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Análise funcional e estrutural da proteína Kin3 em *Saccharomyces cerevisiae*

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"Prove cada um quais são as suas próprias obras, e então terá causa para exultação, apenas com respeito a si próprio e não em comparação com outra pessoa"

Gálatas 6:4

RESUMO

A proteína Kin3 é a única representante da família das *NIMA-related kinases* em *Saccharomyces cerevisiae.* Tendo em vista a existência de poucos estudos sobre a possível função da proteína Kin3 na resposta a danos no DNA e a inexistência de estudos sobre sua estrutura, o objetivo deste trabalho foi elucidar o envolvimento da proteína Kin3 na reposta a danos no DNA *in vitro,* considerando interações genéticas e compreendendo aspectos estruturais em modelos *in silico*. Primeiramente foi avaliado o papel da Kin3 na resposta a metil metano sulfonato (MMS), explorando interações previamente descritas com Mre11 e Srs2. Os resultados mostraram que a estratégia de tratamento de 2 h de exposição seguindo de 1 h de recuperação foi eficiente em induzir quebras no DNA. Adicionalmente, um perfil epistático de Kin3 com Mre11 e Rad52 foi encontrado, sugerindo um possível envolvimento com a via de recombinação homóloga. Esta hipótese foi corroborada com um efeito aditivo na taxa de mutação do duplo mutante *kin3*Δ*rad52*Δ. Posteriormente, foi realizada a modelagem da estrutura da proteína Kin3, com e sem ATP e cofator (Mg^{+2}) e as conformações do loop de ativação após simulação por dinâmica molecular foram exploradas. Os principais achados mostraram a disrrupção de uma pequena estrutura de α-hélice na ausência de ATP, indicando que a ligação com o nucleotídeo é importante para estabilizar esta região. Esta modificação não aproximou os resíduos de serina e treonina localizados no loop de ativação do fosfato γ do ATP, o que permitiria a reação de catálise, não modificou a posição do motivo DFG ou o nucleotídeo para a posição na forma ativa. Assim, neste trabalho foi explorada a função da proteína Kin3 na resposta a DSBs, sugerindo um envolvimento mais significativo com a via da recombinação homóloga. Adicionalmente, foi estabelecida pela primeira vez informações sobre a estrutura da Kin3.

Palavras-chave: Kin3. Função. Resposta a danos. MMS. Estrutura. ATP.

ABSTRACT

Kin3 protein is the only NIMA-related kinase in *Saccharomyces cerevisiae.* Owing the existence of few studies on possible Kin3 function in DNA damage response and no study about Kin3 structure, the aim of this work was to elucidate the involvement of Kin3 protein in response to DNA damage, evaluating genetic interactions and understanding structural aspects by *in silico* models. Firstly, it was evaluated the Kin3 role in response to MMS exploring the interaction already described with Srs2 and Mre11. The results showed that treatment strategy of 2 h of exposition followed by 1 h of recovery was efficient in inducing DNA breaks. In addition, an epistatic profile of Kin3 with Mre11 and Rad52 was observed, suggesting a possible involvement with homologous recombination pathway. This hypothesis was corroborated with an additive effect on double mutant *kin3*Δ*rad52*Δ mutation rate. Posteriorly, it was realized a modeling of Kin3 structure, with and without ATP and cofactor, and explored the conformations of activation loop after a simulation by molecular dynamics. The main findings showed that a small α-helical structure was disrupted in absence of ATP, indicating that the nucleotide binding is important to stabilize this region. This modification did not approximate the serine-threonine residues in the activation loop of γ-phosphate of ATP, allowing the catalysis reaction and did not modify the position of the DFG motif or the nucleotide position for active form. Thus, this work explored the Kin3 function in DSB response, suggesting a more significate involvement with homologous recombination pathway. Additionally, established in the first-time information about Kin3 structure.

Key-words: Kin3. Function. Damage response. MMS. Structure. ATP.

LISTA DE ILUSTRAÇÕES

Figura 1 – Sequência de NRKs..... 17

Tabela 1 – Proteínas relacionadas à função das NEKs descritas neste trabalho 20

Figura 2 – Sequência da Kin3..... 21

Figura 3 – Vias canônicas de reparo de DSBs..... 26

Tabela 2 - Principais proteínas envolvidas na resposta às quebras duplas em Saccharomyces cerevisiae 31

LISTA DE ABREVIATURAS E SIGLAS

ADP – Adenosina di-fosfato

ATM – *Ataxia-telangiectasia mutated*

ATP – Trifosfato de adenosina

ATR – *Ataxia Telangiectasia and Rad3-related protein*

CDKs – *Cyclin-dependent kinase*

CHK1 – *Checkpoint kinase 1*

CHK2 – *Checkpoint kinase 2*

C-Nap1 – *Centrosome-associated protein CEP250*

Csm2 – *Chromosome segregation in meiosis protein 2*

DDR – *DNA damage response*

DNA – D*eoxyribonucleic acid*

Dna2 – *DNA replication ATP-dependent helicase/nuclease DNA2*

DNA-PKs – *DNA dependent protein kinases*

Dnl4 – *DNA ligase 4*

DSBs – *Double strand breaks*

Exo1 – *Exonuclease 1*

Fen1 – *Flap endonuclease 1*

FUN52 – *Function unknown now*

H_2O_2 – Peróxido de hidrogênio

HR – *Homologous recombination*

ICL – *Interstrand crosslink*

IR – *Ionizing radiation*

KIF3A – *Kinesin Family Member 3A*

Ku70 – *ATP-dependent DNA helicase 2 subunit KU70*

Ku80 – *ATP-dependent DNA helicase 2 subunit KU80*

Lif1 – *Ligase-interacting factor 1*

LIG4 – *DNA ligase 4*

Mec1 – *Serine/threonine-protein kinase MEC1*

MMS – Metil Metano Sulfonato

Mre11 – *Double-strand break repair protein MRE11*

Nej1 – *Non-homologous end-joining protein 1*

NHEJ – *Non-homologous end-joining*

NIMA – *Never in mitosis A*

NRK – *NIMA-related kinases*

PCNA – *Proliferating cell nuclear antigen*

PIKK – *3-kinases-related*

PKD – *Polycystic kidney disease*

PP1 – *Protein phosphatase 1*

PP2A – *Protein phosphatase 2A*

PRLR – *Prolactin receptor*

Rad3 – *Serine/threonine-protein kinase RAD53*

Rad50 – *DNA repair protein RAD50*

Rad51 – *DNA repair protein RAD51 homolog 1*

Rad52 – *DNA repair protein RAD52 homolog*

RPA – *Replication protein A*

RNA - *Ribonucleic acid*

Sae2 – *DNA endonuclease SAE2*

Sgs1 – *Slow Growth Suppressor 1*

Shu1 – *Suppressor of HU sensitivity involved in recombination protein 1*

Shu2 – *Suppressor of hydroxyurea sensitivity protein 2*

Srs2 – *ATP-dependent DNA helicase SRS2*

SSB – *Single stranded binding proteins*

ss-DNA – *Single-stranded DNA*

Tel1 – *Serine/threonine-protein kinase TEL1*

TP53BP1 – *TP53-binding protein 1*

TPX2 – *Targeting protein for Xklp2*

XLF – *XRCC4-like factor*

XRCC4 –*DNA repair protein XRCC4*

Xrs2 – *DNA repair protein XRS2*

SUMÁRIO

1. INTRODUÇÃO 15
1.1 NIMA-related kinases (NRK) 15
1.1.1 Neks - mamíferos 17
1.1.2 Kin3 – Saccharomyces cerevisiae 21
1.2 Resposta a quebras duplas em Saccharomyces cerevisiae 23
1.2.1 Junção de extremidades não homólogas 27
1.2.2 Recombinação Homóloga 28
1.3 Resposta às quebras duplas e Kin3 30
1.4 Estrutura 3D de NRKs 33
2. OBJETIVOS 35
3. ARTIGO CIENTÍFICO 1 36
4. ARTIGO CIENTÍFICO 2 58
5. CONSIDERAÇÕES FINAIS 74
6. PERSPECTIVAS 76
7. CURRÍCULO LATTES 77
REFERÊNCIAS 84
ANEXO 1 – Normas da revista Journal of Biomolecular Structure and Dynamics 89
ANEXO 2 – Normas da revista Fungal biology 97

1. INTRODUÇÃO

A molécula constituída de ácido desoxirribonucleico (DNA – *deoxyribonucleic acid*) codifica toda informação necessária à vida. Essa informação é transcrita em ácido ribonucleico (RNA - *ribonucleic acid*) e traduzido em proteínas, as quais são definidas como dispositivos moleculares com uma função biológica. Muitos processos dinâmicos de uma célula, como manutenção dos processos metabólicos, defesa, replicação e reprodução são possíveis através da transmissão de informação gerada por intermédio das interações proteicas. Tendo em vista a relevância da molécula de DNA, assegurar a preservação da informação nela contida é essencial. Neste sentido, a resposta aos danos no DNA (DDR – *DNA damage response*) é um dos processos celulares em que a participação das proteínas é essencial. A interação lesão-proteína pode mediar a reversão direta do dano ou, por vezes, são necessários eventos catalíticos entre múltiplas proteínas (JACKSON; BARTEK, 2010). Compreender quais proteínas estão envolvidas nesta resposta pode elucidar a mecanística de como a detecção, a sinalização e a reparação de lesões ocorrem, e possibilitar a identificação de alvos moleculares em síndromes ocasionadas por falhas neste processo. Além disso, informações sobre sequência e estrutura proteica possibilitam identificar a provável reação de catálise que uma enzima realiza e compreender como as interações com substratos ou parceiros moleculares ocorrem, permitindo então a modulação dessas interações por inibidores ou agonistas sintéticos (MORAES et al., 2015).

1.1 *NIMA-related kinases* (NRK)

A família de proteínas denominada NRK compreende serinas/treoninas cinases que desempenham um papel crucial na regulação e organização de eventos do ciclo celular, sendo, porém, uma das famílias menos estudadas quando comparadas às CDKs, Polo e Aurora cinases. Em uma triagem genética para isolar mutantes de ciclo celular no fungo filamentoso *Aspergillus nidulans* foi identificada a primeira proteína dessa família, denominada *Never in mitosis A* (NIMA). Neste trabalho, os mutantes que paravam a divisão na interfase foram designados como *never in mitosis* (MORRIS, 1975; OAKLEY;

MORRIS, 1983). NIMA regula a transição do ciclo celular entre as fases G2 e M alterando a função do complexo poro nuclear, o que permite a entrada de reguladores mitóticos no núcleo, regula a permeabilidade do envelope nuclear e promove a condensação de cromatina e a fosforilação de histonas. A superexpressão de NIMA induz a rápida entrada na mitose, com disrrupção prematura do envelope nuclear, condensação da cromatina e formação do fuso mitótico (DE SOUZA et al., 2000; FRY, 2002; MONIZ et al., 2011).

As características sequenciais (Figura 1) que definem as proteínas pertencentes a esta família são um domínio catalítico na região N-terminal, onde estão localizados diversos motivos conservados de serina/treonina cinases com alta similaridade de sequência com NIMA, o motivo HRD (His-Arg-Asp) dentro do domínio catalítico e uma serina ou treonina dentro do loop de ativação, sendo este resíduo um provável alvo de modificações estruturais (JOHNSON; NOBLE; OWEN, 1996). A região C-terminal, por sua vez, apresenta alta variabilidade entre as proteínas da família, tanto em termos de sequência, como tamanho e organização de domíneos (FRY et al., 2012). Essa região é comumente responsável pelas interações proteína-proteína, tendo em vista a presença de domínios *coiled-coil* na maioria das proteínas desta família. Essas interações promovem a definição do estado oligomérico da proteína, o qual poderá impactar na forma de ativação da atividade cinase mediando reações de autofosforilação ou possibilitando interações com um segundo interator. Além disso, estão presentes da região C-terminal motivos que indicam degradação proteica, como as sequências PEST para degradação via sistema ubiquitina proteassoma (MONIZ et al., 2011; O'CONNELL; KRIEN; HUNTER, 2003). A partir da descoberta da NIMA, proteínas relacionadas têm sido identificadas e caracterizadas quanto a sua estrutura e função em diversos organismos, como mamíferos e outras classes de fungos (GRALLERT et al., 2012; JONES; ROSAMOND, 1990; SCHULTZ; NIGG, 1993).

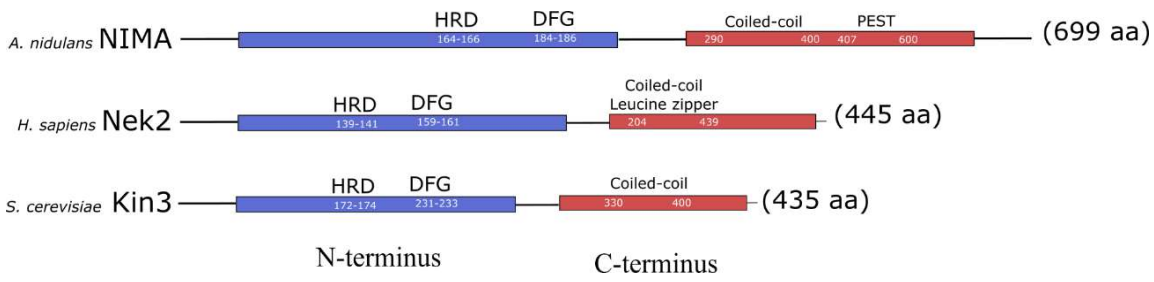

Figura 1 – Sequência de NRKs. Representação esquemática de algumas características sequenciais das proteínas da família NRK. Estão inlustradas a NIMA, primeira proteína descoberta, a Nek2 que possui sequência com maior similaridade com NIMA e a proteína Kin3, foco de estudo deste trabalho.

1.1.1 Neks - mamíferos

Em humanos há onze proteínas ortólogas à NIMA, denominadas Nek1 a Nek11. Elas possuem em torno de 40% de similaridade sequencial ao domínio cinásico da NIMA (SCHULTZ; NIGG, 1993). Uma vez que o número de proteínas aumenta significativamente em humanos e que a região C-terminal possui alta variabilidade, supõe-se que estas onze proteínas desempenham funções celulares adicionais ao papel na regulação do ciclo celular desempenhado pela NIMA (Tabela 1) (MEIRELLES et al., 2014).

A proteína Nek1 é uma das proteínas da família que possui maior descrição de função até o momento, tendo em vista que houve um aumento de interesse da comunidade científica a partir do entendimento que sua deleção possui uma clara associação com a doença do rim policístico (PKD - *polycystic kidney disease*), e de que mutações neste gene estão associadas à Síndrome de Costela Curta e Polidactilia Tipo Majewski (CHEN et al., 2003; UPADHYA et al., 2000). Interessantemente, essa proteína foi descoberta de maneira "serendiptiosa", quando uma linhagem de camundongo com mutação nesse gene ocasionou severos problemas de desenvolvimento, indicando que seu envolvimento em diversos fenótipos poderia ocorrer devido a atuação em alguma função celular básica. Isso foi confirmado a partir dos dados de interatoma da região *coiled-coil* da Nek1 (SURPILI; DELBEN; KOBARG, 2003). Nek1 interage com *Kinesin Family Member 3A* (KIF3A), tuberina e α-catulina, as quais são proteínas relacionados à etiologia da PKD. Além disso, neste trabalho foi demonstrado que Nek1 também interage com proteínas de

controle de ciclo celular, como *TP53-binding protein 1* (TP53BP1) e a Proteína Fosfatase 2A (PP2A – *protein phosphatase 2A)* e está envolvida na DDR, por interagir com a *Double-strand break repair protein MRE11* (MRE11) e A*taxia Telangiectasia and Rad3-related protein* (ATR). Destacando seu papel na resposta a danos genotóxicos, foi demonstrado que a deficiência em Nek1 aumenta a sensibilidade à radiação ionizante (IR - *ionizing radiation*) e, em níveis basais, sua atividade cinase e expressão são aumentadas diante desse mesmo tipo de dano. Além disso, após exposição a IR, ocorre a co-localização de Nek1 com proteínas envolvidas no início da resposta às quebras duplas (DSBs – *double strand breaks)*, como γ-H2AX (POLCI, R., 2004). Adicionalmente, células deficientes em Nek1 possuem um comprometimento nos pontos de checagem do ciclo celular após danos genotóxicos, em função de uma fosforilação prejudicada da *checkpoint kinase 2* (CHK2), resultando, consequentemente, em um reparo defeituoso, o que acarreta em quebras cromossômicas durante a mitose nestas células. Complementarmente, a interação física de Nek1 com ATR foi comprovada e, surpreendentemente, essa interação é necessária para que a sinalização de dano mediada por ATR ocorra eficientemente. O contrário, porém, não é verdadeiro. A inativação de ATR não afeta a atividade cinase, a expressão ou a localização no sítio de dano de Nek1, demonstrando que Nek1 teria um papel independente na DDR (CHEN et al., 2008, 2011; LIU et al., 2013).

Outra proteína desta família com extensa descrição de função e estrutura é a Nek2. A proteína Nek2 possui duas isoformas A e B, derivadas de *splicing* alternativo, sendo que ambas as isoformas apresentam alta similaridade (40 – 45%) do domínio catalítico com a NIMA. Além de possuir detalhes sobre características estruturais, é também a proteína com maior similaridade sequencial com a Kin3, tema da próxima sessão e alvo desse estudo (CHEN et al., 2008; O'CONNELL; KRIEN; HUNTER, 2003). A expressão de Nek2 é modulada ao longo do ciclo celular. Durante a fase G1 há uma baixa expressão, em S ocorre um aumento e, por fim a isoforma A é degradada na transição G2/M, enquanto a isoforma B é degradada na fase G1 seguinte (FRY; MERALDI; NIGG, 1998; SCHULTZ et al., 1994). Apesar da alta similaridade sequencial, existem diferenças de funções entre NIMA e Nek2, o que foi evidenciado pelo fato de que a expressão de Nek2 em *A. nidulans* não complementar a mutação em *nimA* (MEIRELLES et al., 2014). Nek2 participa principalmente na regulação da organização de centrossomos pela fosforilação de *Centrosome-associated protein CEP250* (C-Nap1), possibilitando a separação do

centrossomo, o que é corroborado pelo fenótipo encontrado na sua superexpressão, em que a estrutura do centrossomo é profundamente afetada, promovendo sua divisão e dispersão (FRY; MERALDI; NIGG, 1998; MEIRELLES et al., 2014).

Nek2 atua como um homodímero e pode ser ativada por autofosforilação. A Proteína fosfatase 1 (PP1 – *Protein phosphatase*) é responsável pela desfosforilação de Nek2 a partir da ligação com a região C-terminal não catalítica, acarretando na sua inativação. De maneira similar, Nek2 pode fosforilar PP1 e inativá-la, permitindo que Nek2 permaneça ativa (FRY; MERALDI; NIGG, 1998). A Nek2 possui sua estrutura resolvida por cristalografia de raio-x, o que permite maior compreensão da maneira pela qual ela é ativada. Rellos (2007) e Westwood (2009) avaliaram essas estruturas e concluiriam que a proteína, na presença de um inibidor, possui um motivo helicoidal inibitório que abrange o motivo DFG e o loop de ativação, sendo esta estrutura secundária a uma barreira estérica para a formação da proteína ativa, sugerindo que a dimerização promoveria uma regulação alostérica possibilitando a autofosforilação, como ocorre na interação da *Targeting protein for Xklp2* (TPX2) com Aurora cinase e das ciclinas com as proteínas Cinases Dependentes de Ciclinas (CDKs – *cyclin-dependent kinase*) (RELLOS et al., 2007). Quando a estrutura foi cristalografada com ATγS ou adenosina di-fosfato (ADP), mudanças no posicionamento da hélice inibitória e nas interações foram observadas, mas nenhuma dessas estruturas se mostrou na forma de cinase ativa quando comparada a outras enzimas da mesma classe (WESTWOOD et al., 2009). Isso evidencia que compreender as redes de fosforilação, identificando quais proteínas desempenham um papel crucial na modificação de atividade, localização e estrutura é certamente desafiador quando se trata de cinases.

Embora menos estudas, as demais NRKs de mamíferos também apresentam algumas informações interessantes. Nek3 possui um envolvimento na dinâmica e organização do citoesqueleto, na invasão e motilidade das células através da interação com a *Guanine Nucleotide Exchange VAV2* e o receptor de prolactina (PRLR – *prolactin receptor*) (MILLER et al., 2005, 2007). Nek4 possui envolvimento com DDR, organização de microtúbulos e, mais recentemente, foi descrita uma função do processo de *splicing* de RNA (BASEI et al., 2015). Nek5 possui envolvimento com a via de apoptose mediada por caspase-3, impactando na diferenciação do músculo esquelético, através de alterações em promotores de genes efetores ocasionados pela indução de DSB mediadas pela ativação de DNase por caspase (SHIMIZU; SAWASAKI, 2013). Nek6,

Nek7 e Nek9 contribuem para a organização dos microtúbulos no estabelecimento do fuso mitótico e condensação da cromatina e Nek8, Nek10 e Nek11 estão relacionadas com a replicação do DNA e resposta a estresses genotóxicos (FRY et al., 2012; NOGUCHI et al., 2002; TANG; C POON, 2011). É possível notar que esta família de proteínas possui uma função estabelecida, que pode estar relacionada a três principais contextos, ao controle de mitose, à ciliogênese e à DDR (MEIRELLES et al., 2014). Essa compreensão foi possível quando a ortologia com NIMA não foi decisiva na definição do estudo e um novo contexto biológico ou uma nova função foi considerada, permitindo então compreender o motivo pelo qual são necessárias 11 proteínas em humanos.

Tabela 1 – Proteínas relacionadas à função das NEKs descritas neste trabalho

Proteína	Função
ATR	*Checkpoint* de ciclo celular e reparo de DNA em resposta a danos genotóxicos. Fosforila CHK1, *Cell cycle checkpoint protein RAD17*, *DNA repair protein RAD9* e *Breast cancer type 1 susceptibility protein*
Caspase 3	Ativação da cascata de caspases responsáveis pela ativação da apoptose. Cliva e ativa Caspase 6, 7 e 9
CTNL1 (α-catulina)	Desenvolvimento da doença do rim policístico
Chk2	*Checkpoint* de ciclo celular mediando parada do ciclo, ativação de reparo de DNA e apoptose na presença de quebras duplas
C-Nap1	Coesão de centrossomos durante a interfase
KIF3A	Desenvolvimento de doença do rim policístico
H2AX	Compactação da cromatina, limitando a acessibilidade de maquinarias que interagem com o DNA. Fosforilada na presença de quebras
MRE11	Componente do complexo MRN atuante na resposta às quebras duplas, recombinação no DNA, manutenção da integridade telomérica e meiose.
PP1	Fosfatase associada à regulação de proteínas essenciais ao ciclo celular, metabolismo, contratilidade muscular e síntese proteica
PP2A	Fosfatase de proteínas associadas a microtúbulos.
PRLR	Modulação dos efeitos autócrinos e endócrinos da prolactina. Fator pró-sobrevivência de espermas por suprimir a cinase SRC e estimular AKT

TP53BP1	Supressor tumoral, induzindo parada de crescimento através da inibição de ciclinas dependentes de cinases ou apoptose, pela ativação de BAX e FAX ou repressão de Bcl-2
Tuberina	Desenvolvimento da doença do rim policístico
VAV2	Fator de troca de nucleotídeo de guanina, envolvido na agiogênese

1.1.2 Kin3 – *Saccharomyces cerevisiae*

Em *S. cerevisiae* existe apenas uma proteína identificada como pertencente à família das NRK até o momento, sendo esta denominada Kin3. O gene *KIN3* foi identificado em um trabalho que investigou em uma biblioteca de DNA genômico de leveduras, regiões conservadas do domínio catalítico de serinas/treoninas cinases. A partir da comparação de sequência com outras cinases, foi observado que o gene *KIN3* possui uma sequência consenso de His-Arg-Asp-Leu-Lys-Pro altamente conservada, no qual o resíduo de Asp interage com grupos fosfatos da molécula de trisfosfato de adenosina (ATP); uma sequência, localizada da porção N-terminal, de Gly-X-Gly-X-X-Gly que possui interação com o fosfato terminal e a ribose do ATP; um triplete Asp-Phe-Gly altamente conservado de domínio catalítico; e um elemento representativo de atividade de cinase Asp-X-Trp-Ser-X-Gly (Figura 2). Foi sugerido ainda que o produto do gene *KIN3* não é essencial à mitose, à meiose ou à conjugação, limitando assim seu papel celular para não essencial ou condicional à uma função (JONES; ROSAMOND, 1990). Posteriormente, em uma análise do gene *FUN52,* acrônimo de "*function unknown now*", foi descrito que o mesmo se tratava do gene *KIN3* e estabelecia sua correta localização no Cromossomo I. Por fim, observou-se uma identidade de 42% com o produto do gene *nimA* (BARTON et al., 1992).

MHRRQFFQEYRSPQQQQGHPPRSEYQVLEEIGRGSFGSVRKVIHIPTKKLL
VRKDIKYGHMNSKERQQLIAECSILSQLKHENIVEFYNWDFDEQKEVLYL
YMEYCSRGDLSQMIKHYKQEHKYIPEKIVWGILAQLLTALYKCHYGVELP
TLTTIYDRMKPPVKGKNIVIHRDLKPGNIFLSYDDSDYNINEQVDGHEEV
NSNYYRDHRVNSGKRGSPMDYSQVVVKLGDFGLAKSLETSIQFATTYVG
TPYYMSPEVLMDQPYSPLSDIWSLGCVIFEMCSLHPPFQAKNYLELQTKI
KNGKCDTVPEYYSRGLNAIIHSMIDVNLRTRPSTFELLQDIQIRTARKSLQL
ERFERKLLDYENELTNIEKILEKQAIEYERELSQLKEQFTQAVEERAREVIS
GKKVGKVPESINGYYGKKFAKPAYHWQTRYR

Figura 2 – Sequência da Kin3. A sequência de 435 aminoácidos da proteína Kin3 ilustrada com destaque nas regiões conservadas de serina-treonina cinases.

Nosso grupo de trabalho demostrou que linhagens que possuem o gene *KIN3* deletado (*kin3*Δ) apresentam maior sensibilidade a alguns agentes genotóxicos indutores de adutos e crosslinks (ICL – *interstrand crosslinks*), tais como MMS, cisplatina, doxorubicina e mostarda nitrogenada, mas a proteína não parece ter envolvimento na resposta à radiação UV e exposição a peróxido de hidrogênio (H_2O_2). Além disso, a taxa de mutação parece levemente reduzida na ausência de Kin3. Diante de uma exposição a agentes genotóxicos, a célula tende a ativar mecanismos de pontos de checagem para parada do ciclo celular e remodelamento da cromatina para permitir a atuação das proteínas de reparo. Nesse sentido, a sensibilidade pode estar sendo mediada por uma falha na parada de ciclo celular para *checkpoint* em G2/M, uma vez que a *kin3*Δ não possui acúmulo em G2/M, como ocorre na linhagem selvagem na resposta a um agente genotóxico. Esse prejuízo no ponto de controle do ciclo celular, na parada no ciclo e reparação do dano gerado, promoveu a persistência de quebras. A necessidade da proteína na sinalização e reparo foi evidenciada também pelo aumento de sua expressão após tratamento. Ainda, por esse aumento de expressão de Kin3 ser inibido na presença de cafeína, sugere-se que seu papel é dependente da *Serine/threonine-protein kinase TEL1* (Tel1) e da *Serine/threonine-protein kinase MEC1* (Mec1), uma vez que a cafeína é um inibidor de tais proteínas (MOURA et al., 2010). Este envolvimento ainda não compreendido da proteína Kin3 com o acúmulo de danos ao DNA foi relacionado a tolerância de altas concentrações de etanol de *S. cerevisiae,* sugerindo que a acumulação máxima de etanol pode ser alterada pela modulação da capacidade total de reparo a danos genotóxicos (PAIS et al., 2013). Na tentativa de posicionar a proteína Kin3 na DDR, experimentos de duplo híbrido foram conduzidos e observou-se que Kin3 interage fisicamente com as proteínas no complexo MRX, formado pelas proteínas Mre11, *DNA repair protein RAD50* (Rad50) e *DNA repair protein XRS2* (Xrs2). Esta interação foi confirmada pela epistasia entre os mutantes *kin3*Δ e *mre11*Δ, *rad50*Δ ou *xrs2*Δ em ensaios de sensibilidade a agentes genotóxicos indutores de ICLs, como cisplatina, mostarda nitrogenada e 8-metoxipsoraleno foto-ativado (Moura, 2010). Percebe-se que Kin3 possui um papel no controle de ciclo celular na transição G2/M, mas provavelmente possui uma função adicional no contexto de reparo de DSBs no DNA, tendo em vista a sensibilidade seletiva a agentes indutores desse tipo de dano e a persistência de quebras quando a proteína está ausente. Não existem dados adicionais na literatura a respeito de função no contexto de resposta a danos de DNA ou sobre sua estrutura.

1.2 Resposta a quebras duplas em *Saccharomyces cerevisiae*

O DNA está constantemente exposto a uma série de agentes que causam diversos tipos de lesões em sítios específicos (BOITEUX; JINKS-ROBERTSON, 2013; FINN; FRANCIS; GRENON, 2012). Um dos tipos de lesões mais tóxicos à célula são as quebras duplas, que podem ser decorrentes de processos endógenos como reações metabólicas e estresse replicativo, podem ser consequências de lesões primárias causadas pela supertorção do DNA ou ainda serem induzidas por agentes externos, como drogas quimioterápicas e RI. Esse tipo de lesão possui alta relevância biológica, pois prejudica a atuação da maquinaria de processos celulares básicos, como replicação e transcrição, levando à perda de informação genética e rearranjos cromossomais, ambos associados ao fenótipo de desenvolvimento tumoral (GOBBINI et al., 2013; HANAHAN; WEINBERG, 2011). A fim de evitar que isso ocorra, a célula possui mecanismos para monitorar e reparar tais lesões, o que é denominado de reposta aos danos no DNA (DDR) (CICCIA; ELLEDGE, 2011). A DDR compreende uma complexa rede de subvias que se interconectam e orquestram a preservação da integridade genômica, mantendo a homeostase e a viabilidade celular (FOIANI et al., 2000). Essas subvias envolvem mecanismos de *checkpoint* e processos de remodelamento da cromatina, reconhecimento da lesão, sinalização e transdução de sinal e ativação dos processos de reparo de DNA (BARTEK; LUKAS, 2007; HARTWELL et al., 2016). No entanto, apesar da resposta aos danos genotóxicos ser um processo celular básico para a manutenção da vida, diversas classes de proteínas e enzimas precisam ser ativadas no momento correto e através do substrato adequado. Se essas vias forem ativadas inapropriadamente, a lesão apresentará uma intensidade cada vez maior, tornando a morte a única opção para resolução do dano (BOITEUX; JINKS-ROBERTSON, 2013).

A maquinaria de checkpoint de ciclo celular auxilia no reconhecimento do dano e pode ser influenciada pela fase do ciclo celular e pelo status de condensação da cromatina (FINN; FRANCIS; GRENON, 2012). Os pontos de checagem do ciclo celular compreendem três momentos no qual a célula determinará se está pronta para progredir no ciclo (WEINERT, 1998). Isso acontece entre as fases G1 e S, onde se questiona se o ambiente é favorável para a duplicação do DNA, entre G2 e M, para garantir que a

situação celular está adequada para o início dos eventos mitóticos e entre a metáfase e anáfase, para avaliar se todos os cromossomos estão ligados ao fuso. Se qualquer problema for detectado, a célula interrompe a progressão de ciclo com o objetivo de aumentar o tempo disponível para o reparo do dano antes da replicação ou durante a mitose (FOIANI et al., 2000).

A resposta à DSBs inicia com a sinalização mediada por duas proteínas relacionadas aos mecanismos de pontos de controle do ciclo celular, Tel1 e Mec1 [*Ataxia-telangiectasia mutated* (ATM) e ATR em mamíferos, respectivamente]. Tel1 e Mec1 são proteínas da família das cinases *3-kinases-related* (PIKKs) que fosforilam proteínas efetoras para coordenar a progressão do ciclo celular e o reparo, porém elas são ativadas a partir de diferentes estímulos (GOBBINI et al., 2013). Tel1 é ativada a partir de DSBs, enquanto Mec1 é ativada em diferentes tipos de lesões, pois é recrutada por uma proteína chamada proteína de replicação A (RPA – *replication protein A)* quando esta encontra-se ligada a regiões simples fita, o que pode ser gerado por diferentes tipos de danos primários. Mec1 e Tel1 ativam cinases efetoras importantes para a sinalização de *checkpoint* e amplificação do sinal. Mec1 atua sobre *Checkpoint kinase 1* (Chk1) e sobre *serine/threonine-protein kinase RAD53* (Rad53) (CHK2 em mamíferos), enquanto Tel1 ativa exclusivamente Rad53 (SANCHEZ et al., 1999; STRACKER; USUI; PETRINI, 2010). O remodelamento da cromatina exerce um papel importante na DDR, sendo um dos eventos iniciais nessa resposta a fosforilação de H2A, (H2AX em mamíferos), através de Tel1, possibilitando a retenção estável de proteínas de *checkpoint* e reparo de DNA.

As primeiras proteínas recrutadas para o local da lesão e que atuam como sensoras são o complexo MRX (Mre11, Rad50 e Xrs2/*Nijmegen Breakage Syndrome 1* em mamíferos) e o heterodímero formado por *ATP-dependent DNA helicase 2 subunit KU70* (Ku70)/*ATP-dependent DNA helicase 2 subunit KU80* (Ku80) (LISBY et al., 2004). Ku media a religação das extremidades pela junção de extremidades não homólogas (NHEJ - *non-homologous end-joining*) se houver um mínimo de processamento de extremidade (HANAHAN; WEINBERG, 2011; JACKSON; BARTEK, 2010; PALMBOS et al., 2008). O complexo MRX, por meio de Xrs2, promove o recrutamento de Tel1 levando a ativação do *checkpoint* antes do processamento das extremidades. A ligação do complexo Ku é um dos principais limitantes para que a etapa sequente de ressecção não ocorra durante G1, pois exerce um impedimento físico e assim, a maquinaria de ressecção não consegue atuar. No entanto, durante G2 a atividade de Ku é prevenida por eventos de

fosforilação mediados por CDKs, permitindo que o processamento da extremidade por meio da ressecção ocorra. Sendo assim, a ressecção 5'– 3' para geração de uma cauda 3' de fita simples de DNA (ss-DNA – *single-stranded DNA*) é uma etapa fundamental para a troca de sinalização entre Tel1 e Mec1, bem como para deslocar o reparo de quebras duplas à Recombinação Homóloga (HR – *Homologous Recombination*). A ressecção exige o recrutamento de proteínas ligadoras de simples fintas (SSB – *single stranded binding proteins)*. A RPA atua impedindo a degradação das extremidades de DNA e recrutando Mec1. Isso significa que o passo de ressecção é essencial tanto para iniciar o reparo por HR, bem como para o recrutamento de Mec1 a fim de mediar a resposta de *checkpoint* (LONGHESE et al., 2010).

A etapa de ressecção pode ser realizada em duas etapas, conforme ilustrado na Figura 3. Primeiramente a ressecção no sentido 5' – 3' é realizada por Mre11 juntamente com a *DNA endonuclease SAE2* (Sae2) (CtiP em mamíferos), através das atividades de exonucleases na fita dupla e de endonuclease na fita simples, onde a remoção de aproximadamente 50 – 100 nucleotídeos da extremidade 5' gera uma extremidade 3' de simples fita de DNA. O complexo de proteínas Ku possui baixa afinidade por esta extremidade e, portanto, essa etapa previne a atuação do NHEJ. Além disso, Sae2 é ativada a partir da fosforilação na Ser267 por uma CDK. Este é um dos mecanismos que a célula possui para suprimir a HR durante G1, pois nesta fase do ciclo a atividade de CDK é baixa e as cromátides irmãs não estão expostas para que a recombinação possa ocorrer. Este passo da ressecção remove *hairpins*, adutos proteína-DNA e outras estruturas aberrantes.

A ressecção de extremidades não adulteradas pode ser mediada de maneira independente do complexo MRX e Sae2. Neste caso a atuação de proteínas como a Exonuclease 1 (Exo1), *DNA replication ATP-dependent helicase/nuclease DNA2* (Dna2) e *Slow Growth Suppressor 1* (Sgs1) (*Bloom syndrome protein* em mamíferos) são necessárias para a ressecção mais extensiva. Sgs1, uma helicase da família das RecQ, pode mediar essa etapa através da interação física com Dna2 ou, juntamente com RPA e o complexo MRX, promover a atuação de Exo1 na ressecção da extremidade. Nesse caso, Sgs1 aumentará a afinidade de Exo1 pela extremidade e MRX a processividade (ZHU et al., 2009). Essas duas vias atuam de maneira paralela em *S. cerevisiae,* sendo que a escolha entre as vias parece ser dependente de processos de remodelamento de cromatina mediados por Mec1 (ADKINS et al., 2013).

A partir disso, proteínas de reparo são recrutadas (Tabela 1). Existem duas vias canônicas, mecanisticamente distintas, que competem para reparação de quebras duplas, o NHEJ e a HR. A escolha de cada uma das vias é influenciada pela fase do ciclo celular, natureza e contexto da DSB (DURDÍKOVÁ; CHOVANEC, 2016; GOBBINI et al., 2013). NHEJ é uma via que tem como princípio apenas ligar as extremidades abertas, não levando em consideração a informação genética que pode estar sendo perdida e, por isso, aumenta significativamente as taxas de mutações quando comparada à atuação da HR, via no qual ocorre a busca por outra região do genoma homóloga para realizar a ressíntese de um fragmento de maneira fidedigna (CECCALDI; RONDINELLI; D'ANDREA, 2016).

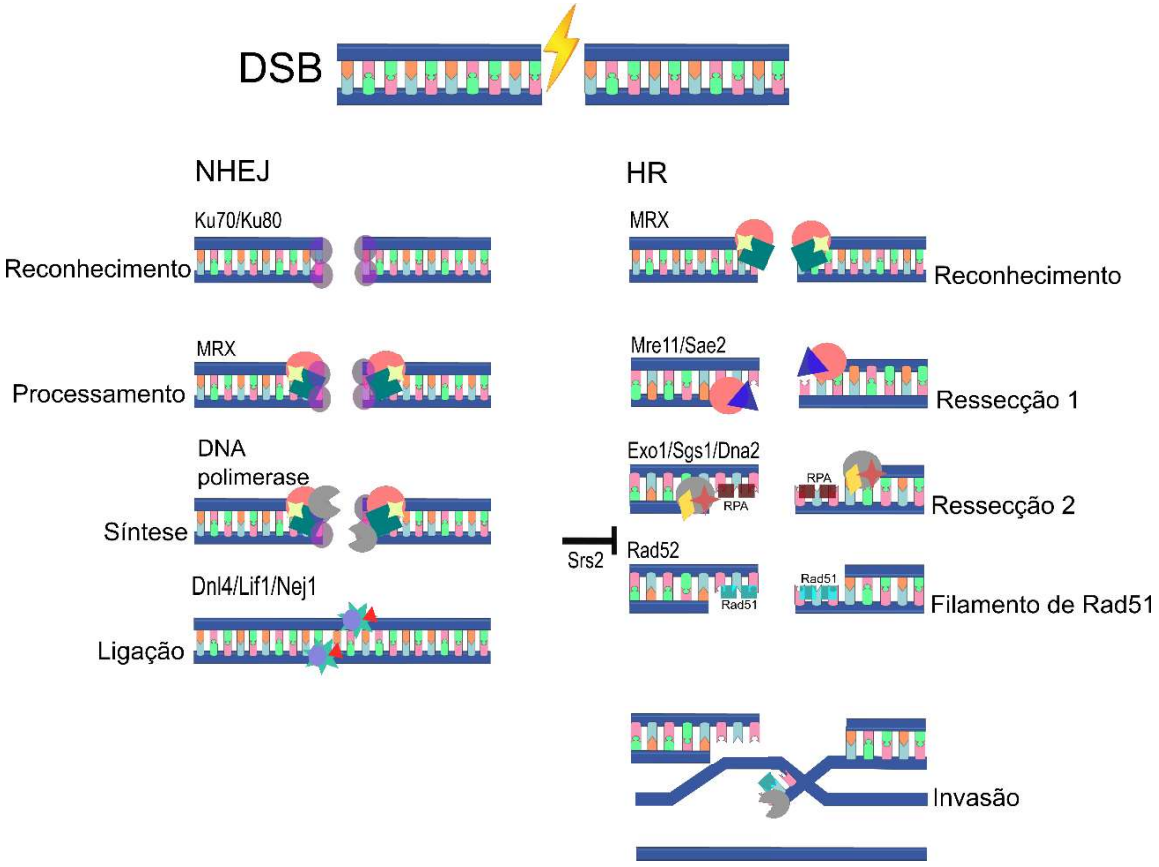

Figura 3 – Vias canônicas de reparo de DSBs. Representação esquemática das vias de reparo clássicas em resposta a DSBs. Ao lado esquerdo está ilustrado as etapas mais importantes do NHEJ e, ao lado direito, da HR. Há um destaque para o papel inibitório de Srs2 na etapa de formação do filamento de Rad51.

1.2.1 Junção de extremidades não homólogas

O NHEJ é caracterizado como uma via de reparação mais rápida, porém passível de erros, uma vez que neste caso ocorre apenas a religação das extremidades com mínimo ou nenhum pareamento de bases na junção. Portanto, o NHEJ está mais associado à instabilidade genômica e à tumorigênese (DURDÍKOVÁ; CHOVANEC, 2016). Existem três etapas principais no NHEJ, reconhecimento, proteção e ancoragem das extremidades (1), processo de retirada e polimerização de nucleotídeos (2) e ligação das extremidades (3) (Figura 3).

Em *S. cerevisiae,* o reconhecimento da lesão e proteção das extremidades contra degradação nucleolítica se dá pela ligação do heterodímero de Ku70/Ku80 nas extremidades, como comentado no tópico anterior. Em mamíferos o complexo KU interage com *DNA dependent protein kinases* (DNA-PKs) para contribuir na detecção do dano e sinalização. Não existem proteínas homólogas às DNA-PKs em *S. cerevisiae*. Assim, esta função de cinase é mediada pelo complexo MRX. Este complexo funciona como adaptador para a maquinaria do NHEJ, coordenando interações entre componentes individuais e mediando a ancoragem na extremidade do DNA (DUDÁŠOVÁ; DUDÁŠ; CHOVANEC, 2004). A atividade de nuclease da Mre11 e de ATPase da Rad50 são necessárias para desacoplar o complexo MRX e o Ku da DSB (WU et al., 2008). Após isso, ocorre a etapa de processamento da extremidade realizado por diversas nucleases (Exo1, Rad27 e *Tyrosyl-DNA phosphodiesterase 1*) e polimerases (Polimerase 2, Polimerase 3 e Polimerase 4) (EMERSON; BERTUCH, 2016) a fim de modificar a extremidade de DNA possibilitando da etapa de ligação. Finalizando o processo de reparo, o complexo de ligação que catalisa a formação da ligação fosfodiéster entre as duas fitas de DNA é composto por *DNA ligase 4* (Dnl4), *Ligase-interacting factor 1* (Lif1) e *Non-homologous end-joining protein 1* (Nej1) [*DNA ligase 4* (LIG4), *DNA repair protein XRCC4* (XRCC4) e *XRCC4-like factor* (XLF) em mamíferos, respectivamente] (KEGEL; SJÖSTRAND; ÅSTRÖM, 2001; PALMBOS et al., 2008). Em humanos, a deficiência nos componentes do NHEJ, além de impactar no reparo de quebras duplas, induz séria imunodeficiência, pois o processo de recombinação somática é prejudicado, impactando o processo de desenvolvimento de linfócitos (TASHER; DALAL, 2012).

O NHEJ pode ser dividido em duas subvias, a canônica e a alternativa. O NHEJ alternativo normalmente é um processo mais lento e menos eficiente, e costuma atuar em células que não estão em processo de divisão (CECCALDI; RONDINELLI; D'ANDREA, 2016). Além disso, trata-se de uma via mais mutagênica, pois necessita de uma região de micro homologia que é gerada pela excisão de nucleotídeos. Existem diferenças nas proteínas recrutadas em algumas etapas da via. A etapa de microressecção é mediada pelo complexo MRX, Sae2 e Rad1/Rad10, pela *Flap endonuclease 1* (Fen1) atuando como nuclease, por algumas polimerases translesão (polimerase H, Rev3/Rev7) e replicativas (Pol3) que participam da etapa de síntese e pela *Dna ligase 1* que é requerida para a ligação (MA et al., 2003). Foi sugerido que a via alternativa seria ativada apenas em situações patológicas ou quando a via canônica está comprometida, porém a completa elucidação desta via ainda está sendo conduzida (DURDÍKOVÁ; CHOVANEC, 2016).

1.2.2 Recombinação Homóloga

A HR, assim como o NHEJ, possui algumas etapas principais (Figura 3). A via inicia com o reconhecimento e ressecção (1), os quais já foram anteriormente descritos, posteriormente há a formação do filamento de *DNA repair protein RAD51 homolog 1* (Rad51) (2), busca pela região de homologia e invasão da dupla fita de DNA (3), finalizando com síntese de nucleotídeos, resolução da estrutura de D-loop e ligação das extremidades (4).

Após a etapa de ressecção, ocorre a ligação de RPA na extremidade 3' gerada, promovendo a estabilização desta região e impedindo a degradação (ADKINS et al., 2013). No entanto, a ligação desta proteína no DNA exerce um impedimento à formação do filamento de Rad51, uma proteína central da HR. Assim, sequencialmente, RPA recruta *DNA repair protein RAD52 homolog* (Rad52) que media o desacoplamento de RPA da fita e estimula a ligação de Rad51, uma ATPase dependente de DNA que forma filamentos de nucleoproteínas, sendo responsável pelo reconhecimento e invasão da fita homóloga (JASIN; ROTHSTEIN, 2013; OHUCHI et al., 2008). Essa etapa é crucial na HR, pois a ligação de Rad51 na fita suprime a atuação de vias mutagênicas e catalisa a formação do D-loop através da invasão da fita intacta. Para isso a estrutura dupla-fita do DNA é aberta e a complementariedade das sequências homólogas serve de *template* para

o reparo (CECCALDI; RONDINELLI; D'ANDREA, 2016; LIBERI et al., 2005) Subsequentemente, as extremidades do D-loop são estendidas por Polimerases δ e ε, com auxílio do *proliferating cell nuclear antigen* (PCNA). A resolução dessa estrutura pode se dar por duas principais formas. O DNA pode se reanelar em outra quebra de terminação cromossomal ou a segunda quebra pode ser capturada formando as duplas junções de *holliday*. No primeiro caso essa estrutura será dissolvida através do complexo STR (Sgs1-Top3-Rm1), levando a produtos sem *crossover*, enquanto no segundo caso, Mms4-Mus81 participam da resolução das junções de holliday com a possibilidade de *crossovers* ocorrerem (MATHIASEN; LISBY, 2014; MEHTA; HABER, 2014).

A modulação da HR é necessária, pois assim como pouca recombinação é prejudicial por aumentar a taxa de mutações, um fenótipo hiperrecombinante também pode ser tóxico à célula e causar rearranjamentos cromossomais (KARPENSHIF; BERNSTEIN, 2012). Uma proteína fundamental que age como regulador negativo de Rad51 é a *ATP-dependent DNA helicase SRS2* (Srs2), uma UvrD helicase. Srs2 estimula a atividade de hidrólise de ATP da Rad51, o que induz sua liberação da fita de DNA (SKONECZNA; KANIAK; SKONECZNY, 2015). A deleção do gene *SRS2* confere à célula um fenótipo hiper recombinante, resultando em letalidade ou severos defeitos no crescimento. Esse perfil pode ser revertido com a mutação em genes do grupo de epistasia com Rad52 (KLEIN, 2001). A atividade antirrecombinacional de Srs2 é promovida pela fosforilação dependente de CDK (KARPENSHIF; BERNSTEIN, 2012). Através de modificações por fosforilação, Srs2 interage fisicamente com Nej1, uma das proteínas do complexo de ligação atuante no NHEJ, indicando um *crosstalk* entre proteínas das vias de reparo de DSBs mediado por inúmeras reações de fosforilação (DURDÍKOVÁ; CHOVANEC, 2016). Existem reguladores positivos da HR, que podem funcionar estimulando a ligação de Rad51 na fita ou inibindo Srs2. Rad52 é uma das proteínas integrantes do primeiro grupo. Por outro lado, um heterodímero composto por Rad55-57, ambos parálogos de Rad51, interage fisicamente com Srs2, bloqueando sua atividade de translocase (LIU et al., 2012). O complexo Shu, composto por Psy3, *Chromosome segregation in meiosis protein 2* (Csm2), *Suppressor of HU sensitivity involved in recombination protein 1* (Shu1) e *Suppressor of hydroxyurea sensitivity protein 2* (Shu2), semelhantemente a Rad55-57, protegem os filamentos de Rad51 inibindo o recrutamento de Srs2 (BERNSTEIN et al., 2011; KARPENSHIF; BERNSTEIN, 2012).

1.3 Resposta às quebras duplas e Kin3

Há poucos achados sobre o envolvimento da proteína Kin3 na resposta a danos no DNA, além dos já mencionados anteriormente. Buscando interatores físicos de Srs2, Chiolo (2005) demonstrou em uma triagem preliminar que essa proteína interage com Kin3, Sgs1 e Mre11. A interação com Mre11 foi investigada pelo autor e a formação de um complexo Sgs1, Srs2 e Mre11 foi evidenciada em condições normais, complexo este que reorganiza em dois sub complexos Srs2-Mre11 e Sgs1-Mre11, seguido da ativação de Mec1 e Tel1, quando há a exposição ao metil metano sulfonato (MMS). No entanto, a interação Srs2 e Kin3 não foi investigada neste trabalho. Como mencionado anteriormente, nosso grupo de trabalho, demonstrou a interação física das proteínas do complexo MRX com Kin3, utilizando o ensaio de duplo híbrido e experimentos de co-imunoprecipitação. Esta interação foi confirmada geneticamente, em que se observou um um fenótipo de epistasia entre essas proteínas e Kin3 na resposta a agentes indutores de adutos e *crosslinks* (MOURA, 2010). Conforme comentado anteriormente, Kin3 parece estar envolvida do *checkpoint* G2/M (MOURA et al., 2010). A sensibilidade a agentes alquilantes aumentada na ausência de Kin3 (*kin3*Δ), o aumento da expressão dessa proteína na exposição a agentes genotóxicos e a interação com proteínas da resposta às quebras duplas indica seu possível papel nessa via de sinalização e reparo.

Radiação ionizante é padrão ouro na indução de quebras duplas, pois esse é o principal tipo de dano causado frente a esta exposição (JASIN; ROTHSTEIN, 2013). No entanto, agentes alquilantes também estão entre as classes de compostos associados à indução de quebras duplas e ativação das vias de reparo deste tipo de lesão (MATHIASEN; LISBY, 2014). A lesão primária é caracterizada pela presença de grupamentos alquil (C_nH_{2n+1}) no DNA, no entanto as modificações estruturais que tais danos causam na dupla hélice promovem a supertorção do DNA que pode levar à indução de quebras duplas. Além disso, se a maquinaria de replicação ou transcrição colidir com esse tipo de dano, existe a clara possibilidade do processo colapsar e quebras serem geradas (MEHTA; HABER, 2014). Assim, o primeiro artigo de dados desta dissertação irá abordar qual a função da Kin3 na reposta a lesões induzidas por MMS, mimetizando um esquema de tratamento que corrobora com a indução de quebras duplas.

Tabela 2 - Principais proteínas envolvidas na resposta às quebras duplas em *Saccharomyces cerevisiae*.

Etapa	Proteína S. cerevisiae	Função	Mamíferos
Sensores	Mec1	Cinase de checkpoint de integridade genômica. Sensora e transdutora de dano afetando parada de ciclo celular e resposta transcricionais.	ATR
	Tel1	Cinase envolvida no controle de checkpoint de ciclo celular em resposta a danos no DNA	ATM
	Mre11-Rad50-Xrs2	Complexo sensor e sinalizador de quebras duplas. Envolvido no processamento de extremidades de DBSs no NHEJ e HR.	MRE11-RAD50-NBS1
Processamento de extremidades	Sae2	Endonuclease envolvida na ressecção 5', atuante em conjunto com o complexo MRX no primeiro passo da ressecção de DSBs	CtIP
	Exo1	Exonuclease 5'-3' envolvida da no segundo passo da ressecção de quebras duplas	EXO1
	Sgs1	DNA Helicase da família das RecQ, atuante manutenção da integridade genômica e no processamento de estruturas da HR em conjunto com Dna2 e Top3	BLM
	Dna2	Nuclease dependente de ATP envolvida no reparo e processamento de quebras duplas	DNA2
Efetores	Rad53	Proteína cinase atuante na resposta a danos no DNA promovendo a parada do ciclo celular	CHK2
	Chk1	Serina/treonina cinase efetora de checkpoint que media a parada de ciclo celular	CHK1
NHEJ	Ku70/Ku80	Complexo de se liga na extremidade do DNA e protege contra ressecção promovendo o reparo através da via NHEJ	KU70/KU80
	Dnl4, Lif1 e Nej1	Complexo importante na etapa de ligação das extremidades no reparo através da via NHEJ	LIG4, XRCC4 e XLF, respectivamente
HR	RPA	Proteína ligadora de simples fita, estabiliza e impede a degradação da cauda 3'	RPA

Rad52	Proteína necessária ao desacoplamento de RPA da fita de DNA e ligação de Rad51	RAD52 e BRCA2
Rad51	ATPase dependente de DNA envolvida no reparo recombinacional através do reconhecimento da sequência homóloga e invasão para formação de estrutura de D-loop	RAD51
Srs2	Helicase e ATPase dependente de DNA envolvida na translocação de Rad51 antes da formação do D-loop	RTEL1

1.4 Estrutura 3D de NRKs

Conforme pode ser concluído a partir da descrição dos processos de sinalização e reparo de danos no DNA realizada acima, proteínas cinases e fosfatases desempenham funções essenciais na DDR. A importância de reações de fosforilação e desfosforilação catalisadas por essas proteínas fica ainda mais evidente quando a mitose é considerada, pois são inúmeras as proteínas cinases que regulam e são reguladas atingindo picos de expressão durante esse processo. Essas famílias em mamíferos incluem Auroras, Cdks, *polo-like kinase 1* (PLK-1) e Neks (TANG; C POON, 2011).

Interessantemente, assim como as sequências de aminoácidos proveem algumas informações sobre a função de determinada proteína pela presença de motivos conservados, as estruturas de cinases mitóticas apresentam características que as definem e que são comuns entre elas. Essas características estruturais determinam o *status* de ativação e possibilitam compreender como a reação de catálise ocorre a nível molecular (BAYLISS et al., 2012). As marcas da arquitetura de cinases ativas envolvem uma estrutura bilobal, composta por uma região N-terminal rica em folhas β e uma região C-terminal constituída principalmente de α hélices. O ATP é o substrato doador dos grupamentos fosfatos e liga-se na fenda existente entre os dois lobos. Os motivos HRD e DFG devem ser corretamente posicionados para promover a interação entre alvo, enzima e ATP (SCHINDLER et al., 2000). Além disso, esses motivos são componentes da cadeia hidrofóbica existente em cinases ativas (KORNEV et al., 2006). Uma conexão entre dois elementos do lobo N-terminal posicionados na folha β3 e na hélice αC é estabelecida através de uma ponte salina entre o grupamento amônio de uma lisina e o grupamento carboxil de um glutamato (JOHNSON; NOBLE; OWEN, 1996). Outro elemento estrutural muito importante é o loop de ativação que inicia no motivo DFG e finaliza na sequência consenso APE. Neste domínio estão localizados os resíduos necessários à catalise e resíduos que normalmente são fosforilados para ativar a proteína, promovendo a correta conformação para ligação do substrato. É importante ressaltar que estas são algumas características e o estado de uma proteína não se define entre inativo e ativo de maneira binária. A catálise é um processo dinâmico, composta por inúmeras modificações estruturais que ocorrem de maneira temporal e espacial (BAYLISS et al., 2012).

Rellos et al. (2007) e Westwood et al. (2009) descreveram algumas destas características com a estrutura cristalográfica da proteína Nek2 complexada à diferentes ligantes, as quais foram previamente descritas no item 1.1.1. Tendo em vista a alta identidade existente entre Nek2 e Kin3, o segundo artigo de dados irá explorar de maneira detalhada o posicionamento destes domínios nas duas proteínas.

2. OBJETIVOS

- OBJETIVO GERAL

Elucidar o envolvimento da proteína Kin3 na resposta a danos ao DNA em *S. cerevisiae* e compreender aspectos estruturais por modelos *in silico*.

- OBJETIVOS ESPECÍFICOS

  - Construir mutantes com os genes de interesse em *S. cerevisiae* (*KIN3*, *SRS2*, *SGS1*, *MRE11*, *RAD52, KU70*).
  - Compreender se perante um estresse genotóxico, os produtos dos genes alvos interagem geneticamente.
  - Verificar se após um dano genotóxico os produtos dos genes modulam a taxa de mutagênese e compreender se há uma interação genética entre eles.
  - Propor uma estrutura 3D para a proteína Kin3.
  - Simular pelo método de dinâmica molecular as modificações na estrutura da proteína Kin3 que possibilitem verificar a interação desta proteína com ATP.
  - Simular pelo método de dinâmica molecular a estrutura da proteína Nek2 na forma complexada e na forma livre para fins comparativos.

3. ARTIGO CIENTÍFICO 1

Contribution of Kin3, a NIMA-related kinase, in double stranded break response in *Saccharomyces cerevisiae*

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Contribution of Kin3, a NIMA-related kinase, in double stranded break response

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ABSTRACT

Kin3 protein is the only one NIMA-related kinase in *Saccharomyces cerevisiae*. Owing the existence of few studies on possible Kin3 function in DNA damage response, here we evaluated the Kin3 role in response to MMS DNA-induced damage by exploring the interaction already described with Srs2 and Mre11. For this, double and triple mutants were constructed and submitted to MMS treatment for 2 h followed by 1 h of recovery and viability, mutation rate and level of DNA fragmentation were evaluated. The results showed that MMS exposure was efficient to induce DNA breaks in *S. cerevisiae* cultures. Additionally, epistatic profile that Kin3 has with Mre11 and Rad52 suggests a possible involvement with homologous recombination. This hypothesis was corroborated with an additive effect on double mutant *kin3*Δ*rad52*Δ mutation rate. This and other published results of our research group has demonstrated that Kin3 protein plays an important role in DNA damage response, by modulating the damage signaling and G2/M arrest, thus contributing to DNA repair process.

Key-words: Kin3. Saccharomyces cerevisiae. DNA damage. MMS. DNA repair.

1. INTRODUCTION

NIMA-related kinases (NRK) are a family of serine threonine kinases that play an essential role in regulation and organization of cell cycle events. Never in mitosis A (NIMA) was the first protein of this family discoveryin *Aspergillus nidulans* (MORRIS, 1975). NIMA protein regulates G2/M transition by modulating the function of nuclear pore complex, allowing the mitotic regulator to enter in the nucleus, altering nuclear envelope permeability and promoting chromatin condensation and histone phosphorylation (DE SOUZA *et al.*, 2000; HAMES; FRY, 2002; MONIZ *et al.*, 2011). The kinases of this family have some sequence characteristics, such as, a N-terminus which consist of a relatively conserved catalytic domain. However, C-terminus is highly variable in size and sequence (FRY *et al.*, 2012; O'CONNELL; KRIEN; HUNTER, 2003) .

In mammals, there are eleven proteins that are part of NRK family, which are called Nek1 to Nek11. Interestingly, the catalytic domain of these proteins presents around 40% similarity to NIMA (SCHULTZ; NIGG, 1993), but these proteins show high C-terminus variability, indicating that they can have additional functions. Meirelles et al. (2014), revised how the interactome contributes to characterize Nek families, suggest that 11 proteins have been classified in three mainly functions, centrioles and mitosis (1), primary ciliary function (2) and DNA damage response (3).

Nek 1 is the most studied protein in this family with descriptions in DNA damage response. Nek1 interacts with TP53, PP2A, MRE11 e ATR, decreasing Ku recruitment. Pellegrini et al. (2010) show that Nek1 knockdown decreases the repair capacity after methyl methane sulfonate (MMS), H_2O_2 and cisplatin-induced injury, fail in arrest G2/M, making the cell more sensitive to these agents. In this sense, Polci et al. (2004) showed that cells without functional Nek1 cannot repair DNA correctly after ionizing radiation. Chen et al. (2008) explored the role of Nek1 in detecting DNA damage and shown that Nek1 knockdown impaired the activation of Chk1 and Chk2 in response to ionizing or UV radiation. Spies et al. (2016) has specified the role of Nek1 in response to double strand breaks and shown that Nek1 is required to phosphorylate Rad54 in the late G2 phase and promote removal of Rad51 from chromatin during homologous recombination (HR) (PELEGRINI *et al.*, 2010; POLCI *et al.*, 2004; SPIES *et al.*, 2016).

In *Saccharomyces cerevisiae*, only one protein represents the NIMA-related kinases and is called Kin3. This protein was discovered in the 1990's and classified as a serine-threonine kinase non-essential to mitosis, meiosis and conjugation (BARTON *et al.*, 1992; JONES; ROSAMOND, 1990). Our research group published in 2010 the role of Kin3 in response to DNA adducts, demonstrating that *KIN3* knockout cells were more sensitive to genotoxic damage such as adducts and crosslinks mediated by a fail of arrest in G2/M checkpoint, which increases DNA breaks persistence. Additionally, it was verified using a two-hybrid assay the interaction between Kin3 and MRX complex proteins (Mre11-Rad50-Xrs2), indicating that Kin3 could be involved in double-stranded breaks response (unpublished dates). Chiolo et al. (2005) show that Mre11 rearranges complexes formed with Sgs1 and Srs2 after damage induced by MMS treatment. Srs2 is a UvrD helicase which acts as a negative regulator of Rad51 stimulating the ATP hydrolysis activity of Rad51 and Sgs1 is a helicase of the RecQ family that interacts with Top3 and resolves homologous recombination structures. Considering that there are few studies about possible Kin3 partners in *S. cerevisiae*, in this article our goal was to evaluate the Kin3 role in response to MMS DNA-induced damage by exploring the interaction already described with Srs2 and Mre11.

2. MATERIALS AND METHODS

Culture media and cell lines: The yeast strains were cultured in YEPD medium (1% yeast extract, 2% peptone, 2% glucose, with suplementation of 2% of bactor-ágar to plates). The growth in selective conditions was conducted in SynCo medium (0.67% of nitrogenates compounds, 1% ammonium sulfate, 2% of glucose and each necessary amino acid at final concentration of 40 $mg.mL^{-1}$) (BURKE, 2000). BY4741 (wild-type; *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*), *kin3*Δ (BY4741; *kin3::kanMX4*), *mre11*Δ (BY4741; *mre11::kanMX4*), *srs2*Δ (BY4741; *srs2::kanMX4*), *rad52*Δ (BY4741; *rad52::kanMX4*) and *ku70*Δ (BY4741; *ku70::kanMX4*) were obtained from *European Saccharomyces cerevisiae Archive for Functional analysis* (EUROSCARF; Johan Wolfgang Goethe-University, Frankfurt - Germany). For this study, the double and triple mutants were constructed by gene disruption, for this study.

Construction of double and triple mutants: for the evaluation of genetic interactions of *KIN3* gene product, double and triple mutants were constructed by disruption of *KIN3*, *MRE11* or *SRS2* genes by homologous recombination, using vector pGADT7 for amplification of *mre11::LEU2*, vector pGBKT7 for amplification of *srs2*::HIS3 and vector YCplac33 for amplification of *kin3*::URA3 disruption cassettes, in accordance to Burke et al (2000). The disruption cassettes were amplified with Platinum high fidelity Taq DNA polymerase (Invitrogen). To cell transformation, cells were inoculated into 5 mL of YEPD and incubated at 30°C overnight. The culture was counted and inoculated into 50 mL of YEPD to a cell density of 5 x 10^6 cells/mL. After growing the cultures to the cell density of 2 x 10^7 cells/mL, cells were transformed with amplify disruption cassettes by a protocol adapted from Gietz and Schiestl (2007). Briefly, the cells were washed with water, concentrated to 1 mL, and 100 µl of pellet was resuspended in a mixture of polyethylene glycol, lithium acetate, denatured salmon DNA and disruption cassette. The mixture was incubated at 42°C for 40 min, washed and plated in selective medium to growth at 30°C for 3-4 days. Colonies were tested with conventional PCR to check the presence of selection gene and the absence of disrupted gene (GIETZ, DANIEL; SCHIESTL, 2007).

Growth curve: for MMS sensitivity assessment, the cells grew overnight and were counted. After, 1x10^8 cells/mL were incubated with increasing concentration of MMS (0.01 – 0.02 – 0.04 – 0.08%) in PBS at 30°C for 2 h. Thereafter, the treatment solutions were withdrawn and the cells were suspended in 1 mL of PBS and incubated for 1 h of recovery. After treatment, the cells were appropriately diluted and plated in triplicate on solid YPD. The plates were incubated at 30 ºC for 2-3 days before the colonies were counted. The data presented represent the mean percentage of survival, in relation to the negative control, of at least three independent experiments, each carried out in triplicate.

Mutagenesis: the mutagenesis yield was measured by resistance to canavanine caused by forward mutations in the *CAN1* gene. Cells were grown overnight and counted. The cells were concentrated to 1x10^9 cells/mL. 100 µl were used for each treatment condition with 1 mL final amount. Cells were incubated with increasing concentration of MMS (0.01 – 0.02 – 0.04 – 0.08%) in PBS at 30°C for 2 h. Thereafter, the treatment solutions were withdrawn and the cells were suspended in 1mL PBS for 1 h of recovery. For the reversion measurement, 100 µl were plated in triplicate on YEPD plated with 60 µg/mL canavanine. For survival assessment, the solutions were diluted to 1x10^3 cell/mL and 100

µl were plated on normal YEPD plates. Colonies were counted after 3-4 days growing at 30°C. The ration of revertant by survivors indicates the mutant yield per 10^7 cells (ECKARDT; HAYNES, 1980; JONES; ROSAMOND, 1990).

DNA fragmentation: alkaline gel electrophoresis was used to visualize DNA fragmentation. After damage induction, the DNA was extracted and denatured with a solution of NaOH (100 mM) and EDTA (4 mM) for 20 min at room temperature. Samples were mixed with loading buffer and SYBR® Gold and run on an agarose gel (2%). Electrophoresis was performed with 1.3v/cm and the samples were visualized under UV light. Samples were quantified using Image J (ABRÀMOFF; MAGALHÃES; RAM, 2004).

Data analysis: Data are expressed as means and standard error of the mean from three independent experiments and were statistically analyzed using analysis of variance (ANOVA) and Dunnett's multiple comparison test or ANOVA two-ways followed of Bonferroni posttest.

RESULTS

3.1 Single mutants are more sensitive than wild type BY4741 *S. cerevisiae*

To evaluate the impact of the *KIN3, MRE11* and *SRS2* genes disruption on the DNA damage induced by MMS, a growth curve with 2 h of treatment followed by 1 h of recovery was performed. As previously showed in literature, deletions of *MRE11* and *SRS2* genes showed high sensitivity compared with BY4741 (Fig. 1A-C). At concentrations of 0.08%, *mre11*Δ was ~60x and *srs2*Δ ~20x more sensitive than BY4741. The *kin3*Δ makes the cells more sensitive at low concentrations (at 0.01% and 0.02% about 1.4 x), indicating that Kin3 is important for the response to DNA damage induced by MMS (Fig. 1D). The *mre11*Δ was the most sensitive mutant, comparing with *kin3*Δ and *srs2*Δ at concentrations of 0.01% and 0.02%. However, at 0.04% and 0.08%, the mutants in *MRE11* and *SRS2* genes showed the same sensitivity (Fig. 1E).

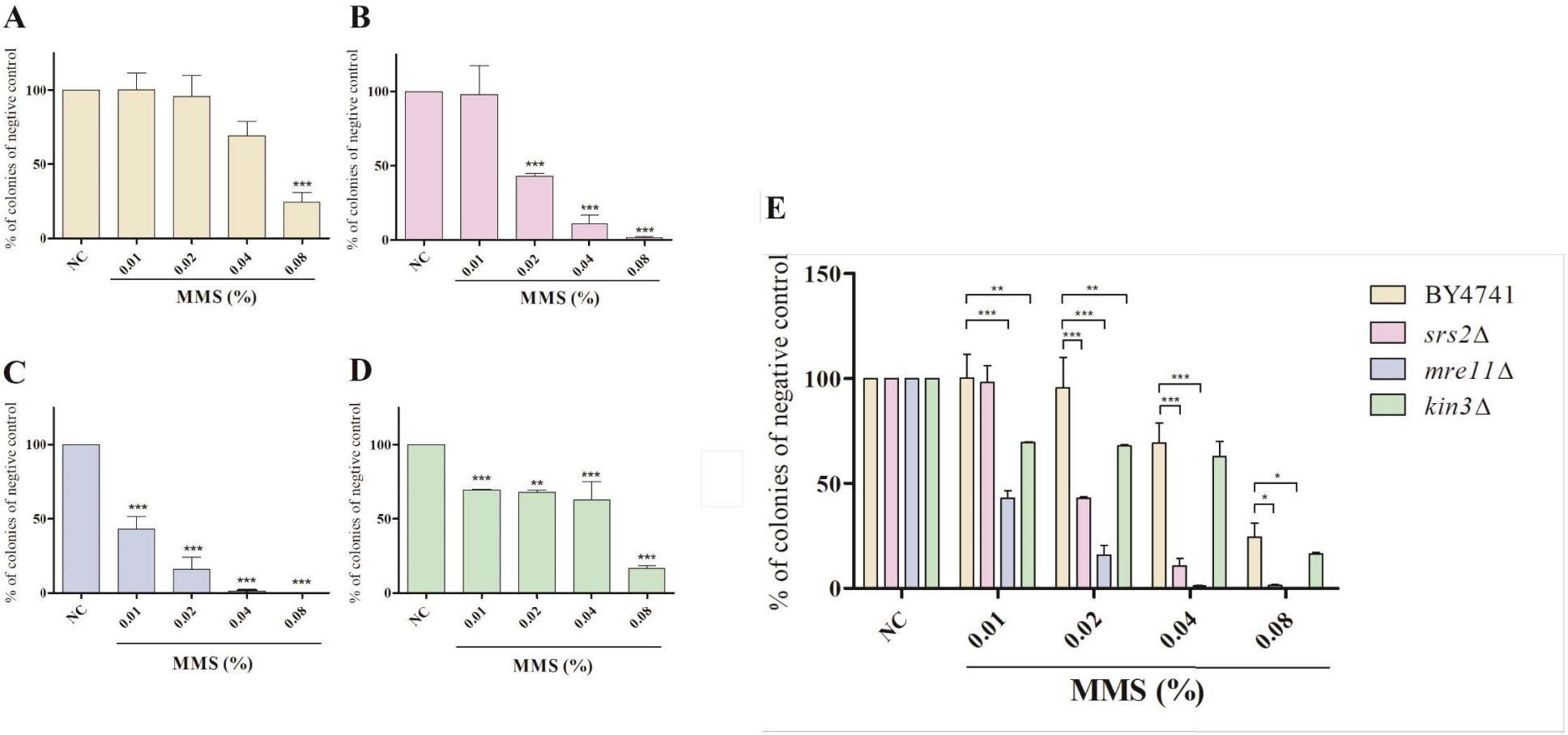

Figure 1. Differential sensitivity to MMS DNA-induced damage in single mutants of *S. cerevisiae*. Cells were treated 2 h with MMS followed by 1 h of recovery. A. BY4741 (wildtype), B. *srs2*Δ*;* C. *mre11*Δ*;* D. *kin3*Δ*;* E. comparison among sensitivity of single mutants in relation to BY4741. Statistical analysis was performed by ANOVA one-way followed of Dunnett's Multiple Comparison Test (A-D) and ANOVA two-ways followed of Bonferroni posttests (E). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2 Genetic interaction of *KIN3* with *SRS2* and *MRE11*

Mre11 is a protein that recognizes double strand breaks and cooperates with the first resection in HR and Non-Homologous End Joining (NHEJ) binding complex. To evaluate the cooperation of these two proteins, a double mutant was subjected to MMS treatment for 2 h followed by 1 h of recovery and cell viability was compared with single mutants. In Figure 2A, it is possible to observe a phenotype of epistasis between *kin3*Δ and *mre11*Δ, because the double mutant was as sensitive as the most sensitive of single mutants. These results show that Kin3 could cooperates with Mre11 in recognizing and repairing the damage induced by MMS.

Srs2 is a translocase that disrupts the Rad51 filament before the invasion step of the homologous sequence in HR (SKONECZNA; KANIAK; SKONECZNY, 2015). Its deletion causes increased death by a hyperrecombinant phenotype, since toxic substrates are accumulated. It has been reported that deletion of *RAD51* decreases the deleterious consequences of in *SRS2* mutation (KLEIN, 2001). To evaluate the phenotype of *KIN3* deletion in *srs2*Δ, the double mutant was subjected to MMS treatment followed by 1 h of recovery and cell viability result was compared with single mutants. The Figure 2 B shows that viability increased in the double mutant, indicating that Kin3 would be involved in the promotion of HR. Given that the phenotype has not been fully recovered, its role in HR seems not to be performed only by Kin3. In addition, Mre11 and Kin3 appear to perform different functions, since the *MRE11* deletion does not show the same phonotype (Fig. 2C). Corroborating with this idea, the triple mutant showed an addictive effect compared to all double mutants (Fig. 2D).

3.3 The double mutant *kin3*Δ*rad52* show a profile of epistasia

Mre11 plays roles in both HR and NHEJ. So, the epistatic profile of *kin3*Δ*mre11*Δ leaves a question of which pathway Kin3 might be cooperating. Thus, a double mutant of *KIN3* and two essential proteins of HR and NHEJ were constructed and exposed to same conditions of treatment with MMS. The results show that the double mutant of *kin3*Δ*ku70*Δ does not have significant differences with single mutants, except at the concentration of 0.01%, in which it was less sensitive (Fig. 3A). However, the double mutant *kin3*Δ*rad52* shows an epistatic profile, as observed in *kin3*Δ*mre11*Δ (Fig. 3B).

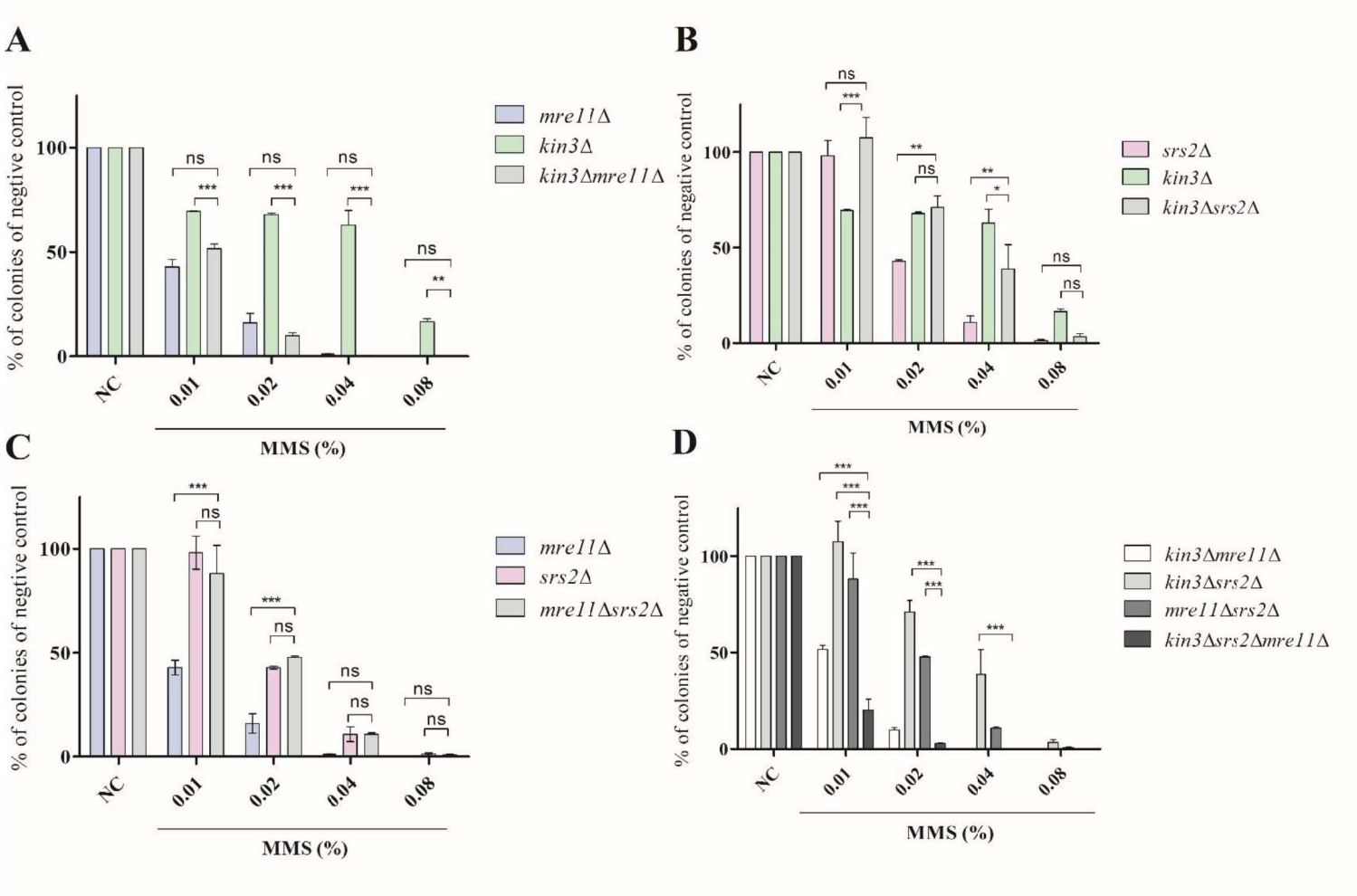

Figure 2. Differential sensitivity to MMS DNA-induced damage in double and triple mutants of *S. cerevisiae*. Cells were treated 2 h with MMS followed by 1 h of recovery. A. *kin3*Δ*mre11*Δ, B. *kin3*Δ*srs2*Δ*;* C. *mre11*Δ *srs2*Δ*;* D. *kin3*Δ*mre11*Δ*srs2.* Statistical analysis was performed by ANOVA two-ways followed of Bonferroni posttest. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

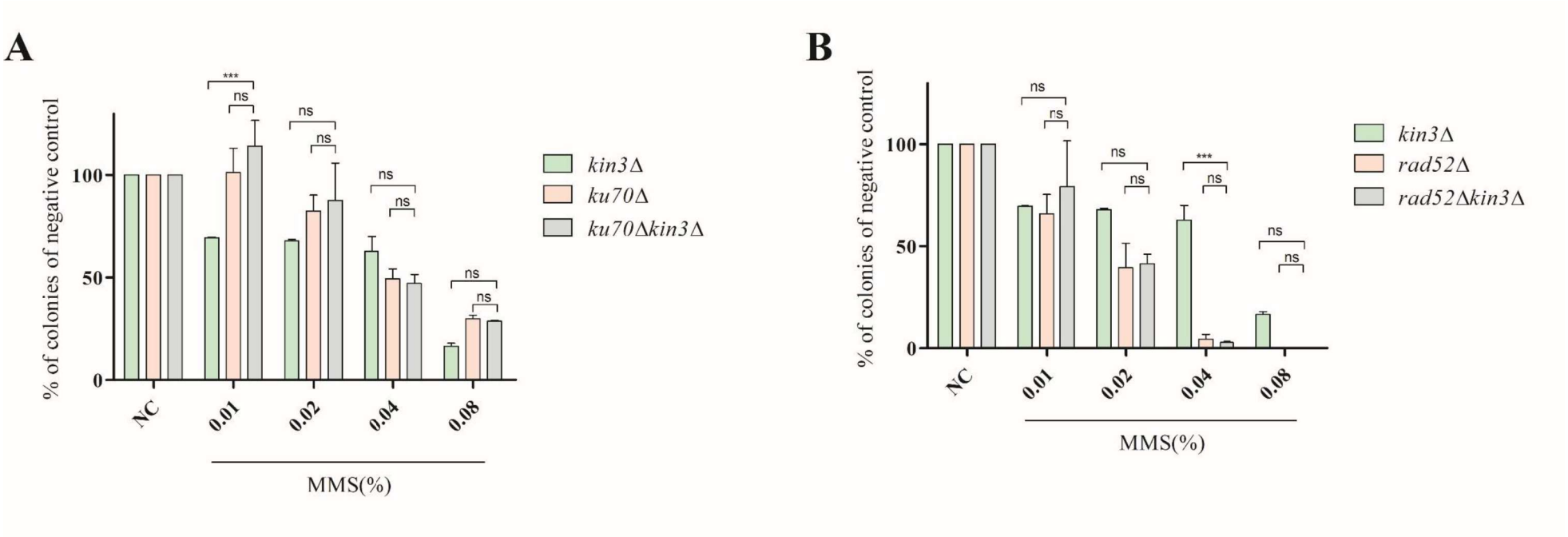

Figure 3. An epistatic phenotype in sensitivity profile to MMS DNA-induced damage between *kin3*Δ and *rad52*Δ. Cells were treated 2 h with MMS followed by 1 h of recovery. A. *kin3*Δ*ku70*Δ, B. *kin3*Δ*rad52.* Statistical analysis was performed by ANOVA two-ways followed by Bonferroni posttest. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.4 Single deletion increase mutation rate in response to MMS

The two canonical pathways responsible for repairing double-strand breaks have different levels of errors during the process. HR is generally considered to be an error-free DNA repair process because it uses a homologous DNA template to repair damaged DNA, whereas NHEJ is considered an error-prone repair process because it simply promotes the reconnection of the ends (DURDÍKOVÁ; CHOVANEC, 2016). To understand how the deletions of the genes *MRE11*, *SRS2, RAD52, KU70* and *KIN3* impact in mutation rate, the canavanine assay was performed, which measure forward mutations in the CAN1 gene from the rate of reversions. Figure 4A shows that deletion of *KIN3,* a gene considered non-essential, tends to increase the mutant yield under basal conditions and increase at the concentration of 0.01%. The Figure 4B shows the deletion of *SRS2* does not modify the reversal rate. However, deletion of *MRE11* promotes an increase in mutation rate when cells were exposed to a mutagen. Interestingly, the most sensitive of single mutants it is also the one with highest mutation rate.

3.5 Deletion of *KIN3* and *SRS2* in *mre11*Δ cells decreases the mutation rate

The result of Figure 5A confirms data shown in Figure 4B, since deletion of *SRS2* did not increase the mutation rate in *kin3*Δ. Figures 5B and C showed that deletions of *KIN3* or *SRS2* in *mre11*Δ cells block induction of mutations. The result of *mre11*Δ *srs2*Δ was correlated with viability data, because in the same way with the mutation rate decreased, viability increased.

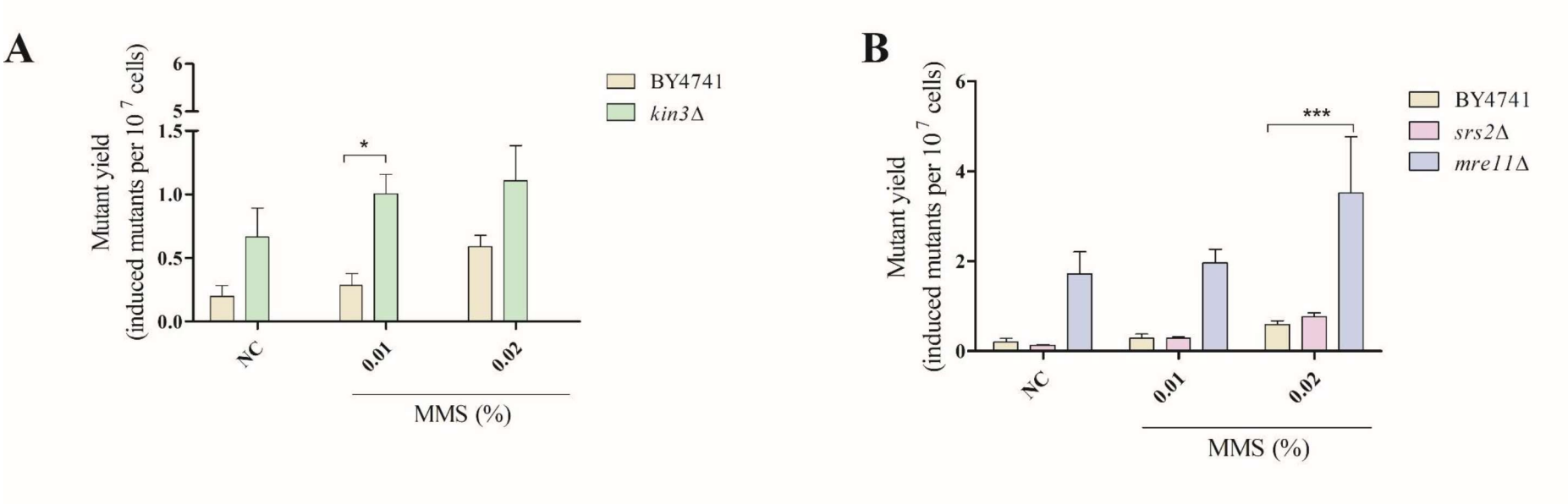

Figure 4. Differential mutant yield caused by single deletions after MMS exposition. For the reversion measurement, cells were plated on YEPD with 60 µg.mL^{-1} canavanine and for sensitivity assessment, the solutions were diluted and were plated on normal YEPD plates. Colonies were counted and the ration of revertant by survivors indicates the mutant yield per 10^7 cells. A. Comparison between wildtype and *KIN3* deletion. B. Comparison among wild type and *MRE11* or *SRS2.* Statistical analysis was performed by ANOVA two-ways followed of Bonferroni posttest. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

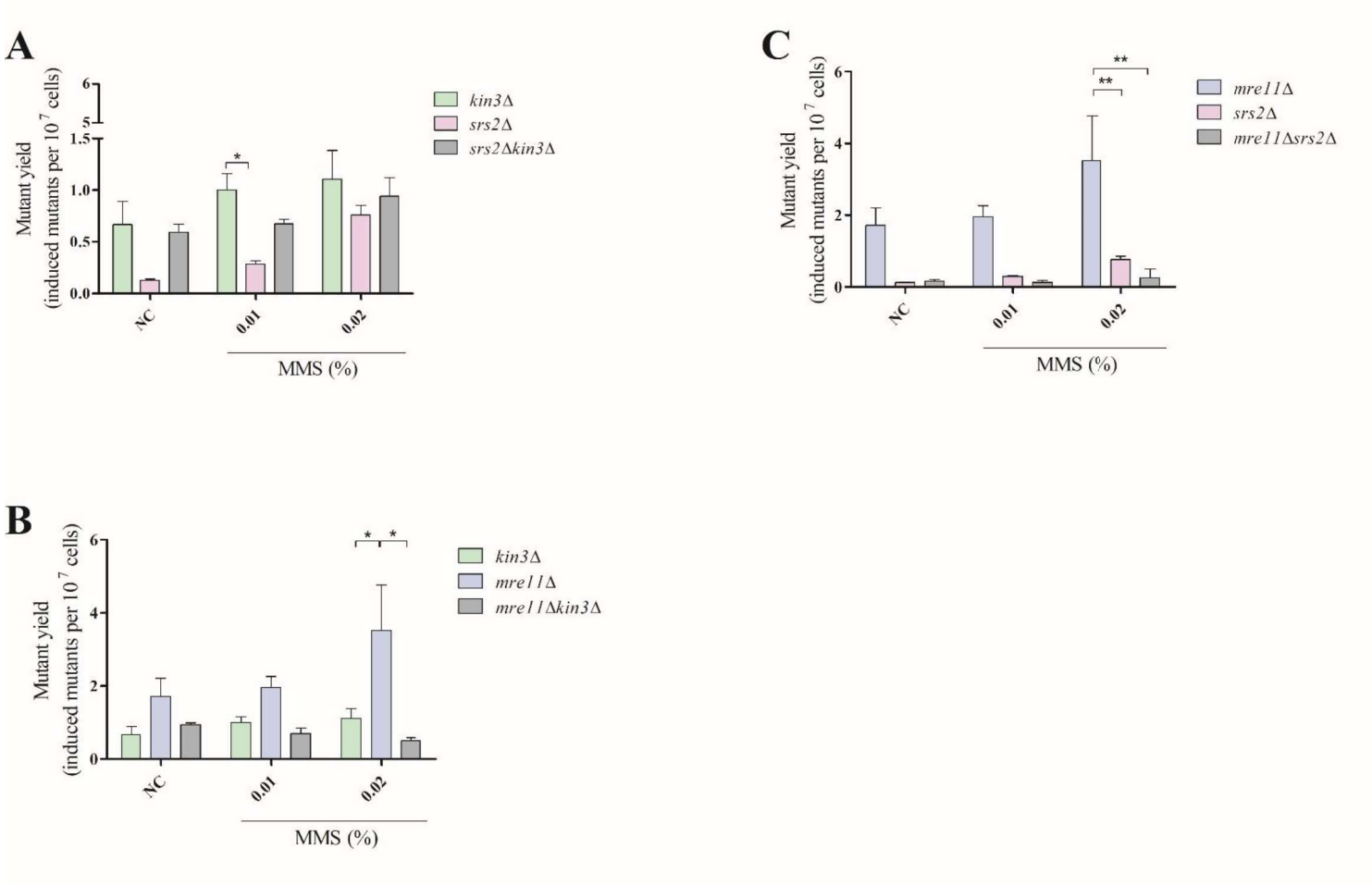

Figure 5. Block of of mutation rate caused by double deletions. Cells were plated in normal YEPD for measure the survival rate and in canavanine YEPD for measure the revert rate. The ration of revert by survival correspond to mutant yield. A. *kin3*Δ*srs2*Δ, B. *kin3*Δ*mre11*Δ*;* C. *mre11*Δ *srs2*Δ. Statistical analysis was performed by ANOVA two-ways followed of Bonferroni posttest. * $p<0.05$, **$p<0.01$, ***$p<0.001$.

3.6 The *KIN3* deletion has an additive effect on *rad52*Δ

Ku and Rad52 are essential proteins in their respective repair pathways. Ku protein mediates the reclosing of the broken DNA ends in NHEJ and Rad52 is responsible for disrupt RPA from 3' end and promotes the binding of Rad51. To understand if deletion of *KIN3* could have an impact on mutation rate in the absence of these two proteins, the canavanine assay was performed on double mutants. Figure 6A shows that *kin3*Δ*ku70*Δ does not increase the mutation rate, as observed in viability. However, deletion of *KIN3* in *rad52*Δ significantly increases the mutation rate under basal and exposed conditions, which indicates an additive effect.

3.7 DNA fragmentation induced by MMS

MMS induce DNA damage by alkylation of bases, which could become DNA breaks. So, to verify the treatment strategy and investigate if DNA fragmentation levels could be modulated by mutations evaluated in this work, cells were treated with 0.02% of MMS for 2 h followed by 1 h of recovery. After treatment, DNA was extracted, denatured and submitted to electrophoresis in agarose gel (Fig. 7A and B). Figure 7C show that BY4741 increase the level in DNA breaks after MMS treatment in approximately 1.5x. All mutants, single and double, in a basal condition has 2x more DNA breaks than BY474. After a damage, cell may attempt to repair the lesion or signalize to death pathways to avoid induction of mutations. In this sense, the breakage rate after MMS treatment does not increase in the mutants, indicating a decrease of repair pathways activation and an increase in death. The *srs2*Δ seems to be the most related to a high rate of DNA breaks. However, this assay was realized once, thus it is necessary more repetitions to verify the reproducibility of this result.

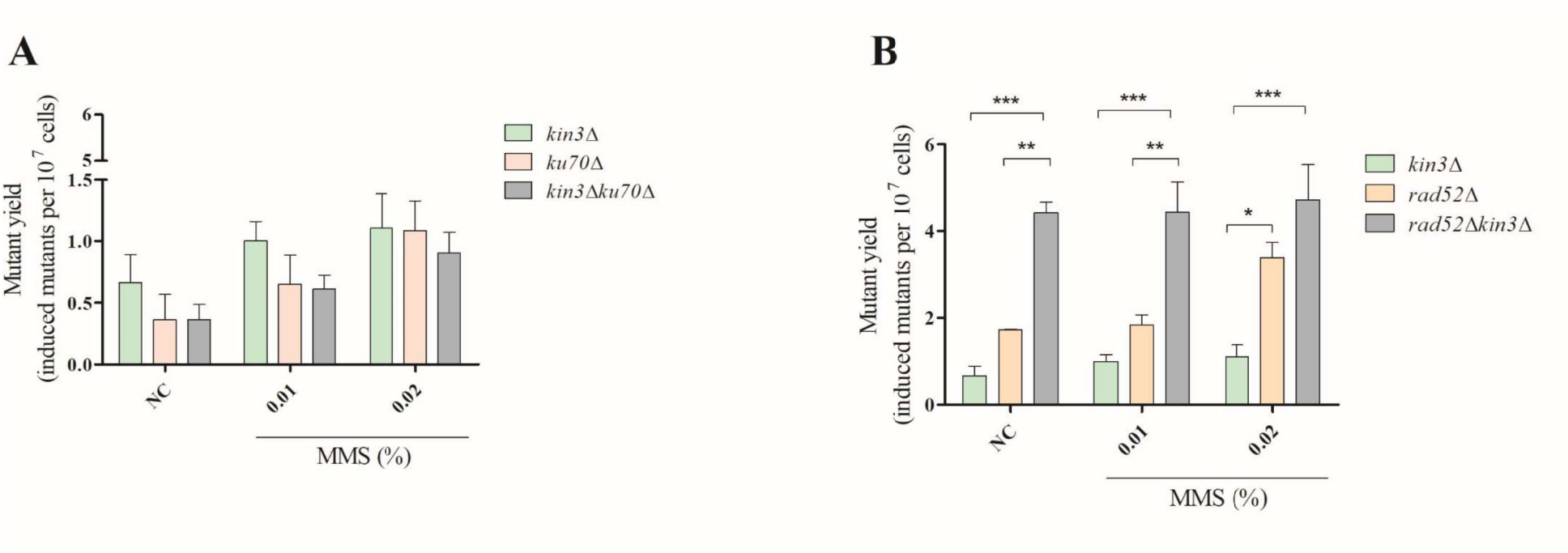

Figure 6. Aditive effect of *KIN3* deletion to mutant yield in *rad52*Δ. Cells were plated in normal YEPD for measure the survival rate and in canavanine YEPD for measure the revert rate. The ration of revert by survival correspond to mutant yield. A. *kin3*Δ*ku70*Δ, B. *rad52*Δ*kin3.* Statistical analysis was performed by ANOVA two-ways followed of Bonferroni posttest. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

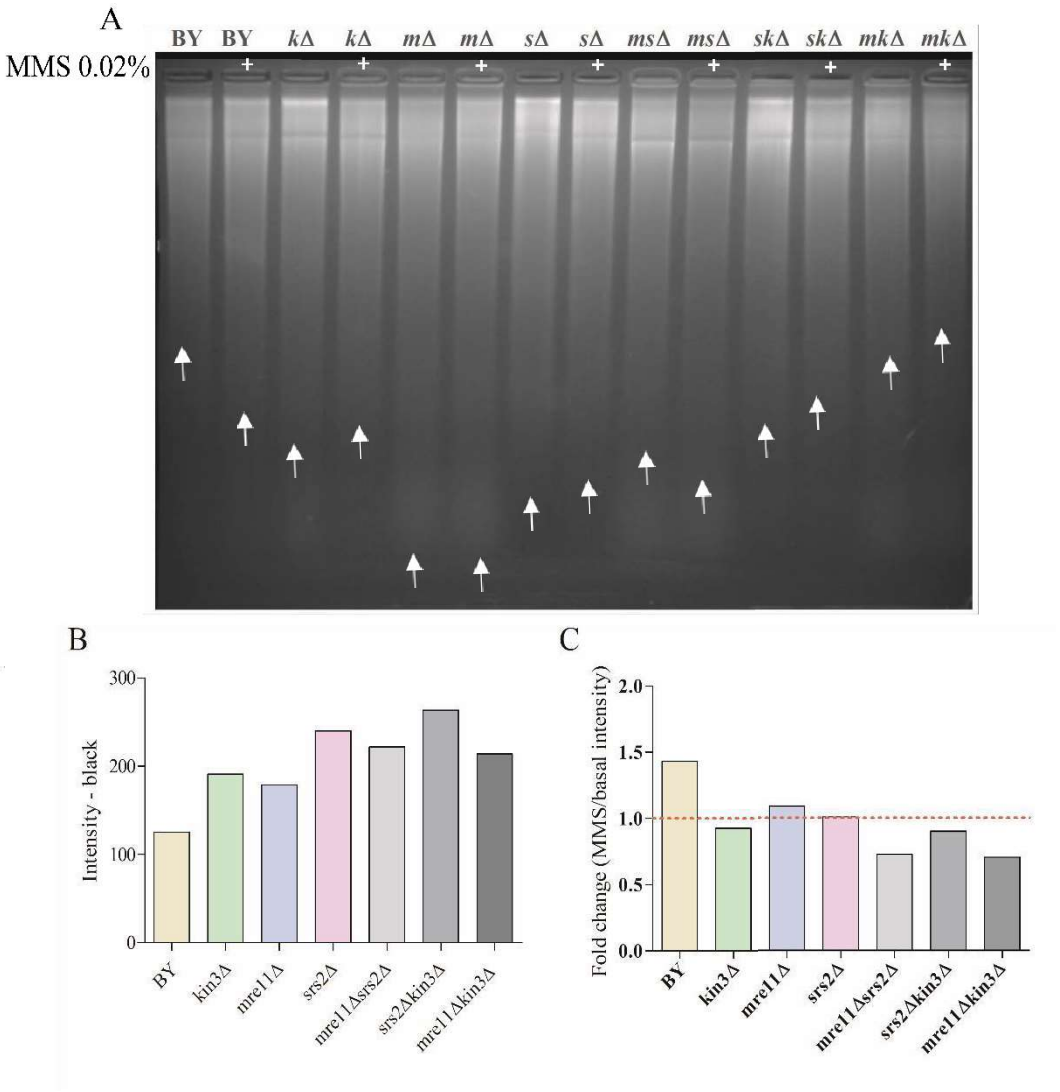

Figure 7. Block of DNA fragmentation after MMS treatment caused by mutations. Cells were treated with 0.02% of MMS for 2 h followed by 1 h of recovery. The DNA was extracted, denatured and subjected to agarose gel electrophoresis. Figure A shows as agarose gel picture, in which the legend of the columns was indicted with the first letter of mutant. Figure B shows the quantification of basal levels of DNA breaks (untreated). Figure C shows the fold change of the ration between the quantification of treated and untreated columns.

3. DISCUSSION

Chemical agents induce several lesions in DNA such as oxidation, alkylation, bulky adduct formation, intercalation and crosslinks. These lesions can generate DNA strand breaks and/or replicate fork collapse. MMS transfer alkyl groups to the DNA, which induce the formation of apurinic sites. These lesions can block DNA replication

and induce double-stranded breaks (STASZEWSKI; NIKOLOVA; KAINA, 2008). Thus, our model of 2 h of treatment followed by 1 h of recovery is intended to stimulate the DNA breaks generation to investigate to role of Kin3 in this response (BREWER; CHLEBOWICZ-SLEDZIEWSKA; FANGMAN, 1984). Alkylation of guanine may increase point mutations or cell death if not repaired.

This article showed that single mutation of *MRE11*, *SRS2* and *KIN3* increase the sensitivity to MMS. In cell lines *mre11*Δ and *kin3*Δ, the decrease of viability was accompanied by an increase in mutation rate. However, *srs2*Δ did not show the same profile. This is very interesting, because Srs2 have an inhibitory role to HR, but Mre11 promotes HR, which indicates that prevent DNA repair increase death rate (GOBBINI *et al.*, 2016; SKONECZNA; KANIAK; SKONECZNY, 2015). The role of Kin3 is not identified, but this results could indicate that this protein has a promoting role in DSB repair. In addition to these hypothesis, Kin3 showed an epistatic profile with Mre11 and Rad52. The genes of RAD52 group of DNA repair are needed to repair DSB and the mutants in these genes were sensitive to the clastogenic chemicals as MMS and bleomycin (KROGH; SYMINGTON, 2004; MCKINNEY *et al.*, 2013).

The mutation rate of double mutant *rad52*Δ*kin3*Δ also corroborates with the hypothesis that Kin3 stimulates HR. The single mutant of *rad52*Δ showed an increase in mutation rate dependent of MMS induction, which was significantly different from *kin3*Δ at the 0.02% concentration. However, deletion of *KIN3* in *rad52*Δ increased the mutation rate by equating the negative control to that found at 0.02% dose. So, if deletion of Kin3 increased the mutation rate, probably this protein its required to DNA damage response of some pathway error-free.

Yeast *S. cerevisiae* is an excellent model to study genetic interactions and understand mechanistically how a repair pathway impacts in viability or mutagenesis induced by clastogenic agent, because it is easily manipulated genetically and the proteins are relatively conserved to mammalians (GIETZ, R. DANIEL *et al.*, 1995; KRAMER *et al.*, 1994). Orthologous of Kin3 in mammalian also are involved in DNA damage response and tumorigênese. Nek1-deficient cells increase the sensibility to MMS by decrease repair capacity, interacts with proteins of double strand response as Mre11 and Nek11 have interesting considerations about cell cycle regulation in DNA damage response after ionizing radiation. At same time, Nek1 is overexpressed in glioblastomas

and downregulation of NEK11 also was associated with drug resistance in ovarian cancer (MEIRELLES *et al.*, 2014; NOGUCHI *et al.*, 2002; PELEGRINI *et al.*, 2010; SURPILI; DELBEN; KOBARG, 2003). Thus, understanding the pathway involved of activity of these proteins, it is important to modulate their action and propose a therapeutically strategy.

In summary, this paper showed results that indicating a role of Kin3 in double stranded breaks response, mainly HR, such as an epistasis between Kin3 and Mre11 and between Kin3 and Rad52, reversion of *srs2*Δ sensitivity after *KIN3* deletion and an additive effect in the mutation rate of *rad52*Δ. Our research group has shown the role of Kin3 in response to adduct strand breaks, mediated by a fail in arrest G2/M, which would allow repair of the lesions (MOURA *et al.*, 2010). Certainly, specific lesion-inducing probes are still required to verify whether modulation found by *KIN3* deletion is due to its role in HR or due to a crosstalk between the repair pathways. In addition to evaluated interactions described in this article, assays that visualize the colocalization or direct physical interaction are necessary to verify the findings referred to in this article and propose the role of the Kin3 protein at a physiological context and under damage.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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4. ARTIGO CIENTÍFICO 2

ATP influence on structure of a Kin3, a NIMA-related kinase from *Saccharomyces cerevisiae*

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ATP influence on structure of a Kin3, a NIMA-related kinase from *Saccharomyces cerevisiae*

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ABSTRACT

The serine/threonine protein Kin3 is the only one representative, so far, of NIMA-related kinase family in yeast *Saccharomyces cerevisiae*. As there is no information about Kin3 structure in literature, the aim of this article was to model a Kin3 structure, with and without ATP and cofactor (Mg^{+2}), and explore the conformations of activation loop after a simulation by molecular dynamics. The conformations and features were compared with the most similar orthologue in mammals, Nek2 protein. Molecular dynamic showed that a small α-helix structure was disrupted in absence of ATP, indicating that the binding to the nucleotide is important to stabilize this region, since the flexibility of an α-helix is smaller than that of loop. This modification didn't approximate the serine-threonine residues in the activation loop of γ-phosphate of ATP, allowing the catalysis reaction and didn't modify the position of the DFG motif or the nucleotide position for active form. Thus, this work establishes in the first-time information about Kin3 structure, suggest that Kin3 could be active after homodimerization and indicate that a rearrangement of the activation loop will be required to approximate the serine and threonine residues to γ-phosphate.

Key-words: structure. Kin3. Nek2. Homology modeling. Molecular Dynamic

List of abbreviations: NRKs - NIMA-related kinases; MTOC - Microtubule Organization Center; SGD - Saccharomyces Genome Database; PDB - Protein Data Bank; MD - Molecular Dynamic; RMSF - Root Mean Square Fluctuation

1. INTRODUCTION

Never in mitosis A (NIMA) is a protein from *Aspergillus nidulans,* characterized as the first protein of the NIMA-related kinases family's (NRKs). The functions of NIMA protein are related to the G2/M transition, modulating the nuclear pore and allowing the entry of mitotic regulators in the core (De Souza, Osmani, Wu, Spotts, & Osmani, 2000; Moniz, Dutt, Haider, & Stambolic, 2011). NIMA is essential to mitosis because, as its name suggest, mutants in *nimA* gene arrest cell cycle and never enter in mitosis (Morris, 1975; Oakley & Morris, 1983). Among humans orthologous of NIMA, Nek2 is the most studied. Its sequence consists of a domain kinase at N-terminus and the non-catalytic C-terminus, which contains essentials coiled-coils motifs, one immediately downstream of the catalytic domain and another at the extreme C-terminus (Rellos et al., 2007). The first motif contain a leucine zipper, through which Nek2 forms homodimers, promoting autophosphorylation and activation (Fry, Arnaud, & Nigg, 1999). There are also degradation domains such as PEST sequences (Rellos et al., 2007). Several observations indicate that Nek2 acts on cell cycle regulating the centrosome organization, being a component of microtubule organization center (MTOC). Nek2 is still important at the spindle assembly checkpoint where it can be involved in the identification of unaligned sister chromatids (Meirelles et al., 2014; O'Connell, Krien, & Hunter, 2003). The serine/threonine protein Kin3 is the representative of NIMA-related kinase family in *S. cerevisiae.* This protein was isolated from a library of cloned genomic fragments from budding yeast *S. cerevisiae,* using a probe with a highly-conserved catalytic domain of serine-threonine kinases and classified as non-essential for mitosis, meiosis, sporulation and conjugation (Barton, Davies, Hutchison, & Kaback, 1992; Jones & Rosamond, 1990). Moura et al. (2010) showed the role of this protein in response to adduct DNA damage, recognizing DNA breaks, mediating G2 cell cycle arrest and impacting of genomic integrity (Moura et al., 2010). Kin3 was also related with maximal ethanol accumulation capacity in yeast through its role in DNA damage response (Pais et al., 2013).

Rellos et al. (2007) resolved the structure of a T175A point mutation of the human Nek2 kinase domain complexed with a pyrrole-indolinone inhibitor SU11652 at 2.2 Å resolution (PDB code 2JAV). The catalytic domain of Nek2 exhibits a typical bilobal protein kinase fold. The structure of Nek2 assumes an inactive conformation that

resembles to of cyclin-free CDK2 bound to ATP and EGFR kinase. When compared with Aurora-A kinase bound to ATP but in inactive form (PDB code 1OL6), the DFG motif plus five residues folds into a small region of α-helix, thus a short sheet structure after DFG motif is lacking, resulting in disorder of region at the end of α-helix. This strongly suggest that αT in the activation loop is a feature of inactive conformation, because this helix promote a salt-bridge between Glu-55 and Arg-164. In this conformation, glutamic acid of C-helix projects away from the active site that prevent efficient catalysis. Autophosphorylation and/or phosphorylation by upstream kinases of residues Thr175, Thr-170 and Ser-171 is required to activate Nek2 and maintain kinase-kinase interactions within the Nek2 dimers (Fry et al., 1999; Rellos et al., 2007). Posteriorly, Westwood et al (2009) resolved Nek2 kinase domain in the APO form, bonding to ADP and a non-hydrolysable ATP analog, at 2.3, 1.55 and 2.4 Å resolution, respectively. They showed that despite three similar structures, the activation loop assumes several conformations dependent on the ligand bound, but as none of the position occupied by D159 and F160 are expect in an active form. Thus, Nek2 exhibited several possibilities of DFG-out conformations. An A136G mutation that disrupts the probably inhibitory α-helix not increase kinase activity, showing that the conversion of the inactive to the active conformation is unlikely to be based purely on the unfolding of this putative inhibitory helix. The nucleotide positioning resembles one of others inactive kinase structures.

In contrast to Nek2, no structural information was reported about Kin3. Thus, the objective of this article was to model a structure of Kin3, with and without ATP and cofactor (Mg^{+2}), and explore the conformations of activation loop by molecular dynamics. The results showed that ATP is necessary to maintain an α-helix of activations loop, but the stabilization of this conformation didn't change the conformation to active form of mitotic kinases.

2. MATERIALS AND METHODS

2.1 Template-based homology modeling

Homology modeling is a theoretical method to predict the 3D structure of a protein when the structure has not been resolved by an experimental method (Fiser, 2010). A sequence of target (Kin3 protein), obtained from Saccharomyces Genome Database

(SGD), was matched against a library of 3D structures, the Protein Data Bank (PDB). The comparative modeling process was conducted on MODELLER 9v14 (Sali & Blundell, 1993). The template was choose considering the highest identities with Kin3, a resolution and R-factor on crystallography structure and the deposit year . The NEK2 bound with ATPgammaS (PDB ID: 2W5B) and APO form (PDB ID: 2W5H) was used as template. To build a model, alignment was produced and the protocol of homology modeling was applied to generation of a thousand models. Two Kin3 structures were built, one with substrate and cofactor (scKIN3:ATP and Mg^{+2}) and a second in free form (scKIN3). Four of each protocol were select considering the lowest comparative energy potential function and the assessment of stereochemistry of a model was check through Ramachandran plot.

2.2 Simulations by Molecular Dynamic (MD) method

Kin3 models were shown as a rigid and unique structure. However, it is known that the physiologic behavior involves many conformational changes. In this context, MD simulations have been used to investigate structures and evaluate functions of biomolecules, incorporating flexibility receptor-ligand, improving the interactions e reinforcing the complementarity between them. In this technique, its possible incorporate solving molecules in the simulations, allowing evaluate water effect about stability of receptor-ligand interactions.

MD simulations were performed using GROMACS v.4.6.7 package (Van Der Spoel et al., 2005) employing the systems scKin3, scKIN3:ATP Mg^{+2}, Nek2APO and Nek2ATP). For ATP, hydrogen atoms were added through Avogadro program (Hanwell et al., 2012) and were parameterized employing CHARMM27 all-atom force field (Vanommeslaeghe et al., 2009). Each complex was immersed in a dodecahedron-shaped box (x, y, and z) with minimum distance of 1 nm between the protein surface and the box walls, followed by solvation in a TIP3P water model molecules. Periodic boundary conditions were assigned in all directions. The system net charge was neutralized by adding both Na^{+} and Cl^{-} ions in order to simulate physiological buffer (0.15 mM NaCl). These ions randomly substituted water molecules in suitable electrostatic potential positions. Then, the whole system was submitted to a first energy minimization employing the steepest descent algorithm with a tolerance of 1000 kJ/mol/nm followed by a second energy minimization with a conjugate gradient algorithm with a tolerance of

500 kJ/mol/nm. To relax strong solvent-solvent and solvent-protein non-bonded interactions, 1 ns (from 0 to 1 ns at 50 K) of MD simulation was performed restraining the protein structure. Initial velocities were assigned according to Maxwell distribution. All simulations were performed for 200 ns using an integration time step of 2 fs. Each system was heated with gradual increments in the following temperatures: 50 K (from 1 to 2 ns), 75 K (from 2 to 3 ns), 100 K (from 3 to 4 ns), 150 K (from 4 to 5 ns), 200 (from 5 to 6 ns), 250 K (from 6 to 7 ns), 300 K (from 7 to 8 ns) and after that (from 8 to 100 ns) the temperatures of the systems were adjusted to 303.15 K. CHARMM27 force field was assigned to the system. Berendsen barostat and thermostat were applied to keep the pressure and temperature constant with a coupling time of $\tau p = 0.500$ ps, and $\tau T = 1.00$ ps, respectively. Long-range electrostatic interactions were calculated with the Ewald particle mesh method. The bond lengths were restrained employing Linear Constraint Solver algorithm (LINCS) (Hess, Bekker, Berendsen, & Fraaije, 1997), allowing an integration step of 2 fs. Evaluations on the influence of ATP on the structure, the positioning of serines and threonine to ATP, classical folds of active kinases and interactions of DFG and HRD motifs was made during simulations.

Structure figures were produced using PyMOL (Delano, 2002) and organized using Inkscape 0.91 (Harrington, B. et al (2004-2005), Inkscape. http://www.inkscape.org/).

3. RESULTS

3.1 Three-dimensional structures

As mentioned before Nek2 and Kin3 are protein of NRKs family. These proteins present a very similar sequence with 38% of identity, and the classical sequences of serine-threonine cinases are conserved (Fig. 1). Both proteins have on the catalytic domain an triplet Asp-Phe-Gly and the consensus sequence His-Arg-Asp, as well as, Asp-X-Trp-Ser-X-Gly that are commonly used as indicators of kinase activity (Jones & Rosamond, 1990). The two structures generated by modeling homology shown a classical bilobal structure of kinases, which have predominantly β-sheets in N-terminus and α-helix in C-terminus (Fig. 2).

3.2 ATP influence on structure stability

To evaluate if ATP can modify the stability of the Kin3 structure, we simulated by molecular dynamic the protein, with and without ATP, under 200 ns. Our results showed that the measure of the average atomic mobility, RMSF, increases in the region between residues 200 and 250 in the APO form (Fig. 3A). This region corresponds to Kin3 loop activation, which contains serine and threonine residues to promote catalysis of γ-phosphate transfer from ATP to substrate. A short region of α--helix was disrupted in the structure after 200 ns of simulation, showing that ATP has influence on the stabilization of the activation loop (Fig. 3B).

3.3 Profile of autophosphorylation

The Nek2 is activated by autophosphorylation in a dimeric form. Then, we asked whether stabilization of the α-helix could approximate the serine and threonine residues from γ-phosphate of ATP. As shown in Figure 4, five residues of activation loop (Thr 221, 227, 228, 232 and Ser 222) are between 13 and 18 Å away of γ-phosphate, which does not make possible for a catalysis reaction. This result indicates that this conformation needs some additional modifications to be active.

3.4 Features of active kinases

Bayliss et al. (2012) described some features of the activation of mitotic kinases and Westewood et al. (2009) evaluated in Nek2 some of these characteristic, comparting with inactive or active structures of Aurora and of Cdk2 by function and structure resemblance. To evaluate this in the same context, Nek2APO and Nek2ATP were simulated by 200 ns, and some structures evaluations were performed with Nek2 and Kin3, before and after dynamics.

Westwood et al. (2009) described four DFG potions of this motif in Nek2 structures bound in distinct ligands (ATP, ADP and SU11652) and overlap together with the position adopted by fully active conformation of Aurora-A showed that all conformations were DFG-out. In active forms, DFG adopts the DFG-in conformation and coordinate the magnesium ions that active the ATP as well as is necessary to form a

hydrophobic spine (Coxon et al., 2017). In Figure 5 is shown that neither at the beginning nor at the end of dynamic simulation, Kin3 or Nek2 reached a DFG-in conformation, however, an approximation of this position has been visualized.

The positioning of nucleotide within the cleft is different between active and inactive kinases. In Figure 6, the inactive (PDB code 1B39) and active (PDB code 1QMZ) forms of two CDK2 structures showed the conformation difference adopted by nucleotide, which will modify the molecule after solvent exposure. So, we wonder if after 200 ns of molecular dynamics, the nucleotide could be in active form. Figure 6A and B show the superposition of Kin3 and Nek2 structures before and after simulations, respectively, together with CDK2 in both forms. It is possible to visualize, the nucleotide did not reach the active form in any of the structures.

4. DISCUSSION

In this work a structure to Kin3 protein was proposed, with and without ATP and cofactor, by molecular modeling and achieved the conformations by the activation loop, DFG motif and nucleotide position at the start and finish of the molecular dynamics were explored. Whereas there is no information about Kin3 structure in literature, the conformations and features were compared with the most similar orthologue in mammals, the Nek2 protein.

The molecular dynamic results showed that a small α-helix structure was disrupted in ATP absence, indicating that the binding to the nucleotide is important to stabilize this region, since the flexibility of an α-helix is less than one loop. The hypothesis that this would be enough to stabilize the protein on active form was not confirmed. This modification did not approximate the serine-threonine residues present in the activation loop of γ-phosphate of ATP, allowing the catalysis reaction, did not modify the position of the DFG motif or the nucleotide position for active form present in active Aurora or CDK2.

It is known that Nek2 has two coiled-coil motifs in the structure, which promote the homodimers formation and activation of the protein by autophosphorylation (Fry et al., 1999). Our research group investigated Kin3 interactors in the context of DNA and

observed in a two-hybrid assay a physical interaction of Kin3 with itself (date not published). Thus, our hypothesis is that Kin3 could be active after homodimerization. One limitation of this work is the absence of an experimental Kin3 structure that allows a modeling of dimers with high confidence. Other mitotic kinases such as CDK2 need the presence of another molecule to stabilize the movement of activation loop (Tang & C Poon, 2011). Aurora is activated by phosphorylation and through the binding with partner proteins (Bayliss, Fry, Haq, & Yeoh, 2012). Thus, further studies are needed to understand the exact mechanism of Kin3 activation.

Westwood et al. (2009) evaluated in Nek2 some characteristic of the inactive and active structures of mitotic kinases, as was described by Bayliss et al. (2012). The authors showed that DFG positions in the Nek2 structures bound in different ligands (ATP, ADP and SU11652) were DFG-out conformations, that positioning of the nucleotide within the cleft was equal to CDK2 in inactive conformation. In addition, an A136G mutation that disrupts the probably inhibitory α-helix does not increase kinase activity, showing that the conversion of the inactive to the active conformation is not based purely on the unfolding of this putative inhibitory helix. We show that α-helix disruption is a feature of APO structures, because the disruption of the first α-helix occurs in both Kin3 and Nek2 after 200 ns of molecular dynamic simulations. Therefore, it is probably not the disruption of this structure that will be present in the active binding structure, but rather a rearrangement of the activation loop will be required to approximate the serine and threonine residues of this region of γ-phosphate.

Thus, our work establishes information about Kin3 structure and suggest that are modifications of structures promoted by ligations with ATP. In addition, this article showed a similar behavior of the free forms of both Nek2 and Kin3. Obviously, the experimental method for analyze Kin3 still is necessary to check the findings referred to in this article and to propose the exact activation mechanism.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ACKNOWLEDGMENTS

This work was supported by the CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil), FAPERGS (Fundação de Amparo à Pesquisa do Rio Grande do Sul), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and FINEP (Financiadora de Estudos e Projetos).

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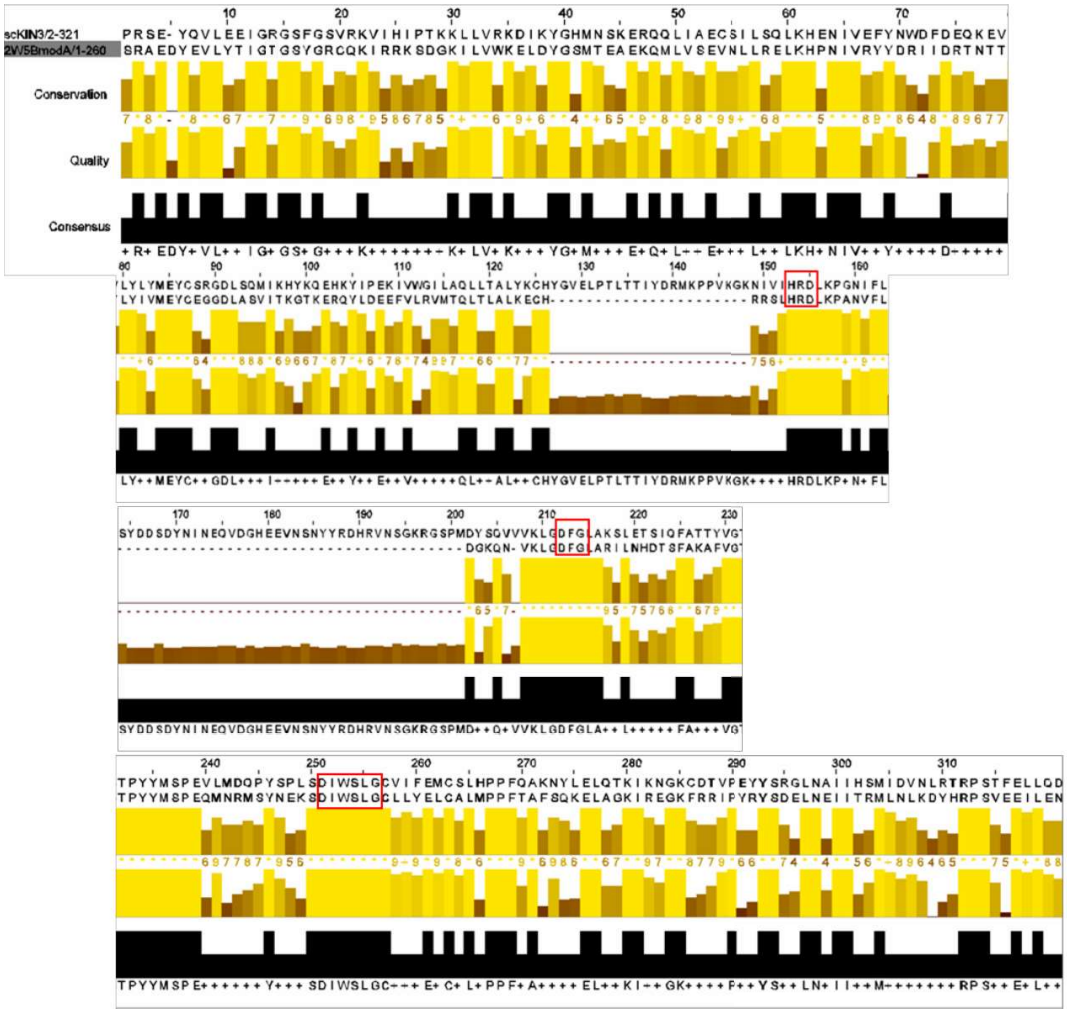

Figure 1. Alignment of the amino acid sequences of Kin3 and Nek2. Alignment results indicate the high conservation between these two proteins. Kinases motifs as His-Arg-Asp, Asp-Phe-Gly and Asp-X-Trp-Ser-X-Gly revealed conservation between two sequences.

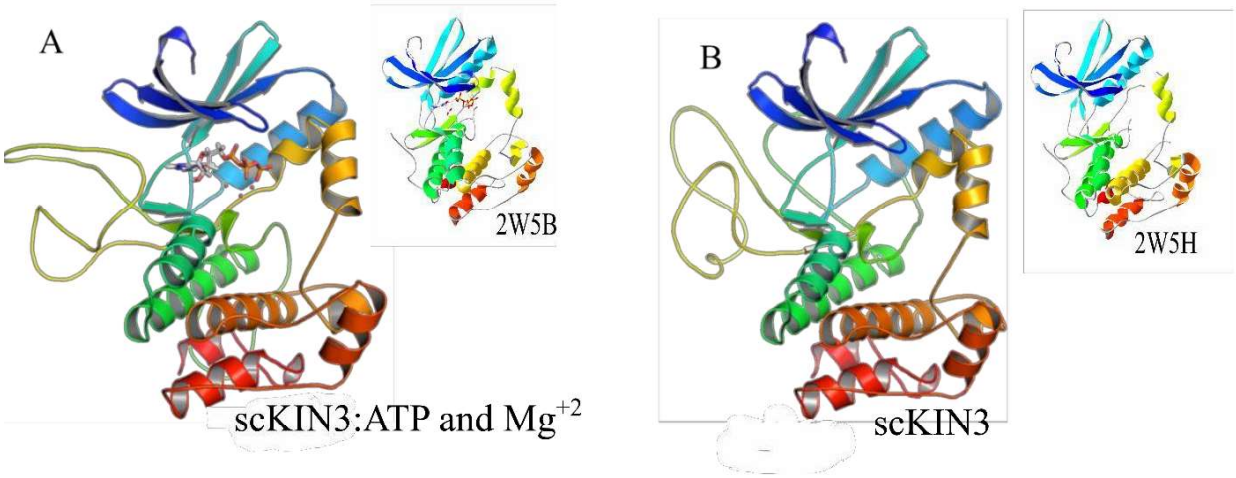

Figure 2. Three-dimensional molecular models. Ribbon diagrams of three-dimensional structures of Kin3 generated by homology modeling with the human NEK2 as template. A – Kin3 bound to ATP and Mg^{+2}. B – Kin3 in the free form (APO).

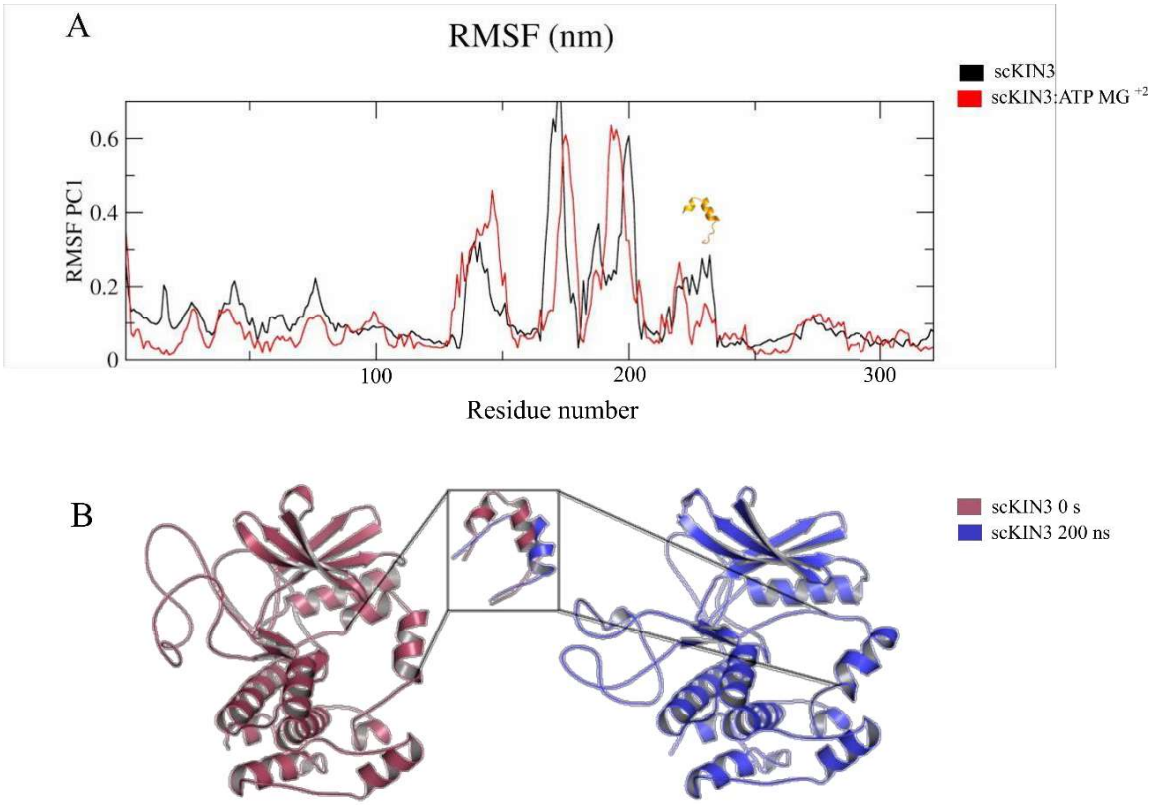

Figure 3. Molecular dynamics of Kin3 structures. scKin3 and scKin3:ATP the protein bound and free forms were simulating by molecular dynamic by 200 ns. A – Root Mean Square Fluctuation (RMSF) of two proteins in the end of simulation. B – Comparison of activation loop between sc-KIN3 in the beginning (red) and in the end (blue) of simulation.

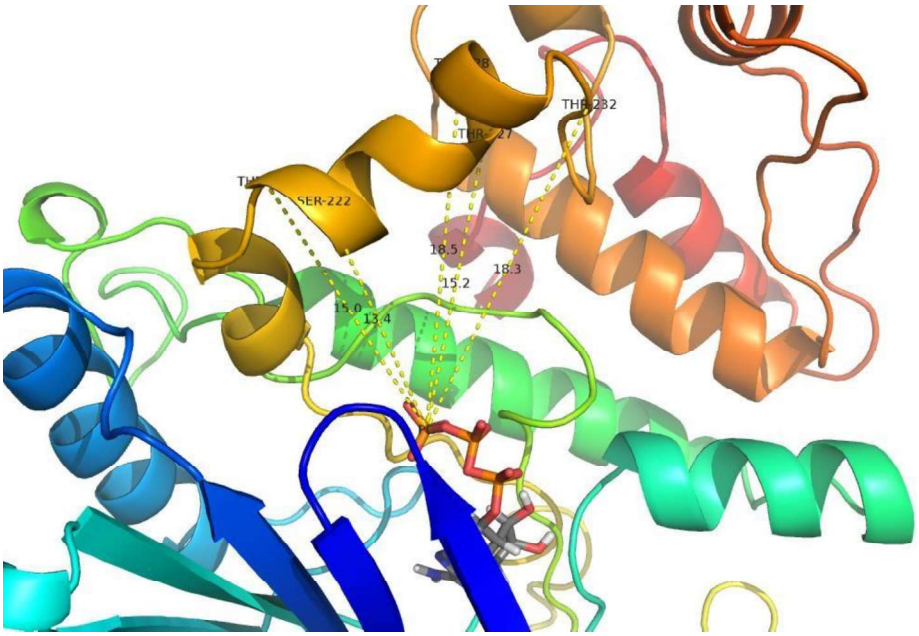

Figure 4. Distance of Thr and Ser residues from γ-phosphate from ATP. The distance in Å was measure between Thr 221, 227, 228, 232 and Ser 222 from γ-phosphate of ATP in the end of dynamic simulation.

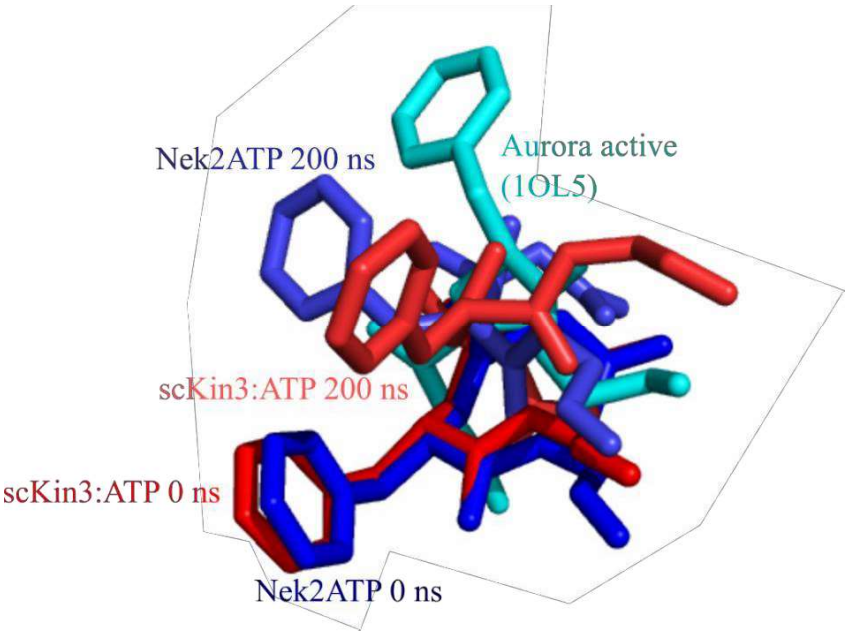

Figure 5. Conformation of DFG motif. Superposition of DFG motif of Kin3 and Nek2, before and after molecular dynamic of 200 ns, compared with an active form of Aurora.

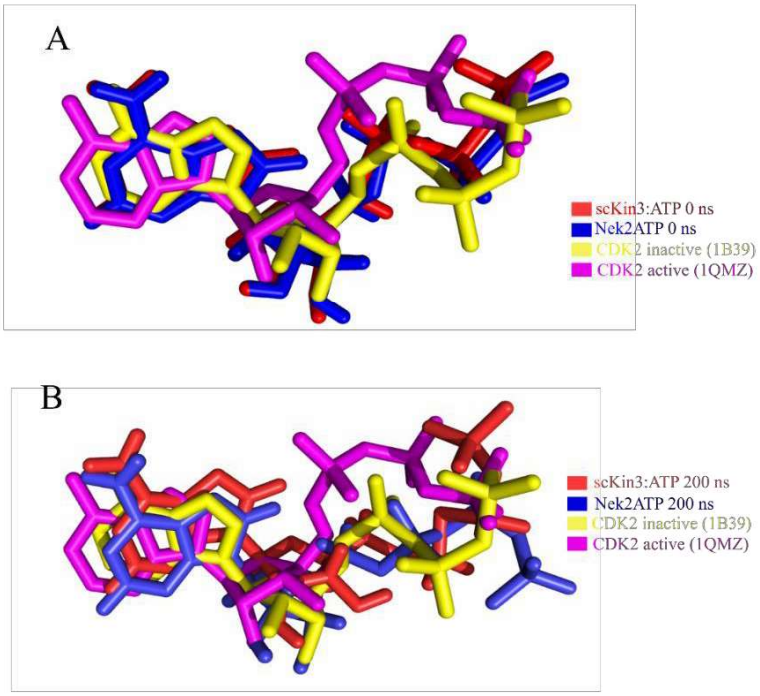

Figure 6. Nucleotide conformation. Superposition of nucleotide position of two CDK2 conformations, active and inactive, together with Kin3:ATP and Nek ATP, before (A) and after (B) 200 ns of molecular dynamic simulations.

5. CONSIDERAÇÕES FINAIS

Esta dissertação objetivou compreender qual a função da proteína Kin3 após a indução de danos no DNA induzidos pelo MMS, em um esquema de tratamento para geração de quebras. As principais conclusões desta etapa são:

- A proteína Kin3 está no grupo de epistasia das proteínas Mre11 e Rad52;
- A deleção do gene *KIN3* reverte a sensibilidade causada pela deleção de *SRS2;*
- A proteína Kin3 apresenta um efeito aditivo na taxa de mutação causada pela deleção de *RAD52.*

Essas conclusões, somado aos demais resultados produzidos pelo nosso grupo de pesquisa permitem sugerir que a função de Kin3 está relacionada à resposta de quebras duplas que ocorre durante G2/M, sumariamente a Recombinação Homóloga. Esta primeira etapa contém duas principais limitações, a primeira é a heterogeneidade existente na população avaliada frente as posições no ciclo celular, o qual pode influenciar na atuação das diferentes vias de reparo relacionadas às DSBs. Além disso, o tipo de dano induzido indiretamente leva à geração de quebras no DNA. Porém, este não é o tipo de lesão primária. MMS é primordialmente um agente alquilante. O tempo de recuperação de 1 h foi estabelecido para permitir a transição entre as fases dos ciclos da *S. cerevisiae* estimulando a geração de quebras. Essas questões implicarão em algumas perspectivas, abordadas a seguir.

A segunda etapa deste trabalho procurou avaliar aspectos estruturais da proteína Kin3, o que possibilitou a geração das seguintes considerações:

- Estabelecimento de uma estrutura 3D para Kin3;
- O ATP é necessário para aumentar a estabilidade do loop de ativação;
- O aumento da estabilidade do loop de ativação não aproxima os resíduos de serina e treonina do fosfato γ;
- Sem substrato, ocorre a disrrupção da α-hélice inicial do loop de ativação após 200 ns de dinâmica molecular;
- A presença do ATP não posiciona o motivo DFG na conformação ativa após 200 ns de dinâmica molecular;

- O ATP não se posiciona na conformação ativa após 200 ns de dinâmica molecular.

Tendo em vista os dados existentes na literatura, esta é a primeira vez que avaliações estruturais com e sem a presença de ATP são realizadas com a proteína Kin3, indicando a relevância do trabalho. Porém, algumas limitações inerentes ao modelo utilizado também devem ser consideradas. Primeiramente a proteína utilizada como molde possui uma mutação do resíduo 175, em que uma treonina foi substituída por uma alanina. Esse é um dos principais resíduos de ativação da proteína e pode impactar em uma variação na resposta. Além disso, a comprovação da ativação pelo homodimerização necessitaria da resolução experimental da proteína. Alguns esforços neste sentido foram conduzidos durante este projeto, porém não houve sucesso em finalizar esta etapa.

6. PERSPECTIVAS

- Concluir a triplicata experimental do ensaio de fragmentação de DNA.
- Realizar a co-imunoprecipitação de Kin3 com Mre11 e Srs2 numa condição com e sem danos induzidos por MMS.
- Avaliar a influência das fases de ciclo celular nos perfis epistáticos encontrados neste trabalho.
- Construir experimentalmente a estrutura da proteína Kin3.
- Avaliar o estado oligomérico da proteína.
- Verificar características de cinases ativas a partir da construção de um homodímero de Kin3.

7. CURRÍCULO LATTES

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Última atualização do currículo em 07/06/2017

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Nome em citações bibliográficas	MORÁS, A. M.;Ana Moira Morás;MORÁS, A.;MORÁS, ANA MOIRA

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2015	Mestrado em andamento em Programa de Pós-Graduação em Biociências. Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil. Orientador: Dinara Jaqueline Moura.
2011 - 2012	Aperfeiçoamento em Jovem Aprediz. (Carga Horária: 1000h). Banco Cooperativo Sicredi, SICREDI, Brasil. Título: Programa Jovem Aprendiz. Ano de finalização: 2012. Bolsista do(a): Serviço Naciolnal de Aprendizagem do Cooperativismo do Estado do Rio Grande, SESCOOP/RS, Brasil.
2012 - 2014	Graduação em Toxicologia Analítica. Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.
2009 - 2011	Ensino Médio (2º grau). Escola Estadual de Ensino Médio Elisa Tramontina, EEEMET, Brasil.

Formação Complementar

2016 - 2016 I Encontro do Programa de Pós-Graduação em Biociências. (Carga horária: 12h).
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.

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2015 - 2015 Análise de Risco de Organismos Geneticamente Modi. (Carga horária: 8h).
Associação Nacional de Biossegurança, ANBIO, Brasil.

2014 - 2014 Estudo da sinalização celular no câncer. (Carga horária: 15h).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

2014 - 2014 Toxicogenomics and Genotoxicity. (Carga horária: 8h).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

2013 - 2013 Elaboração de documentos do Sistema de Gestão. (Carga horária: 8h).
Associação Rede de Metrologia e Ensaios do Rio Grande do Sul, REDE METROLOGICA, Brasil.

2013 - 2013 Espécies genotóxicas em fármacos. (Carga horária: 3h).
Sociedade Brasileira de Toxicologia, SBTOX, Brasil.

2012 - 2012 Toxicologia Analítica: aplicações clínica e forens. (Carga horária: 7h).
Associação Bras. de Centros de Info Assist Toxic. e Toxicologistas Clínicos, ABRACIT, Brasil.

Atuação Profissional

Green Lab - laboratório de análises químicas e toxicológicas, GREEN LA, Brasil.

Vínculo institucional
2014 - 2014 Vínculo: Bolsista, Enquadramento Funcional: Analista, Carga horária: 30
Outras informações Estágio realizado na área de química analítica, onde foram desenvolvidas análises de orgânicos voláteis e íons inorgânicos, tanto em amostras biológicas como ambientais, por técnicas cromatográficas (GC-FID e IC). Participação na implantação da recertificação do laboratório pela Rede Metrológica do RS e acreditação na norma ISO17025 pelo INMETRO.

Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.

Vínculo institucional
2014 - 2015 Vínculo: Bolsista, Enquadramento Funcional: Iniciação Científica, Carga horária: 20
Outras informações Aluna voluntária no laboratório de genética toxicológica da Universidade Federal de Ciências da Saúde de Porto Alegre

Vínculo institucional
2014 - 2015 Vínculo: Bolsista, Enquadramento Funcional: Bolsista de Apoio Técnico, Carga horária: 40
Outras informações Bolsista de apoio técnico pela FAPERGS, cumprindo carga horária semanal de 40h, no laboratório de Genética Toxicológica, na Universidade Federal de Ciências da Saúde de Porto Alegre. A bolsa abrange o desenvolvimento de funções relacionadas à rotina de laboratório, como limpeza e esterilização de materiais, bem como organização de estoque e preparação de soluções. Além disso, envolve o auxílio na execução de ensaios com detecção por citometria de fluxo e espectrofotometria, cultivo de células de mamíferos e levedura.

Vínculo institucional
2014 - 2014 Vínculo: Bolsista, Enquadramento Funcional: Iniciação Científica, Carga horária: 20
Outras informações Projeto de iniciação científica onde foi avaliado o efeito citotóxico sobre linhagens tumorais do veneno bruto de B. jararaca.

Vínculo institucional
2013 - 2014 Vínculo: Bolsista, Enquadramento Funcional: Iniciação Científica, Carga horária: 20

Fundação Estadual de Proteção Ambiental Henrique Luís Roessler, FEPAM, Brasil.

Vínculo institucional
2012 - 2013 Vínculo: Bolsista, Enquadramento Funcional: Iniciação Científica, Carga horária: 24

Projetos de pesquisa

2014 - 2016

Avaliação da atividade antibacteriana, antifúngica e antitumoral do veneno de serpentes do gênero Bothrops
Descrição: O Brasil apresenta uma ampla biodiversidade com grande potencial para inovações na área de biotecnologia e para o descobrimento de biomoléculas com atividade farmacológica. Os venenos de animais peçonhentos são uma rica fonte de compostos protéicos e não protéicos que apresentam inúmeras atividades farmacológicas, e de onde cada vez mais, estão sendo isolados proteínas e peptídeos de interesse farmacológico. A partir de componentes presentes no veneno de serpentes, já foram isoladas substâncias utilizadas no tratamento das doenças hematológicas, cardiovasculares, hipertensão, atividade antitumoral, entre outras. Considerando que a resistência antimicrobiana, antifúngica, antiviral e antitumoral representa uma ameaça para a saúde pública nos países em todo o mundo, sendo esta, associada à maior mortalidade, morbidade e custos mais elevados de tratamentos, este projeto tem como objetivos avaliar o efeito farmacológico dos venenos de serpentes do gênero Bothrops frente a bactérias e fungos, assim como a ação antitumoral, em sistemas in vitro. Este projeto envolverá a cooperação de pesquisadores da UFCSPA (avaliação da atividade antitumoral), da Universidade de Passo Fundo UPF (avaliação da atividade antimicrobiana) e do Centro de Informações Toxicológicas do Rio Grande do Sul CIT/RS (fornecimento dos venenos)...
Situação: Concluído; Natureza: Pesquisa.
Alunos envolvidos: Graduação: (2) .

Integrantes: Ana Moira Morás - Integrante / Dinara Jaqueline Moura - Integrante / Eliane Dallegrave - Coordenador / Sandra Monoela Dias Macedo - Integrante / Jenifer Saffi - Integrante / Luciana Grazziottin Rossato - Integrante / Maria da Graça Boucinha Marques - Integrante / katia Rosana de Lima Moura da Silva - Integrante.

Desenvolvimento de um extrato padronizado de Plantago major L. em ácidos triterpênicos e determinação do potencial cicatrizante, anti-inflamatório e da sua segurança toxicológica
Descrição: ste projeto tem como objetivo elaborar um extrato padronizado de P. major, em ácidos triterpênicos, e determinar o potencial cicatrizante e anti-inflamatório deste extrato em modelos in vitro e in vivo. Também serão conduzidos os ensaios biológicos in vitro com células T-helper (TH-1), para avaliação da atividade anti-inflamatória, com células de queratinócitos (HaCaT), para avaliação da atividade cicatrizante. Paralelamente serão realizados ensaios in vitro para determinação do potencial mutagênico. Os ensaios toxicológicos in vivo complementarão os ensaios biológicos e incluirão as avaliações farmacológicas (anti-inflamatória e cicatrizante) e os ensaios de toxicidade aguda, toxicidade de doses repetidas e genotoxicidade utilizando ratos Wistar. Adicionalmente, este projeto prevê a identificação e a caracterização morfoanatômica desta espécie, visando à elaboração de uma descrição detalhada de suas características macroscópicas e microscópicas...
Situação: Concluído; Natureza: Pesquisa.
Alunos envolvidos: Graduação: (2) / Mestrado acadêmico: (2) .

Integrantes: Ana Moira Morás - Integrante / Dinara Jaqueline Moura - Coordenador / Valéria F. Peres - Integrante / Eliane Dallegrave - Integrante / Sandra Monoela Dias Macedo - Integrante / Nathalia Denise de Moura Sperotto - Integrante / Jenifer Saffi - Integrante / Jeferson Gustavo Henn - Integrante.

2013 - Atual

A proteína Kin3 de Saccharomyces cerevisiae: estudo estrutural e funcional
Descrição: O projeto tem como objetivo alterar a sequência primária da proteína Kin3 através de experimentos de mutagênese sítio dirigida, e analisar o perfil de fosforilação de Kin3 utilizando espectrometria de massas, avaliando possíveis sítios importantes para resposta a estresse genotóxico e interação com outras proteínas. Também, definir quais proteínas são substrato para Kin3 através de ensaios de fosforilação in vitro, e avaliar a interação de Kin3 com outras proteínas, desta forma identificar novos parceiros que possam atuar em conjunto com a mesma e o complexo MRX na resposta a estresse genotóxico...
Situação: Em andamento; Natureza: Pesquisa.
Alunos envolvidos: Graduação: (2) / Mestrado acadêmico: (2) / Doutorado: (1) .

Integrantes: Ana Moira Morás - Integrante / Dinara Jaqueline Moura - Coordenador / Jenifer Saffi - Integrante / Guido Lenz - Integrante / Bruna Frielink Immich - Integrante / Paula Pellenz Tomasini - Integrante / Helen T da Rosa - Integrante / João Antonio Pegas Henriques - Integrante.

Áreas de atuação

1. Grande área: Ciências Biológicas / Área: Genética / Subárea: Genética Toxicológica.
2. Grande área: Ciências Exatas e da Terra / Área: Química / Subárea: Quimica Analítica.
3. Grande área: Ciências Sociais Aplicadas / Área: Administração / Subárea: Cooperativismo.

Idiomas

Português Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.
Inglês Compreende Razoavelmente, Fala Razoavelmente, Lê Bem, Escreve Razoavelmente.
Espanhol Compreende Bem, Fala Pouco, Lê Bem, Escreve Pouco.

Prêmios e títulos

2014 Destaque na categoria pesquisa - toxicologia e farmacologia do evento de extensão "III Semana acadêmica da UFCSPA", Universidade Federal de Ciências da Saúde de Porto Alegre.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

Ordenar por

Ordem Cronológica

1. BIANCINI, GIOVANA BRONDANI ; **MORÁS, ANA MOIRA** ; REINHARDT, LUIZA STEFFENS ; BUSATTO, FRANCIELE FACCIO ; DE MOURA SPEROTTO, NATHALIA DENISE ; SAFFI, JENIFER ; MOURA, DINARA JAQUELINE ; GIUGLIANI, ROBERTO ; VARGAS, CARMEN REGLA . Globotriaosylsphingosine induces oxidative DNA damage in cultured kidney cells. NEPHROLOGY JCR, v. 22, p. 490-493, 2017.

Apresentações de Trabalho

1. Nordio, B. E. ; **MORÁS, A.** ; DALLEGRAVE, E. ; MOURA, D. J. . Avaliação in-vitro da atividade antitumoral do veneno da serpente Bothrops jararaca. 2015. (Apresentação de Trabalho/Outra).
2. **MORÁS, A. M.**; MOURA, D. J. . Avaliação do efeito antitumoral do veneno bruto de Bothrops jararaca. 2014. (Apresentação de Trabalho/Outra).
3. **MORÁS, A. M.**; SOARES, M. F. . Avaliação de parâmetros de validação analítica na determinação de Cd, Cr, Pb e Ni por espectrometria de emissão ótica com plasma indutivamente acoplado.. 2013. (Apresentação de Trabalho/Outra).

Eventos

Participação em eventos, congressos, exposições e feiras

1. I Encontro do Programa de Pós-Graduação em Biociências, XIX Encontro de Geneticistas do Rio Grande do Sul e I Encontro Regional Sul da Sociedade Brasileira de Genética Médica.Estudo de estrutura e função do produto do gene KIN3 em saccharomyces cerevisiae. 2016. (Encontro).
2. XII Congresso da MutaGen-Brasil. 2016. (Congresso).
3. Cellularised Scaffolds, Co-cultures and Bioreactors for Engineering the Vascular Wall: Potential for Tissue Regeneration. 2015. (Seminário).
4. IX Congresso brasileiro de biossegurança. 2015. (Congresso).
5. Toxicologia em debate: acidentes em massa. 2015. (Outra).
6. 1st Latin American Congress of Clinical ans Laboratorial Toxicoloy. Cytotoxic, genotoxic and antioxidant properties of Tripodanthus acutifolius aqueous extract in V79 cells. 2014. (Congresso).
7. 1st Latin American Congress of Clinical ans Laboratorial Toxicoloy. 2014. (Congresso).
8. III Semana acadêmica da UFCSPA.Avaliação do efeito antitumoral do veneno de Bothrops jararaca. 2014. (Encontro).
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10. Curso pré-congresso 54: Espécies genotóxicas em fármacos: Origens, detecção e regulamentação. 2013. (Congresso).
11. IX JORNADA DE INICIAÇÃO CIENTÍFICA: MEIO AMBIENTE FZBRS/FEPAM. 2013. (Encontro).
12. XVIII Congresso Brasileiro de Toxicologia - CBTOX2013. 2013. (Congresso).
13. I Semana Acadêmica da UFCSPA 2012. 2012. (Encontro).
14. IV Congresso Brasileiro de Toxicologia Clínica e I Simpósio Brasileiro de Toxicologia Analítica. 2012. (Congresso).
15. Rally Científico - UCS. 2011. (Olimpíada).
16. Rally Científico - UCS. 2011. (Olimpíada).

Organização de eventos, congressos, exposições e feiras

1. SAFFI, J. ; MOURA, D. J. ; **MORÁS, A. M.** . I Encontro do Programa de Pós-Graduação em Biociências, XIX Encontro de geneticistas do Rio Grande do Sul e I Encontro Regional Sul da Sociedade Brasileira de Genética Médica. 2016. (Outro).
2. **MORÁS, A. M.**. IX Congresso Brasileiro de Biossegurança. 2015. (Congresso).
3. **MORÁS, A. M.**; MOURA, D. J. ; SAFFI, J. ; SPEROTTO, N. D. M. ; HENN, J. G. ; ROSA, H. T. . I Curso de Toxicidade Genética: Causas, Consequências e Ensaios Pré-Clínicos. 2015. (Outro).
4. **MORÁS, A. M.**. XVIII Congresso Brasileiro de Toxicologia - CBTOX2013. 2013. (Congresso).
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Orientações

Orientações e supervisões concluídas

Orientações de outra natureza

1. Rick Shandler Rodrigues da Cunha. Estágio Curricular. 2016. Orientação de outra natureza. (Biomedicina) - Fundação Universidade Federal de Ciências da Saúde de Porto Alegre. Orientador: Ana Moira Morás.
2. Luiza Steffens Reinhardt. Estágio Curricular. 2016. Orientação de outra natureza. (Toxicologia Analítica) - Fundação Universidade Federal de Ciências da Saúde de Porto Alegre. Orientador: Ana Moira Morás.

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Organização de eventos, congressos, exposições e feiras

1. SAFFI, J. ; MOURA, D. J. ; **MORÁS, A. M.** . I Encontro do Programa de Pós-Graduação em Biociências, XIX Encontro de geneticistas do Rio Grande do Sul e I Encontro Regional Sul da Sociedade Brasileira de Genética Médica. 2016. (Outro).
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Contents List

Manuscript preparation

1. General guidelines
2. Style guidelines
3. Figures
4. Publication charges
   - Submission fee
   - Page charges
   - Colour charges
5. Reproduction of copyright material
6. Supplemental online material
7. Proteopedia

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ANEXO 2 – Normas da revista *Fungal biology*

Fungal Biology - Instructions to Authors

INTRODUCTION

Fungal Biology is an international research journal and the flagship journal of the British Mycological Society http://www.britmycolsoc.org.uk/

It publishes original contributions in all fields of basic and applied research involving fungi and fungus-like organisms (including filamentous fungi, yeasts, lichen fungi, oomycetes, and slime moulds). These fields include biochemistry, biodeterioration, biotechnology, cell biology, developmental biology, disease control, ecology, environment, evolution, physiology, genetics, genomics, geomycology, insect pathology, medical mycology, molecular genetics, mutualistic interactions, physiology, plant pathology, secondary metabolites, taxonomy and systematics.

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Fungal Biology publishes papers reporting original research which make a significant contribution to mycology. Review articles on themes of topical interest will also be considered.

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New scientific names

These are placed in bold Roman type, followed by the author citation in normal type, and then an indication of the rank or transfer in bold (e.g. **sp.nov.; comb.nov**.) as shown in Fig. 1.

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In formal taxonomic presentations, synonyms should be listed in order of date, and grouped as that homotypic (obligate, nomenclatural) synonyms occur together. Each should appear on a new line, with any over-run indented (see Figure 1A), and the type details can be placed immediately after the appropriate name or under *Specimens examined* at the end of the account of that fungus.

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The place of publication of scientific names given in the body of the paper must be abbreviated, following Stafelu & Cowans's *Taxonomic Literature* (for books), and any of *Botanico Periodicum Huntianum* or *The World List of Scientific Periodicals* or the *Catalogue if Scientific Periodicals in the British Museum (Natural History)* for journals.

Contrary to the general practice in the journal, abbreviations of authors' names are always to be cited after scientific names in formal presentations of synonymy. Abbreviations must follow the system used in the *Index Fungorum* database (available free on the internet). If an author's name is not in the database, the surname should be given in full, and also full initials if there is a different person with the surname already in the system (in some cases forenames are spelled out in full to avoid confusion).

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Where epitypes, neotypes or lectotypes are being designated for the first time, the phrase '***lectotypus hic designatus***' (or with a different prefix depending on the kind of type) should appear in bold italic type after the citation of the name or specimen being selected as the nomenclatural type. This is not necessary where taxa are being described for the first time and a holotype (and any isotypes) is being designated.

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Fig 1 – *Examples of presentation of taxonomic information.*

(A) Synonymy. (B) New combination. (C) New species.

A

Xerocomus parasiticus (Bull.) Quél., *Fl. Mycol.:* 418 (1888).

Synonyms: Boletus parasiticus Bull., Herb. Fr.: 371 (1790) : Fr., Syst. Mycol. **1:** 389 (1821).

Pseudoboletus parasiticus (Bull.) Šutara, *eská Mykol.* **45**: 2 (1991).

B

Colletogloeopsis zuluense (M.J. Wingf., Crous & T.A. Court) M.N. Cortinas, M.J. Wingf. & Crous, **comb. nov.**

Basionym: *Coniothyrium zuluense* M.J. Wingf., Crous & T.A. Court, *Mycopathologia* **136**: 142 (1997).

C

Chaetosphaeria tortuosa Réblová, Seifert & G.P. White, **sp. nov.**

MycoBank No.: MB497232 MycoBank No.: MB492056 **Fig 2** – *Extract from a portion of a dichotomous key.*

3(2) Conidia 3-septate 4

Conidia 0-septate 5

4(3) Phialides arising singly, terminal on 1–3-septate metulae; phialide apex strongly curved downwards away from the main stipe; conidia 16–26 x 3–4.5 μm; Phialophora-like synanamorph; ascospores fusiform, 3- septate, 21–29 x 4–5.5(-6) μm; asci (100-)115–150 x 8.5–11.5 μm

. . . . . glauca

Phialides arising in groups of 2–9, the cluster having a digitate appearance, terminal or lateral on short, branched or unbranched metulae; phialide apex strainght or curved very gently; conidia 18–25 x 3.5–4 μm; ascospores fusiform, 3-septate, 19–24 x 5–6 μm; asci (110-)120–133(-

145) x 12–14 μm . . . . . tortuosa

Specimen citations

Details of specimens and cultures examined are grouped at the end of species accounts and printed in smaller type. The information should be organized in the following way: *Specimen examined*: **United Kingdom**: *South Devon* (V.C. 3): Slapton, Slapton Ley National Nature Reserve, 'The Causeway', 20(SX)/821442, alt. 10 m, on thallus of *Parmelia sulcata on Salix* sp., 25 Jun. 1974, *D. L. Hawksworth* 3762 (IMI 186831 - holotype of *Cornutispora lichenicola*).

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