt 1999). Accordingly, *SNM1/PSO2* homologs have been found in mammals and humans. Based on its homology with the yeast *PSO2* locus, human gene *KIAA0086* is a likely

candidate for a role in DNA ICL repair (Demuth and Digweed). Mouse embryonic stem cells with a *PSO2* (-/-) genotype are sensitive to mitomycin C but not to other ICL-inducing agents (Dronkert et al. 2000). The recent linking of mutant alleles of the human *ELAC2* and Artemis genes, encoding Pso2p-like proteins, with hereditary pre-disposition for prostate cancer and severe immune deficiency/radio-sensitivity, due to defects in V(D)J recombination respectively, points to a contribution of human *PSO2*-like genes to DNA or chromosome stability and genome integrity in man (Tactigian et al. 2001, Moshous et al. 2001, Simard et al. 2002). The conserved region of the C-terminal half of most Pso2p-like orthologues was named β-CASP motif (after metallo-**β**-lactamase-associated **C**PSF **A**rtemis **S**NM1/**P**SO2) and marks a new family of enzymes involved in nucleic acid metabolism (Callebaut et al. 2002).

Several proteins encoded by genes of the 3 epistasis groups of yeast DNA repair are involved in ICL repair (Brendel and Henriques 2001, Henriques and Moustacchi 1981, McHugh et al. 2000, Grossmann et al. 2001). Enzymes of nucleotide excision repair (NER) start processing ICL-containing DNA but ICL repair is post-incisionally blocked in absence of functional Pso2p (and probably at a post excision step as well). However, the role of Pso2p in ICL removal and/or in subsequently reconstituting high m.w. DNA is not well defined. The role of Pso2p in ICL-repair in yeast could be clarified by knowing its interaction with other proteins. Therefore, with the aim to better understand the role of this protein in ICL repair , we have initiated a screen for potential Pso2p binding partners using the yeast THS. In this report we present a collection of potential binding partners of Pso2p and demonstrate that Pso2p is a phosphorylation target of the protein kinase Pak1p, a temperature-sensitive suppressor of DNA polymerase α mutations (Hovland et al. 1997).

Material and Methods

Yeast strains

All yeast strains used in this study are listed in Table 1. Phenotypes of the haploid ascospore-derived strains were determined by establishing the respective deletion markers: geneticin resistance (*YER129w::kanMX4*) and uracil prototrophy (*pso2::URA3*).

Genetic and molecular biological methods

Techniques in yeast genetics were according to Rose *et al.* (Rose et al. 1990) and standard molecular techniques were performed according to Ausubel *et al*. (Ausubel et al. 1996).

E. coli strains and plasmids

Escherichia coli strains XL1-blue (Stratagene), KC8 (Watt et al. 1995), and TOP10 (Invitrogen) were used as recipients for cloning procedures. The following constructions were used: *LexAPSO2-A* (contains the *SNM1/PSO2* coding ORF from aa 47 to 661, in frame fused to the LexA DNA binding domain of pEG202 using an internal *Eco*RI restriction site); *LexAPSO2-B* (contains the *SNM1/PSO2* coding ORF from aa 47 to 466, fused in frame to the LexA DNA binding domain of pEG202 via two internal *Eco*RI restriction sites). pMS3141I containing the *PSO2* coding ORF disrupted with *URA3* was used to obtain *pso2Δ* strains by single step gene replacement (Rothstein 1983).

Over-expression and Purification of recombinant GST-Pso2p and GST-Pak1p proteins

For expression and purification of Pso2 protein, a 2734 bp *Sna*BI-*Sac*I- fragment from pDR3141 (Richter et al. 1992) was firstly subcloned into the *Sma*I and *Bam*HI sites of pUC18. From the resulting plasmid, an internal 2014bp *Bgl*II-*BamH*I fragment containing the *PSO2* ORF was then cloned into the *Bam*HI site of the vector pGEX-KG (Amersham Biosciences). The GST-

Pak1p recombinant protein was obtained from pPH601 (kindly provided by R. Sclafani (Hovland et al. 1997) expression. The GST-Pso2p and GST-Pak1p fusion proteins were expressed in *E. coli* strain BL21(DE3)pLysS (Novagen) and affinity-purified on glutathione-Sepharose 4B (Amersham Biosciences). Pso2p was obtained by thrombin (Amersham Bioscences) cleavage of GST-Pso2p according to the manufacturer's instructions.

In vitro phosphorylation assay

Pso2p (8ug) was incubated with either GST-Pak1p (2ug) or GST (3ug) in a protein phosphorylation buffer containing 50mM Tris-HCl pH7.5, 15mM $MgCl_2$, 1mM DTT, 5uM unlabeled ATP and 2 uCi of γ-^{32}P-ATP (3000Ci/mmol; Amersham Bioscences), at room temperature for 30 minutes. Alternatively, GST-Pak1p was incubated alone in order to identify the autophosphorylation activity. The reactions were stopped by adding 10uL of 2X SDS sample buffer (0.125M Tris-HCl pH6.8, 4% SDS, 20% glycerol, 0.3 M β-mercaptoethanol, 0.05% bromophenol blue) and boiling for ten minutes before loading onto a 10% SDS- polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue G 250, dried, and subjected to autoradiography.

Media and growth conditions

Yeast strains were grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30^{o}C. For selective growth, either YEPD plus geneticin (G418, Calbiochem) 0.2 mg/mL or SynCo (0.67% yeast nitrogen base from DIFCO/USB, 2% glucose, 1% ammonium sulfate) supplemented with the appropriate essential nutrients (40 μg/mL) was used. For detection of canavanine-resistant mutants, canavanine sulfate (Sigma) was added at 40 μg/mL to appropriately supplemented SynCo media. The sporulation medium (KAC) contained 1% potassium acetate, 0.1% yeast extract and 0.05% glucose. Diploids were sporulated on KAC agar for 3-5 days. Sporulation efficiency was calculated by microscopically determining the frequency of asci in a counting chamber. Plating medium was solidified with 2% agar.

Chemical and mutagen treatments

Bleomycin (BLE, Calbiochem) was dissolved in water at a concentration of 10 mg/mL, filter-sterilized and stored at –20°C prior to use. BLE containing YEPD plates were prepared by diluting BLE solution at given concentrations in warm YEPD (see Fig.1). The plates were stored overnight at 4°C before use.

UVC: different concentrations of cells in exponential phase of growth (~10^7cells/mL) were spread immediately in triplicate on YEPD plates and, after drying, irradiated with UVC (Stratalinker, Stratagene) with doses ranging from 0 to 60 J/m^2. For drop tests, stationary cultures were serially diluted in 1:10 steps and 5 or 10 μL aliquots were spotted onto YEPD medium. After drying the plates were UVC-irradiated as described above.

8-MOP+UVA: suspensions of 5x10^6 cells/mL in exponential phase were treated with photo-activated 8-methoxypsoralen (8-MOP+UVA; Sigma) according to Henriques and Moustacchi (1980). After dilution and plating all plates were incubated for 3-5 d at the appropriate temperature in the dark. Survival data represent the average of at least three experiments.

Two-hybrid analysis

Two-hybrid analysis was essentially as described by Gyuris *et al*. (Gyuris et al. 1993). Two in-frame fusions of the *PSO2* ORF to the LexA DNA binding domain plasmid (pEG202) were constructed for the screening and interaction analysis using the THS. The *LexAPSO2-A* fusion contains the portion from aa 47 to 661 and the *LexAPSO2-B* is a truncated construction from aa 47 to 466, lacking the last 195 aa of the C-terminal part. Both were constructed using internal *Eco*RI restrictions sites and displayed no intrinsic transcriptional activation when transformed into strains containing reporter constructs regulated by LexA DNA-binding sites.

Yeast strain EGY48 containing the reporter plasmid pSH18-34 and the bait plasmid LexAPSO2-A was transformed with a yeast genomic library cloned into the prey plasmid pJG4-5 (Watt et al. 1995). Plasmids were isolated from yeast that survived selection for leucine prototrophy on galactose and showed *lacZ* expression on X-Gal-galactose plates. *E.coli* strain K12 KC8 *pyrF::Tn5, hsdR, leuB600, trpC9830, lacD74, strA, galK, hisB436* was used for the rescue of the plasmids as described by Gyuris *et al.* (Gyuris et al. 1993). Plasmid DNA was sequenced with an Applied Biosystems sequencer A377 (Foster City, CA). All obtained sequences were submitted to a Blast search at MIPS (Munich Information Center for Protein Sequences (Mewes et al. 2000). The expression of the LexA fusion baits were analyzed by western blotting using a monoclonal antibody against LexA (Clontech Laboratories Inc.) and with complementation assays in *pso2Δ* mutants.

β-galactosidase activity of the pSH18-34 two-hybrid reporter plasmid was assayed and quantified according to Ausubel *et al.* (Ausubel et al. 1996) using exponential phase cultures (approx. $2x10^7$ cells/mL). Three to four individual transformants were assayed in liquid SC that lacked the appropriate nutrients.

Results

Isolation of potential molecular partners of Pso2p using the THS

A two-hybrid screen was used to identify proteins that may physically interact with Pso2p. The full complementing *LexAPSO2-A* bait (Fig.1) was used in the screening and a population of $2x10^6$ independent transformants was obtained after transformation with the prey library. Aliquots were pooled and plated on selective medium containing galactose for induced expression of the activation domain fusion library. Some 320 clones were identified that allowed growth on SD-Leu as a result of the activation of the LexAop**::***LEU2* reporter construct. These clones were colony-purified, molecularly characterized by restriction mapping, and re-tested after re-transformation for their ability to activate Gal-inducible transcription of two reporter constructs (*LexAop::LEU2* and *pSH18-34*; (Gyuris et al. 1993). We found 47 transformants containing putative interactors, able to activate transcription of the 2 independent reporter constructs in the presence of *LexA-PSO2-A*. Further sequencing of the library plasmids revealed 13 different fusion protein products with 4 of them containing no real ORF sub-fragments or incorrect orientation. The remaining 9 different fusion protein products were unable to induce transcription when co-expressed with unrelated LexA DNA-binding domain fusions (p*RFHM1* (Hovland et al. 1997), *LexAPSO5* - yeast *RAD16* allele and *LexAHDF1* - yeast Ku70 protein), indicating that the interaction with *LexA-PSO2-A* was specific (Table 2). Three of these putative Pso2p interacting proteins are encoded by ORFs with as yet unknown function. The remaining 6 are encoded by the genes *NRK1, PAK1*, *APL6, FIR1, IFH1* and *REH1* their encoded proteins can roughly be grouped into five functional classes: (a) cell cycle and cell wall maintenance (*NRK1*); (b) DNA synthesis (*PAK1*); (c) RNA metabolism (*FIR1* and *IFH1*) (d) an APL3-complex associated protein (*APL6*) and protein degradation (*REH1*).

We focused our efforts on the study of Pso2p interaction with most relevant candidate based

on the available literature and published data, corresponding to the gene *PAK1*. Encoding a protein kinase capable of suppressing DNA polymerase alpha mutations, Pak1p was suggested to modify/stabilize thermo-labile DNA polymerases during DNA repair (Hovland et al. 1997). The same C-terminal third of *PAK1* ORF was independently isolated 6 times via THS. The domain isolated in the fusion protein encoded by *PAK1* is preceded by the kinase consensus motifs (aa 131-450 (Hovland et al. 1997) and corresponds to the last C-terminal third of the protein (aa 618-1142). Using this part of the *PAK1* coding region as query, the non-redundant database (NRDB) and domain databases at NCBI were searched using PSI-BLAST and RPS-BLAST, respectively, and no significant similarity to known deposited domains/proteins were highlighted within this region.

Pak1p interact with the conserved C-terminal β-CASP domain of Pso2p

To evaluate which part of Pso2p is required for interaction with its putative binding partner Pak1p, we firstly tested the ability of the Pak1p prey-fusion protein to induce the expression of *lacZ* and *LEU* reporters in presence of the full *pso2* complementing bait LexAPSO2-A and the truncated construction LexAPSO2-B lacking the C-terminal 195 aa. Deletion of the last 195 aa within the β-CASP domain in LexAPSO2-B fails to complement *pso2Δ* mutants (Fig.2) and, therefore, was used to determine if the conserved β-CASP domain, essential for WT-like DNA repair, also mediates interaction between its potential molecular partners. The *PAK1* prey fusion product was able to activate both reporters (*lacZ* and *LEU*) in the presence of LexAPSO2-A bait (Leu prototrophy and blue colour in galactose-containing SynCo) whereas interaction with LexAPSO2-B was considerably reduced (Table 3). This implies that the deletion of the last 195 aa within the conserved β-CASP domain specifically affects the interaction between Pso2p with the prey fusion encoded by *PAK1*. Figure 2 shows a multiple protein alignment of this recently characterized domain amongst potential orthologues of *PSO2* in various organisms, where the deleted portion of *LexAPSO2 B* bait is highlighted. In addition to the three characteristic conserved polar aa

constituting motifs A, B and C (Callebaut et al. 2002), another polar aa residue (His502 in Pso2p) was found to be conserved in a predicted extended strand region within aa sequence 491-509 that has significant similarity in all Pso-like proteins but one, the human Artemis protein.

Genetic interaction between PSO2 and PAK1 after induced DNA damage

Three putative *PSO2* interactors *PAK1, YJL084c* and *YHR080c* were characterized for their response to mutagen treatment. The haploid mutants *yjl084cΔ* and *yhr080cΔ* showed WT-like sensitivity to 8-MOP+UVA, UVC, MMS, Trenimon, and HN2 (data not shown), whereas the *pak1Δ* strain was sensitive to UVC and to 8MOP+UVA. Since all known *pso2* mutant alleles confer high sensitivity to the ICL-producing 8-MOP+UVA treatment but are only moderately sensitive to UVC (Brendel and Ruhland 1984, Henriques and Moustacchi 1980, Ruhland et al. 1981), survival was determined in single and double mutants after both treatments. Figure 3 shows that *pak1Δ* mutation confers sensitivity to both treatments and that combination of *pso2Δ* and *pak1Δ* yields double mutants with a *pso2Δ*-like sensitivity phenotype, implying an epistatic interaction of the two mutant alleles. The survival data clearly indicates that gene *PAK1* is necessary for normal resistance to UVC and 8-MOP+UVA and its epistatic interaction with *pso2Δ* suggests that it has a role in the same pathway of controlling sensitivity to DNA damage agents as *PSO2*, i.e. NER.

Pso2p is phosphorylated by Pak1p in vitro.

Since Pak1p was found to interact with Pso2p in the yeast two-hybrid system and taking into account that the combination of both mutations conferred an increased sensitivity to 8-MOP +UVA and, we aimed to investigate whether this interaction could be confirmed with an *in vitro* protein interaction assay. As a kinase activity was described for Pak1p (Hovland et al. 1997) we hypothesized whether Pso2p could be phosphorylated by Pak1p. The Fig. 4 shows the results obtained in the *in vitro* phosphorylation assay. The Pak1p autophosphorylation activity, firstly

reported by Hovland et al. (Hovland et al. 1997), was confirmed (105 kDa GST-Pak1p autophosphorylation signal; Fig.4 lanes 1 and 2). When purified Pso2p was added, an additional phosphorylated protein signal was observed at the predicted size of Pso2p (76kDa) (Fig. 4, lane 3). When the concentration of the purified Pso2 protein was increased in relation to Pak1p (Fig. 4 lanes 4, 6 and 7) an inhibition of the Pak1p phosphorylation activity was observed, suggesting a possible substrate inhibition effect or an allosteric mechanism. When Pso2p was incubated with GST (encoded by pGEX-KG empty plasmid) no detectable Pso2p phosphorylation occurred thus showing that Pak1p is necessary for Pso2p phosphorylation *in vitro* (Fig. 4, lane 5). Also no detectable phosphorylation was observed when Pso2p was incubated with an unrelated caseine kinase from *Catharanthus roseus* (data not shown). These results suggests that an association of Pso2p with Pak1p is possible and could direct its enzymatic activity on a DNA post-incision substrate (ICL repair intermediate) for normal DNA repair function in yeast.

Discussion

The *pso2* and *snm1* mutants were amongst the first yeast isolates found to be specifically sensitive to highly cytotoxic bi– or poly-functional mutagens (e.g. 8MOP+UVA, nitrogen mustard, Trenimon) that, apart from mono-functional adducts, also produce bi-functional DNA lesions, amongst them DNA-ICL (Brendel and Ruhland 1984, Henriques and Moustacchi 1980, Ruhland et al. 1981, Cassie and Moustacchi 1981). Although being extensively studied (Brendel and Henriques 2001) the role of *PSO2* as well as of that other *PSO2*-like orthologues (Dronkert et al. 2000, Tactigian et al. 2001, Moshous et al. 2001, Li et al. 2005, Zhang et al. 2004, Barber et al. 2005, Yu et al. 2004, Bonatto et al. 2005) in the removal of ICL from DNA is still not well understood. By identifying new phenotypes for *pso2Δ* by and isolating set of putative interactors of Pso2p using the THS we could extend the genetic and molecular characterization of *PSO2*

involvement in repair of DNA-ICL.

The abortive repair of DNA-ICL in *pso2* mutants leads to DNA degradation, apparently via DNA double strand breaks (DSB) (Henriques and Moustacchi 1980, Magaña-Schwencke et al. 1982, Magaña-Schwencke and Averbeck 1991). These DSB must be different from those introduced by ionizing radiation, as *pso2* has WT-sensitivity to ionizing radiation (Brendel and Ruhland 1984, Henriques and Moustacchi 1980). Bleomycin (BLE) is used as an X-ray mimetic and is known to introduce DNA degradation via DSB through the production of free radicals (Absalon et al. 1995). Since *pso2* mutants are also sensitive to BLE (Fig.1) we may deduce that BLE-induced DSB are also different from those produced by ionizing radiation. DSB in DNA-ICL containing *pso2* mutants may be the consequence of initiated repair events that were not successfully terminated (abortive repair). This would also indicate that X-ray-induced DNA DSB are not a substrate for Pso2p (and putative protein partners) and that Pso2p will process DSB generated by other (repair) proteins as intermediates in ICL excision repair.

PAK1, was the most interesting amongst the putative interactors found for Pso2p, and thus was further investigated. *PAK1* stands for a DNA *P*olymerase *A*lpha suppressing protein *K*inase, which, when over-expressed, was shown to act as a *RAD9*-dependent, allele-specific suppressor of thermo-labile DNA polymerase α mutations, a feature which suggested a possible involvement in cellular responses to DNA damage (Hovland et al. 1997). It is worth noting that not even a single gene known to be directly involved in DNA repair was found in two independent two-hybrid screenings (this work and M. Strauss, unpublished data). Perhaps our prey libraries were not representative or assembly of the protein complexes involving Pso2p relies on weak indirect interactions specific for the cell repair response state. On the other hand, it may be an indication of the complex processes of as yet unknown mechanisms and protein complexes acting in ICL repair.

The integrity of the conserved domain existing between Pso2p-related proteins of several species at the C-terminal third of the protein, baptized as β-CASP by Callebaut et al. (2002), was

clearly shown to be essential for WT-like DNA repair, confirming the previous findings reported by Li & Moses (Li and Moses 2003) (Fig. 1). Additionally, we have shown that the β-CASP is necessary to keep interaction with Pak1p (Table 3). Deletion of the last 195 aa in LexAPSO2-B bait includes the two conserved motifs B and C described by these authors and another conserved histidine motif identified in our study (Fig. 2). While motifs A and B are conserved in all members of the β-CASP family, motif C distinguishes those members involved in RNA metabolism, where it is always a histidine, from proteins acting on DNA, where histidine is substituted by the hydrophobic valine.

Of the putative Pso2p interactors selected for testing for sensitivity to DNA damaging agents only *pak1Δ* mutant strain was sensitive to UVC and to 8-MOP+UVA (Fig. 3). This is in contrast to early observations reported by Ostroff and Sclafani (1995) where strains harboring *pak1::LEU2* mutant alleles were not sensitive to UVC at doses up to 70 J/m². The caffeine sensitivity for *pak1* mutant strains reported by two independent investigations (Ostroff and Sclafani 1995, Hovland et al. 1997) could not be confirmed in repeated assays, neither by us nor by M. Strauss (data not shown, personal communication). The above-mentioned phenotypic differences could be due to different genetic backgrounds since clear phenotypic differences were often encountered during gene function analysis among the yeast strains used (Smith et al. 1994).

As the *pak1Δ* mutant had a DNA repair-deficiency phenotype, we tested its genetic interaction with *pso2Δ* mutant alleles in double mutant strains (otherwise isogenic with the corresponding single mutants), assaying for UVC and 8MOP+UVA sensitivity (Fig. 3). According to survival analysis the *pso2Δ* mutant allele showed epistatic interaction with *pak1Δ* since double mutants had the same sensitivity as the single *pso2Δ* mutant. The survival data thus indicate that Pak1p and Pso2p might function in the same pathway of controlling DNA sensitivity to DNA damage, in late step(s) of ICL repair (Fig.5). Hence, yeast *PAK1* could be defined as a new *locus* involved in cellular responses to DNA damage.

The Pso2p-like human Artemis protein is part of the DNA double strand break (DSB) repair machinery, as inferred from the phenotype of patients with severe immune deficiency combined with increased radio-sensitivity (RS-SCID) who have defects in V(D)J recombination leading to arrest of B and T-cell maturation (Moshous et al. 2001). With the potential enzymatic function of its metallo-β-lactamase/β-CASP domain, Artemis has been shown to be involved in the opening of hairpin-sealed coding ends generated by the RAG1/RAG2 complex and nucleolytic activity on 5' and 3' overhangs (Ma et al. 2002). Moreover, Ma and co-workers (2002) demonstrated that the Artemis hydrolase catalytic activity depends strictly on the continuous association of Artemis with the catalytic subunit of the DNA dependent-protein kinase (DNA-PKcs). More recently, Zhang et al. (2004) reported that Artemis is a phophorylation target of the checkpoint protein kinases ATM (Ataxia Telangiectasia Mutated) and ATR (Ataxia Telangiectasia and RAD3-related) and DNA-PK after genotoxic stress *in vivo*. Therefore, the isolation of a known protein kinase via THS (Pak1p) was meaningful and allowed us to extend these studies, demonstrating that Pso2p is a putative phosphorylation target of the protein kinase Pak1p *in vitro*.

In accordance with the model we have recently proposed (Brendel et al. 2003), an association of Pso2p with Pak1p kinase may direct its possible enzymatic activity on a DNA post-incision substrate intermediate to help remove the DNA-ICL. This model was partially corroborated when Yu et al. (2004) and Li et al (2004) reported that Pso2p could function in DNA repair of hairpins induced by transposition of Ac/Ds elements from *Zea mays* in *S. cerevisiae* and that purified Pso2p has an *in vitro* 5′ exonuclease activity, indicating that Pso2p may recognize a DNA hairpin as a structure similar to a covalent ICL lesion substrate and may bind to it, as the Artemis protein of vertebrates does during V(D)J recombination (Ma et al. 2002). Although biochemical data about Pso2p-like proteins is becoming available, there is no evidence reporting that the yeast *PSO2* encoded protein associates or is part of a multi-protein complex for the sequential processing and removal of ICL lesions, or if the Pso2p protein may act in physically

separated complexes like the repairosome and recombinosome (Araujo and Wood 1999). In this report, we have gathered the first preliminary evidence describing an interaction of the yeast Pso2p protein that resembles to the already characterized Artemis interactions in human cells (Ma et al. 2002, Ma et al. 2005).

There are a number of reports, that in yeast, further processing of intermediate structures generated during ICL-repair involves proteins of NER as well as of recombination, non-homologous end-joining (NHEJ) and error-prone repair (Henriques and Moustacchi 1981, Grossmann et al. 2000, Magaña-Schwencke et al. 1982, Wilborn and Brendel 1989, McHugh et al. 2000, Grossmann et al. 2001, Yu et al. 2004, Dronkert and Kanaar 2001, Barber et al. 2005). However, one difficult question that arises from all the models presented elsewhere so far is the paucity of knowledge concerning the steps occurring after ICL incision. In view of this, while future studies are required to elucidate the role of the Pso2p and Pak1p in ICL repair, the data presented here may lead to new biochemical approaches to understand how the various sub pathways contribute to the post incision steps of ICL repair in yeast.

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Figure legends

Figure 1. LexAPSO2 bait constructs and complementation assays after mutagen treatment.

(**A**) Length in aa of the *LexAPSO2*-A (PSO2-A) and *LexAPSO2*-B (PSO2-B) bait constructs and the position of predicted conserved domains: putative zinc finger domain (Zn); β-lactamase (β-lac); and the β-CASP region with the three conserved motifs represented for β-CASP proteins acting on DNA substrates [A (aspartic acid), B (histidine) and C (valine)]. (**B**) BLE treatment: EH3714-2B strain containing the plasmid pEG202 empty plasmid (1), EH3846-4A strain containing the LexAPSO2-A bait construction (2, 3), EH3846-4A strain containing the *LexAPSO2*-B bait construction (4), EH3846-4A strain containing the pEG202 empty plasmid (5); 8-MOP + UVA treatment: BY4742 (WT) strain containing the pEG202 empty plasmid (6), BY*pso2Δ* strain containing the pEG202 empty plasmid (7), BY*pso2Δ* strain containing the *LexAPSO2*-A bait construction (8, 9), BY*pso2Δ* strain containing the LexAPSO2-B bait construction (10, 11).

Figure 2. Amino acid alignment of Pso2p C-terminal half of the protein containing the β-CASP domain and other potential orthologs from various organisms. The conserved motifs A, B and C among the members of the β-CASP family are indicated. Identical residues are shown in black and conserved residues in dark and light gray. The dotted line denotes the last 195 aa, which were deleted *in LexAPSO2*-B bait. The line indicates the newly identified region of similarity containing a conserved polar amino acid histidine in a predicted extended strand region (E) within aa sequence 491-509. The accession numbers at the NCBI are: PSO2Sc (*S. cerevisiae* P30620) YDH2Sp (*Schizosaccharomyces pombe* 19862928), KIAA0086Hs (*Homo sapiens* 577303), SNM1Mm (*Mus musculus* 9055350), orf12At (*Arabidopsis thaliana* 1495267), CG10018Dm (*Drosophila melanogaster* 7296732) FLJ12819Hs (*H. sapiens* 12383082), ArtemisHs (*H. sapiens* 13872809).

Figure 3. Survival of haploid WT (BY4742), *pak1Δ* (Y16128), *pso2Δ* (BY*pso2Δ*) and *pso2Δ/pak1Δ*

(BY*pso2Δ/pak1Δ*) mutants after UVC and 8-MOP+UVA treatment. (A) survival of *pso2Δ* in combination with *pak1Δ* for UVC. (B) survival of *pso2Δ* in combination with *pak1Δ* for 8-MOP+UVA.

Figure 4. *In vitro* phosphorylation of Pso2p by Pak1p. The arrow indicates position of the phosphorylated Pso2p protein. Lane 1: Pak1p; lane 2: Pak1p + trombin; lane 3: Pak1p + Pso2p (8 μg Pso2p + 2 μg Pak1p); lane 4: Pak1p + Pso2p (16 μg Pso2p + 2 μg Pak1p); lane 5: Pak1p + Pso2p (8 μg Pso2p + 3 μg GST - control); lane 6: Pak1p + Pso2p (24 μg Pso2p + 4 μg Pak1p); lane 7: Pak1p + Pso2p (40 μg Pso2p + 4 μg Pak1p).

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

Strain	Relevant genotype	Source
BY4742 (WT)	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	EUROSCARF
Y16128 (*pak1Δ*)	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yer129w::kanMX4*	EUROSCARF
BY*pso2Δ*	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pso2::URA3*	this work
BY*pso2Δ/pak1Δ*	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yer129w::kanMX4 pso2::URA3*	this work
EH3714-2B	*Mata ade2-1 lys1-1 his5-2 ura3-52 leu2-3, 112*	Niegemann and Brendel 1994
EH3846-4A	*Mata ade2-1 lys1-1 his3 ura3-52 leu2-3, 112 trp1 pso2-11*	Niegemann and Brendel 1994
Y11339	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yjl084c::kanMX4*	EUROSCARF
Y11908	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yhr080c::kanMX4*	EUROSCARF
EGY48	*Mat*a *ura3 his3 trp1 LexAOp(6)::LEU2*	R. Brent[a]

[a] Massachusetts General Hospital, Boston, MA, USA.

Table 2. Genes isolated in a two-hybrid screen using LexAPSO2-A as bait.

Gene[a]	Gene information[b,c]	Extent of fusion[d]
NRK1/KIC1 (22)	Ser/Thr protein kinase that interacts with Cdc31p; required for cell integrity and morphogenesis; involved in cell wall biogenesis; 3.8 fold induction after MMS treatment; cytoplasmic localization; CH139, CH361; CH539	Amino acids 832-1432
PAK1 (6)	DNA synthesis; protein kinase capable of suppressing DNA polymerase alpha mutations (*cdc17-1*); *RAD9* gene product is required for the suppression; CG166	C-terminal third of the protein
REH1 (4)	Unknown function; possibly involved in proteasome function; similarity to Rei1p; cytoplasmic localization	Amino acids 60-327
YKR015c (4)	Unknown function; similarity to hypothetical protein YJL043w	C-terminal third of the protein
APL6 (3)	AP-3 complex subunit; probably involved in vesicle transport from the plasma membrane	C-terminal 90 amino acids
FIR1 (1)	3' RNA processing/modification; interacts with *REF2*	Starts at amino acid 251
IFH1 (1)	Protein controlling rRNA expression	C-terminal half of the protein
YHR080c (1)	Unknown function;; 6.2-fold induction after MMS treatment; mitochondrial localisation belongs the recently defined family of GRAM proteins (GRAM motif from aa 548-617)	Amino acids 340-713
YJL084c (1)	Protein of unknown function localised to cytoplasm; 1.7-fold induction after MMS treatment	C-terminal half of the protein

[a] Frequency of independent isolation is shown in parentheses.

[b] According to Guldener et al. (2005) and SGD (http://genome-www.stanford.edu/Saccharomyces/).

[c] CH# indicates association of the respective isolate with the protein complex reported by Ho et al. (2002) and CG# indicates association of the respective isolate with the protein complex reported by Gavin et al. (2002).

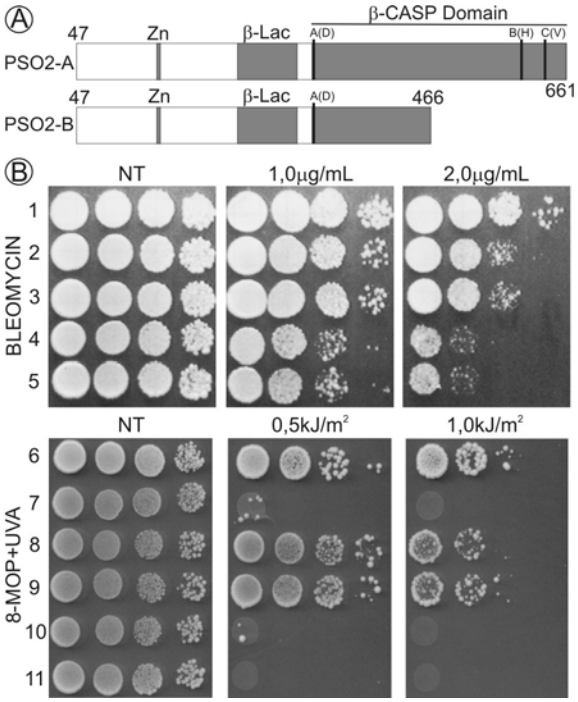
[d] Segment of each ORF fused to the transcriptional activation domain constructs.

Table 3. Comparison of β-Galactosidase activity between Pso2p and Pak1p[a] with either LexAPSO2-A or the LexAPSO2-B baits.

DNA binding domain plasmid (pEG202)	Activation domain plasmid (pJG4-5)	Galactose	
		Leu	β-Galactosidase activity[b]
pLexA GAL4	pJG4-5 (HA-tagged)	++	697±54.4
pLexA Bicoid	pJG4-5 (HA-tagged)	-	6.5±2.0
LexAPSO2-A	pJG4-5-PAK1 (aa 618-1142)	++	82.82±17.6
LexAPSO2-B	pJG4-5-PAK1 (aa 618-1142)	-	3.5±2.3

[a] Plasmids that directed the synthesis of the DNA-binding domain (pEG202) and activation domain (pJG4-5) fusion proteins were introduced into EGY48. In addition to the LexAop(6)-*LEU2*, the strain also harbored pSH18-34, a very sensitive LexAop-*lacZ* reporter. All constructions expressed full length fusion proteins except when indicated. pLexA GAL4 is a transcription activator and was used as positive control; pLexA Bicoid contains residues 2-160 of the *Drosophila* bicoid gene product and was used as negative control.

[b] Expressed in Miller Units. The values are averages from three to four independent transformants each assayed in duplicate. BLUE indicates strong coloring in solid galactose SynCo media.


Ⓐ
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Zn
β-Lac
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B(H)
C(V)
PSO2-A
661
47
Zn
β-Lac
A(D)
466
PSO2-B
Ⓑ
NT
1,0μg/mL
2,0μg/mL
1
2
3
4
5
BLEOMYCIN
NT
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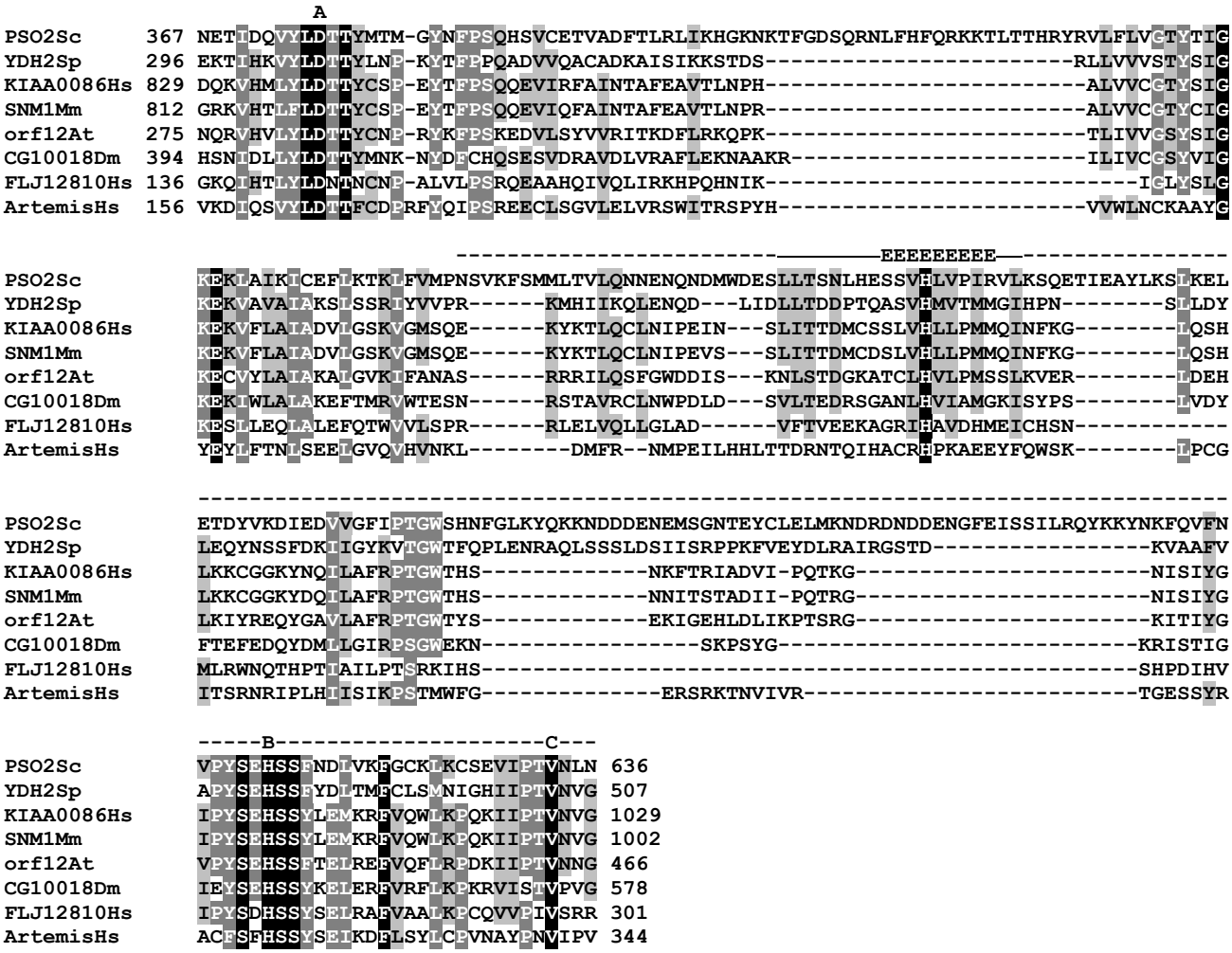
1,0kJ/m²
6
7
8
9
10
11
8-MOP+UVA

Figure 1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KIAA0086Hs 829 DQKVHMLYLDTTYCSP-EYTFPSQQEVIRFAINTAFEAVTLNPH----------ALVVCGTYSIG
SNM1Mm 812 GRKVHTLFLDTTYCSP-EYTFPSQQEVIQFAINTAFEAVTLNPR----------ALVVCGTYCIG
orf12At 275 NQRVHVLYLDTTYCNP-RYKFPSKEDVLSYVVRITKDFLRKQPK----------TLIVVGSYSIG
CG10018Dm 394 HSNIDLLYLDTTYMNK-NYDFCHQSESVDRAVDLVRAFLEKNAAKR----------ILIVCGSYVIG
FLJ12810Hs 136 GKQIHTLYLDNTNCNP-ALVLPSRQEAAHQIVQLIRKHPQHNIK----------IGLYSLG
ArtemisHs 156 VKDIQSVYLDTTFCDPRFYQIPSREECLSGVLELVRSWITRSPYH----------VVWLNCKAAYG
EEEEEEEEE
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YDH2Sp KEKVAVAIAKSLSSRIYVVPR------KMHIIKQLENQD---LIDLLTDDPTQASVFMVTMMGIHPN--------SLLDY
KIAA0086Hs KEKVFLAIADVLGSKVGMSQE------KYKTLQCLNIPEIN---SLITTDMCSSLVFLLPMMQINFKG--------LQSH
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orf12At KECVYLAIAKALGVKIFANAS------RRRILQSFGWDDIS---KNLSTDGKATCLFVLPMSSLKVER--------LDEH
CG10018Dm KEKIWLALAKEFTMRVWTESN------RSTAVRCLNWPDLD---SVLTEDRSGANLFVIAMGKISYPS--------LVDY
FLJ12810Hs KESLLEQLALEFQTWVVLSPR------RLELVQLLGLAD------VFTVEEKAGRIFAVDHMEICHSN------------
ArtemisHs YEYLFTNLSEELGVQVHVNKL--------DMFR--NMPEILHHLTTDRNTQIHACRFPKAEEYFQWSK--------LPCG
PSO2Sc ETDYVKDIEDVVGFIPTGWSHNFGLKYQKKNDDDENEMSGNTEYCLELMKNDRDNDDENGFEISSILRQYKKYNKFQVFN
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KIAA0086Hs LKKCGGKYNQILAFRPTGWTHS-------------NKFTRIADVI-PQTKG----------------------NISIYG
SNM1Mm LKKCGGKYDQILAFRPTGWTHS-------------NNITSTADII-PQTRG----------------------NISIYG
orf12At LKIYREQYGAVLAFRPTGWTYS-------------EKIGEHLDLIKPTSRG----------------------KITIYG
CG10018Dm FTEFEDQYDMLLGIRPSGWEKN-----------------SKPSYG----------------------------KRISTIG
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ArtemisHs ITSRNRIPLHIISIKPSTMWFG-------------ERSRKTNVIVR----------------------------TGESSYR
-----B----------------------C---
PSO2Sc VPYSEHSSFNDLVKFGCKLKCSEVIPTVNLN 636
YDH2Sp APYSEHSSFYDLTMFCLSVNIGHIIPTVNVG 507
KIAA0086Hs IPYSEHSSYLEMKRFVQWLKFQKIIPTVNVG 1029
SNM1Mm IPYSEHSSYLEMKRFVQWLKFQKIIPTVNVG 1002
orf12At VPYSEHSSFTELREFVQFLRFDKIIPTVNNG 466
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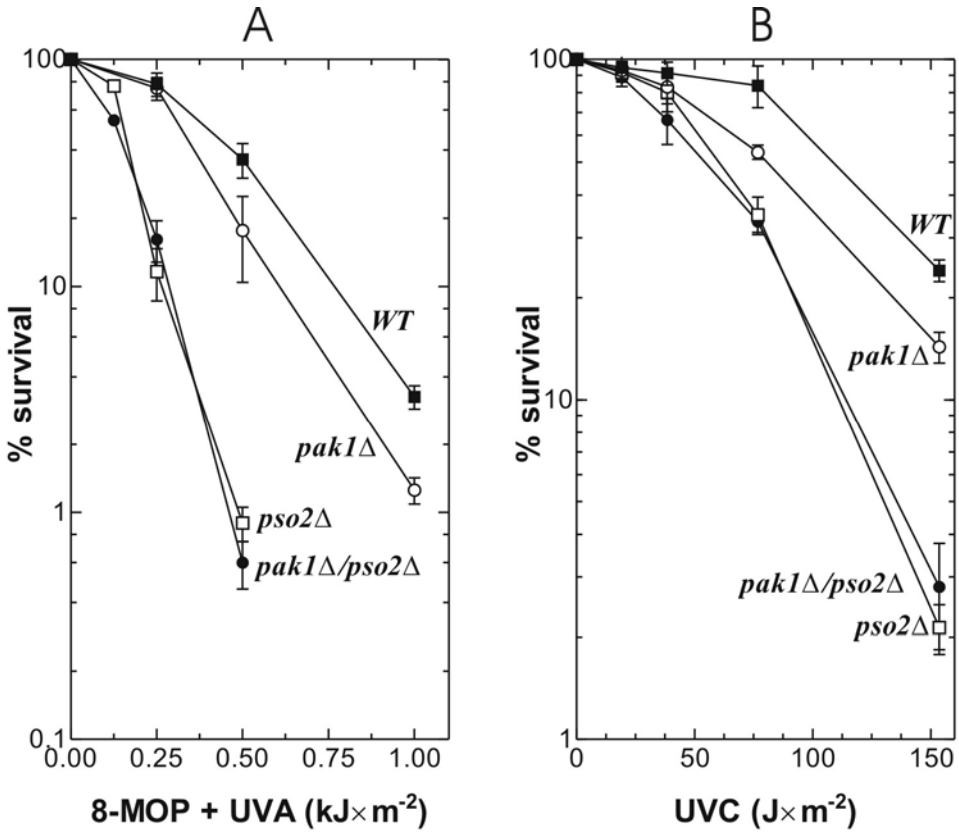
Figure 2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10
1
0.1
% survival
WT
pak1Δ
pso2Δ
pak1Δ/pso2Δ
0.00
0.25
0.50
0.75
1.00
8-MOP + UVA (kJ×m-2)
0
50
100
150
UVC (J×m-2)

Figure 3

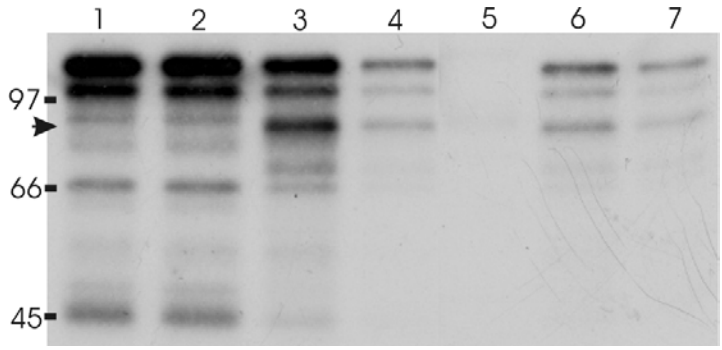

Figure 4

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