UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE CIÊNCIAS FARMACÊUTICAS

ISADORA CAROLINA BETIM PAVAN

ESTUDO FUNCIONAL E MOLECULAR DA QUINASE HUMANA NEK6 COMO ALVO DE DROGAS CONTRA CÂNCER DE PRÓSTATA

FUNCTIONAL AND MOLECULAR STUDY OF HUMAN KINASE NEK6 AS A TARGET OF DRUGS AGAINST PROSTATE CANCER

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Tese apresentada à Faculdade de Ciências Farmacêuticas da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Ciências, na área de Ciências Farmacêuticas - Insumos Farmacêuticos Naturais, Biotecnológicos e Sintéticos.

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Orientador: Prof. Dr. Jörg Kobarg

ESTE TRABALHO CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA ISADORA CAROLINA BETIM PAVAN, E ORIENTADA PELO PROF. DR. JÖRG KOBARG..

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RESUMO

Introdução: NIMA (Never In Mitosis, gene A)-related kinase-6 (NEK6) pertence a uma superfamília de proteínas composta por 11 membros de quinases relacionadas a NIMA. As funções da NEK6 são pouco estudadas, exceto por sua participação já evidenciada no ciclo celular. A literatura tem mostrado sua relevância no câncer de próstata resistente à castração (CPRC), evidenciando seu papel central no crescimento do tumor independente de andrógeno, sugerindo que NEK6 regula vias de sobrevivência celular, as quais não estão relacionadas com sua função no ciclo celular. No entanto, ainda não está claro quais são essas vias e mecanismos regulados por NEK6 que a fazem participar do CPRC. **Objetivo:** O objetivo desse estudo foi explorar a NEK6 no CPRC, avaliando os efeitos celulares na ausência da sua expressão e inibição, bem como identificar os mecanismos moleculares de sobrevivência que NEK6 regula nesse câncer. Além disso, também avaliamos a relevância da depleção e/ou inibição de NEK6 no contexto do tratamento de quimioterápicos utilizados no CPRC, tais como cisplatina e docetaxel, e do composto natural anti-tumorigênico, resveratrol. **Material e métodos:** Foram geradas células nocautes de NEK6 nas linhagens celulares DU-145 e PC-3 utilizando o sistema CRISPR/Cas9. O uso de um inibidor específico de NEK6 também foi testado nas células DU-145 e PC-3. Ensaios funcionais para avaliar viabilidade, capacidade clonogênica, morte celular, migração, geração de Espécies Reativas de Oxigênio (ROS), permeabilidade da membrana mitocondrial foram realizados nessas células. Avaliação da expressão a nível de RNA mensageiro e proteico de genes relacionados à progressão e quimioresistência no câncer de próstata foram realizados. **Resultados:** Os nocautes de NEK6 diminuiram a capacidade clonogênica, proliferação, viabilidade celular e a atividade mitocondrial. A depleção de NEK6 aumentou o nível de ROS intracelular; diminuiu a expressão das defesas antioxidantes (SOD1, SOD2 e PRDX3); aumentou a fosforilação de JNK, uma quinase responsiva ao estresse; e aumentou os marcadores de dano ao DNA (p-ATM e γH2AX). A superexpressão exógena de NEK6 também aumentou a expressão dessas mesmas defesas antioxidantes e diminui γH2AX. A depleção de NEK6 induziu a morte celular por apoptose e reduz a proteína anti-apoptótica Bcl-2. As células com falta de NEK6 têm mais sensibilidade à cisplatina. NEK6 regulou a localização nuclear de NF-κB2, sugerindo que NEK6 pode regular a atividade transcricional de NF-κB2. Portanto,

NEK6 altera o equilíbrio redox, regula a expressão de proteínas antioxidantes e danos ao DNA, e sua ausência induz a morte de células DU-145. Foi observado que a inibição de NEK6 sensibilizou as células DU-145 ao tratamento com docetaxel, induziu marcadores significativos de apoptose (clivagem de PARP1, BAK), reduziu as vias de sobrevivência (p65 fosforilado) e migração celular (TJP1). O inibidor de NEK6, individualmente, foi capaz de reduzir significativamente as expressões de Bcl-xL, Bad fosforilado e TJP1. O inibidor de NEK6 exibiu um IC_{50} relativamente baixo em DU-145 e PC-3, podendo ser um composto interessante a ser explorado no câncer de próstata. O co-tratamento com docetaxel e inibidor de NEK6 reduziu significativamente a formação de colônias e a migração celular quando comparado com o grupo tratado somente com o docetaxel. O nocaute de NEK6 em células DU-145 e PC-3 sensibiliza as células ao tratamento com docetaxel. Resveratrol também foi capaz de inibir a expressão de NEK6 e HK2. Foi observado que NEK6 regula a expressão da enzima glicolítica HK2, conectando NEK6 com vias metabólicas no CRPC. Sugere-se que o eixo de sinalização NEK6-HK2 pode ser um possível mecanismo de regulação do resveratrol. **Conclusão:** NEK6 pode ser um alvo estratégico no câncer de próstata e os inidores de NEK6 devem ser explorados, bem como seu uso na combinação com quimioterápicos.

ABSTRACT

Introduction: NIMA (Never In Mitosis, gene A)-related kinase-6 (NEK6) belongs to a superfamily of 11 members of NIMA-related kinases. The functions of NEK6 are little studied, except for its already evidenced participation in the cell cycle. The literature has shown its relevance in castration-resistant prostate cancer (CRPC), evidencing its central role in androgen-independent tumor growth, suggesting that NEK6 regulates cell survival pathways. However, it is still unclear which these pathways and mechanisms regulated by NEK6 make it participate in the CPRC. **Aim:** This study aimed to explore NEK6 in CRPC, evaluating the cellular effects in the absence of its expression and inhibition, as well as identifying the molecular survival mechanisms that NEK6 regulates in this cancer. In addition, we also evaluated the relevance of NEK6 depletion and/or inhibition in the chemotherapy treatment used in CPRC, such as cisplatin and docetaxel, and the natural anti-tumor compound, resveratrol. **Material and methods:** NEK6 knockout cells were generated in DU-145 and PC-3 cell lines using the CRISPR/Cas9 system. The use of a specific NEK6 inhibitor was also tested on DU-145 and PC-3 cells. Functional assays to evaluate viability, clonogenic capacity, cell death, migration, generation of Reactive Oxygen Species (ROS), and permeability of the mitochondrial membrane were performed in these cells. Evaluation of expression at the level of messenger and protein RNA of genes related to progression and chemoresistance in prostate cancer cells were performed. **Results:** NEK6 knockouts decreased clonogenic capacity, proliferation, cell viability, and mitochondrial activity. NEK6 depletion increased the level of intracellular ROS, decreased expression of antioxidant defenses (SOD1, SOD2, and PRDX3), increased phosphorylation of JNK, a stress-responsive kinase, and increased DNA damage markers (p-ATM and γH2AX). Overexpression of NEK6 also increased the antioxidant defenses and decreased DNA damage. NEK6 lack induced apoptosis reduced Bcl-2 protein. DU-145 cells lacking NEK6 are more sensitive to cisplatin treatment. NEK6 modulated the nuclear localization of NF-κB2. Therefore, NEK6 alters redox balance, regulates antioxidant protein expression and DNA damage, and its absence induces DU-145 cell death. We observed that NEK6 inhibition sensitized DU-145 cells to docetaxel treatment, induced significant markers of apoptosis (PARP1 cleavage, BAK), reduced survival (phosphorylated p65) and cell migration (TJP1) pathways. The NEK6 inhibitor, individually, was able to significantly reduce Bcl-xL, phosphorylated Bad, and TJP1

expressions. The NEK6 inhibitor exhibited an attractive IC_{50} in DU-145 and PC-3 and could be an interesting compound to be explored in prostate cancer. Co-treatment with docetaxel and NEK6 inhibitor significantly reduced colony formation and cell migration when compared to docetaxel group. Knockout of NEK6 in DU-145 and PC-3 cells sensitizes the cells to docetaxel treatment. Resveratrol was also able to inhibit the expression of NEK6 and HK2. It was observed that NEK6 regulates the expression of the glycolytic enzyme HK2, connecting NEK6 with metabolic pathways in CRPC. It is suggested that the NEK6-HK2 signaling axis may be a possible resveratrol regulation mechanism. Conclusion: NEK6 may be a strategic target in prostate cancer and NEK6 inhibitors should be explored, as well as their use in combination with chemotherapy drugs.

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LISTA DE ABREVIATURAS E SIGLAS

NEK: *NIMA-related kinase*

NIMA: *Never In Mitosis, gene A*

DNA: *Deoxyribonucleic acid*

SAC: *Spindle assembly checkpoint*

ATR: *Ataxia telangiectasia and Rad3-related protein*

ATM: *Ataxia telangiectasia mutated*

CHK1: *Checkpoint kinase-1*

CHK2: *Checkpoint kinase-2*

Bcl-2: *B-cell lymphoma protein 2*

PARP1: *Poly [ADP-ribose] polymerase*

JNK: *c-Jun N-terminal kinase 1*

***Nim*:** *Never in mitosis*

Bim: *Blocked in mitosis*

RCC1: *Regulator of Chromosome Condensation*

CPRC: Câncer de próstata resistente à castração

CRPC: Castration-resistant prostate cancer

NEK6-KO: Nocaute de NEK6

Eg5: *Kinesin-5*

Hsp72: *Heat shock 70 kDa protein 1A*

EML4: *Echinoderm microtubule-associated protein-like 4*

SgRNA: *Single-guide RNA*

MAPK: *Mitogen Activated Protein Kinases*

ERBB2: *Receptor tyrosine-protein kinase erbB-2*

Src: *Proto-oncogene tyrosine-protein kinase Src*

ROS: *Reactive oxygen species*

PRDX3: *Peroxiredoxin 3*

ERK: *Extracellular signal-regulated protein kinases*

PI3K: *Phosphatidylinositol 3-kinase 3*

Akt: *Protein kinase B alpha*

Cdc25*: Cell division cycle 25*

p34-Cdc2: *Cyclin-dependent protein kinase p34-Cdc2*

LHSR-AR: *Androgen-dependent prostate epithelial cells*

NF-κB: *Nuclear Factor-kappa-B*

RelB: *Transcription factor RelB*

MMP-2: *Matrix Metalloproteinase-2*

FOXOJ2: *Forkhead box protein J2*

NCOA5: *Nuclear receptor coactivator*

Nrf2: *Nuclear factor erythroid 2-related factor 2*

KMT2D: *Histone-lysine N-methyltransferase 2D*

KLF4: *Krueppel-like factor 4*

FOXO3: *Forkhead box protein O3*

IκB: *IkappaB kinase*

IKKα: *IκB Kinase α*

IKKβ: *IκB Kinase β*

NIK: *Nuclear Factor-κB (NF-κB)-inducing kinase*

ATF4: *Activating Transcription Factor 4*

TRIP4: *Thyroid Hormone Receptor Interactor 4*

NAC: *N-Acetyl Cysteine*

TJP1/ZO1: *Tight junction protein 1/ Zonula occludens-1*

PTEN: *Phosphatase and tensin homolog*

ATP: *Adenosine triphosphate*

HK2: *Hexokinase 2*

SIRT1: *Sirtuin 1*

BCL-xL: *B-cell lymphoma-extra large*

Mcl-1: Myeloid leukemia cell differentiation protein

PRAD: *Prostate adenocarcinoma*

SUMÁRIO

1. INTRODUÇÃO

1.1 O ciclo celular

O ciclo celular é um mecanismo conservado em eucariontes que engloba um conjunto de eventos coordenados que tem como objetivo promover a duplicação da célula (Schafer, 1998). Esse processo é dividido em duas grandes etapas: a interfase e a mitose. Em média, a célula eucariótica demora 24 horas para completar o ciclo celular, sendo que, aproximadamente, 23 horas são utilizadas durante o processo da interfase, etapa na qual ocorre o crescimento celular e duplicação do DNA e 1 hora somente é utilizada para a etapa de mitose (Cooper, 2020).

A interfase é subdividida em 3 fases, sendo estas: G1 (pré-síntese de DNA), S (síntese de DNA) e G2 (pré-mitótica). Durante o G1, a célula é metabolicamente ativa e cresce continuamente, mas não replica seu DNA. Nessa fase, estímulos externos comandam o caminho que a célula tomará, uma vez que sinais mitogênicos contribuem para o avanço da fase G1 para S, e as citocinas antiproliferativas podem levar a célula a entrar no estado quiescente, conhecido como fase G0. No estado quiescente, as células ainda são metabolicamente ativas, porém param de crescer e possuem uma taxa reduzida de síntese proteica. Uma vez que fatores mitogênicos estão presentes, o ciclo celular avança do chamado “ponto de restrição” presente no final da fase G1, o que significa que a progressão do ciclo celular não será mais afetada por fatores extracelulares nas fases seguintes. Desregulações nesse ponto de restrição podem levar a uma proliferação descontrolada e ao surgimento de neoplasias. Em células cancerosas, os estímulos externos são incapazes de controlar a transição da fase G1 para S, permitindo que as células permaneçam no ciclo celular e tenham uma proliferação descontrolada (SHERR, 1996; MALUMBRES; BARBACID, 2001).

A fase S é o período geneticamente mais vulnerável, pois é nesse momento que a célula duplicará o DNA, gerando uma cópia única e fiel de seu material genético. Nessa fase, estresses genotóxicos, tais como radiação ionizante ou luz ultravioleta, compostos químicos, drogas e metabólitos reativos da célula podem causar lesões no DNA, alterando, portanto, a integridade do material genético dessa célula. As células que sofrem algum tipo de estresse genotóxico durante a replicação do DNA ativam o *checkpoint* dessa fase, ocorrendo uma inibição reversível dos disparos das origens de replicação de DNA, desacelerando a síntese de DNA em andamento. Dessa forma, torna-se possível a correção dos erros genéticos nesse período (Palou *et al.*, 2010;

Shackelford, Kaufmann e Paules, 1999).

Na fase G2, a célula produz proteínas e componentes do fuso mitótico, tornando viável a execução da próxima etapa, a mitose. O *checkpoint* dessa fase impede que a célula avance no ciclo celular se ainda houverem danos no DNA (Stark e Taylor, 2004).

A mitose é o processo de divisão celular, na qual uma célula origina duas células-filhas geneticamente idênticas. Para um melhor entendimento dessa fase, ela é sub-dividida em cinco fases: prófase, prometáfase, metáfase, anáfase e telófase (Zimmerman e Doxsey, 2000).

Durante a replicação do DNA na intérfase, o material genético apresenta-se como cromatina. Entretanto durante a fase de prófase, o DNA precisa ser condensado em cromossomos, o qual é composto por duas cromátides geneticamente idênticas, unidas por um centrômero. Esse processo leva ao desaparecimento gradual dos nucléolos. Na prometáfase, o envelope nuclear desintegra-se e o cromossomos se direcionam para a região central do citoplasma, na onde se ligam nos fusos polares. Na metáfase, os centrossomos se orientam para lados opostos da célula, os cromossomos estão ligados nos fusos mitóticos através do cinetócoro, uma região específica do centrômero. Essa tensão criada nos microtúbulos faz com que os cromossomos se alinhem na região equatorial da célula, chamada de placa metafásica. Na anáfase, as fibras do fuso diminuem de tamanho de forma a separar as cromátides irmãs. Na telófase, os cromossomos, já em lados opostos da célula, descompactam-se e produzem novamente os nucléolos, além de ocorrer a formação da membrana nuclear. A divisão celular então é finalizada com a citocinese, um processo que se inicia na anáfase e termina na telófase. Esse processo divide a membrana plasmática através da formação de um anel contrátil (MOIR *et al*., 2000; ZIMMERMAN; DOXSEY, 2000).

A compreensão do ciclo celular é de extrema importância para estudos relacionados a câncer, bem como para o desenvolvimento de novos fármacos antineoplásicos.

Em linhas gerais, a proteína NIMA-related Kinase 6 (NEK6) participa da progressão da metáfase para anáfase, participando especialmente na formação adequada do fuso mitótico e do processo de citocinese (O'Regan, L. e Fry, 2009; Yin *et al.*, 2003). A seguir, as funções de NEK6 no ciclo celular serão mais aprofundadas.

1.2. NIMA em *Aspergillus nidulans*

A espécie de fungos *Aspergillus nidulans* é um modelo clássico para avaliar eventos bioquímicos no processo mitótico. Em 1975, Ronald Morris utilizou esse modelo para identificar mutantes sensíveis à temperatura que falhavam na progressão do ciclo do celular (Morris, 1975). A partir dessa análise, os mutantes foram classificados em dois grupos, sendo estes: *Bim* e *Nim*. O grupo *Bim* (***B****locked* ***i****n* ***m****itosis*) comportava os mutantes que eram bloqueados na mitose ou que sofriam um prolongamento na etapa mitótica. Sendo assim, os índices mitóticos desses mutantes eram aumentados a uma temperatura de 42 °C. Nesse grupo foram alojados os componentes do complexo promotor da anáfase (APC), que são genes importantes envolvidos na mitose. Já os mutantes classificados no grupo *Nim* (***N****ever* ***i****n* ***m****itosis*) nunca entravam em mitose, visto que mutações nesses genes sempre afetavam algum pré-requisito para a mitose ocorrer, tais como alterações na síntese de DNA, na condensação dos cromossomos e na formação do fuso mitótico. No grupo *Nim* estavam incluídos a fosfatase Cdc25, DNA polimerases, a ciclina do tipo B e também os quatro alelos do gene *NimA* (*Never in mitosis, gene A* (Morris, 1975).

Em 1988, Stephen Osmani observou uma indução da mitose após o aumento de expressão de *NimA* em *Aspergillus nidulans*. As análises da sequência do cDNA de *NimA* deram indícios de que tal gene poderia codificar uma proteína quinase, sugerindo então que o controle do processo mitótico pela proteína NIMA poderia ser dado através da sua capacidade de fosforilar outras proteínas (Osmani, Pu e Morris, 1988).

Nos anos seguintes, Aysha Osmani evidenciou que a ausência da ativação da proteína NIMA leva a grandes defeitos mitóticos em *Aspergillus nidulans*, sendo essencial para o início da mitose (Osmani, McGuire e Osmani, 1991). Mutações em NIMA ocasionam a parada do ciclo celular, sem alterar a ativação de p34-Cdc2, um conhecido regulador do ciclo celular, mostrando que NIMA possui um papel importante na transição da fase G2 para a mitose (Osmani, McGuire e Osmani, 1991).

Outros estudos revelaram que o início da mitose requer a ativação de p34-Cdc2 e NIMA (Osmani e Ye, 1996). Mutações no gene *NinX3,* o qual codifica Cdc2, levam a parada do ciclo celular em G2 e ausência de ativação de NIMA, indicando que NIMA poderia ser fosforilada e ativada por Cdc2 (Ye *et al.*, 1995).

Além da sua importância na fase inicial do processo mitótico, a degradação da proteína NIMA também é necessária para o término correto da mitose em *Aspergillus*

nidulans (Pu e Osmani, 1995). Acredita-se que NIMA pode ser degradada pela via proteolítica da ubiquitina-proteassoma ou por apresentar sequências PEST na sua porção C-terminal, as quais são motivos ricos em resíduos de prolina (P), ácido glutâmico (E), serina (S) e treonina (T), envolvidos no direcionamento de proteínas para degradação (Osmani e Ye, 1996; Rogers, Wells e Rechsteiner, 1986).

A atividade da proteína NIMA é regulada através das etapas do ciclo celular (Ye *et al.*, 1995). Esse estudo revelou que a atividade e o conteúdo proteico de NIMA são baixos durante as etapas G1 e S, entretanto, observou-se um aumento da sua atividade na fase G2, acompanhado de um aumento dos níveis proteicos. O pico máximo de atividade e níveis proteicos ocorre na fase de mitose. Após a fase mitótica, como já mencionado, NIMA é encaminhada para degradação (Ye *et al.*, 1995).

De forma geral, a proteína NIMA contribui para múltiplos aspectos da progressão mitótica, incluindo a entrada para o processo mitótico, condensação da cromatina, organização do fuso e citocinese (O'Regan, Blot e Fry, 2007). O envolvimento da proteína NIMA no controle mitótico em eucariotos superiores indica que existem vias de sinalização importantes que a envolvem, sugerindo a existência de proteínas homólogas à NIMA conservadas durante a evolução.

1.3. Membros da família NEK em mamíferos

As NEK quinases formam uma família altamente conservada de 11 proteínas quinases (NEK1 à NEK11), que compartilham 40-45% de identidade com a proteína NIMA de *A. nidulans* no domínio catalítico N-terminal (O'Connell, Krien e Hunter, 2003). Em 1992, LETWIN *et al* (1992) descreveram a primeira proteína quinase relacionada à NIMA em humanos, a NEK1. Posteriormente, outros trabalhos descrevendo membros dessa família foram publicados, mas nem todos possuem sua função bem caracterizada (Tabela I).

Tabela I. Funções e interações propostas para as quinases NEK de mamíferos.

Proteína	Função proposta	Referência
NEK1	Alterações no gene *NEK1* em animais são modelos para a doença policística renal em humanos.	(Upadhya *et al.*, 2000)

	Interage com proteínas envolvidas no ciclo celular e no reparo do DNA. Mecanismo de reparo por homologia. Papel na resposta a dano ao DNA induzido por radiação ionizante.	(Surpili, Delben e Kobarg, 2003) (Polci *et al.*, 2004)
NEK2	Localizada em centrossomos e cinetócoros. Fosforila a C-Napland (Nlp) no centrossomo. Regula a separação do centrossomo na transição G2/mitose. Possível papel na regulação do fuso mitótico.	(Fry *et al.*, 1995) (Fry *et al.*, 1995) (Rapley *et al.*, 2005) (Chen *et al.*, 2002)
NEK3	Modula a sinalização dos receptores de prolactina. Aumento da expressão está relacionado com baixo prognóstico de pacientes com câncer gástrico.	(Miller *et al.*, 2005) (Cao *et al.*, 2018)
NEK4	Reparo de DNA, *splicing* de RNA.	(Basei *et al.*, 2015)
NEK5	Respiração mitocondrial. Substrato da caspase 3, promovendo diferenciação das células do músculo esquelético.	(Melo Hanchuk *et al.*, 2015) (Shimizu e Sawasaki, 2013)
NEK6	Regulação dos telômeros através da fosforilação de TPP1. Estabilização das fibras do cinetócoro através da fosforilação de Hsp72. Relacionado com câncer de próstata resistente a castração. Fosforila o fator de transcrição FOXJ2.	(Hirai *et al.*, 2016) (O'Regan *et al.*, 2015) (ATISH et al., 2017)
NEK7	Ativação do inflamassoma NLRP3.	(He *et al.*, 2016)

	Participa da mesma cascata de sinalização na mitose que NEK6. Interactor de RGS2.	(Belham *et al.*, 2003) (Souza, De *et al.*, 2015)
NEK8	Aumento da expressão em câncer de mama. Estabilidade na forquilha de replicação mediada por RAD51.	(Bowers e Boylan, 2004) (Abeyta *et al.*, 2017)
NEK9	Associação ao Bicd2 *in vivo*, fosforilação de Bicd2. Regula o alinhamento e a segregação dos cromossomos na mitose. Mediador da organização dos cromossomos e do centrossomo. Ativa a NEK6 durante a mitose. Regula a progressão de G1 e S através da interação com o complexo facilitador da transcrição da cromatina (FACT).	(Holland *et al.*, 2002) (Roig *et al.*, 2002) (Tan e Lee, 2004)
NEK10	Resposta a dano no DNA, UV.	(Moniz e Stambolic, 2011)
NEK11	Quinase responsiva a estresse replicativo e a danos ao DNA. É ativada pela NEK2a em células com defeito na progressão do ciclo celular em G1/S.	(Noguchi *et al.*, 2002) (Noguchi *et al.*, 2004)

Os 11 membros da família das NEKs humanas estão envolvidos em três grandes contextos funcionais: 1) Regulação do ciclo celular, especialmente na mitose (NEK2, 5, 6, 7, 9) (Fry *et al.*, 2012); 2) Função do cílio primário (NEK1, 4, 8) (Mahjoub, Trapp e Quarmby, 2005; Zalli, Bayliss e Fry, 2012); e 3) envolvimento com a resposta a danos de DNA (NEK1, 4, 5, 6, 7, 10, 11) (Pavan *et al.*, 2021). Ainda existem novas funções pouco exploradas, tais como o envolvimento das NEKs na atividade mitocondrial, visto para NEK1, NEK5 e NEK10 (Martins *et al.*, 2021; Melo Hanchuk *et*

al., 2015; Peres de Oliveira *et al.*, 2020), ambos os trabalhos gerados por nosso grupo de pesquisa.

As NEKs compartilham um domínio de quinase, geralmente localizado no N-terminal de sua seqüência, o qual as caracteriza como membros da família. A maioria das NEKs também possui um domínio não-catalítico, o qual não apresenta semelhança, de acordo com sua seqüência, tamanho e organização entre os membros, e, portanto, essa região é capaz de conferir mecanismos específicos de regulação e seletividade dos substratos a jusante destas quinases (Fry, Bayliss e Roig, 2017).

Na maioria das NEKs, a extremidade C-terminal constitui seu domínio regulatório e apresenta maior variabilidade, estando envolvida na determinação da especificidade por substratos e na interação com outras proteínas (Meirelles *et al.*, 2014), como demonstrado na figura 1. Entretanto, sabe-se que as regiões desordenadas no N-terminal de NEK6 e NEK7, consistem na maior diferença estrutural entre essas quinases e definem seus interactores e funções não redundantes na célula (Souza, De *et al.*, 2014).

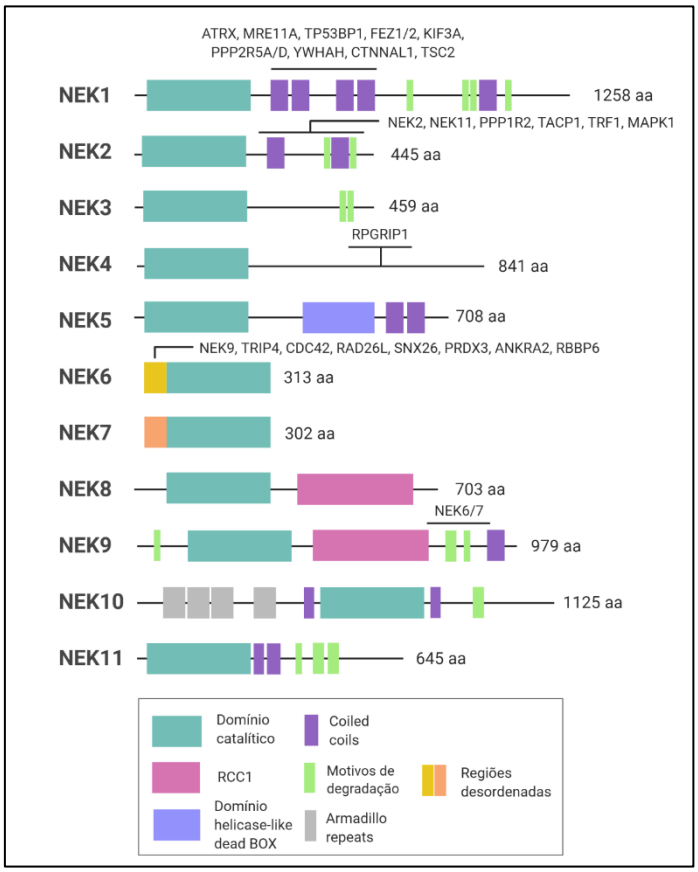

Figura 1. Representação dos domínios das 11 NEKs humanas e suas regiões de interação com outras proteínas. Alguns símbolos dos genes correspondentes aos

interactores de NEKs são mostrados acima das regiões que medeiam essa interação. Diferentes domínios repetidos foram indicados pela legenda de cores na parte inferior da figura. Os tamanhos das proteínas completas são indicados pelo número de aminoácidos (aa) no C-terminal (Figura adaptada de MEIRELLES *et al*., 2014).

1.4. A quinase NEK6, seu envolvimento na progressão do ciclo celular e outros processos biológicos

A proteína NEK6 é uma quinase serina/treonina com 313 aminoácidos que é codificada em humanos pelo gene *NEK6* localizado no cromossomo 9. NEK6 possui uma pequena região N-terminal desordenada, que é essencial para sua interação com parceiros celulares, e um domínio catalítico no C-terminal (Vaz Meirelles *et al.*, 2010). Sua conformação globular e monomérica, embora ligeiramente alongada, foi revelada por MEIRELLES *et al* (2011). A expressão e a atividade de NEK6 aumentam durante a mitose, sendo que sua atividade é dependente de sua fosforilação na serina 206 por NEK9. A ativação de NEK6 durante a mitose é importante para a formação e manutenção do fuso mitótico (Belham *et al.*, 2003).

A ativação de NEK6 não é causada apenas pela fosforilação direta de NEK9, mas também pela interação com o domínio C-terminal não catalítico de NEK9, com a consequente dimerização de estruturas de NEK6 e inibição de resíduos auto-inibitórios (tirosina 108) (Richards *et al.*, 2009).

NEK6 é intimamente relacionada com a NEK7, uma vez que compartilham, aproximadamente, 86% de identidade em seus domínios C-terminais e somente 20% de identidade nas extremidades N-terminais (BELHAM et al., 2003; *MEIRELLES et al*., 2011). Estudos indicam que as duas proteínas têm diferentes funções biológicas, apontando à sua extremidade desordenada do N-terminal como um possível fator para as funções diferenciais dessas quinases (Souza, De *et al.*, 2014). Além disso, NEK6 e NEK7 mostram diferenças na localização subcelular, na distribuição tecidual espaço-temporal e no controle enzimático (O'REGAN; FRY, 2009; FEIGE; MOTRO, 2001; MINOGUCHI; MINOGUCHI; YOSHIMURA, 2003). Dessa forma, NEK6 e NEK7, apesar de algumas funções redundantes, atuam em diversos processos de maneira independente.

Embora existam alguns dados contraditórios sobre a função de NEK6 e muito pouco se sabe sobre seus alvos *downstream*, acredita-se que interferir com a função de NEK6, seja por superexpressão do *kinase dead* ou reduzir sua expressão por RNA de interferência (siRNA), causa parada mitótica e desencadeia apoptose (Yin *et al.*,

2003).

Um estudo realizado por YIN *et al* (2003) identificou que NEK6 é uma quinase crítica para a progressão do ciclo celular. Foi identificado que sua atividade de quinase é up-regulada na mitose. As células transfectadas com siRNA de NEK6 apresentam parada na metáfase e indução de apoptose. A diminuição de expressão de NEK6 por siRNA também causou o aumento de células com dois ou mais núcleos, sugerindo um defeito na finalização da mitose. Nesse mesmo estudo, foi realizado a superexpressão da quinase morta "*kinase dead*" de NEK6, obtendo resultados semelhantes. Com isso, evidenciou-se que atividade de quinase de NEK6 é requerida para a finalização da metáfase e início da anáfase (Yin *et al.*, 2003).

Outro estudo demonstrou que NEK6 e NEK7 são ativadas na mitose e que interferir com suas atividades por depleção ou expressão de mutantes de atividade de quinase reduzida leva à parada mitótica e apoptose (O'Regan, Laura e Fry, 2009). Mutantes inativos de NEK6 e NEK7 atrasam as células na metáfase, promovendo células com fusos mitóticos frágeis. A parada da metáfase foi o resultado da ativação do SAC (*checkpoint* da montagem do fuso). Além disso, a ausência de NEK6 e NEK7 atrasam também as células na citocinese. Sendo assim, NEK6 e NEK7 atuam dentro da mitose em dois pontos: na metáfase e citocinese. Ambas as quinases se localizam nos polos do fuso mitótico, porém apenas NEK6 se localiza nos microtúbulos do fuso na metáfase e anáfase e no corpo médio durante a citocinese. Esses dados sugerem que NEK6 e NEK7 desempenham papéis independentes não apenas na formação robusta do fuso mitótico, mas também na citocinese (O'Regan, Laura e Fry, 2009).

A proteína Eg5 é necessária para a separação pré-mitótica do centrossomo, formação e separação do polo do fuso, translocação dos microtúbulos em direção ao polo e movimento do centrossomo pós-mitótico e, portanto, é um dos motores necessários para a organização e função adequada do fuso (Walczak *et al.*, 1998). NEK6 fosforila um subconjunto de resíduos conservados de Eg5 durante a mitose, sendo este evento crítico para a função mitótica de Eg5 (Rapley *et al.*, 2008).

EML4 é uma proteína associada a microtúbulos que promove a estabilidade dos microtúbulos (Houtman *et al.*, 2007). As quinases mitóticas NEK6 e NEK7 fosforilaram o domínio N-terminal de EML4 em Ser144 e Ser146 *in vitro*, e a depleção dessas quinases em células levou ao aumento da ligação de EML4 aos microtúbulos na mitose. Foi observado que a fosforilação de EML4, dependente de NEK6 e NEK7, promove a dissociação de EML4 dos microtúbulos, o que é necessário para o

alinhamento dos cromossomos na placa equatorial durante a metáfase (Adib *et al.*, 2019).

A família de proteínas Hsp70 representam chaperonas que regulam a homeostase celular e são necessárias para a sobrevivência das células cancerígenas. Raramente há associação dessas proteínas com a mitose, entretanto o estudo de O'REGAN *et al* (2015) mostrou que a proteína Hsp72 é necessária para a montagem de um fuso bipolar robusto capaz de alinhar os cromossomos na placa equatorial da célula de forma eficiente na fase de metáfase. O direcionamento de Hsp72 para o fuso mitótico é dependente da fosforilação em treonina 66 pela quinase NEK6. A Hsp72 fosforilada concentra-se nos polos do fuso e no cinetócoro. NEK6 facilita a associação de Hsp72 ao fuso mitótico, onde promove a montagem estável da fibra K através do recrutamento do complexo ch-TOG-TACC3 (O'Regan *et al.*, 2015).

A maioria das células cancerosas tem centrossomos extras ou "amplificados", inativando ou agrupando-os em dois polos para formar um fuso pseudo-bipolar (Ganem, Godinho e Pellman, 2009). O tempo adicional necessário para organizar tal fuso e a presença de mais de dois centrossomos na mitose favorece a geração de erros no qual um único cinetócoro é ligado aos microtúbulos que saem de ambos os fusos. Esse erro não é reconhecido pelo SAC, portanto, centrossomos amplificados são tolerados, promovendo erros de segregação cromossômica, instabilidade do genoma e progressão do câncer (Silkworth *et al.*, 2009). O estudo de SAMPSON *et al* (2017) revelou que a atividade de quinase NEK6 e Hsp72 são necessárias para o agrupamento dos centrossomos em células cancerosas. Uma terapia interessante para matar células de câncer pode ter como alvo terapêutico os centrossomos agrupados, uma vez que esse evento ocorre de forma seletiva em células cancerosas.

Um estudo identificou a relação de NEK6 com o *checkpoint* ativado pelo dano no DNA (Lee *et al.*, 2008). Foi mostrado que NEK6 é fosforilada no seu N-terminal pela CHK1 e CHK2 após dano no DNA por radiação e luz ultravioleta. Essa fosforilação inibe totalmente a ativação de NEK6 durante a mitose, induzindo a parada do ciclo celular na fase G2/M. Esse estudo sugere que NEK6 é um novo alvo do ponto de verificação de danos ao DNA e que a inibição da atividade de NEK6 é necessária para a interrupção adequada do ciclo celular na fase G2/M após dano ao DNA (Lee *et al.*, 2008). O resumo do envolvimento de NEK6 no ciclo celular está ilustrado na figura 2.

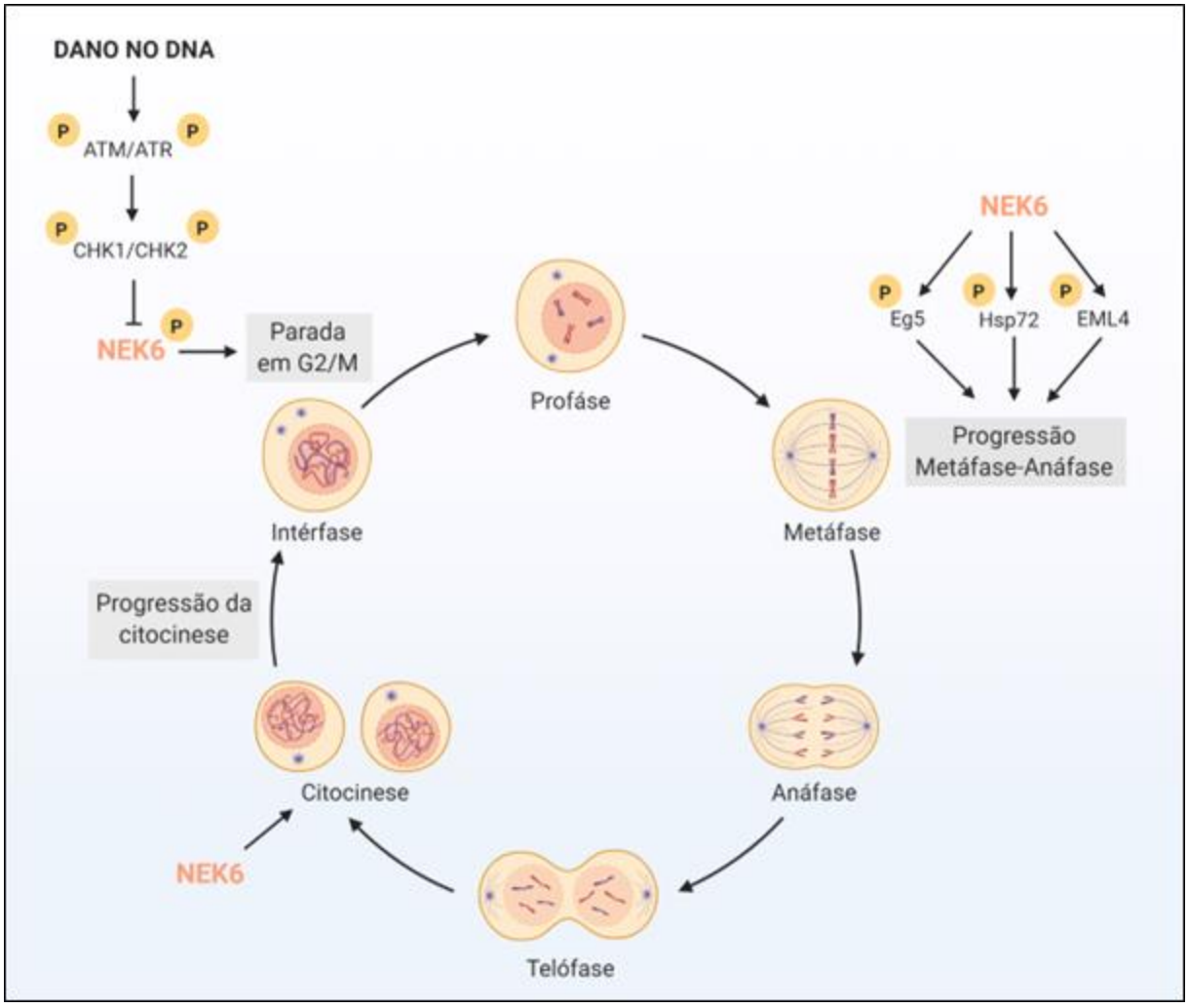

Figura 2. Participação da quinase NEK6 no ciclo celular. NEK6 é uma quinase responsiva a dano no DNA, sendo fosforilada por CHK1/2 tendo sua atividade inibida. Esse evento leva a parada do ciclo na fase G2/M. NEK6 também tem função essencial na finalização correta da metáfase e início da anáfase, através da fosforilação de seus substratos Eg5, Hsp72 e EML4. Essas fosforilações são importantes para o alinhamento dos cromossomos na placa equatorial durante a metáfase. A inibição de NEK6 também atrasa as células na citocinese, mostrando seu papel na progressão dessa etapa do ciclo celular.

Além das funções de NEK6 relacionadas ao ciclo celular, existem indícios que NEK6 pode ter relevância em outros processos biológicos. Um estudo de nosso grupo identificou por ensaio de duplo híbrido alguns parceiros de interação que sugerem que NEK6 possui um envolvimento em processos biológicos que vão além do ciclo celular, tais como metabolismo, reparo de DNA, resposta à estresse, transcrição, resposta ao dano no DNA, entre outros processos (Vaz Meirelles *et al.*, 2010). O ensaio de duplo hidrido revelou que NEK6 interage com o fator de transcrição RelB, o qual é um membro da família Fator Nuclear-Kappa B (NF-κB), e está envolvida na inflamação, imunidade, crescimento celular, tumorigênese e apoptose (Ryseck *et al.*, 1992). Nesse

mesmo estudo, também foi identificado que NEK6 também interagiu com PRDX3, uma proteina mitocondrial que desempenha um papel na proteção celular contra o estresse oxidativo através da detoxificação do peróxido (Whitaker *et al.*, 2013). MMP-2, proteína intimamente associada com o crescimento do tumor, invasão e metástase, também foi identificada no estudo como interactora de NEK6 por ensaio de duplo híbrido (XIE *et al.*, 2016).

O estudo de Atish *et al.*, 2017 reveleu pela primeira vez a NEK6 como sendo uma quinase essencial para a resistência à castração no câncer de próstata, tópico esse que será abordado com mais detalhes a seguir. Curiosamente, esse mesmo estudo evidenciou que NEK6 não mediava CPRC através da sua função mais conhecida, a de participação no ciclo celular (Atish *et al.*, 2017). A superexpressão de NEK6, após a castração de camundongos, aumentou a expressão de genes envolvidos nas vias de sinalização mediada por citocinas. Foi comparada a expressão de NEK6 selvagem induzida por doxiciclina com NEK6 com dominio catalitico inativo em um contexto de câncer de próstata resistente a castração *in vivo*. Algumas proteínas envolvidas em diversos processos biológicos tiveram fosfopeptídeos significativamente enriquecidos, tais como ACLY (biossíntese de ácidos graxos), ATM (resposta à danos no DNA), TRA2B, SRSF1, SRSF3, PRPF3, HNRNP's (*splicing* de RNA mensageiro), FOS/JUN, FOXO3, FOXA1 (fatores de transcrição), dentre outros, sugerindo que essas proteínas podem ser alvos de fosforilação de NEK6 (Atish *et al.*, 2017).

Dessa forma, torna-se evidente que NEK6 apresenta uma relevância em outros processos biológicos que vão além do ciclo celular, sendo um interessante tópico para investigação, já que, nos últimos anos, NEK6 vem sendo relevada uma quinase promissora para a terapia do câncer, em especial o câncer de próstata, o qual será discutido a seguir.

1.5. O câncer de próstata e vias de sinalização envolvidas no câncer de próstata resistente à castração (CPRC).

O câncer de próstata (CaP) é o principal câncer diagnosticado em homens e também a segunda maior causa de mortes relacionadas à câncer. Em estágios iniciais, o câncer de próstata tem um bom prognóstico, entretanto a taxa de sobrevivência diminui drasticamente em estágios avançados e metastáticos. Cerca de 20% dos pacientes com CaP são diagnosticados ou evoluem para fase avançada, sem

possibilidade de terapia curativa. A castração cirúrgica ou química, na qual ocorre a privação androgênica é o tratamento de escolha principalmente nos CaP metastáticos, entretanto é muito comum o retorno da neoplasia em 3 anos, progredindo para uma patologia letal conhecida como câncer de próstata resistente à castração (CPRC), que está associada ao óbito entre 18 e 48 meses (Chandrasekar *et al.*, 2015). Na figura 3, evidencia-se o padrão geral de progressão do câncer de próstata à medida que eles se desenvolvem nas modalidades de tratamento.

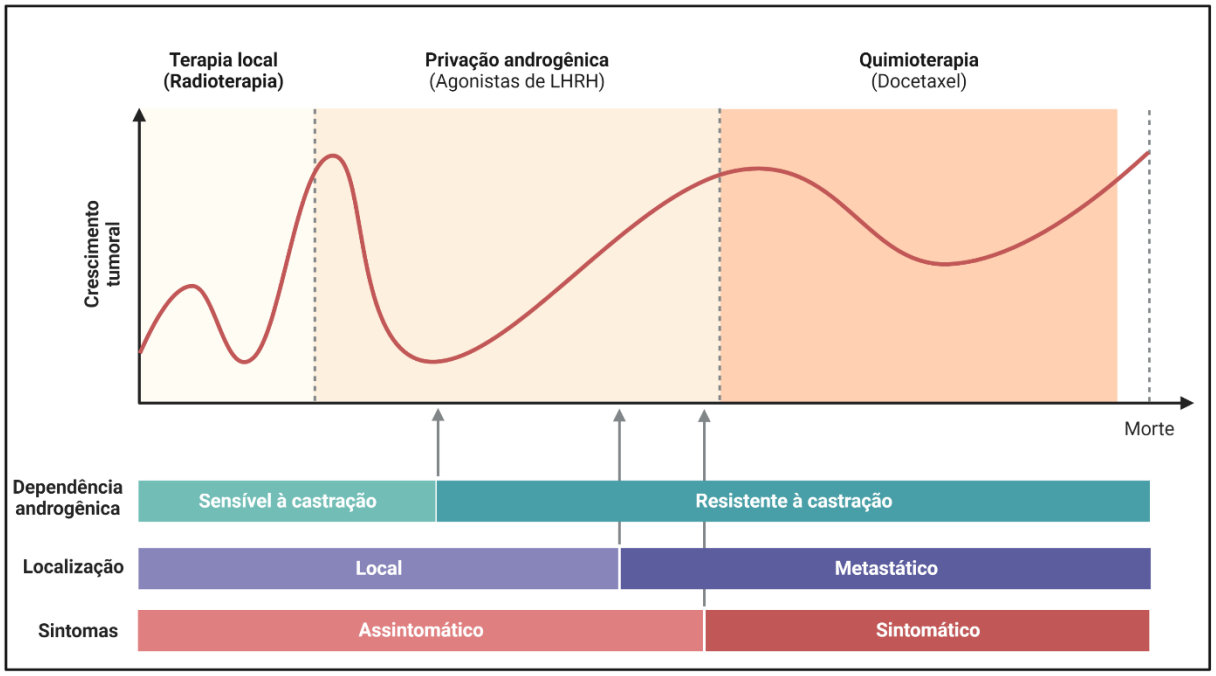

Figura 3. Evolução dos tratamentos no CPRC. Em estágio inicial e, muitas vezes assintomático, o câncer de próstata que não está em processo de metástase é tratado com radioterapia, a fim de destruir as células cancerígenas. Quando não há sucesso nesse tratamento, o tumor cresce e a terapia de privação androgênica, tal como o uso do agonista do hormônio liberador de hormônio luteinizante (LHRH), é iniciada a fim de reduzir a produção de testosterona, e assim, reduzir a proliferação e crescimento desse tecido tumoral. Ainda assim, as células tumorais são capazes de se proliferarem independente da presença de hormônios, tornando-se células resistêntes à castração, e com caráter metástatico. Diante deste cenário, a quimioterapia de primeira linha tem como base o docetaxel, o qual é capaz de aumentar a sobrevida do paciente em poucos meses.

Estudos de tecido metastático de pacientes revelam que tumores podem tornar-se resistentes à castração através da ativação persistente da via do receptor andrógeno (RA) ou pela progressão para um estado descrito como câncer de próstata independente da via de andrógeno (Chandrasekar *et al.*, 2015). Segundo Pienta e Bradley (2006), os mecanismos que levam a geração de independência de

andrógenos são: 1) as células de câncer de próstata desenvolvem a capacidade de usar baixos níveis de androgênio para a sobrevivência por aumento da produção do receptor de androgênio (AR; geralmente por amplificação do gene); 2) as mutações do receptor de andrógeno permitem a ligação de moléculas de esteróides não androgênicas, ativando o receptor; 3) ativação de vias “fora da lei”, visto que moléculas não esteróides ativam o receptor de andrógeno por ligação dependente de ligante ou ativam a sinalização *downstream* do receptor de andrógeno por mecanismos independentes de ligante; 4) as células do câncer de próstata desenvolvem a capacidade de sobreviver independentemente do receptor de andrógeno. A via de contorno mais conhecida é através da modulação da apoptose pela regulação positiva da molécula Bcl-2 por células de câncer de próstata andrógeno-independentes que as protegem da apoptose ou morte celular programada quando são expostas à falta de testosterona; 5) alterações no equilíbrio entre coativadores e corepressores, que funcionam como intermediários de sinalização entre o receptor de andrógeno e a maquinaria transcricional e, por fim, 6) regeneração de células-tronco. As células-tronco de câncer de próstata, que não dependem do receptor de andrógeno para sobreviver, reabastecem continuamente a população de células tumorais, apesar da terapia (Pienta e Bradley, 2006).

A figura 4 demonstra dois modelos mais aceitos para a geração de uma subpopulação de células de câncer de próstata resistente à castração após a privação androgênica.

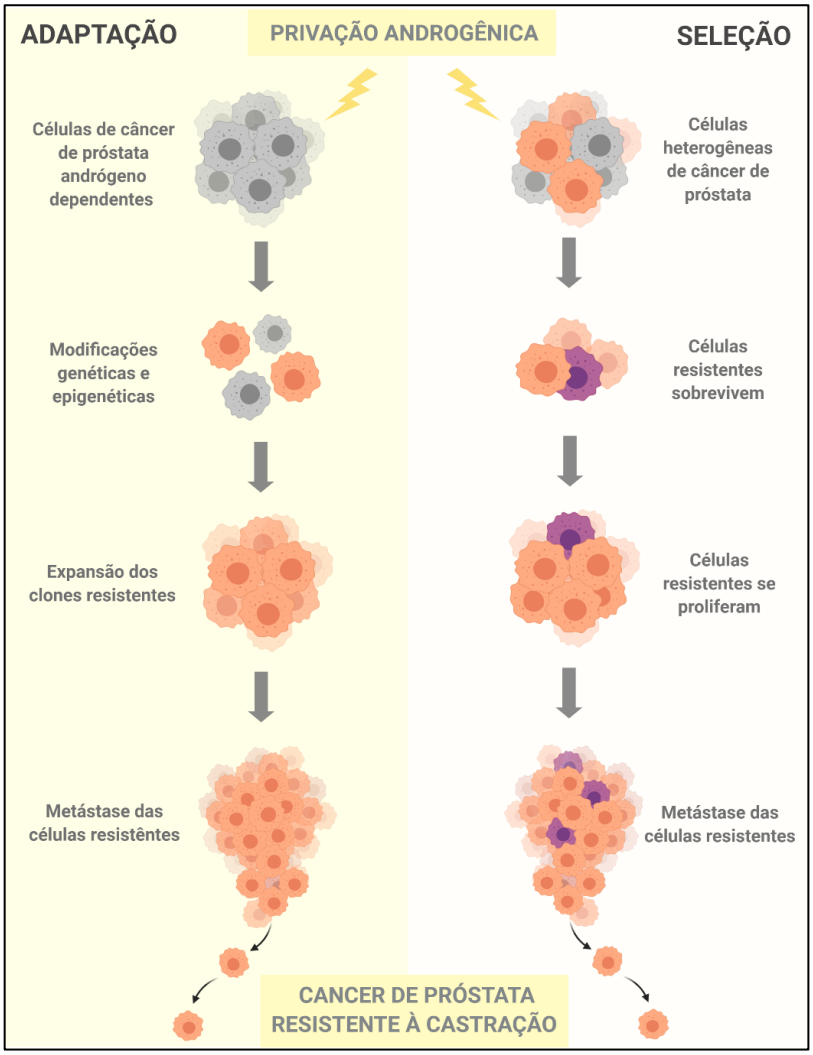

Figura 4. Modelos propostos para a geração do CPRC. A terapia de privação de androgênio leva à apoptose da maioria das células do câncer de próstata, mas algumas células sobrevivem e dão origem ao câncer de próstata resistente à castração. O modelo de adaptação propõe que um subconjunto de células adquira modificações genéticas ou epigenéticas adicionais que levam à sua sobrevivência, enquanto o modelo de seleção propõe que existem células pré-existentes independentes de andrógeno e que o tratamento de privação de androgênio não é capaz de matar essas células, as quais se proliferam, formando o CPRC. A metástase dessas células resistentes ao tratamento é um evento comum no CPRC. Figura adaptada de ZONG; GOLDSTEIN, 2013.

Múltiplos mecanismos de resistência ajudam a contribuir para a progressão para doença resistente à castração. A ativação de quinases como ERBB2, MAPK, PI3K/Akt e Src foi implicada na mediação da resistência a terapias hormonais por meio de mecanismos andrógeno dependentes e independentes. No entanto, os inibidores de muitas dessas quinases não conseguiram demonstrar benefício clínico significativo em populações de pacientes não selecionados (Pienta e Bradley, 2006).

Existe uma relação próxima entre o estresse oxidativo e a resistência à castração no câncer de próstata. O estresse oxidativo é causado quando o sistema de defesa antioxidante celular é sobrecarregado por um aumento nos níveis de espécies reativas de oxigênio (ROS) ou uma diminuição na capacidade antioxidante celular (Trachootham, Alexandre e Huang, 2009). As ROS são um grupo de moléculas contendo átomos de oxigênio e que são derivadas do metabolismo do oxigênio dentro das células (Apel e Hirt, 2004). Essas moléculas incluem os radicais livres superóxido (O^{2-}), hidroxila (OH^{-}) e o peróxido de hidrogênio (H_2O_2) (Liou e Storz, 2010). Nas células eucarióticas, as ROS são geradas durante a respiração mitocondrial ou em reações catalisadas por enzimas como NADPH oxidase (NOX), xantina oxidase e citocromo P450 (Dansen e Wirtz, 2001). A respiração mitocondrial é a principal fonte de ROS como resultado da produção de O^{2-} dos complexos I e III da cadeia transportadora de elétrons. O superóxido O^{2-} é posteriormente convertido em outras espécies reativas de oxigênio, como OH^{-} e H_2O_2 (Stowe e Camara, 2009).

O sistema antioxidante é composto principalmente pela superóxido dismutase (SODs), catalase, peroxirredoxina (Prx) e glutationa peroxidase (GPx) (Birben *et al.*, 2012). A Superóxido Dismutase 2 (SOD2) desempenha um papel central na aquisição de independência de androgênio em células de câncer de próstata (Quiros-Gonzalez *et al.*, 2011). SOD2 também é capaz de aumentar a proteína anti-apoptótica Bcl-2 e previnir a morte celular induzida por quimioterápicos utilizados no câncer de próstata (Quiros-Gonzalez *et al.*, 2011). A peroxirredoxina 3 (PRDX3), a qual já foi citada aqui como sendo interactora de NEK6, catalisa a redução de peróxidos, protegendo a célula contra a morte celular induzida por estresse oxidativo. PRDX3 é regulada positivamente em vários tumores relacionados ao sistema endócrino, em particular no câncer de próstata. Células de câncer de próstata que são resistentes ao tratamento androgênico apresentam um aumento da expressão de PRDX3 e são mais resistentes ao estresse oxidativo e apoptose induzida por H_2O_2 (Whitaker *et al.*, 2013). Além disso, PRDX3 é capaz de ativar o fator de transcrição NF-κB, a qual é uma via de sinalização que tem se mostrado muito importante para a progressão e desenvolvimento de CPRC, através da indução de sobrevivência celular (Basu *et al.*, 2011; Whitaker *et al.*, 2013).

O NF-κB consiste em uma família de fatores de transcrição que participa de vários processos biológicos, incluindo resposta imune, inflamação, crescimento e sobrevivência celular (Liu *et al.*, 2017; Sun, 2011). A família NF-κB de mamíferos é

composta por cinco membros, incluindo RelA (também denominada p65), RelB, c-Rel, NF-κB1 p50 e NF-κB2 p52, que formam vários complexos diméricos que regulam a expressão de vários genes por meio da ligação ao intensificador κB (Sun e Ley, 2008). Existem duas vias de ativação de NF-κB, a canônica e a não-canônica, as quais estão ilustradas na figura 5. Na via canônica, IκB é fosforilada, principalmente por IKKβ, causando sua ubiquitinação e liberação do dímero p50/p65 (NF-κB), o qual saíra do citoplasma e migrará para o núcleo. Os dímeros p50/p65 no núcleo induzirão a transcrição de diversos genes, dentre eles genes antioxidantes (SOD1, SOD2), anti-apoptóticos (Bcl-2, Bcl-xL), proliferativos (ciclina D1), genes relacionados a metástase (MMP-2), dentre outros (Galante *et al.*, 2004; Guttridge *et al.*, 1999; Morgan e Liu, 2011). Estudos mostram que os dímeros p50/p65 de NF-κB estão constitutivamente ativos em linhagens celulares de câncer de próstata independentes de andrógenos DU145, PC3, JCA1 e CL2, conferindo vantagens de crescimento e sobrevivência para essas células (Gasparian *et al.*, 2002; Lessard *et al.*, 2005).

Já na via não canônica, NIK catalisa a fosforilação de IKKα e p100 para conduzir a subsequente ubiquitinação de p100 e degradação mediada por proteassoma para liberar p52 (Liao *et al.*, 2004). Sob condições basais, p100 existe tipicamente em complexos de dímeros com RelB e após sua degradação gera dímeros p52-RelB capazes de se translocar para o núcleo para estimular a transcrição de genes distintos. Ambos NIK e IKKα desempenham papéis críticos na fosforilação de p100 para liberar dímeros maduros da proteína p52-RelB (Paul *et al.*, 2018). A ativação de p52:RelB aumenta a sobrevivência e a proliferação das células PCa ao regular positivamente Bcl-xL e ciclina D1 (Wang *et al.*, 2020). A inibição da translocação dos dímeros p52:RelB leva a redução de SOD2 em células de câncer de próstata (Xu *et al.*, 2008).

A expressão de NF-κB2/p52 mediada por adenovírus em células de câncer de próstata aumenta o crescimento tumoral em camundongos machos nude e também induz o crescimento tumoral em camundongos machos nude castrados, sugerindo que a superexpressão de NF-κB2/p52 induz crescimento independente de andrógeno no câncer de próstata (Nadiminty *et al.*, 2008). Lessard *et al* (2005) mostraram a expressão constitutiva de p52 nos núcleos de células de câncer de próstata (Lessard *et al.*, 2005). Uma expressão elevada de p52 em tecidos de câncer de próstata em comparação com tecidos benignos correspondentes foi detectada por prostatectomia radical (Nadiminty *et al.*, 2008).

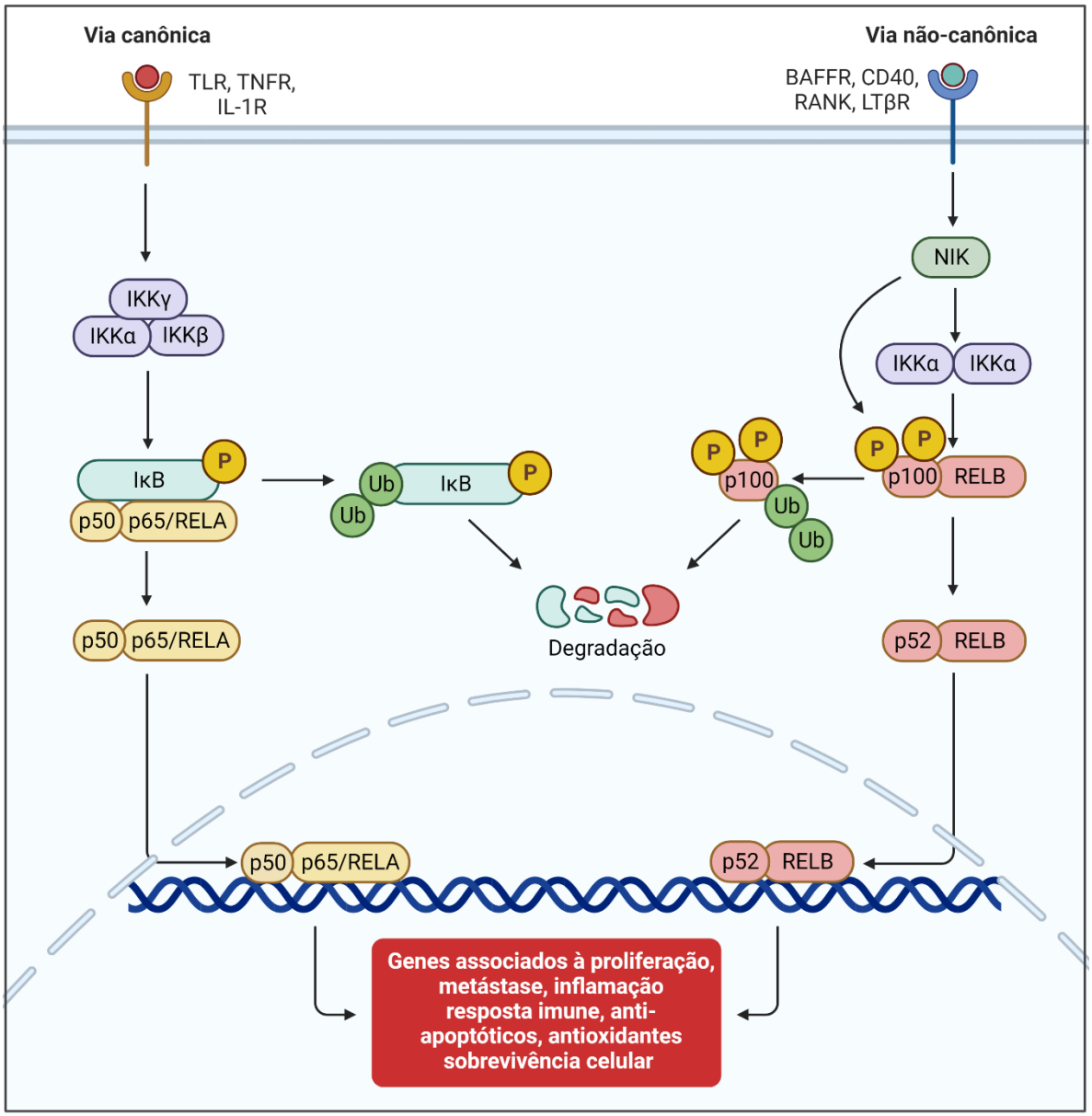

Figura 5. Via canônica e não-canônica de NF-κB. A sinalização canônica de NF-κB é estimulada por citocinas pró-inflamatórias como IL-1β, TNF-α e LPS, induzindo a ativação do complexo IKK. O complexo IKK fosforila a quinase IκB, causando a degradação via ubiquitinação e translocação do heterodímero do p50/p65 para o núcleo. A sinalização NF-κB não canônica é ativada pela superfamília de receptores TNFR. A ativação dos receptores aciona NIK para fosforilar e ativar o complexo IKKα, o qual fosforila p100, levando ao processamento e liberação do heterodímero ativo p52/RelB. NIK também fosforila p100. Os dímeros p50/p65 e p52/RelB vão para o núcleo e transcrevem genes relacionados à proliferação, metástase, inflamação, resposta imune, sobrevivência celular, genes anti-apoptóticos e antioxidantes.

A glicólise é uma via bioenergética que está ligada diretamente com a sobrevivência de tumores (Bolaños, Almeida e Moncada, 2010). A via glicolítica, seguido da fermentação de lactato em vez da fosforilação oxidativa, é muito ativada em vários tipos de câncer, incluindo em câncer de próstata associado a metástase.

Esse processo preferencialmente escolhido por essas células cancerosas é comumente conhecido como efeito Warburg (Zhou *et al.*, 2010). Entretanto, observa-se que o câncer de próstata na fase inicial é dependente de lipídios e outras moléculas de energia para a produção de ATP e somente em estágios posteriores, mais avançados, o efeito Warburg se torna a rota metabólica proeminente (Ahmad, Cherukuri e Choyke, 2021). O ambiente ácido resultante dessa sinalização promove a agressividade e a metástase do câncer de próstata, devido a ativação de proteinases que degradam a matriz extracelular (Estrella *et al.*, 2013).

As hexoquinases catalisam a primeira etapa essencialmente irreversível da via glicolítica, e um alto nível de expressão de HK2 está associado a um prognóstico ruim em pacientes com câncer de próstata (Anderson *et al.*, 2017). Estudos mostram que mutações em PTEN e p53 aumentam a expressão de hexoquinase 2 e induz a glicólise aeróbica, estimulando o câncer de próstata agressivo (Wang *et al.*, 2014). Além disso, o efeito Warburg pode apoiar o metabolismo anabólico indiretamente, mantendo alta a expressão dos intermediários glicolíticos que favorecem o envolvimento da via das pentoses fosfato e outras vias biossintéticas dentro da célula (Newsholme, Crabtree e Ardawi, 1985).

Em suma, existem muitas vias de sinalização que podem conferir vantagens para as células de câncer de próstata sobreviverem de maneira dependente e independente de andrógenos. Entretanto, pouco se sabe sobre como NEK6 pode atuar nesse contexto. Indícios obtidos de trabalhos anteriores de nosso grupo sugerem que NEK6 pode ter interagir com proteínas envolvidas na ativação de NF-kB, como por exemplo, PRDX3, ATF4, TRIP4, e até mesmo um componente do NF-kB, o RelB (Jung *et al.*, 2002; Schmitz *et al.*, 2018; Vaz Meirelles *et al.*, 2010). O estudo de Atish *et al* 2017 mostrou NEK6 como uma quinase central na mediação de resistência à castração no câncer de próstata, de maneira independente de andrógenos e também não havendo relação com sua participação no ciclo celular (Atish *et al.*, 2017). Levando em consideração todos esses indícios, hipotetizou-se que NEK6 poderia estar envolvida na ativação de vias de sobrevivência celular no CRPC.

1.6. NEK6 como potencial alvo terapêutico em câncer, em especial, o de próstata

NEK6 parece desempenhar um papel no desenvolvimento e progressão do câncer. Foi demonstrado que o RNA mensageiro de NEK6 está aumentado em 70%

dos carcinomas hepáticos (Chen *et al.*, 2006). Outros estudos mostram o aumento da transcrição de NEK6 em câncer de mama, colorretal, pulmão e laringe (Capra *et al.*, 2006).

TAKENO *et al* (2008) realizaram um estudo dos perfis de transcrição comumente ativados em diferentes estágios de câncer gástrico usando uma abordagem integrada combinando o perfil de expressão gênica de 222 amostras humanas. NEK6 estava entre os sete candidatos envolvidos na carcinogênese gástrica, sendo relatado níveis mais elevados da proteína NEK6 em cânceres gástricos avançados em comparação com amostras do estágio inicial da doença.

A hipóxia ativa uma resposta adaptativa que torna as células cancerosas derivadas de tumores sólidos menos quimio e radiossensíveis. Esta resposta é impulsionada principalmente pelo fator de transcrição Hif-1α (Semenza, 2013). A inibição de Hif-1α está sob investigação ativa em ensaios clínicos. Um estudo demonstrou que NEK6 é um alvo *downstream* de Hif-1α, tendo sua expressão aumentada em situações de hipóxia em células de câncer de ovário (Donato, De *et al.*, 2015). Além disso, NEK6 tem sua expressão aumentada em câncer de ovário em comparação com tecidos normais. A depleção de NEK6 torna as células mais sensíveis ao tratamento com cisplatina e paclitaxel, que são quimioterápicos de primeira linha utilizados em câncer de ovário (Donato, De *et al.*, 2015).

O estudo de NASSIRPOUR *et al* (2010) demonstrou que a inibição de NEK6 não altera o ciclo celular de células normais, somente de células cancerosas, indicando que a inibição de NEK6 pode fornecer vantagens terapêuticas no tratamento do câncer.

Um estudo evidenciou o envolvimento crucial de NEK6 com o câncer de próstata (Atish *et al.*, 2017). O câncer de próstata é o segundo diagnóstico de câncer mais frequente feito em homens e a quinta causa de morte em todo o mundo (Rawla, 2019). A terapia de privação androgênica é o tratamento padrão para o câncer de próstata (CaP). Embora quase todos os pacientes respondam ao tratamento, a maioria acabará progredindo para um estágio fatal da doença, denominado câncer de próstata resistente à castração (CPRC) (Chandrasekar *et al.*, 2015). Estudos de tecido metastático desses pacientes com CPRC revelam que os tumores podem se tornar resistentes por meio da ativação persistente da via do receptor de andrógeno ou pela progressão para um estado descrito como câncer de próstata independente da via androgênica (Nelson, 2012). Na figura 3 está ilustrado dois modelos propostos para a

geração de CPRC, o modelo de seleção e de adaptação.

As amostras de tumor de pacientes com CPRC mostram níveis aumentados de fosforilação de tirosina em comparação com amostras de câncer de próstata na fase inicial, e a análise de eventos de fosforilação de serina/treonina e tirosina em CPRC demonstrou um número de quinases que são diferencialmente expressas ou ativadas na metástase desse câncer. No entanto, existem evidências limitadas para a ativação de mutações pontuais de quinase em CPRC, sugerindo que as vias de quinase são ativadas por outros mecanismos (genéticos, epigenéticos e microambientais) (Faltermeier *et al.*, 2016).

A partir de uma triagem genômica funcional *in vivo* para identificar novas vias que podem estar envolvidas no CPRC, o estudo de ATISH *et al* (2017) identificou a proteína NEK6 como uma quinase que medeia o crescimento tumoral independente de andrógenos. O estudo identificou também que NEK6 é expressa de forma aberrante no câncer de próstata humano e em diversas linhagens celulares de câncer de próstata. Mais de 600 quinases foram superexpressas na forma de lentivirus em células LHSR-AR, as quais foram introduzidas em camundongos nude BALB/C fêmeas. Poucos genes foram capazes de promover o crescimento de tumor na ausência de hormônios, um destes foi a quinase NEK6. A superexpressão de NEK6 em camundongos fêmeas e machos castrados levou a formação de tumores, sendo que a superexpressão de sua quinase morta, "*kinase-dead*", não foi capaz de gerar tumores (Atish *et al.*, 2017). Após a superexpressão de NEK6 com doxiciclina por 35 dias, os camundongos foram castrados e os tumores foram monitorados quanto à resposta. Foi observado que os tumores que expressam NEK6 continuaram a crescer mesmo em camundongos machos castrados. No entanto, quando a superexpressão de NEK6 foi anulada pela retirada da doxiciclina no momento da castração, os tumores resultantes eram tão sensíveis à castração quanto os tumores controle. Estas observações demonstram que NEK6 pode levar à resistência à castração em tumores e sugere-se que o direcionamento terapêutico de NEK6 pode restaurar a sensibilidade à castração em tumores onde sua atividade é aumentada. Por fim, foi realizado fosfoproteômica dos tumores e foram encontrados que 59 fosfopeptídeos de 50 proteínas foram regulados positivamente pela expressão de NEK6 WT em comparação com o controle e NEK6 *kinase dead*, dentre elas FOXJ2 e NCOA5. O estudo sugeriu que a atividade de quinase de NEK6 tem um papel central em medear a resistência à castração em tumores, de forma andrógeno independente, e acredita-

se que as vias reguladas por NEK6 são relacionadas a sobrevivência celular (Atish *et al.*, 2017).

1.7. Inibidores de NEK6 e sua relevância nos diversos tipos de câncer

Nos últimos anos, vários compostos químicos foram explorados contra as NEK quinases. Alguns deles específicos para NEK6 serão descritos a seguir, bem como suas estruturas químicas estão representadas na Figura 6.

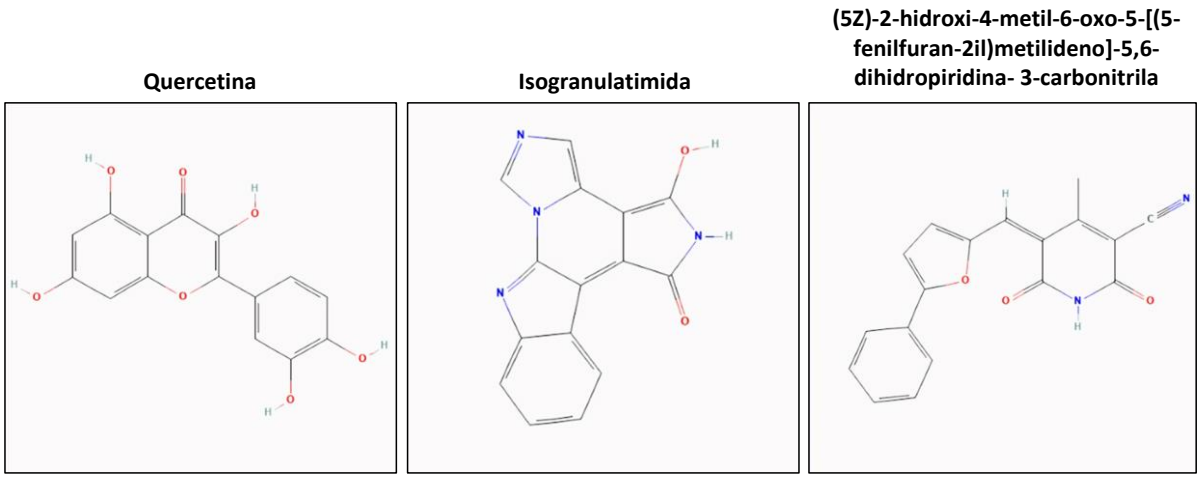

Figura 6. Inibidores de NEK6. A figura apresenta a estrutura química de da quercetina, isogranulatimida e (5Z)-2-hidroxi-4-metil-6-oxo-5-[(5-fenilfuran-2il)metilideno]-5,6-dihidropiridina- 3-carbonitrila.

A quercetina foi identificada como um potente inibidor tumoral, pois é capaz de reduzir os níveis globais de metilação das células HeLa de maneira dependente da dosagem e do tempo. Os achados também relataram uma diminuição dependente da dose de quercetina na expressão de NEK6 (Kedhari Sundaram *et al.*, 2019).

A triagem por Ensaio de Mudança Térmica de Inibidores Competitivos de ATP indicou a isogranulatimida como um inibidor promissor de NEK6 (S206A). O composto à 0,625 µM pode causar até 74,9% de redução na atividade de NEK6 (Moraes *et al.*, 2015). Como a NEK6 tem função na parada G2/M e na resposta ao dano do DNA por meio da interação com CHK1 e CHK2, e também identificada como um alvo da isogranulatimida, a NEK6 pode ser uma boa opção para regular as respostas de *checkpoint* em câncer.

Um potente inibidor de NEK6 direcionado ao sítio de ATP identificado por triagem virtual, adotando técnicas baseadas em estrutura e ligante, foi identificado por Donato e colaboradores (Donato, De *et al.*, 2018). Usando um modelo de NEK6 construído por homologia, bem como as características farmacofóricas de inibidores

de NEK6 conhecidos, foram identificados novos sítios de ligação. 25 compostos dos foram submetidos a ensaios de quinase *in vitro* e o composto 8, ((5Z)-2-hidroxi-4-metil-6-oxo-5-[(5-fenilfuran-2-il)metilideno]-5,6-di-hidropiridina-3-carbonitrila), também chamado de NEK6-I no decorrer dessa dissertação, foi capaz de inibir NEK6 com baixo valor de IC_{50} e também exibiu atividade anti-proliferativa contra um painel de linhagens celulares de câncer humano, tais como câncer de mama (MDA-MB-231), de cólon (HCT15), de ovário (PEO1) e de pulmão (NCI-H1299), utilizando um valor de IC_{50} abaixo de 100 μM (Donato, De *et al.*, 2018). Como já mencionado, a estrutura de NEK6 exibe uma identidade significativa com NEK7 e, portanto, o desenvolvimento de inibidores seletivos de NEK6 é árduo devido aos domínios catalíticos serem altamente semelhantes, de forma a diferirem somente em um resíduo no sitio ativo da enzima. O composto identificado por Donato não foi capaz de alterar significativamente a atividade de NEK7, sendo bem específico e interessante em termos terapêuticos. Além disso, o composto 8, ou NEK6-I, exerceu um efeito sinérgico com cisplatina e paclitaxel em uma linhagem de células de câncer de ovário, suportando um possível uso para terapia personalizada (Donato, De *et al.*, 2018).

O câncer próstata resistente a castração apresenta a regulação de várias vias de sobrevivência e muitas dessas vias são reguladas por quinases. A atividade de quinase de NEK6 parece ter um envolvimento com vias que regulam a sobrevivência celular no contexto do CPRC à terapia convencional, sendo uma atuação independente de hormônios. Nesse sentido, identificar alvos e novas funções de NEK6 é interessante para o desenvolvimento de novas terapias mais direcionadas que consigam controlar o crescimentos das células cancerosas resistentes ao tratamento convencional. O uso do novo inibidor de NEK6 identificado por Donato *et al* (2018) parece ser interassante tendo em vista a especificade obtida pelo composto e relevância de NEK6 para esse tipo de câncer.

1.8. Quimioterapia e vias de resistência quimioterápica no câncer de próstata.

O paclitaxel é um composto alcalóide isolado da *Taxus brevifolia* (Francis *et al.*, 1995). Já o docetaxel é um análogo semi-sintético derivado do paclitaxel. Ambos, paclitaxel e docetaxel, são pertencentes ao grupo farmacológico dos taxanos (Clarke e Rivory, 1999). O docetaxel é um agente antineoplásico utilizado no tratamento de múltiplos tipos de tumores metastáticos (Lyseng-Williamson e Fenton, 2005; Puente

et al., 2017), e, desde 2004, é utilizado para aumentar a taxa de sobrevivência e qualidade de vida de pacientes com câncer de próstata resistente à castração (Tannock *et al.*, 2004). À medida que o fenótipo do câncer de próstata progride, há a expressão de fatores de sobrevivência, tais como Bcl-2 (Feldman e Feldman, 2001), cIAP-1, cIAP-2, XIAP, survivina (McEleny *et al.*, 2004), clusterina, proteinas de choque térmico (Gibbons *et al.*, 2000), entre outros, ocasionando na resistência a uma série de gatilhos apoptóticos, agravamento da doença e falhas nas terapias propostas para cada fase da doença.

O principal mecanismo de ação do docetaxel é ligar-se à β-tubulina, estabilizando sua conformação e inibindo a montagem adequada de microtúbulos no fuso mitótico, de forma a interromper o ciclo celular durante G2/M (Ramaswamy e Puhalla, 2006).

Um mecanismo pelo qual o docetaxel induz morte celular é através da redução da expressão gênica de Bcl-2, um conhecido oncogene anti-apoptótico, o qual é geralmente superexpresso em células cancerígenas (Cao *et al.*, 2008). Estudos mostram que a inibição de Bcl-2 sensibiliza as células cancerosas de próstata ao tratamento com docetaxel, induzindo, portanto, morte celular por apoptose (Tamaki *et al.*, 2014). Estratégias para bloquear vários membros da família Bcl-2 estão em andamento com AT-101, um pequeno inibidor molecular de Bcl-2, Bcl-xl, Bcl-w e Mcl-1 (clinicaltrials.gov ID: NCT00571675) (O'Neill *et al.*, 2011).

Além do docetaxel, o câncer de próstata também pode ser tratado com cisplatina, entretanto, assim como no contexto do câncer colorretal e de pulmão, o maior problema de seu uso é a resistência gerada à própria droga (Galluzzi *et al.*, 2012). Contornar a resistência à cisplatina permanece, portanto, um objetivo crítico para a terapia do câncer e esforços consideráveis foram realizados para resolver este problema ao longo das últimas três décadas. Estratégias de combinação quimioterápica foram exploradas para reverter a resistência à cisplatina em tumores (Galluzzi *et al.*, 2012). A cisplatina induz danos no DNA, que culminam na apoptose mediada pelas mitocôndrias, além de aumentar a produção de ROS. Evidências mostram que aumentar o conteúdo mitocondrial ou a produção de ROS é uma estratégia interessante para aumentar a sensibilidade às terapias anticancerígenas baseadas em cisplatina (Kleih *et al.*, 2019).

A homeostase celular de ROS é mantida através da produção de ROS e a atividade do sistema antioxidante (Snezhkina *et al.*, 2019). Em situações em que

ocorre uma produção excessiva de ROS ou uma diminuição do sistema antioxidante, inevitavelmente a homeostase de ROS é desfeita, culminando em um excesso dessa molécula dentro da célula (Snezhkina *et al.*, 2019).

As ROS são importantes reguladoras de funções celulares, como o próprio metabolismo celular e também estão envolvidas na transdução de sinais em resposta a estresse (Finkel, 2011). Quando há um aumento na produção de ROS intracelular, esse ROS é responsável pela oxidação de macromoléculas, como DNA, proteínas e lipídios, bem como o aumento de mutações e danos em organelas celulares (Imlay, 2003; Imlay, Chin e Linn, 1988).

As células cancerosas apresentam um nível aumentado de ROS quando comparado com células normais. O aumento de ROS em câncer gera alterações moleculares e bioquímicas que favorecerem o início, promoção, progressão do tumor e resistência quimioterápica (Tafani *et al.*, 2016). Entretanto, o aumento de ROS em células cancerosas pode ser utilizado como uma estratégia terapêutica seletiva para matar estas células, visto que drogas que tendem a elevar ainda mais esses níveis de ROS culminam em uma extrema toxicidade e, consequentemente, morte celular induzida por ROS (Trachootham *et al.*, 2006). As células tumorais tendem a aumentar as atividades de enzimas antioxidantes, para que o tumor consiga sobreviver aos níveis de ROS, também representando um importante mecanismo de resistência quimioterápica em vários tipos de câncer, inclusive o de câncer de próstata (Huang *et al.*, 2000). Esse processo é ilustrado na figura 7.

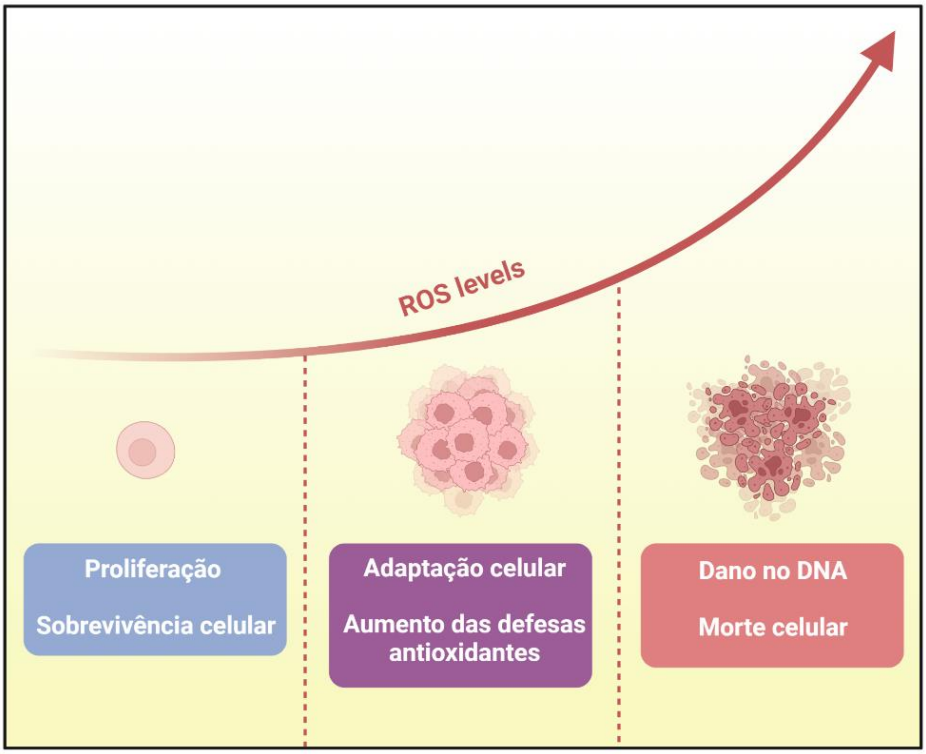

Figura 7. O efeito das espécies reativas de oxigênio no destino da celula depende do nível em que as ROS estão presentes. Baixos níveis de ROS fornecem um efeito benéfico, apoiando a proliferação celular e as vias de sobrevivência em células normais. Já as células cancerígenas têm um aumento nos níveis de espécies reativas de oxigênio quando comparado com as células normais. As células de câncer sofrem adaptações celulares que visam aumentar as defesas antioxidantes, e, assim reduzir o ROS intracelular a fim de não alcançar um nível de ROS exacerbado a ponto de levar a morte celular. Altos níveis de ROS causam danos a proteínas, membranas, organelas, e, principalmente aos ácidos nucléicos, levando à morte celular. A manipulação dos níveis de ROS, de forma a aumentar seus níveis e causar morte celular, é uma estratégia promissora para o tratamento do câncer.

A literatura mostra que existe um papel significativo dos genes antioxidantes no desenvolvimento da resistência ao estresse oxidativo no câncer de próstata (Luo *et al.*, 2019; Lv *et al.*, 2019; Mancini *et al.*, 2022). A superexpressão do gene antioxidante KLF4 restaura o equilíbrio redox das células de câncer de próstata, reduzindo a morte celular dependente de ROS induzida por drogas quimioterápicas (Luo *et al.*, 2019; Lv *et al.*, 2018). O silenciamento de KMT2D induz danos ao DNA mediados por ROS no câncer de próstata, suprimindo a atividade do fator de transcrição antioxidante FOXO3 (Lv *et al.*, 2019). O Nrf2 é um dos principais fatores de transcrição que regula as enzimas antioxidantes (He, Ru e Wen, 2020). O nocaute de Nrf2 desencadeia a morte das células do câncer de próstata através do aumento dos níveis de ROS, sensibilizando essas células à cisplatina (Mancini *et al.*, 2022).

Um estudo revelou que a senescência induzida por doxorrubicina é inibida pela superexpressão de NEK6 em células H1299 de câncer de pulmão (Jee *et al.*, 2011). A parada do ciclo celular em G2/M e redução de ciclina B e Cdc2 induzidas por doxorrubicina foram significativamente revertidos pela superexpressão de NEK6. Curiosamente, o aumento nos níveis intracelulares de ROS em resposta a doxorrubicina também foi inibido em células que superexpressam NEK6 (Jee *et al.*, 2011). Esses resultados sugerem que NEK6 torna as células cancerígenas resistentes à senescência induzida por doxorrubicina, sendo que a regulação de ROS pela presença de NEK6 pode ter sido crucial para esse fenótipo.

Sendo assim, uma elevação adicional no nível de ROS, mediado por agentes indutores de ROS ou por compostos que anulam o sistema antioxidante, resulta em vários tipos de morte celular (Han *et al.*, 2020). A este respeito, a maioria das terapias de câncer de próstata atualmente disponíveis são altamente dependentes da citotoxicidade desenvolvida por ROS.

Considerando que o aumento das defesas antioxidantes é um mecanismo de sobrevivência celular e também de resistência à quimioterápicos, é de interesse terapêutico descobrir as proteínas e vias que regulam o balanço redox e os componentes do sistema antioxidante.

Por fim, os resultados presentes nessa dissertação trazem novas informações sobre como a NEK6 pode estar envolvida na sobreviência de células de CPRC. Os resultados podem ser agrupados em 3 seções: 1) Efeito da NEK6 no estresse oxidativo, dano no DNA, apoptose e via de sinalização de NF-κB em células DU-145 tratadas ou não com cisplatina; 2) Efeito da depleção ou inibição de NEK6 no tratamento com docetaxel em células DU-145 e PC-3; e 3) Envolvimento de NEK6 na regulação da hexoquinase 2, proteína crítica na via glicolítica, em células DU-145.

2. OBJETIVOS

2.1. Objetivo geral

O objetivo geral desse estudo foi explorar a NEK6 no contexto do câncer de próstata avançado, identificando novas vias de sinalização que NEK6 está envolvida, as quais às relacionam ao processo de sobrevivência em modelo de células metastáticas de câncer de próstata resistentes à castração (DU-145 e PC-3). Além disso, também tivemos como objetivo avaliar o papel de NEK6 na quimioterapia utilizada no CPRC.

2.2. Objetivos específicos

- Depleção da NEK6 em células DU-145 e PC-3 utilizando o sistema CRISPR-Cas9;
- Caracterização a nível genômico e proteíco dos nocautes de NEK6;
- Ensaios funcionais comparando as células WT e nocautes de NEK6, a fim de avaliar viabilidade celular, capacidade clonogênica, morte celular com marcação de anexina V e iodeto de propídio, migração celular, produção de lactato e glicose, quantificação das espécies reativas de oxigênio;
- Ensaios moleculares para avaliar a expressão de genes a nível de RNA mensageiro (RTqPCR) e proteina (*western blotting*). Avaliação da translocação do citosol e núcleo do NF-kB utilizando técnicas de fracionamento núcleo/citosol.
- Avaliação de vias moleculares (estresse oxidativo, dano no DNA, apoptose) nas células WT e nocaute de NEK6 tratadas com o quimioterápico cisplatina.
- Avaliação do efeito na viabilidade celular da depleção de NEK6 por CRISPR-Cas9 na promoção de quimiossensibilidade ao tratamento com docetaxel;
- Ensaios funcionais comparando as células tratadas com docetaxel, o inibidor de NEK6, e o co-tratamento dos dois compostos (viabilidade celular, capacidade clonogênica e migração celular).
- Avaliação das vias moleculares por *western blotting* afetadas com o tratamento de docetaxel, inibidor de NEK6 e o co-tratamento com esses dois compostos.
- Avaliação da relação da NEK6 com proteínas da via glicolítica, especificamente a hexoquinase 2.

3. ARTIGOS PUBLICADOS

3.1. Artigo de revisão

cells

MDPI

Review

On Broken Ne(c)ks and Broken DNA: The Role of Human NEKs in the DNA Damage Response

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Abstract: NIMA-related kinases, or NEKs, are a family of Ser/Thr protein kinases involved in cell cycle and mitosis, centrosome disjunction, primary cilia functions, and DNA damage responses among other biological functional contexts in vertebrate cells. In human cells, there are 11 members, termed NEK1 to 11, and the research has mainly focused on exploring the more predominant roles of NEKs in mitosis regulation and cell cycle. A possible important role of NEKs in DNA damage response (DDR) first emerged for NEK1, but recent studies for most NEKs showed participation in DDR. A detailed analysis of the protein interactions, phosphorylation events, and studies of functional aspects of NEKs from the literature led us to propose a more general role of NEKs in DDR. In this review, we express that NEK1 is an activator of ataxia telangiectasia and Rad3-related (ATR), and its activation results in cell cycle arrest, guaranteeing DNA repair while activating specific repair pathways such as homology repair (HR) and DNA double-strand break (DSB) repair. For NEK2, 6, 8, 9, and 11, we found a role downstream of ATR and ataxia telangiectasia mutated (ATM) that results in cell cycle arrest, but details of possible activated repair pathways are still being investigated. NEK4 shows a connection to the regulation of the nonhomologous end-joining (NHEJ) repair of DNA DSBs, through recruitment of DNA-PK to DNA damage foci. NEK5 interacts with topoisomerase IIβ, and its knockdown results in the accumulation of damaged DNA. NEK7 has a regulatory role in the detection of oxidative damage to telomeric DNA. Finally, NEK10 has recently been shown to phosphorylate p53 at Y327, promoting cell cycle arrest after

exposure to DNA damaging agents. In summary, this review highlights important discoveries of the ever-growing involvement of NEK kinases in the DDR pathways. A better understanding of these roles may open new diagnostic possibilities or pharmaceutical interventions regarding the chemo-sensitizing inhibition of NEKs in various forms of cancer and other diseases.

Keywords: DNA damage response; cell cycle; kinase; protein kinase

1. Introduction

Human NEKs (never in mitosis A (NIMA)-related kinases) are a family of protein kinases composed of 11 members that share structural homology with *Aspergillus nidulans* NIMA proteins [1,2]. NEKs are predominantly related to the cell cycle (mitosis and meiosis), centrosome organization, and primary cilia functions, but also to gametogenesis [3], mRNA splicing [4,5], myogenic differentiation [6,7], inflammasome formation [8], intracellular protein transport [2,9], mitochondria homeostasis [5,10–15], and DDR [2,9]. Later studies extended this role to DDR for NEK1 to NEK4, 5, 8, and 10 and then to all other NEKs. There are several classical [16,17] and recent reviews on NEKs functions [2,18] and their role in different diseases [9]. Characteristics of NEKs at the gene and protein levels, such as gene location, number of amino acids, molecular weight, functions, subcellular location, protein domains, and other structural information, are shown in Table 1. In this review, we focus on the emerging family-wide functions of NEKs in DDR.

Genome replication and maintenance are extremely important for cellular functions and the survival of all living organisms. However, during normal cellular metabolism, the DNA is susceptible to chemical modifications by reactive molecules that can cause DNA damage. The sources of spontaneous DNA damage include reactive oxygen species (ROS), deamination, and the inherent susceptibility of DNA to depurination and depyrimidination. In addition, many environmental factors can cause DNA injuries, such as ionizing radiation (IR), ultraviolet (UV) radiation, or chemical agents. These injuries can induce cell death or lead to mutations, which are consequently related to several human disorders, including cancer and age-related diseases. To avoid this situation, cells are equipped with anti-DDR mechanisms and systems capable of repairing DNA damage [19,20].

Based on phosphorylation signaling pathways, the DDR maintains genome integrity through a set of DNA repair mechanisms, damage tolerance processes, and cell-cycle checkpoint pathways. The major functions against DNA damage are DNA repair mechanisms that include: O6-methylguanine-DNA methyltransferase (MGMT) [21], base excision repair (BER) [22], nucleotide excision repair (NER) [23], mismatch repair (MMR) [24], DNA single-strand break repair (SSBR) [25], which relate to the TDP1, APTX, and PNKP proteins and DNA double-strand break repair (DSBR), with two different pathways—the homologous recombination (HR) and non-homologous end-joining (NHEJ) [19].

In eukaryotic cells, cell division undergoes two successive processes. During the S-phase, the DNA is replicated and, during mitosis, the chromosomes are segregated. The gap phases (G1 and G2) take place between S-phase and mitosis. The cell cycle checkpoints control cell cycle

events, which include cell size control, chromosome replication and integrity, and the correct mitosis segregation [26].

The DNA damage promotes cell cycle arrest for proper DNA repair. The main proteins related to the checkpoint are ATR, ATM, Chks (checkpoint kinase), CDKs (cyclin-dependent kinases), and p53. Upon DNA damage, these proteins orchestrate the cell cycle arrest through an organized network to properly repair DNA, or to lead the cell to apoptosis [26]. The G1 checkpoint is controlled by ATM-CHK2-p53, while control of the S and G2/M checkpoints are performed by ATR-CHK1-Wee1 [27,28]. The phosphatases Cdc25A and Cdc25C are phosphorylated by CHK2, promoting cell cycle arrest through CDKs [27], which are inactivated after DNA damage until the damage is resolved [26].

The protein kinase mutated (ATM) and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK) can recognize DNA damage and start the protein kinase cascade. It is known that double-strand breaks (DSBs) induced by IR are mostly recognized by ATM while UV or stalled replication forks are recognized by ATR [29]. The histone H2AX is phosphorylated by DNA-PK, ATM, and ATR, resulting in the accumulation of γH2AX around the DNA lesion (DSBs) [27,30,31]. The γH2AX induction plays a crucial role in DDR, where the histone H2A variant H2AX is phosphorylated at the serine 139 and recruits DNA repair proteins [29,32,33].

The p53 protein, a tumor suppressor, plays a crucial role in DDR by regulating a large number of genes, related to DNA damage repair, cell cycle arrest, apoptosis, and senescence [34,35]. The CDK inhibitor p21 is transcriptionally activated by p53 and promotes cell cycle arrest and tumor growth suppression [36]. The choice between DNA repair or apoptosis relies on DNA damage type and extent [34,35].

Aside from transcriptional and post-translational mechanisms for DDR, a large part of the DDR is mediated by post-transcriptional mechanisms regulating mRNA processing and metabolism [37,38]. Alternative splicing plays a major role in the control of DDR-related gene expression by affecting both mRNA stability and protein activity. These genes that encode sensing factors, as well as components of the DNA repair, cell-cycle, and apoptotic machinery, often produce splice variants that harbor distinct and sometimes opposite activities [39,40].

Splice variants for CHK1, MDM2 (an important regulator of p53 activity), and CDC25C [41–43], among others, have been described in the literature. They are important for the control of cell fate after DNA damage, driving cell cycle arrest for DNA repair or apoptosis.

In addition to their roles in alternative splicing and the expression of DDR genes, a number of splicing factors are recruited at DNA damage sites, indicating their direct participation in the DNA repair process [39]. Among the members of the human NEKs group, the involvement of NEK2 and NEK4 has been observed in the regulation of alternative splicing examples. Most members of the NEK family have demonstrated direct involvement in DDR or interference with partners of paramount importance. We describe below how each member of this family acts in DNA damage and repair. Before presenting each NEK member, will present their main molecular, structural and functional aspects in a table which can be consulted for comparison and overview (Table 1).

Table 1. Summary of the main molecular features of the members of the NEK family.

NEK Members	Gene Location (Chromosome)	Amino Acids; Molecular Weight	Functions	Subcellular Location	Protein Domains	3D Structure; Method; PDB Entry	Ref.
NEK1	4q33	1258 aa, 143 kDa	Primary cilium formation, meiosis I spindle assembly, mitochondrial membrane permeability, cell cycle control, DNA damage response	Cytoplasm, cilia, centrosome, and nucleus upon DNA damage	Catalytic domain, coiled-coil, degradation motif (PEST sequence)	Yes; X-ray; PDB: 4APC	[3,11,36,44–48]
NEK2	1q32.3	NEK2A: 445 aa, 48 kDa NEK2B: 384 aa, 44 kDa NEK2C: 437 aa, 50 kDa	Centrosome integrity and separation, cell cycle regulation, primary cilia, splicing	Centrosome, cytoplasm, nucleus Centrosome, cytoplasm Centrosome, nucleus	Catalytic domain, coiled-coil, degradation motif (PEST sequence) Catalytic domain, coiled-coil Catalytic domain, coiled-coil, degradation motif (PEST sequence)	Yes; X-ray and electron microscopy; PDB: 2W5H	[4,49–59]
NEK3	13q14.2	506 aa, 56 kDa	Cell cycle regulation	Cytoplasm	Catalytic domain, degradation motif (PEST sequence)	No	[60]
NEK4	3p21.1	NEK4 I1: 841 aa, 94 kDa NEK4 I2: 781 aa, 88 kDa NEK4 I3: 752 aa, 84 kDa	Microtubule stabilization, primary cilia stabilization, DNA damage response, splicing	Cilia, basal bodies, nucleus, mitochondria	Catalytic domain	No	[13,61–63]
NEK5	13q14.3	708 aa, 81 kDa	Centrosome disjunction, DNA damage response, mitochondrial respiration, mtDNA maintenance	Cytoplasm, centrosome, mitochondria	Catalytic domain, dead-box helicase-like domain, coiled-coils	No	[14,64]
NEK6	9q33.3	313 aa, 35 kDa	Mitotic spindle and kinetochore fiber formation, metaphase-anaphase transition, cytokinesis, checkpoint regulation	Cytoplasm, nucleus, mitotic spindle, centrosome, central spindle, midbody	Short unfolded interaction region, catalytic domain	Yes; SAXS	[65–70]
NEK7	1q31.3	302 aa, 34 kDa	Mitotic spindle formation, centrosome separation, cytokinesis, NLRP3 inflammasome activation, DNA telomeric integrity	Centrosome, spindle midzone, midbody	Short unfolded interaction region, catalytic domain	Yes; X-ray and electron microscopy; PDB: 6S76	[65,71–77]
NEK8	17q11.2	692 aa, 74 kDa	Stability and function of the primary cilium, DNA damage response	Cytoplasm, centrosome, cilia, nucleus, perinuclear compartments	Catalytic domain, Regulator of Chromosome Condensation (RCC1) domain	No	[78–81]
NEK9	14q24.3	979 aa, 120 kDa	Mitotic spindle formation, centrosome separation, replication stress response	Spindle poles, centrosome, cytoplasm, nucleus	Catalytic domain, Regulator of Chromosome Condensation (RCC1) domain, degradation motif (PEST sequence), coiled-coil	No	[82–87]

NEK10	3p24.1	1172 aa, 133 kDa	DNA damage response, mitochondrial metabolism	Associated with mitochondria	Armadillo repeats, coiled-coil, catalytic domain, degradation motif (PEST sequence)	No	[15,88]
NEK11	3q22.1	NEK11 Long (L): 645 aa, 74 kDa NEK11 Short (S): 470 aa, 54 kDa NEK11C: 482 aa, 56 kDa NEK11D: 599 aa, 69 kDa	DNA replication and DNA damage response	Cytoplasm Nucleus, cytoplasm Nucleus, cytoplasm Cytoplasm	Catalytic domain, coiled-coil, degradation motif (PEST sequence)	No	[89,90]

2. NEK1

NEK1 is the first ortholog of NIMA kinases described in mammalian cells [36]. Understanding the interaction profile of a kinase is essential to postulate possible investigation pathways. Surpili et al. (2003) performed a yeast two-hybrid (Y2H) screening of a human fetal brain cDNA library and identified NEK1 interactors. In the context of cell cycle regulation and DNA damage repair mechanisms, the authors identified interacting partners, such as 14-3-3 protein, ATRX, MRE11, 53BP1, and PP2A subunit B56 [91]. Later, in 2017, Melo-Hanchuk et al. (2017) performed immunoprecipitation followed by liquid chromatography—tandem mass spectrometry (LC-MS/MS) to study NEK1 interactome after DNA damage induced by cisplatin. The authors revealed interactions with important DNA repair pathways, such as homology recombination, nucleotide excision repair, mismatch repair, and the Fanconi anemia pathway [46] (Figure 1).

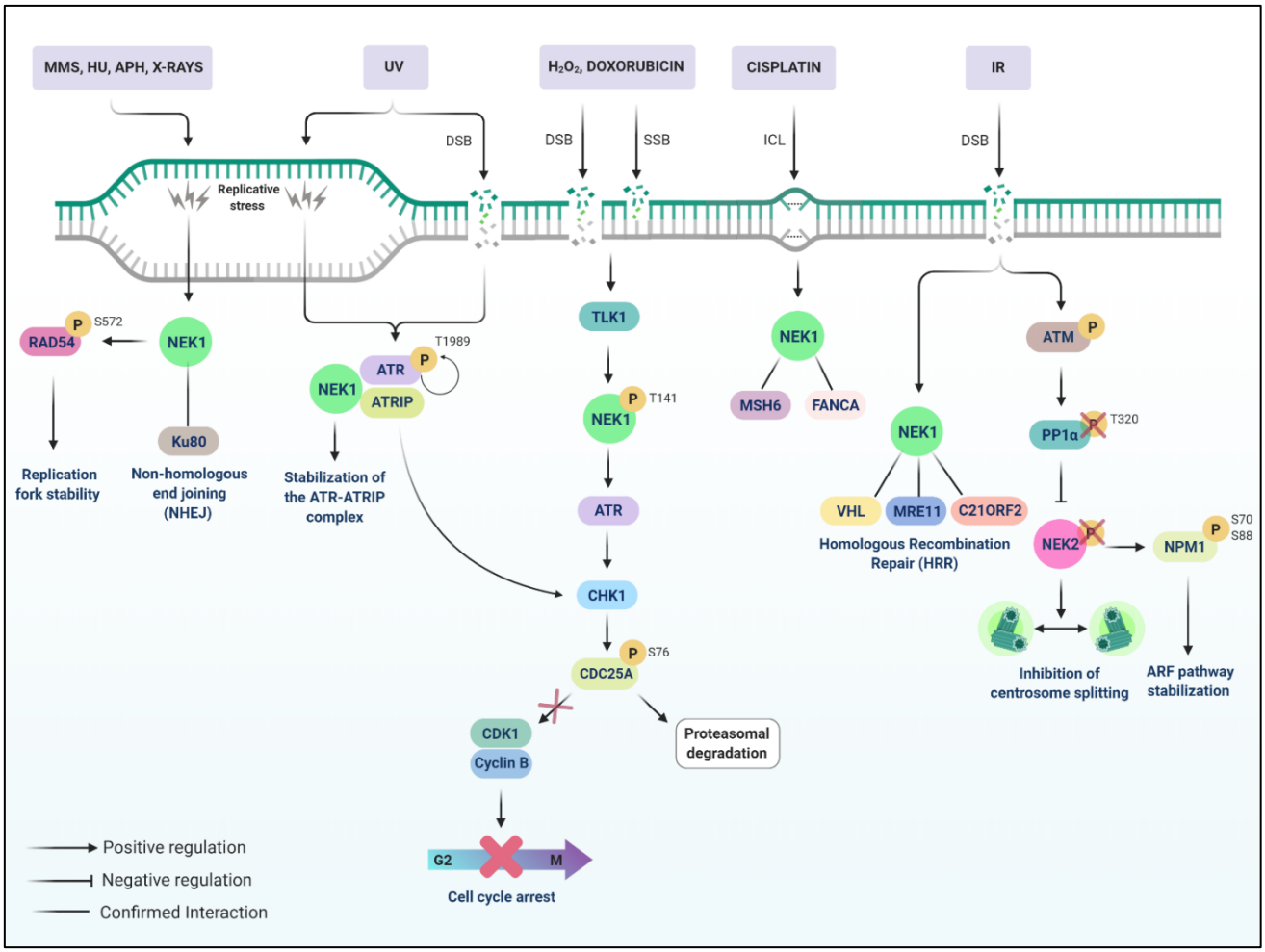

Figure 1. NEK1 and NEK2 play key roles in the DDR pathway. NEK1 is required for replication fork stability by phosphorylating Rad54 at S572. Moreover, the TLK1 > NEK1 > ATR > CHK1 axis plays a major role in DDR, involving cell cycle arrest through CDC25A phosphorylation and CDK1 inhibition. TLK1 phosphorylates NEK1 at T141 and NEK1 interacts with ATR, promoting the stabilization of ATR-ATRIP complex and autophosphorylation of ATR at T1989. NEK2 regulates centrosome disjunction since ATM activates the PP1 phosphatase, which in turn dephosphorylates NEK2, leading to the inhibition of centrosome splitting. The PP1a phosphatase activation also counteracts the NEK2-dependent phosphorylation of NPM/B23, which stabilizes the ARF pathway. NEK2 also upregulates the Wnt1/β-catenin pathway.

Polci et al. (2004) first revealed that NEK1 is involved in early DDR after IR, and plays an important role in repair mechanisms, translocating from the cytoplasm to nuclear foci, where it co-localizes with γH2AX and NFBD1/MDC1 [92]. They extended those findings in 2008 by inducing DNA damage into NEK$^{-/-}$ kat2J mice cells, which failed to arrest cells in G_1/S or G_2/M and properly repair DNA after damage induced by IR [45]. In 2011, they demonstrated that NEK1$^{-/-}$ and NEK1$^{+/-}$ mutant cells suffered from nuclei abnormalities and aberrant chromosome segregation, therefore upholding the importance of NEK1 for mitotic control and function in centrosome duplication and stability [93]. In this context, to further explore NEK1 in cell cycle control, Patil et al. (2013) demonstrated that loss of NEK1 leads to severe proliferation defects due to a delay in the S-phase and the interaction with Ku80 [94]. Moreover, Spies et al. (2016) revealed that NEK1 is required for the replication fork stability and homologous recombination repair by phosphorylating Rad54 at serine

572 in late G_2 and that phosphorylation is required for degradation of stalled replication forks [95] (Figure 1).

In humans, ATM and Rad3-related (ATR) kinases play a major role in DNA damage signaling by activating repair pathways after DSBs, replication stress, and other impairments [96]. Interestingly, a primary study demonstrated that NEK1 activity in DDR and checkpoint control might not depend on ATM and ATR, since inactivation of ATM or ATR did not affect NEK1 expression or translocation to the nucleus after DNA damage. Additionally, neither ATM nor ATR activity was altered in NEK1 deficient cells [97]. In contrast, Liu et al. (2013) demonstrated that NEK1 is required to stabilize the ATR-ATRIP complex for efficient DNA damage signaling, stimulating the autophosphorylation of ATR (T1989) and interacting even before DNA damage induction [44]. However, NEK1 depletion does not show the same effects of ATR depletion since ATR deletion leads to early embryonic lethality [98] and NEK1 depletion to pleiotropic effects, as mentioned before [99]. Thus, the DNA damage signaling functions of NEK1 are more likely to be critical in specific tissues.

A novel pathway suggested by Singh et al. (2020) provides a better understanding of the DDR mechanisms of NEK1. The TLK1 > NEK1 > ATR > CHK1 axis resides on the interaction and activation of NEK1 by TLK1 in threonine 141 (T141) to promote an efficient DDR [12,100–102] via cell cycle arrest through CDC25A/CDK1 (Figure 1). Human tousled-like kinases (TLK) are involved in processes of replication, transcription, repair, and chromosome segregation [103]. A protein screen to identify interactors of TLK1B revealed NEK1 as a potential target, which was confirmed by immunoprecipitation and in vitro kinase assays to determine that TLK1 phosphorylates NEK1 at T141. They also found that thioridazine (THD), an inhibitor of TLK1, significantly reduced the activation of NEK1 after DNA damage induction by doxorubicin [100]. These findings led to three other studies exploring the relevance of TLK1 > NEK1 axis for prostate cancer progression, suggesting a novel target for treatment [12,101,102].

In the context of other human diseases, NEK1 was found to be involved in the development of amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder that causes the death of motoneurons (MN) [104]. Subsequent studies also demonstrated that NEK1 loss-of-function mutations conferred susceptibility to ALS [105,106]. Higelin et al. (2018) demonstrated that ALS-associated NEK1 mutations led to dysregulation of DDR machinery and increased cell death to motor neurons, suggesting possible novel therapeutic strategies to reduce DNA damage in neurodegenerative diseases such as ALS [107]. Interestingly, a study demonstrated the interaction between C21orf2 (Chromosome 21 open reading frame 2) and NEK1 related to efficient DNA damage repair [108]. Furthermore, C21orf2 was also found as a risk gene associated with ALS [109], which may indicate a possible novel axis to be explored as a treatment for the disease.

3. NEK2

The human NEK2 is the most closely related to NIMA (never in mitosis A), and is one of the most studied proteins in the NEK family [2,49].

DNA damage induces cell arrest in the G1, S, or G2 phase of the cell cycle, depending on the phase in which the damage is sensed, allowing

the cells to repair their damaged DNA [110,111]. Based on the fact that NEK2 regulates the separation of the centrosomes during the G2 phase, Fletcher et al. (2004) demonstrated that NEK2 activation and expression are decreased in response to radiation in HeLa cells [59]. Moreover, NEK2-induced centrosome splitting is also inhibited after irradiation in a dose-dependent manner and in different cell lines, as well as in response to other DNA-damaging agents such as etoposide. Thus, NEK2 might act as a downstream target of the DDR pathway and decreased NEK2 activity after irradiation inhibits centrosome separation and contributes to the G2 arrest [59].

ATM, as mentioned, is a central mediator of the cellular response to DNA damage produced by IR, and it is also responsible for activating Protein Phosphatase 1 (PP1), a serine/threonine phosphatase, acting in cell metabolism, transcription, and progression of the cell cycle [112,113]. The PP1α subunit is located in the centrosome and has proven to be a regulator of the NEK2 function, the premature division of the centrosome is triggered both by the overexpression of NEK2 and by the inhibition of PP1 [114], suggesting PP1 as a physiological antagonist of NEK2 [113]. The inhibition of NEK2 activity can be observed in HeLa exposed to IR, and this response proved to be dependent on ATM. the activation of ATM leads to dephosphorylation of the inhibitory residue (T320) of PP1, followed by the increase in its activity and its binding to NEK2. The authors proposed that PP1a maintain NEK2 dephosphorylated, which in turn prevents centrosome splitting [113].

The telomeric repeat binding factor 1 (TRF1) is a double-stranded DNA-binding protein [115] that plays a role in controlling the cell cycle and maintaining telomeres [116,117]. Mitotic aberrations, such as centrosome amplification and chromosome instability caused by NEK2 overexpression, require the TRF1 protein [118]. Misaligned chromosomes, due to overexpression of NEK2 during metaphase, were prevented by depletion of TRF1. Exogenous TRF1, added to cells that overexpressed NEK2, caused the cytokinetic failure, showing that mitotic aberrations caused by NEK2 overexpression are probably dependent on TRF1. NEK2 can directly interact and phosphorylate the TRF1 N-terminal, which contains a D/E-rich and the dimerization domain. It is hypothesized that NEK2 positively regulates the stabilization of the shelterin complex or the TRF1 dimerization. Another possibility is that NEK2 can positively regulate the stability of TRF1 through competition with a ligand that negatively regulates TRF1, Tankyrase 1. Therefore, the association between the overexpression of TRF1 and NEK2 is a mechanism that protects cells against aneuploidy and is related to cancer cell progression [118].

Studies have shown overexpression of NEK2 in several human cancers, acting in malignant transformation, tumor progression, metastasis, and drug resistance [119–121]. NEK2 functionally suppresses p53-mediated apoptosis to induce tumorigenesis through p53 phosphorylation in S315 and its reduced stability [122]. In addition, NEK2 depletion impairs drug resistance in multiple myeloma cells through inhibition of the PP1/AKT/NF-κB signaling pathway [50,123].

The role of NEK2 in radioresistance was evaluated in HeLa, where NEK2 depleted cells show a significant increase in the tail comet and yH2AX foci formation, indicating that the NEK2 knockdown accelerates DNA damage [124].

Rad51 is an essential modulator of the HR pathway [125]. The formation of Rad51 foci decreased substantially after exposure to radiation in silenced cells for NEK2, indicating that the loss of NEK2 hinders DNA repair. Finally, the knockdown of NEK2 leads to negative regulation of WNT4/1, both on the mRNA and protein levels. This in turn leads to a down-regulation of beta-catenin and, consequently, to greater radioresistance and oncogenesis progression [124] (Figure 1).

Depending on the extent of the DNA damage, apoptotic pathways can be activated or inhibited to direct cell response for DNA repair or apoptosis, and one of the mechanisms responsible for that is alternative splicing. Genotoxic agents may induce either pro-apoptotic or anti-apoptotic splice variants. For example, cyclophosphamide and UV damage may favor the production of pro-apoptotic caspase 9a and Bcl-xS, while several topoisomerase I and II inhibitors increase the synthesis of anti-apoptotic caspase 2S transcripts [126]. Naro et al. (2014) suggested that NEK2 plays a role in the alternative splicing regulation of several SRSF1 target genes involved in cell viability. Characterization of the subcellular distribution of NEK2 highlighted its co-localization with SRSF1 and SRSF2 in nuclear speckles. Moreover, NEK2 interacts and phosphorylates SRSF1, similarly to SRPK1. The knockdown of NEK2 induced expression of pro-apoptotic Bcl-X, BIN1, and MKNK2 splice variants [37]. The role of NEK2 in alternative splicing may involve additional regulation of cell fate after DNA damage.

4. NEK3

NEK3 is one of the less-studied members of the family, and its involvement in DDR is still unknown. However, our group showed evidence of a possible role of NEK3 in DDR [127]. Using the Matchmaker Gold Yeast Two-Hybrid system, a screening for NEK3 interaction partners was performed and 65 clones were obtained, whose cDNAs encode 27 different proteins, functionally involved in sumoylation, ubiquitinylation, regulation of transcription, DNA repair, RNA processing, regulation of cell proliferation, invasiveness, and metastasis [127]. One of the identified interactors is PCNA [127], which is involved in the recruitment of proteins in the DNA replication and damaged DNA repair processes [128]. This study therefore proposes that NEK3 may be involved in DDR through PCNA interaction. However, further studies need to be carried out to elucidate the role of NEK3 in DDR.

5. NEK4

In 2012, Nguyen and peers described the interaction between NEK4 and DNA-PK (cs) and its substrates Ku70 and Ku80 [63]. The study was looking for proteins important for replicative senescence in human cells. They found that NEK4 loss of function nearly doubles the cell lifespan. As the suppression of NEK4 did not change telomere length, the observed effects could be attributed to the proliferation rate. Indeed, a reduction of p21 transcription was observed in NEK4 knockdown cells associated with the higher proliferation ratio. As the senescence induced by oncogene H-RasV12 was not affected by NEK4 depletion, they tested if DNA damage could induce cell cycle arrest in these cells and, surprisingly, NEK4 knockdown cells were resistant to DSB agents, such as etoposide and gamma radiation. After etoposide treatment, the fraction of DNA-PK (cs) bound to DNA decreased in NEK4-depleted cells, such as H2AX phosphorylation and p53 activation, despite the fact that DNA damage

was found unaltered, nor was there any change in ATM activation [63]. This demonstrated an important role of NEK4 in DDR, mainly linked to DNA- PK recruitment (Figure 2). The mechanism proposed by the authors is that NEK4 acts as a scaffold protein that can maintain the interaction between DNA-PK (cs) and Ku70/Ku80, and can also regulate the distribution of DNA-PK [63].

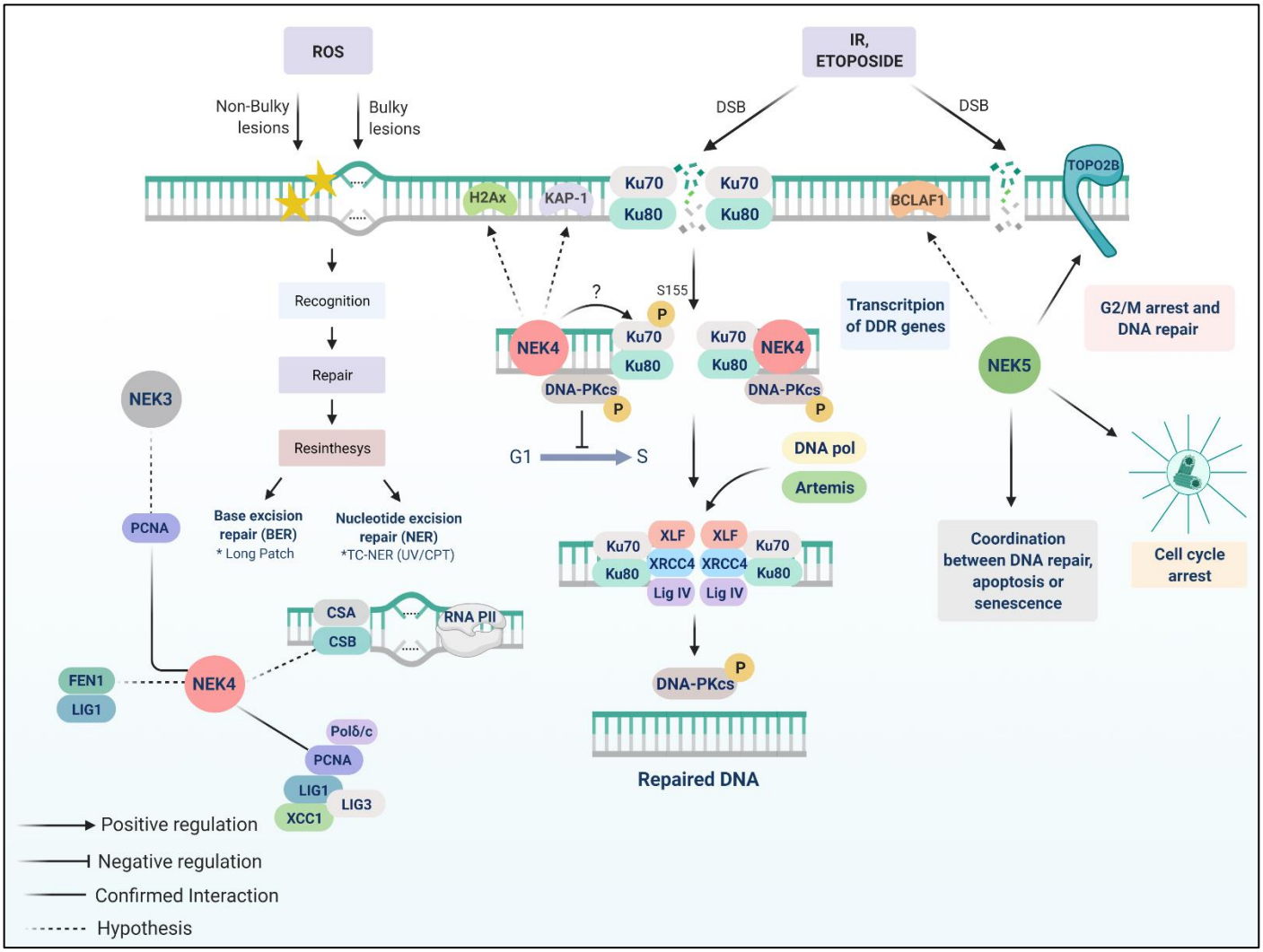

Figure 2. NEK3, NEK4, and NEK5 roles in DDR. The interaction of NEK3 and PCNA is a possible link between NEK3 and DDR. NEK4 interacts with the DNA-PK complex and is important for efficient DNA-PKcs recruitment to DNA damage foci, as well as the activation of pathways essential to induce cell cycle arrest after DSBs with IR or etoposide. We propose that NEK4 acts very early in DNA break recognition, phosphorylating Ku70 (possibly at S155 residue) and stabilizing the DNA-PK complex. Also, NEK4 possibly interacts with other NHEJ-related proteins, such as H2AX, and KAP-1, and PCNA, and FEN1. The interaction between NEK5 and topoisomerase IIβ might be related to cell cycle halting due to DNA damage, as this interaction increases during the first stage of DDR. NEK5-depleted cells overcome the G2/M checkpoint upon DNA damage, indicating that NEK5 is required for an appropriate DDR.

In 2014, our group characterized a new isoform of human NEK4 (GeneBank: KJ592714) [13]. In screening to identify NEK4 isoforms new interaction partners, we have found Ku70/Ku80/DNA-PK (cs) (PRKDC) and PCNA, as previously reported by Nguyen et al., and several other proteins related to DDR, such as TRIM28 (or KAP-1), HERC2, PALB2, PRPF19, H2AX, MDC1, PML, and MCMs (MCM 3, 4 and 5). Aside from

NHEJ-related proteins, various proteins that are classical to other types of DDR were found, such as PARP1, XRCC-6 or CSB, and FEN1, indicating that the role of NEK4 is not exclusive to NHEJ. We have also observed NEK4 and PCNA colocalization after UVC irradiation, as well as colocalization with H2AX after IR exposure. In addition, NEK4 presented PML body localization, a nuclear structure enriched with DDR-related proteins [13]. Nguyen and peers, however, did not exploit the molecular role of NEK4 in the DDR context, but one possibility can be related to DNA-PK (cs) or Ku70/Ku80 phosphorylation. We observed no Ku70 phosphorylation in NEK4 kinase-dead expressing cells compared to control or NEK4 wild type expressing cells [13].

We therefore hypothesize two mechanisms by which NEK4 may participate in the NHEJ pathway via maintenance of the DNA-PK complex: (A) In the NEK4 absence, Ku70/Ku80 does not remain assembled at DSB sites and DNA-PK (cs) cannot bind. As a consequence, H2AX phosphorylation decreases, and so does signaling for DNA repair, culminating in a reduction in p53 activation and cell cycle arrest (Figure 2). (B) Ku70/XRCC6 can be a NEK4 substrate after extensive DNA damage and could activate cell cycle arrest. In NEK4 absence, Ku70 would not be phosphorylated, which would lead to a decrease in DDR signaling and ensuing transcriptional upregulation of p21, followed by cell cycle progress, despite DNA damage.

Fell and colleagues (2016) observed Ku70 phosphorylation at S155 after IR and the S155D phosphomimetic expression led to DDR activation, Aurora B inhibition, and cell cycle arrest. Conversely, the constitutive phosphorylation of Ku70 strongly activated p21 and cell senescence was also observed. The authors proposed that Ku70 phosphorylation at S155 occurs under overwhelming DNA damage to prevent cell cycle progress until the cell completes DNA repair. In case the repair is not possible, the persistent Aurora B inhibition would lead to senescence entry or apoptosis [129].

NEK4 could also directly phosphorylate H2AX, KAP1, and MDC1-promoting signal amplification, or these interactions, could occur in a complex bound to DNA. Surprisingly, our interactome study shows NEK4 interaction with other very early sensors of DNA damage, such as PARP-1, CSB, and ERCC-6 [13], suggesting that NEK4 is closely located at DNA damage sites. PARP-1 is common among all DDR pathways. Based on Nguyen and colleagues' work and our findings, we propose the NEK4 role in multiple DNA repair pathways, mainly NHEJ through DNA-PK complex, long-patch BER by interacting with FEN1 and PCNA, and TC-NER through its interaction with CSB and PCNA (Figure 2).

When analyzing the NEK4 protein-protein interaction network, we found that several of its interactors play important roles in the mRNA splicing process. Our study also revealed endogenous NEK4 localization to nuclear speckles, regions enriched in splicing factors [13]. Liu and colleagues (2019) demonstrated that DNA-PK (cs) translocates from DNA damage foci to nuclear speckles 30 min after DNA damage [130]. Although additional studies must be performed, NEK4, similar to NEK2, can be a cell fate regulator after DNA damage, regulating the alternative splicing.

6. NEK5

Prosser et al. (2015) described the implications of NEK5 knockdown in pericentriolar material (PCM) recruitment, microtubule nucleation,

centrosome linker disassembly, and timely centrosome disjunction [64]. NEK5 knockdown showed a fourfold increase of interphase cells, which presented micronuclei and contained 0, 1, or 2 centromeres. An increase in binucleated cells and in the frequency of anaphase cells with lagging chromosomes and unsolved sister chromatids that gave rise to chromosome bridges were also observed. These data suggest that spindle assembly is less efficient in NEK5-depleted cells, which is consistent with the increase in prophase/prometaphase duration. This was the first-ever described evidence that NEK5 could participate in the cell cycle and genomic stability [64].

Considering this information, the role of NEK5 in DDR was further explored, as it is centrosome localized and its altered expression leads to chromosome instability. A recent publication from our research group demonstrated that NEK5 affects DDR mediated by etoposide (Figure 2). NEK5-depleted cells escape the G2/M checkpoint arrest and progress to mitosis, even upon DNA damage induced by etoposide treatment. Moreover, NEK5-depleted cells showed a significant increase in DNA breaks compared to control cells, while NEK5-overexpressed cells attenuated the DNA breaks upon etoposide treatment, demonstrating that NEK5 plays an important role in DDR [131].

Indeed, a yeast two-hybrid screening performed by our research group identified two proteins interacting with NEK5 that are involved with the DDR: topoisomerase IIβ and BCLAF1. Topoisomerase II (TOP2) is an evolutionarily conserved enzyme capable of generating reversible DSBs in DNA, which enables the resolution of problematic DNA topological structures that arise during normal cellular processes, such as transcription, replication, and mitosis [132].

Drugs known as TOP2 poisons, such as etoposide, stop the TOP2 activity and trap it, covalently, to the DNA, leading cells to accumulate DNA breaks and eventually dying; those TOP2 poisons are widely used as anti-cancer drugs [133]. The interaction between NEK5 and Topoisomerase IIβ was confirmed by immunoprecipitation and the dynamic of this interaction was evaluated by proximity ligation assay (PLA). Upon etoposide treatment, the interaction between Topoisomerase IIβ and NEK5 is considerably increased, especially during the early DDR [131].

During DNA replication, topoisomerase II decatenates newly replicated sister chromosomes and aids in relaxing positive supercoils that accumulate ahead of the replication forks. Topoisomerase IIβ is normally present uniformly throughout the cell cycle and it may sustain a catenated state of sister chromosomes to assist chromosome condensation and cohesion without subsequently interfering with segregation [134].

Those data indicate that NEK5 might interact with topoisomerase IIβ to halt the cell cycle upon DNA damage, as this interaction is increased in the early response to damage, leading cells to arrest at the G2/M checkpoint, although this hypothesis should be further explored.

Another NEK5 interactor related to DDR is Bcl-2-associated transcription factor 1 (BCLAF1). BCLAF1 was first identified as a transcriptional repressor that interacts with the anti-apoptotic proteins, Bcl-2 and Bcl-xL, promoting apoptosis when overexpressed. Studies potentially support a role for BCLAF1 in apoptosis through events that control transcription. Despite its role in cell death, it is known that BCLAF1 also participates in other cellular processes, such as T-cell

activation, lung development, and RNA metabolism, including DDR [135,136].

BCLAF1 first manifested association with DDR through its interaction with γH2Ax in the IR-induced DSBs model. BCLAF1 promoted apoptosis of irreparable cells through disturbing p21-mediated inhibition of Caspase/cyclin E-dependent, mitochondrial-mediated pathways. BCLAF1 also co-localized with γH2AX foci in nuclei and stabilized the Ku70/DNA-PK (cs) complex, which are DNA damage sensory proteins, facilitating NHEJ-based DSB repair in surviving cells [137]. Later, BCLAF1 manifested its involvement in the selective splicing and export of a large subset of transcripts related to DDR, such as BRCA2, FANCD2, FANCL, and RAD51 [138]. Since NEK5 has been related to both apoptosis and DDR processes, and the roles of BCLAF1 are based on whether cells will correct the DNA damage or undergo senescence or apoptosis, more experiments are needed to better understand the relationship between these two proteins. NEK5 could either activate BCLAF1 to trigger the transcription of DDR genes in an early phase of the DDR, or interact with BCLAF1 to promote cell death via mitochondrial-intrinsic pathways, in which NEK5 had already manifested its role [14].

Finally, NEK5 also proved to interact with Cyclin A2 in breast cancer cells. NEK5 silencing promoted downregulation of Cyclin A2 and Cyclin B1 [139], two of the main mitotic cyclins that are strictly related to mitotic commitment [140].

7. NEK6

The knowledge regarding NEK6 involvement in DDR and DNA repair is still sparse. Lee et al. (2008) found that NEK6 kinase activity is negatively regulated in HeLa cells after genotoxic stress [70]. The authors observed phosphorylation of ectopic NEK6 and inhibition of its kinase activity after IR and UV-irradiation, and this phosphorylation was hampered with caffeine treatment 30 min before irradiation. Caffeine is a known inhibitor of the ATM/ATR-Chk1/Chk2 signaling [141] (Figure 3).

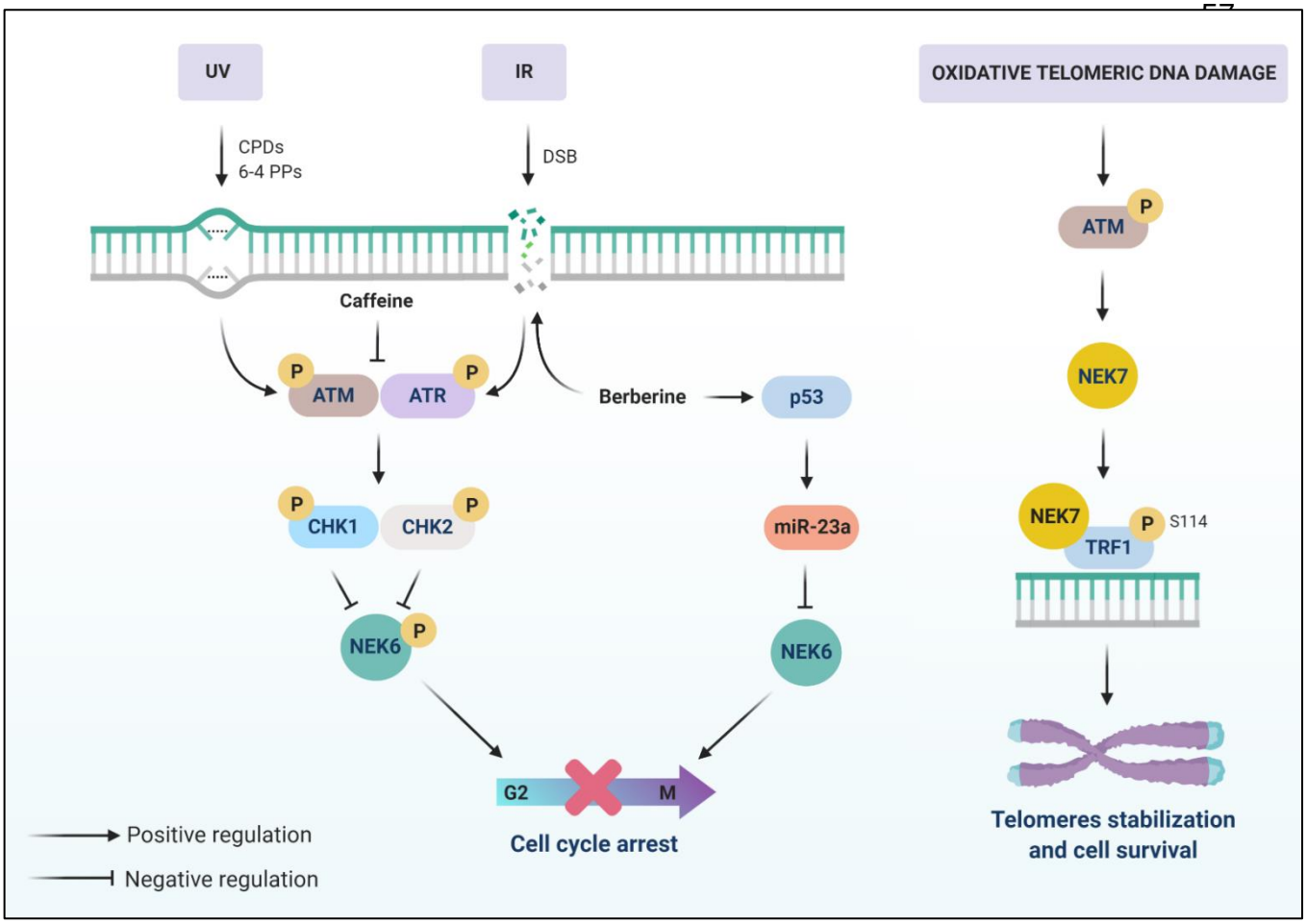

Figure 3. The possible involvement of NEK6 and NEK7 in DDR. NEK6 kinase activity is inhibited by ATM/ATR-Chk1/Chk2 caffeine-sensitive signaling pathway after DSBs induced by IR and UV treatment. NEK6 kinase activity inhibition by DDR is necessary for G2/M arrest. NEK6 is repressed by miR-23a, which is induced by berberine, a genotoxin that increases the accumulation of DSBs, in a p53-dependent manner. NEK7 is related to a protective function of telomeres in response to oxidative DNA damage. ATM activation mediates the function of NEK7 in telomeres. NEK7 is recruited to the telomeres and binds and phosphorylates TRF1 in S114, preventing its degradation. The integrity of telomeres is linked to the stability of TRF1.

In HeLa cells synchronized with nocodazole, when ectopically expressed, immunoprecipitated NEK6 was more phosphorylated and showed higher activity compared to non-synchronized cells [70]. Nocodazole disrupts microtubule dynamics and mitotic spindle function by binding to β−tubulin and inducing cell cycle arrest at G2/M phase [142], and NEK6 transcription and kinase activity is upregulated during the M phase [65]. However, after IR and UV-irradiation, the phosphorylation of ectopic immunoprecipitated NEK6 was increased in both synchronized and non-synchronized cells, but the kinase activity diminished when compared to synchronized non-irradiated cells [70]. NEK6 phosphorylation by the DDR checkpoint proteins Chk1 and Chk2 were also described in an in vitro assay [70].

The necessity of NEK6 kinase activity for mitosis is known since kinase-dead NEK6 expression blocks chromosome segregation at the metaphase-anaphase transition before increasing apoptosis in more than half of the cells [69]. Conversely, flow cytometry analysis of HeLa cells overexpressing NEK6 demonstrates a loss of cell cycle arrest in G2/M after IR-irradiation [70]. NEK6 overexpression induces a reduction of the G2/M population with an increase of G1 and sub-G1 population after IR compared to irradiated wild-type cells, while overexpressing only NEK6

does not change cell cycle distribution [70]. The same is observed with etoposide treatment [143].

NEK6 expression promotes cell proliferation by regulation of cyclin B transcription levels mediated by Cdc2 [144] Additionally, NEK6 expression counteracts cell cycle arrest, ROS production, and the reduction of cyclin B and Cdc2 induced by topoisomerase inhibitors, doxorubicin, and camptothecin treatments, and by p53 expression [145,146].

Therefore, this evidence indicates that inhibition of NEK6 kinase activity during the G2/M phase after genotoxic stress is necessary for proper cell cycle arrest, which is at least partly explained by the phosphorylation of NEK6 by the caffeine-sensitive ATM/ATR-Chk1/Chk2 signaling pathway (Figure 3).

Several miRNAs are predicted and some are confirmed to target NEK6, such as miR-23a [147], which is induced by DNA damage [148]. MiR-23a is induced by berberine treatment, a potent genotoxin that induces the accumulation of DSBs [149,150], in a p53-dependent manner in the HCC HepG2 cell, repressing NEK6 and inhibiting p53 repression induced by NEK6 [147] (Figure 3).

Although NEK6 can be classified as a high confidence hub kinase and the interactors are related to several signaling pathways, including DDR and repair [2,151], to date, no study has identified molecular signaling implications related to DDR after the NEK6 kinase activity inhibition by genotoxic stress. However, NEK6 was found to colocalize at the centrosome with RAD26L, CDC42, and TRIP4, which demonstrated to be NEK6 substrates, as shown by in vitro kinase assays using NEK6 WT and NEK6 S206A (inactive mutant) [151]. In the same study, in a pull-down assay using NEK6 WT and NEK6 Δ1-44 (mutant lacking N-terminal regulatory domain), the authors showed that the NEK6 N-terminal is important for RAD26L, CDC42, and TRIP4 interaction, but not the C-terminal kinase domain [151].

NEK6 phosphorylation by Chk1 and Chk2 is described between amino acids 1-80 [70] since NEK6 S37 was the only residue found to be phosphorylated in vivo in this region [83]. It is therefore likely that NEK6 kinase activity inhibition mediated by Chk1 and Chk2 occurs via the N-terminal regulatory domain [70]; this is the same region that is important for RAD26L, CDC42, and TRIP4 interaction [151]. Thus, it is reasonable to speculate some functional regulation induced by DDR through NEK6 phosphorylation by Chk1 and Chk2, impacting cell cycle, cytoskeleton organization, DNA repair, and NF-κB signaling. It is also dependent on the interaction with CDC42, RAD26L, and TRIP4 at the N-terminal regulatory domain [151].

Zuo et al. (2015) found a NEK6 kinase activity-independent role in the SMAD4 transcriptional function [152]. The authors reported that NEK6 kinase-dead overexpression increased the nuclear localization and transcriptional function of SMAD4 using CAGA-reporter when compared to control cells. In contrast, the overexpression of NEK6 blocked nuclear localization and transcriptional function of SMAD4 in cells treated, or not, with TGFβ when compared to control cells, inhibiting cell cycle arrest induced by TGFβ in a kinase activity-dependent manner [152]. TGFβ is activated after IR-induced DNA damage, and TGFβ treatment increases XPA and XPF interaction and nuclear localization with ERCC1, requiring SMAD4 signaling [153–155].

Therefore, NEK6 kinase activity-independent roles observed by Zuo et al. (2015) [152] may have some relationship with the inhibition of NEK6 kinase activity after DNA damage [70]. This might also be related to the regulation of the NEK6 interaction with several proteins, such as SMAD4, RAD26L, CDC42, and TRIP4, as described above.

Cisplatin resistance is associated with an increase of NER and HR and with a reduction in MMR [156]. De Donato et al. (2015) found that NEK6 overexpression decreased cisplatin sensibility in A2780 cells, a human ovarian carcinoma cell line [157]. Another study by De Donato et al. (2018) found a compound named compound 8, that binds to NEK6 and NEK1 kinase domains and inhibits their kinase activities, increasing cisplatin sensibility in PEO1 cells, another ovarian cancer cell line [158].

Jeon et al. (2010) [159] identified that the ectopic expression of NEK6 in JB6 Cl41 cells phosphorylates S727 of STAT3 (Signal transducer and activator of transcription 3), increasing transcriptional activation activity [159]. The transcription factor STAT3 is a member of the STAT signaling family and mediates cytokines and growth factor signaling, promoting transcription of genes related to proliferation, invasion, angiogenesis, and anti-apoptosis [156,159–161]. STAT3 also modulates ATM-Chk2 and ATR-Chk1 pathways by MDC1 transcriptional regulation [162], and it contributes to cisplatin resistance [163].

8. NEK7

The stability of telomeres is very important to protect against diseases such as cancer and prevent premature aging [164]. Cancer cells have altered oxidative metabolism, promoting telomeric DNA damage [165]. Telomeric repeat binding factor 1 (TRF1) is an essential component of the shelterin complex and acts to protect the integrity of telomeres after oxidative damage to DNA [166].

One study showed that NEK7 regulates the integrity of telomeres [77] (Figure 3). It is recruited and accumulated at telomeres in situations of oxidative DNA damage, protecting telomeres against this type of damage. Moreover, γ-H2AX and association of tumor suppressor p53 binding protein 1 (53BP1) to telomeres, after DNA damage, was diminished in NEK7-deficient cells when compared to control cells. NEK7-deficient cells also presented telomere aberrations. Mechanistically, NEK7 can bind and phosphorylate TRF1 on serine 114, which limits its interaction with F-box only protein 4 (Fbx4) and prevents its degradation via the proteasome. Activation of the ATM pathway is required for NEK7 recruitment and its role in maintaining telomeric integrity. Thus, NEK7 regulates the stability of TRF1, thereby protecting the DNA telomeres from oxidative DNA damage [77].

A mass spectrometry analysis identified that RAD50 co-immunoprecipitated with NEK7 in HEK293 cells [167]. RAD50 acts in the repair of DSB [168] and also in the telomeres' integrity [169]. Therefore, NEK7 may have several functions in response to DNA damage and the integrity of telomeres. Nevertheless, more studies need to be carried out to fully understand the role of this kinase in this context.

9. NEK8

Studies have shown that the maintenance of the genome requires NEK8. By employing comet assay, NEK8 knockdown in HeLa cells shows evidence of DSBs. H2AX phosphorylation was also induced by the absence of NEK8 in these cells, especially after aphidicolin treatment, an

inhibitor of the B-family DNA polymerases [170]. NEK8$^{-/-}$ MEFs (mouse embryonic fibroblasts) show hypersensitivity to this treatment, presenting a decline in cell viability [171].

NEK8 prevents DSB accumulation by suppressing cyclin A-associated CDK activity, and the interaction of NEK8 with ATR, ATRIP (ATR-interacting protein), and CHK1 proteins are stronger in the presence of aphidicolin compared to control [81] (Figure 4). NEK8 proved to be important for the progression of replication forks, since the low expression of NEK8 in these cells is associated with deficient S-phase progression, therefore indicating its relationship with DNA replication [81].

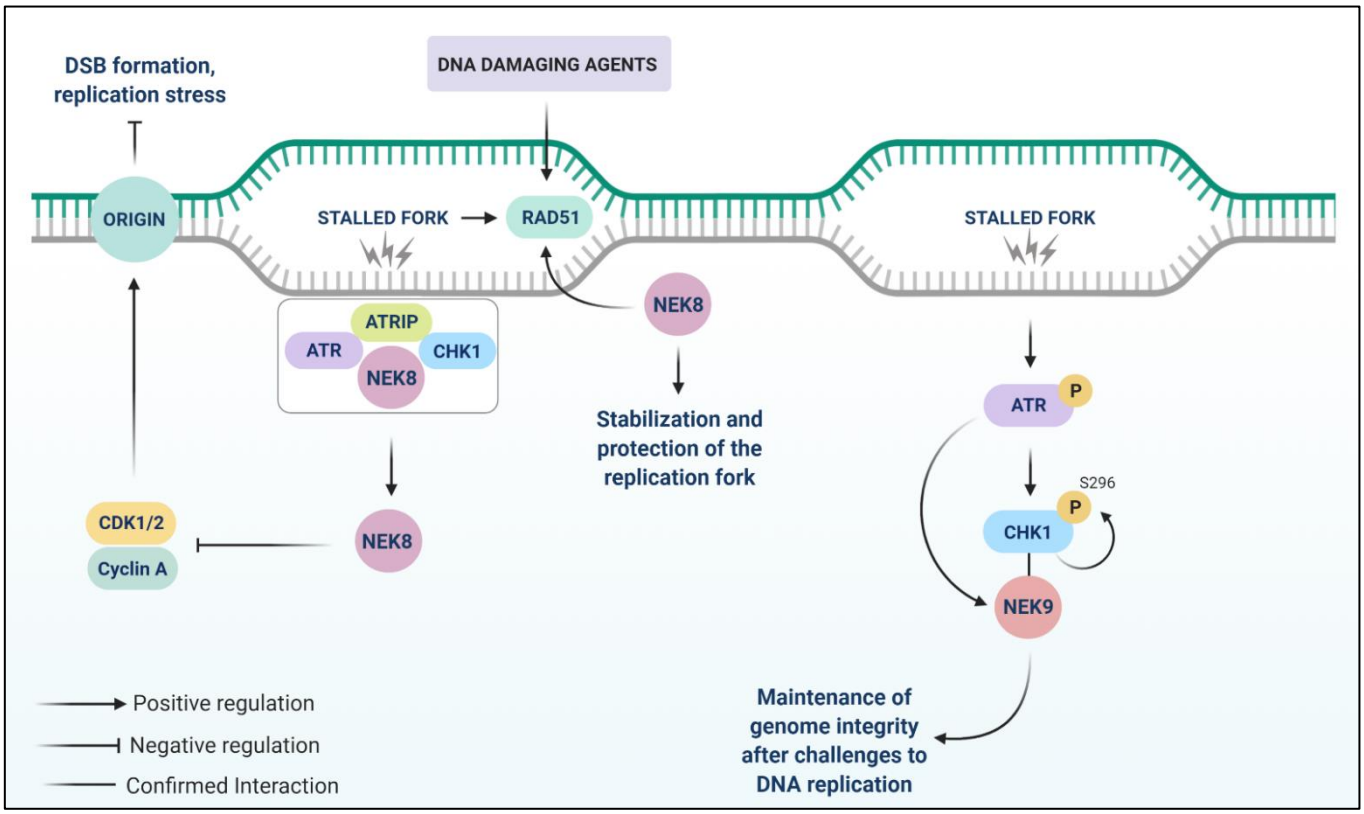

Figure 4. Signaling pathways involving NEK8 and NEK9 in response to replication stress. NEK8 prevents DSB accumulation by suppressing cyclin A-associated CDK activity. NEK8 interacts with ATR, ATRIP (ATR-interacting protein), and CHK1 proteins after DNA damage and is important for the progression of replication forks. NEK8 also regulates RAD51 foci formation upon DNA damage. NEK9 is a replication stress response (RSR) protein since agents involved in replication stress increase its expression. NEK9 interacts with CHK1 and its knockdown decreases CHK1 autophosphorylation (S296) and its kinase activity in response to replicative stress.

During Homologous Repair and Recombination, RECA, homolog to RAD51, is the major protein that performs homology search and DNA strand invasion. Additionally, HR is associated with the replication fork support, since RAD51 plays a role in the replication fork protection [19,172] and promotes the connection between the invading DNA and homologous duplex DNA template [173].

NEK8 appears to play a role in HR [174]. Abeyta et al. (2017) demonstrated the regulation of RAD51 focus formation by NEK8 upon DNA damage, using NEK8-depleted human osteosarcoma cells (U2OS) and NEK8$^{-/-}$ MEFs. The lack of NEK8 promotes inhibition of RAD51 foci formation after treatment with MMC (mitomycin C, a DNA cross-linking agent,), IR, and HU (hydroxyurea, an inhibitor of ribonucleotide

reductase) [174]. Employing a specific siRNA against NEK8 in U2OS cells, the authors also showed that these cells presented a mild decrease in the RAD51 expression, demonstrating the role of NEK8 in RAD51 foci formation, regardless of the damage type or cell type [92]. To further assess the effect of NEK8 depletion in MEFs after DNA damage, the cells were treated with HU, MMC, PARP inhibitor (AZD2281), ATR inhibitor (VE-821), etoposide, or microtubule inhibitor (paclitaxel). NEK8-depleted cells saw a decrease in cell survival after treatment with HU, VE-821, MMC, and AZD2281 [174]. $NEK8^{-/-}$ cells presented a decrease in HR efficiency, thus showing that NEK8 plays a role in the response to stalled replication forks, mediated by RAD51, and in the maintenance of genomic stability [174]. All these data imply the involvement of NEK8 in DDR.

10. NEK9

To date, few studies have observed the association of NEK9 with DDR. NEK9 was identified as a possible ATM/ATR substrate [175]. A proteomic approach has shown that NEK9 is a putative interactor of DDR and DNA replication proteins, such as RFC3, RRM1, MCM5 CHK1, Ku70, and Ku80 [176].

A study employed a siRNA library to identify genes involved in the replication stress response, which sensitizes to gemcitabine treatment [87]. Gemcitabine is a nucleoside analog used as a chemotherapeutic agent and its incorporation into replicating DNA causes a halt in replication since new nucleosides can no longer be added, leading to the arrest of tumor cell growth and induction of apoptosis [177]. Smith et al. (2014) showed that NEK9 knockdown caused hypersensitivity to gemcitabine in a non-cell-type-specific manner [87]. The knockdown of NEK9 also caused hypersensitivity to other agents involved in replication stress, such as MMC, HU, and camptothecin, a topoisomerase I inhibitor. Moreover, NEK9 may be considered a component of the replication stress response (RSR), since its knockdown causes a spontaneous accumulation of γH2AX and RPA70 foci and a decrease in the recovery from replication stress. Upon replicative stress caused by HU, gemcitabine, and MMC treatments, NEK9 expression usually increases. Finally, NEK9 also interacts in a complex with CHK1 and its knockdown decreased CHK1 autophosphorylation (S296), and its kinase activity in response to replication stress. Thus, NEK9 and other NEKs, such as NEK1, NEK6, NEK8, and NEK11, are involved in RSR and DDR activities [87]. The inhibition of NEK9, concomitant to conventional chemotherapy treatments may be an advantageous strategy since the reduction of its expression sensitizes cells treated with DNA damaging agents.

A previous phylogenetical analysis of all NEKs at large and, specifically, of the vertebrate NEKs had shown the clustering of human Neks in subfamilies [178]. Most interestingly, a distinct subfamily cluster is formed by human NEKs 8,9,1,3, and 5. It is remarkable that three of these NEKs: NEK1, 8, and 9, are all somehow functionally involved with the ATR/ATM axis (Figures 1 and 5).

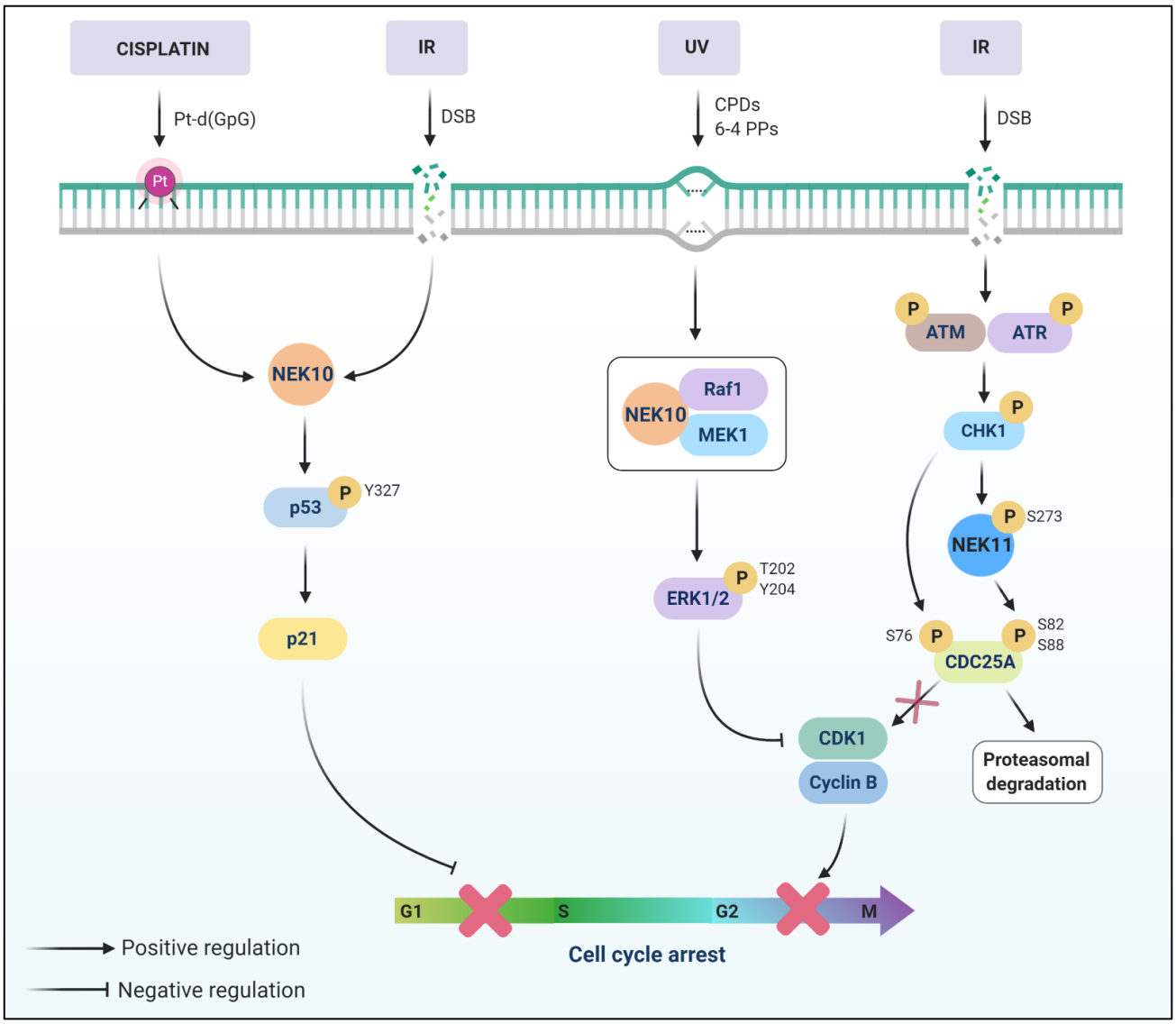

Figure 5. The signaling pathway of NEK10 and NEK11 upon DNA damage. NEK10 assembles in a ternary complex with RAF-1 and MEK1 and mediates ERK1/2 activation, playing a role in the maintenance of the G2/M checkpoint after UV light irradiation. Upon Cisplatin and IR treatments, NEK10 phosphorylates p53 at Y327, increasing p21 levels. p21 is a cyclin-dependent kinase inhibitor that suppresses the cell cycle G1/S phase. NEK10 participates in the control of cell cycle progression through p53 phosphorylation. Upon DNA damage, NEK11 is involved with ATM/ATR checkpoint pathway. Degradation of CDC25A is regulated by NEK11 through the phosphorylation of S82 and S88 of CDC25A, in vivo. CHK1 is responsible for phosphorylating NEK11 at the S273 site in vitro.

11. NEK10

Moniz and colleagues (2011) showed the role of NEK10 in DDR after irradiation with UV light [88]. UV light usually generates two photoproducts, CPDs (cyclobutane pyrimidine dimer) and 6-4 PPs (6-4 pyrimidine-pyrimidone), which are, in general, repaired by the NER system [20,179–181] (Figure 5).

The activation of ERK1/2 (Extracellular Signal-Regulated Kinases) is dependent on MEK1/2 (Dual specificity mitogen-activated protein kinase 1/2) activity, which is in turn activated by the kinase RAF-1 (RAF proto-oncogene serine/threonine-protein kinase) [182]. Moniz et al. (2011) observed an increase of ERK1/2 phosphorylation in HEK293T cells with NEK10 overexpression after UV irradiation, which was dependent on MEK activity [88]. Since NEK10-depletion impairs the activation of ERK1/2 after UV irradiation, NEK10 was implied in mediating ERK1/2 activation. Further investigations showed that NEK10, RAF-1, and MEK1

form a ternary complex. NEK10 also plays a role in the maintenance of the G2/M checkpoint upon irradiation with UV light [88].

Recently, by employing mass spectrometry interactomics, our group found possible NEK10 interactors related to DDR, such as SMC3 (structural maintenance of chromosomes protein 3), UBC (SUMO-conjugating enzyme UBC9), ATRX (transcriptional regulator ATRX), PRKDC (DNA-dependent protein kinase catalytic subunit), and SUMO1 (small ubiquitin-related modifier 1) [15].

Haider and coworkers (2020) showed the importance of NEK10 in p53 phosphorylation [183], which is a tumor suppressor that regulates cell cycle arrest, senescence, apoptosis, autophagy, metabolic reprogramming [35,184], and downregulates genes needed for DNA repair [185]. NEK10 phosphorylated p53 at Y327, increasing the levels of p21 in a NEK10-dependent manner [183]. The knockout of NEK10 by CRISPR promoted greater proliferation, colony formation, and DNA replication. The increase in G1/S phase arrest was also related to NEK10-dependent growth suppression. In the presence of genotoxic stress caused by cisplatin, the lack of NEK10 impaired the induction of p21 and, upon IR, the levels of p21 increased in control cells compared to NEK10 knocked-down cells [183]. These data show the importance of the role of NEK10 in the maintenance of DDR upon genotoxic stress, promoted through UV light, cisplatin, or IR.

12. NEK11

During the cell cycle, the NEK11 subcellular localization is altered and this implies the different roles of NEK11 in the interphase and mitotic phase, in addition to its role in DDR, participating in DNA replication in response to genotoxic stress [89] (Figure 5). NEK11 activity is shown to increase two-fold after treatment with inhibitors of DNA replication (aphidicolin, thymidine, and hydroxyurea) compared to asynchronous cells. After the treatment with DNA damaging agents, such as etoposide, adriamycin/doxorubicin, and cisplatin, the activation of NEK11 was observed, implying its role in DDR [89].

The treatment with caffeine decreased NEK11 activation by aphidicolin and other genotoxic agents, suggesting the involvement of NEK11 in the ATM/ATR checkpoint, in addition to a possible role of NEK11L in the phase S checkpoint [89].

The cell division cycle 25 (CDC25) family members are phosphatases responsible for activating cyclin-dependent kinase (CDK) complexes, key regulators of cell cycle progression. CDC25 is involved in cell cycle and checkpoint control [186]. The degradation of the CDC25A is mediated by CHK1, controlling normal cell cycle progression [187]. In mammalian cells, three isoforms are found: CDC25A, CDC25B, and CDC25C [186]. As shown by Melixitian and colleagues, degradation of CDC25A was regulated by NEK11 [177]. They employed an shRNA library to screen for genes that lead to abnormal cell progression into mitosis after IR. In addition to the classical genes responsible for the G2/M checkpoint, they also found NEK11.

The role of NEK11 in DDR of CRC (colorectal cancer) cells was evaluated by Sabir et al. (2015) [90]. Using flow cytometry, they show that NEK11-depleted cells had a decrease in the G2/M phase after IR, suggesting that it may be partially p53-dependent. NEK11 has also been related to the prevention of apoptosis and cell survival [90].

Normal expression of NEK11 leads to increased G2/M arrest, which results in DNA integrity control. However, NEK11 depletion in HCT116 cells prevented G2/M arrest after irinotecan treatment [90], a topoisomerase I inhibitor [188]. This suggests the role of NEK11 in the G2/M checkpoint. In this same study, these cells showed p53-dependent apoptosis, both in the presence and absence of DNA damage, indicating that the loss of NEK11 leads to the induction of an exacerbated p53-dependent apoptosis after IR exposure [96]. Jointly, these data show the importance of NEK11 kinase in DDR.

13. Conclusions

NEKs are known for their role in the cell cycle, mitosis, centrosome disjunction, and primary cilia, but not for their participation in DDR. We reviewed the literature and presented important information associating the activity of NEKs in the context of DNA damage, induced by IR or chemotherapeutic agents, for example. NEKs are associated with several known DDR pathways, such as ATM/ATR, CHK1, CDKs, p53/p21, and RAD51. By regulating the cell cycle, NEKs appear to be an important link between DNA damage and cell cycle arrest, guaranteeing a proper response for cells to repair the damage. Considering recent advances in synthetic lethetic as a strategy for cancer treatment, the role of NEKs in DDR shines a light on these kinases. Therefore, NEKs may not only be pivotal for the maintenance of cell homeostasis but may also represent novel opportunities for therapeutic interventions of NEK-related diseases such as cancer.

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3.2. Artigo envolvendo trabalho experimental

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NEK6 Regulates Redox Balance and DNA Damage Response in DU-145 Prostate Cancer Cells

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Abstract: NEK6 is a central kinase in developing castration-resistant prostate cancer (CRPC). However, the pathways regulated by NEK6 in CRPC are still unclear. Cancer cells have high reactive oxygen species (ROS) levels and easily adapt to this circumstance and avoid cell death by increasing antioxidant defenses. We knocked out the NEK6 gene and evaluated the redox state and DNA damage response in DU-145 cells. The knockout of NEK6 decreases the clonogenic capacity, proliferation, cell viability, and mitochondrial activity. Targeting the NEK6 gene increases the level of intracellular ROS; decreases the expression of antioxidant defenses (SOD1, SOD2, and PRDX3); increases JNK phosphorylation, a stress-responsive kinase; and increases DNA damage markers (p-ATM and γH2AX). The exogenous overexpression of NEK6 also increases the expression of these same antioxidant defenses and decreases γH2AX. The depletion of NEK6 also induces cell death by apoptosis and reduces the antiapoptotic Bcl-2 protein. NEK6-lacking cells have more sensitivity to cisplatin. Additionally, NEK6 regulates the nuclear localization of NF-κB2, suggesting NEK6 may regulate NF-κB2 activity. Therefore, NEK6 alters the redox balance, regulates the expression of antioxidant proteins and DNA damage, and its absence induces the death of DU-145 cells. NEK6 inhibition may be a new strategy for CRPC therapy.

Keywords: NEK6; CRISPR/Cas9; ROS; DDR; apoptosis

1. Introduction

Prostate cancer is the second most frequent cancer diagnosis in men and the fifth leading cause of death worldwide [1]. Androgen deprivation therapy is the standard treatment for prostate cancer. Unfortunately, although nearly all patients respond to treatment, most of these individuals will eventually progress to a fatal stage of the disease called castration-resistant prostate cancer (CRPC) [2].

NIMA (Never In Mitosis, gene A)-related kinase-6 (NEK6) belongs to a protein kinase superfamily composed of 11 members of NIMA-related kinases [3]. Although NEK proteins are poorly studied, they are known to be involved in cell cycle regulation [4], primary cilium function [5,6,7], and DNA damage response [8]. Additionally, a few recent studies have emerged exploring the relationship between NEKs and mitochondrial activity [9,10] and also emphasized the family of NEKs as biomarkers of several types of cancer [11].

NEK6 is a 313 amino acid serine/threonine kinase encoded in humans by the NEK6 gene located at chromosome 9 [9,10]. Regarding its known functions, NEK6 participates in mitotic spindle kinetochore fiber formation, metaphase-anaphase transition, cytokinesis, and the checkpoint [8]. NEK6 is also involved in liver, breast, colorectal, gastric, and retinoblastoma [12,13,14,15,16,17,18,19]. NEK6 inhibition does not alter the cell cycle of normal cells, only cancer cells, indicating that the inhibition of NEK6 may provide therapeutic advantages in cancer treatment [20]. A recent study emphasized that NEK6 is an executable target in cancer [21]. A high-throughput genetic screen designed to establish new kinases involved in CRPC [22] identified the NEK6 protein as a central kinase that mediates androgen-independent tumor growth and determined that NEK6 is aberrantly expressed in human prostate cancer and several prostate cancer cell lines. The kinase activity of NEK6 appears to be directly involved with cell survival in CRPC [22]. However, it is still unclear what mechanisms and pathways NEK6 may participate in CRPC.

Reactive oxygen species (ROS) are a class of highly reactive, oxygen-containing molecules involved in survival signaling [23,24]. An excess or deficient level of ROS improves the chances of cell death or inhibition of cell growth through mediating ROS-dependent signaling, representing a novel anticancer therapeutic strategy based on ROS regulation [25]. Usually, cancer cells have higher levels of ROS compared to normal cells [26]. However, they suffer an adaptation of increased antioxidant capacity to maintain nonlethal ROS levels, then avoid cancer cell death [27]. Therefore, targeting the ROS signaling pathways and redox mechanisms in cancer progression are new potential approaches to cancer therapy. Additionally, the association of ROS inductors with conventional therapy (chemotherapy or radiotherapy) elevates the ROS levels above the suitable threshold, allowing a better efficiency and specificity to kill cancer cells [27,28,29]. ROS can also induce DNA damage and activate the DNA damage response (DDR), which culminates in DNA repair, cell cycle arrest, and cell death [30].

NEK6 roles are poorly studied, except for its well-characterized participation in the cell cycle [31,32]. However, only recently, the literature has shown its relevance as a possible potential therapeutic target in CRPC [9,21]. Thus, understanding the mechanisms that NEK6 regulates in prostate cancer models becomes essential for the design of NEK6 inhibitors. For these reasons, we targeted NEK6 depletion using the CRISPR/Cas9 system and evaluated the survival pathways NEK6 may regulate in DU-145 cells. We found that NEK6 regulates the redox balance and DNA damage response, inducing cell death. Furthermore, we show that NEK6 depletion is sensitive to cisplatin treatment. Therefore, NEK6 is an attractive target for developing new anticancer drugs.

2. Materials and Methods

2.1. Cell Culture

DU-145 (human prostate cancer cell line) was cultivated in RPMI 1640 (Thermo Scientific, #11875093) medium supplemented with 10% fetal bovine serum (FBS, #12657029) and 1% penicillin/streptomycin (Gibco, #15140-122). Cells were maintained at 37 °C in a humidified atmosphere containing 5% carbon dioxide. Cells were used for experiments within 10-25 passages from thawing.

2.2. NEK6 Knockout (NEK6-KO) Generation Using CRISPR-Cas9 System

To generate the NEK6-KO in DU-145 cells, two sgRNA sequences were designed using CRISPOR [33]. First, we chose guides that have high specificity and efficiency scores, which criteria were determined by Doench, Hsu, and colleagues [34,35]. One selected sgRNA targets exon 3 of the NEK6 gene, called 83 forward sgRNA (sg83), sequence: 5′ AGGCCGAGGACAGTTCAGCG 3′. The other sgRNA targets exon 7 of the NEK6 gene, named sgRNA 56 reverse (sg56), sequence: 5′ TGAAGAAGCGGCCCAGACCA 3′. Oligos containing the sequence were cloned into the PX459 vector (SpCas9(BB)-2A-Puro V2.0, Addgene, #62988); transfected in DU-145 cells using 2500 ng DNA, lipofectamine (Thermo Scientific, #18324012), and plus reagent (Thermo Scientific, #11514015); and cultured with 1 μg/mL puromycin for 72 h for select positive transfected cells. An empty PX459 vector was used as a mock control of transfection. Positively transfected cells were then isolated into 96-well plates by seeding 0.5 cells in 100 μL of medium per well using serial dilutions. This protocol was performed as described by Ran and colleagues [36]. The resulting monoclonal cultures were screened by Western blot for the loss of NEK6 expression using a NEK6 antibody (Santa Cruz, sc-50752). Cells transfected with the empty PX459 vector were also isolated in 96-well plates and used as mock control in the following experiments. To evaluate the indels generated by the CRISPR-Cas9 system, the targeted genomic region for NEK6 was amplified by PCR from genomic DNA and sequenced. The indels were characterized by cloning the amplicons in the pGEM-T vector (Promega, #A3600), followed by Sanger-type sequencing and detection of up to four different chromatograms (Supplementary Materials, Figure S1).

2.3. DNA Genomic Extraction, Conventional PCR and RT-qPCR

Genomic DNA was extracted using the PureLink™ Genomic DNA Mini Kit (Invitrogen; Thermo Fisher Scientific, Inc., K182001). The PCR reaction was performed following the manufacturer's instructions (Invitrogen™, #10966018) using 10 mM dNTP, 10× buffer, a mix of 10 mM primers each, 50 mM $MgCl_2$, 500 ng of genomic DNA, 1.5 U of Platinum Taq DNA Polymerase®, and ultrapure water. The reaction was carried out under the following conditions: initial denaturation at 94 °C for 2 min, followed by 35 cycles with 3 steps: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C (1 min per kB of the gene). The primers used for genomic screening were:

- NEK6 genomic 83fwdF 5′ CAGCAGAGTCCCTCCTTCACCTTAGAG 3′;
- NEK6 genomic 83fwdR 5′ GATGGGTGGACATGGTATGAACCTCAG 3′;
- NEK6 genomic 56revF 5′ ACGTAGGCTGCTTCATGGAC 3′;
- NEK6 genomic 56revR 5′ GCCACAGCTGATTCCCTTCT 3′.

For real-time PCR, RNA was extracted from DU145 cells (WT and NEK6-KO) using TRIzol® (Invitrogen™, #15596018). An amount of 2000 ng of RNA was used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc., #4368814). SYBR Green PCR Master Mix (Applied Biosystems, #1725121) was used to perform the RT-qPCR. The quantification was measured according to the comparative threshold (Ct) cycle method using β-actin as a housekeeping gene. Samples, in triplicate, were arranged in a 96-well plate (MicroAmp, Applied Biosystems, #4306737) for amplification and were run in the Step One Plus Real-Time PCR System (Applied Biosystems). The primers used for RT-qPCR were:

- SOD1_Forward: 5′ GTTTCCGTTGCAGTCCTCG 3′;
- SOD1_Reverse: 5′ GGTCCATTACTTTCCTTCTGCTC 3′;
- SOD2_Forward: 5′AAGGAACGGGGACACTTACAAA 3′;
- SOD2_Reverse: 5′AGCAGTGGAATAAGGCCTGTTG 3′;
- PRDX3_Forward: 5′ GCCACATGAACATCGCACTCTTG 3′;
- PRDX3_Reverse: 5′ ACTGGGAGATCGTTGACGCTCA 3′;
- β-actin_Forward: 5′ GCCGCCAGCTCACCAT 3′;
- β-actin_Reverse: 5′ CCACGATGGAGGGGAAGAC 3′.

2.4. Cell Treatment

DU-145 cells (WT and NEK6-KO) were plated at a density of 2×10^5 cells/well in 6-well plates. On the next day, the cells were treated with cisplatin (Calbiochem, CAS 15663-27-1) at 30 μM for 24 h. Western blotting was performed to analyze the expression of antioxidant proteins, DNA damage, and antiapoptotic proteins.

2.5. Colony Formation Assay

For the colony formation assay, cells were seeded in triplicate in 60 mm dishes (5×10^2 cells/dishes). Cells were incubated for 8 days at 37 °C and stained with violet crystal solution (0.05% violet crystal *w*/*v*, 1% formaldehyde, 1% PBS, 1% methanol, and deionized water) for 20 min at room temperature. The number of colonies was manually quantified. Demonstrative images of the colonies were obtained under an optical microscope (Optika Italy) and using Optika Proview software.

2.6. Measurement of Mitochondrial Membrane Potential (ΔΨm)

Tetramethylrhodamine, ethyl ester (TMRE, Invitrogen™, #T669), is a red–orange fluorescent used to measure ΔΨm and selectively stain mitochondria. DU-145 cells (WT and NEK6-KO) were plated at a density of 1×10^4 in a black 96-well microplate and incubated overnight in an incubator. TMRE treatment at 500 nM was performed for 30 min at 37 °C in a 5% CO_2 incubator. The medium was removed, and in each well was added 100 µL of PBS. The fluorescence signal was analyzed at 549/575 nm in a microplate reader (Epoch, Biotek).

2.7. Proliferation and MTT Assay

DU-145 cells (WT and NEK6-KO) were plated at a density of 8×10^3 cells/well in 96-well plates in triplicate. The proliferation was measured 4, 24, 48, and 72 h after seeding by the MTT assay. To evaluate cell viability, DU-145 cells (WT and NEK6-KO) were plated at a density of 8×10^3 cells/well in 96-well plates in triplicate. The cells reached confluence in 3 days, and only then was the viability measured by adding 10 µL of 12 mM MTT (Invitrogen™, M6494) to each well and incubating for 2 h at 37 °C. The formazan crystals were solubilized in HCl and isopropanol solution for 15 min at 37 °C. The optical density was measured at 570 nm.

2.8. ROS Detection

The total amount of ROS present in cells was measured using dihydroethidium (DHE, Invitrogen™, D11347). Cells were trypsinized and centrifugated, and the pellets were resuspended in 500 µL of medium with DHE (5 µM) and incubated for 20 min at 37 °C. The cells were analyzed in BD Accuri C6™ flow cytometry, with the acquisition of 5000 events. The fluorescence of DHE was analyzed in the FL2-A channel.

2.9. NEK6 Overexpression

DU-145 cells (WT and NEK6-KO) were plated at a density of 2×10^5 cells/well in 6-well plates, and at 80% confluency, the cells were transfected using Lipofectamine 3000 (Invitrogen™, L3000001), following the manufacturer's instructions. An amount of 2500 ng of pFLAG-NEK6 plasmid was used to overexpress NEK6 in cells. The same DNA mass of pFLAG-Ø, an empty vector, was used as the control of the assay. In the immunofluorescence assay, an amount of 400 ng of GFP-NEK6 plasmid was used to overexpress NEK6 in NEK6-KO cells.

2.10. Apoptosis Assay

Cell death was measured using the FITC Annexin V Apoptosis Detection kit (BD Pharmingen™, #556547). DU-145 cells (WT and NEK6-KO) were plated at a density of 2×10^5 cells/well in 6-well plates and kept for 24 h at 37 °C in the incubator. The cells were washed with PBS, trypsinized, and 1×10^6 cells were centrifuged for 150× *g* for 5 min, rewashed with PBS, centrifuged, and resuspended in 100 µL 1× Binding buffer. A small amount (3 µL) of FITC-Annexin V and 3 µL of propidium iodide (PI) were added to the cells, mixed, and incubated for 15 min at room temperature in the dark. An additional 100 µL of 1× Binding buffer was added to each sample and analyzed within 1 h. Controls were performed by staining with annexin only, another one with PI only, and another one in the absence of both reagents. The cells were analyzed in BD Accuri C6™ flow cytometry, with the acquisition of 5000 events.

2.11. Subcellular Fractionation

Cells were trypsinized, centrifuged 150× *g* for 5 min, washed with PBS, and centrifuged again. The cell pellet was resuspended in a cytoplasmic extraction buffer (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% (*v*/*v*) NP-40, 1 mM DTT, and protease inhibitor cocktail); incubated for 3 min on ice; and centrifuged in 1300× *g* for 30 min. The supernatant (cytoplasmic fraction) was transferred to new tubes. The nuclear pellet was washed with cytoplasmic buffer five times and resuspended in nuclear extraction buffer (20 mM Tris-HCl, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (*v*/*v*) glycerol, and protease inhibitor cocktail); incubated for 10 min on ice; and vortexed every 2–3 min. Cytoplasmic and nuclear fractions were centrifuged at 12,000× *g* for 10 min, and the supernatants were collected.

2.12. Western Blotting

The proteins were collected from DU-145 cells using a cell lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease, and phosphatase inhibitor cocktail), and samples containing 30 μg of total protein were separated by SDS-PAGE. The gels were electrotransferred to 0.45 μm nitrocellulose membranes (Bio-Rad Laboratories, Inc.) and incubated for 1 h at RT with 5% nonfat powdered milk dissolved in TBS-Tween-20 (TBS-T; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20). Membranes were incubated with primary antibodies overnight at 4 °C, washed three times with TBS-Tween-20, and incubated with secondary antibodies for 1 h at room temperature, followed by washing three times with TBS-Tween-20. Protein bands were visualized using the Pierce ECL Western Blotting substrate (Thermo Scientific, #32106) or Clarity Max Western ECL substrate (Bio-Rad, #1705063) in the ChemiDoc Imaging System (Bio-Rad Laboratories, Inc.), and densitometry was performed using ImageJ software v1.53. Primary antibodies PRDX3 (Abcam, Ab73349), p-JNK (Cell Signaling, #4668), JNK (Cell Signaling, #3708), SOD1 (Cell Signaling, #4266), SOD2 (Cell Signaling, #13141), Bcl-xL (Cell Signaling, #2764P), Bcl-2 (Cell Signaling, #2870P), ATM (Cell Signaling, #2873P), pATM (Cell Signaling, #5883P), γH2AX, (Cell Signaling, #9718), Lamin A/C (Bethyl, #A303-430A), GAPDH (Santa Cruz, sc-25778), NEK2 (Santa Cruz, sc-33167), NEK3 (Santa Cruz, sc-100402), NEK4 (Santa Cruz, sc-81332), NEK5 (Santa Cruz, sc-84527), NEK6 (Santa Cruz, sc-50752), NEK7 (Abcam, ab133514), NEK8 (Santa Cruz, sc-50760), NEK9 (Santa Cruz, sc-50763), NEK11 (Santa Cruz, sc-100429), NF-κB2 (Cell Signaling, #4882), vinculin (Abcam, Ab18058), and α-tubulin (Calbiochem, CP06). All antibodies were used at 1:2000 stoichiometry. Secondary antibodies: HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich, AP308P, 1:2000), goat anti-rabbit IgG (Sigma-Aldrich, AP307P, 1:5000), and goat (Sigma-Aldrich, A5420, 1:5000).

2.13. Immunofluorescence Assay

The assay was performed as described by Pavan and colleagues [37]. DU-145 NEK6-KO cells were transfected with GFP-NEK6, and after 24 h, the cells were fixed in 3.7% formaldehyde for 20 min, washed three times with PBS, permeabilized with 0.5% Triton X-100 for 10 min, and blocked with PBS containing 0.2% Triton X-100 and 3% bovine serum albumin (BSA) for 30 min. The cells were incubated with the NF-κB2 primary antibody rabbit (Cell Signaling, #4882) overnight at 4 °C, washed three times with PBS, and incubated with the secondary antibody anti-rabbit

Alexa Fluor 594 (Invitrogen, A-11012). The cells were further incubated with Hoechst diluted 1:5000 in PBS for 10 min for nuclei staining. Coverslips were finally mounted using Prolong (Invitrogen™, P36980).

2.14. Statistical and Biostatistical Analysis

GraphPad Prism 8.01 software (https://www.graphpad.com/, accessed on 8 November 2022) was used to perform a statistical analysis. Data were presented as the means and SD. The mean difference was tested by the Student's unpaired t-test or one-way ANOVA, followed by Tukey's or Dunnett's post-test, considering * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ as statistically significant. The GEPIA platform was used for the correlation of gene expression in prostate adenocarcinoma (PRAD). Spearman's correlation coefficient was calculated to measure the strength of the correlation between two genes. The correlation degree was classified according to Schober and colleagues [38].

3. Results

3.1. Generation of NEK6-KO in DU-145 Cells Using the CRISPR-Cas9 Gene-Editing System

We first generated *NEK6* gene knockout in DU-145 cells using the CRISPR/Cas9 system to evaluate the effects of the lack of the NEK6 protein. We designed two sgRNAs against exons 3 and 7 of the *NEK6* gene, named sgRNA 83 and sgRNA 56, respectively (Figure 1A). We used two sgRNA to avoid the nonspecific effects of the CRISPR/Cas9 system. The specificity and efficiency scores were important for choosing the most appropriate sgRNA. Therefore, four NEK6-KO DU-145 cell lines were generated, named 83.7, 83.14, 56.3, and 56.5. Genomic DNA PCR products from each NEK6-KO cell line were demonstrated (Figure 1B). It is possible to visualize 631 and 754 base pair amplicons for sgRNA 83 and 56, respectively. We also observed an increase of a few base pairs in the 56.5 NEK6-KO cell line, which generated a second higher band in the gel. The genomic DNA PCR products of the NEK6-KO cell lines were sequenced, and the chromatogram of each clone was obtained (Figure 1C). To characterize these indels, we cloned the genomic DNA PCR products into a pGEM-T vector and sequenced the mutated locus of several clones (Supplementary Materials, Figure S1). For all NEK6-KO cell lines, we obtained indels that generated premature stop codons. In the specific case of the 56.5 cell line, we detected an addition of 91 base pairs that explains the second-highest band shown in Figure 1B. We also analyzed the protein expression of NEK6 by Western blotting (Figure 1D). As a result, no detection of the NEK6 protein was observed in the NEK6-KO cell lines. WT cells were obtained using the same selection and isolation steps as the NEK6-KO cells.

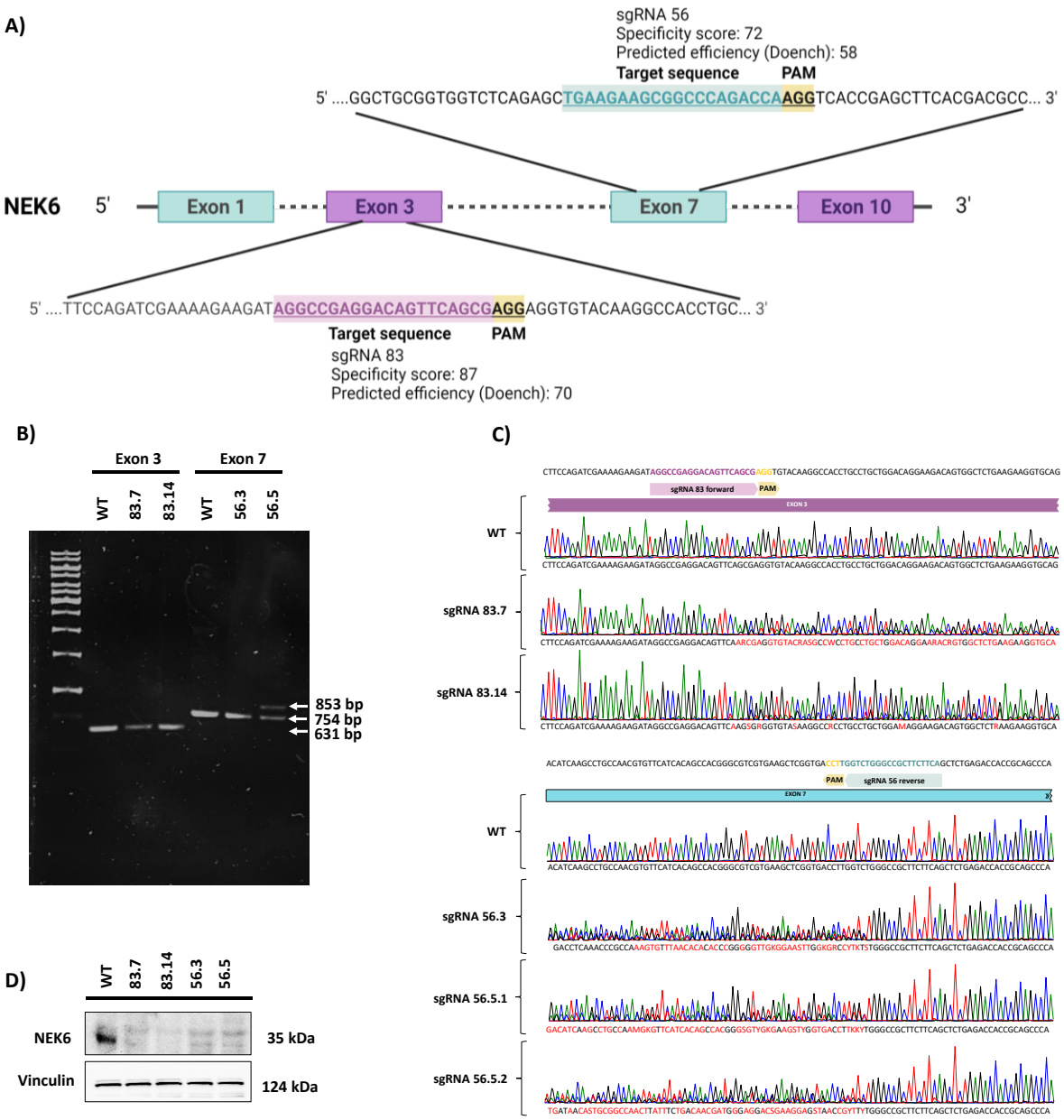

Figure 1. Generation and validation of NEK6-KO in DU-145 cells. (**A**) Gene targeting strategy for generating NEK6-KO in DU-145 cells. (**B**) PCR amplification of the mutated locus of NEK6-KO cell lines (83.7, 83.14, 56.3, and 56.5). (**C**) DNA sequence analysis showed the presence of indels adjacent to the PAM sequence in NEK6-KO cells. (**D**) Western blot analysis of NEK6 protein expression in WT and NEK6-KO cells.

As part of the NEK6 knockout characterization, we evaluated the expression of other NEKs in NEK6 knockout cells (Supplementary Materials, Figure S2). NEK6 knockout increased NEK7 and NEK11 and decreased NEK2 and NEK9 expression at the protein level. NEK7 is a protein that has high structural similarity to NEK6, sharing 86% amino acid sequence identity at the C-terminal and only 20% identity at the N-terminal regions [39,40]. Thus, an increase in NEK7 expression was expected to partially compensate for the NEK6 lack. Additionally, together, NEK2, NEK6, NEK7, and NEK9 contribute to the establishment of the microtubule-based mitotic spindle [41], while NEK11 has been implicated in the DNA damage response [42]. NEK1 and NEK10 were not

evaluated due to technical issues with antibodies. These data suggest new regulations between the NEKs in the cell cycle and DNA damage response.

3.2. Targeted Deletion of NEK6 in DU-145 Cells Reduces Clonogenic Capacity, Cell Proliferation, and Mitochondrial Membrane Potential

Malignant tumor cells can survive and grow without their neighboring cells and anchorage to the extracellular matrix (ECM) [43]. Therefore, we evaluated the clonogenic potential of NEK6-KO cell lines in the colony formation assay (Figure 2A). NEK6-KO cells exhibited a reduction in clonogenic potential. We also observed a decrease in the cell spread in NEK6-KO compared to WT colonies (Figure 2B), which may be related to a reduction in migration capacity. Quantification of the colony numbers was also manually performed, and the statistical analysis was shown (Figure 2C). We evaluated the proliferation rates of WT and NEK6-KO cell lines for 4, 24, 48, and 72 h (Figure 2D). All NEK6-KO cell lines, at 48 and 72 h, showed a significant decrease in proliferation: for 48 h (WT vs. 83.7 **, WT vs. 83.14 ***, WT vs. 56.3 **, and WT vs. 56.5 ***) and for 72 h (WT vs. 83.7 ****, WT vs. 83.14 ****, WT vs. 56.3 ****, and WT vs. 56.5 ****), with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. The cells were maintained in the culture until reaching 100% cell confluence, and the MTT assay was performed to evaluate the cell viability (Figure 2E). We found that NEK6-KO cells presented a lower viability than WT cells, suggesting that mitochondrial activity may be downregulated in NEK6-KO cells. We stained the cells with TMRE to measure the mitochondrial membrane potential (Figure 2F) to prove this hypothesis. NEK6-KO cells demonstrated a diminished mitochondrial membrane potential, suggesting that NEK6 depletion alters the mitochondrial activity and viability.

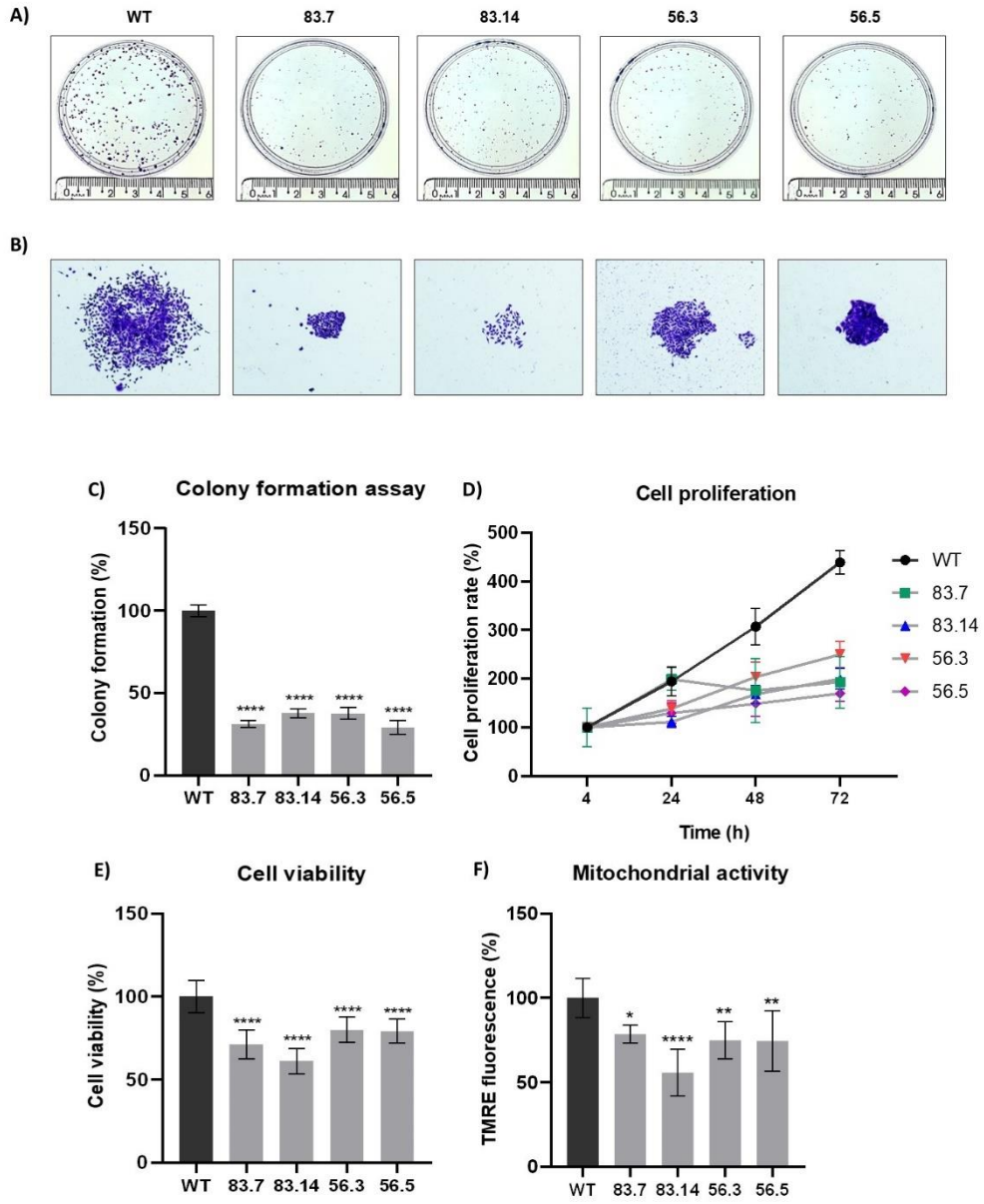

Figure 2. NEK6-KO reduces clonogenic capacity, cell proliferation, and mitochondrial membrane potential in DU-145 cells. (**A**) Deletion of the NEK6 gene significantly reduces the clonogenic potential in DU-145 cells. (**B**) Demonstrative images reveal the visual difference in colony size and its spread. (**C**) Quantification of colonies numbers obtained in WT and NEK6-KO cells. (**D**) Cells were evaluated for proliferation at 4, 24, 48, and 72 hours after seeding, and the results showed all NEK6-KO clones significantly proliferated less than WT cells at 48 and 72 hours. (**E**) Cells were maintained in an incubator (3 days) until reaching confluency. **The** MTT assay was performed to evaluate **the viability of** these cells. NEK6-KO showed a reduction in cell viability when compared to WT cells. (**F**) The target deletion of NEK6 in DU-145 cells also reduced the mitochondrial membrane potential using TMRE staining. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$, and **** $p < 0.0001$.

3.3. Modulation in NEK6 Expression Alters ROS Levels and Antioxidant Defenses in DU-145 Cells

One of the reasons that could lead to a decrease in cell viability and depolarization of the inner mitochondrial membrane is the production of reactive oxygen species (ROS) [44]. Functionally compromised mitochondria generate even more ROS, mainly superoxide anion (O2−) and hydrogen peroxide (H2O2) [44]. Thus, we evaluated the presence of ROS in WT and NEK6-KO cells by DHE labeling (Figure 3A). NEK6-KO showed increased ROS levels, which could explain a reduction in the NEK6-KO cell viability and increased mitochondrial membrane depolarization observed in Figure 2E,F. Increased ROS levels usually result from an imbalance between the production of oxidants and default in their elimination by the antioxidant defense system [45]. Several enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and peroxiredoxin (PRDX), act in defense against oxidative stress [46,47]. Superoxide dismutase (SOD) converts superoxide anion (O2−) to hydrogen peroxide (H2O2), and PRDXs catalyze the reduction of H2O2, alkyl hydroperoxides, and peroxynitrite to water, alcohol, and nitrite, respectively [47]. The GEPIA platform was used to measure the correlation between the antioxidant protein expression: Superoxide Dismutase 1 and 2 (SOD1 and SOD2) and peroxiredoxin 3 (PRDX3) with the NEK6 gene expression in prostate adenocarcinoma samples. Spearman's correlation coefficient showed a moderated correlation between the gene expression of SOD2 and NEK6 and PRDX3 and NEK6 based on coefficients of 0.5 and 0.44, respectively (Figure 3B).

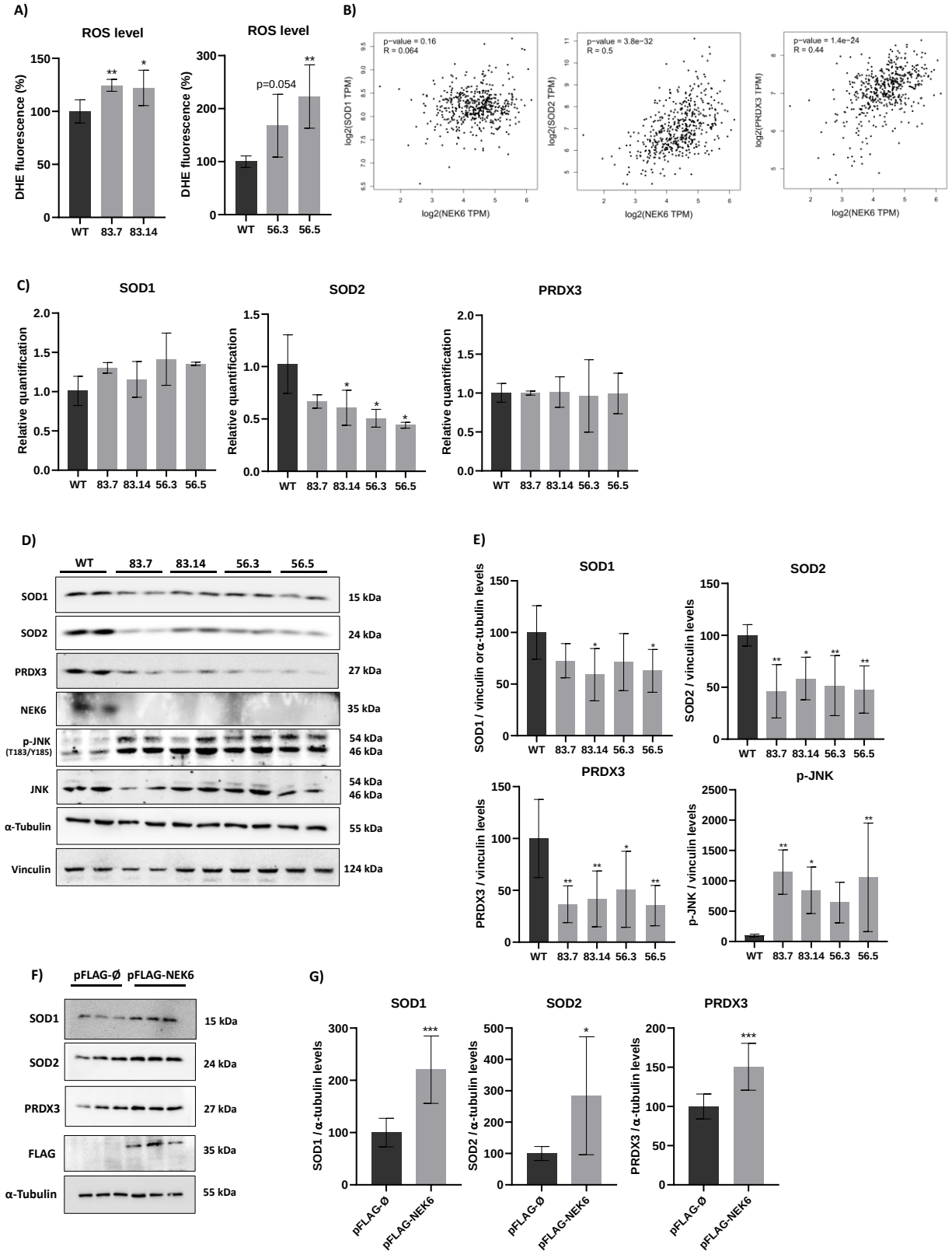

Figure 3. NEK6-KO increases ROS levels and its expression modulates antioxidant defenses in DU-145 cells. (**A**) DU-145 WT and NEK6-KO's ROS levels were measured by DHE staining in flow cytometry. NEK6-KO showed an increase in ROS levels when compared with WT cells. (**B**) Correlation of the gene expression in prostate adenocarcinoma (PRAD) using the GEPIA platform. Spearman's coefficient was used to measure the intensity of the correlation between genes. (**C**)

mRNA levels of SOD1, SOD2, and PRDX3 were analyzed through RT-qPCR. A significant reduction in mRNA of SOD2 was observed. (**D**) The expression of antioxidant proteins (SOD1, SOD2, and PRDX3) was evaluated by Western blotting. Target deletion of the NEK6 gene reduces the expression of SOD1, SOD2, and PRDX3. Additionally, high levels of JNK phosphorylation were observed in NEK6-KO cells. (**E**) Quantification of antioxidant protein expression in WT and NEK6-KO cells. (**F**) NEK6 overexpression in DU-145 WT cells upregulated the SOD1, SOD2, and PRDX3 protein expression. (**G**) Quantification of antioxidant protein expression in FLAG-NEK6 expressing cells. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$, and **** $p < 0.0001$.

Therefore, we evaluated the SOD1, SOD2, and PRDX3 expression at mRNA levels in WT and NEK6-KO cells by RT-qPCR (Figure 3C). The expression of SOD2 was significantly diminished at transcriptional levels in NEK6-KO cells; however, we did not observed any statistical difference in the SOD1 and PRDX3 mRNA levels. SOD1, SOD2, and PRDX3 protein expression were also evaluated by Western blotting (Figure 3D). A significantly reduced protein expression of SOD2 and PRDX3 was observed in all NEK6-KO cell lines, and a reduction of SOD1 expression was noted in 83.14 and 56.5 NEK6-KO cells lines, suggesting that increased ROS levels may be related to a lack of antioxidant defenses, such as SOD1, SOD2, and PRXD3. ROS also promotes the activation of c-Jun N-terminal kinase (JNK), a protein activated by a wide range of cellular stresses [48]. Then, we also evaluated the phosphorylation levels of JNK (T183/Y185), which revealed a statistically significant increase in its phosphorylation in NEK6-KO cells (Figure 3D,E). We also overexpressed FLAG-NEK6 in DU-145 WT cells and evaluated the SOD1, SOD2, and PRDX3 protein levels (Figure 3F). These antioxidant proteins had a statistically significant higher protein expression in NEK6 overexpression DU-145 cells, revealing that NEK6 regulates the antioxidant system through the SOD1, SOD2, and PRDX3 levels (Figure 3G). Here, we suggest that NEK6-KO cells showed higher oxidative stress, since a NEK6 lack causes a reduction in antioxidant defenses, which may be involved with the decrease in cell viability and mitochondria activity.

3.4. Targeted Deletion of NEK6 Increases DNA Damage Markers in DU-145 Cells

ROS are well known as mediators of DNA damage. Ataxia–telangiectasia mutated (ATM) is a sensor kinase of a double-stranded break (DSB), followed by a signaling cascade that culminates in the phosphorylation of histone H2AX [30]. ATM is directly activated by oxidative stress, leading to its autophosphorylation in serine 1981 [49]. Since NEK6 is involved in the cell redox balance, we explored whether NEK6 could be related to the DNA damage response. We analyzed the phosphorylation levels of ATM and H2AX in WT and NEK6-KO cells (Figure 4A,B). Western blotting confirmed that the levels of ATM phosphorylation in serine 1981 and γH2AX were increased in NEK6-KO cells compared to WT cells, suggesting NEK6 is involved in DDR. We confirmed this result by overexpressing NEK6 in WT cells, revealing that NEK6-overexpressing cells presented lower levels of γH2AX (Figure 4C,D). Additionally, we performed the rescue assay by overexpressing NEK6 in all NEK6-KO cell lines and observed that H2AX recovered its phosphorylation (Figure 4E,F). These results indicated that NEK6 regulates DDR pathways, which may be related to the reduction in

viability, proliferation, and alteration in the redox balance in NEK6-KO cells (Figures 2 and 3).

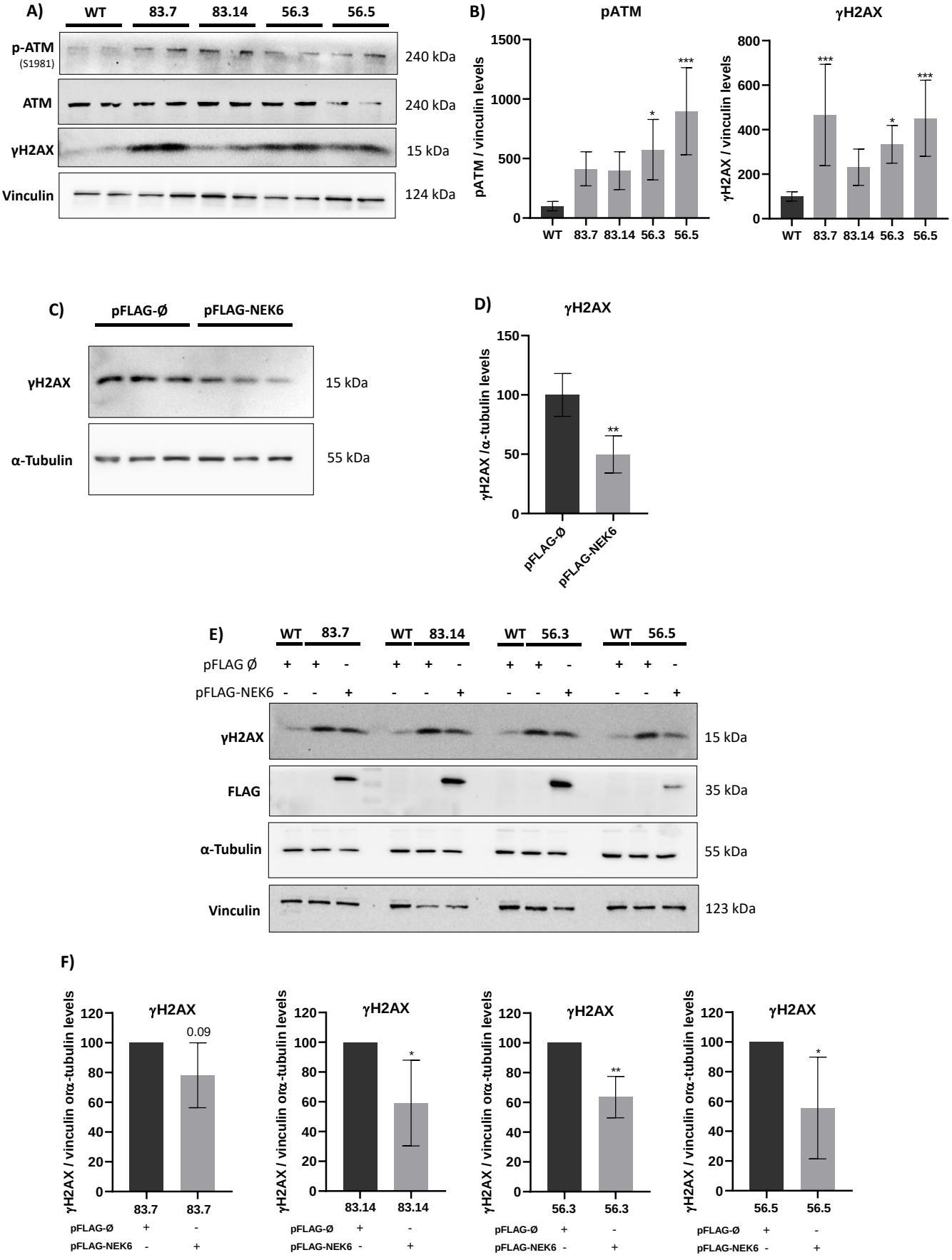

Figure 4. NEK6-KO increases DNA damage response in DU-145 cells. (**A**) NEK6-KO showed an increase in the phosphorylation of DNA damage markers, such as ATM and H2AX. (**B**) Quantification and statistical analyses of the results of Figure 4A. (**C**) NEK6 overexpression in DU-145 WT cells downregulated the phosphorylation of H2AX. (**D**) Quantification and statistical analyses of the results of Figure 4C. (**E**) NEK6 overexpression in NEK6-KO cells partially restores the H2AX phosphorylation levels. (**F**) Quantification and statistical analyses of the results of Figure 4E. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$, and **** $p < 0.0001$.

3.5. Targeted Deletion of NEK6 Induces Death in DU-145 Cells

ROS can activate several signaling pathways that can trigger the process of cell death [25]. One of them is the activation of JNK kinase, leading to apoptosis [50]. We already demonstrated in Figure 3 that NEK6-KO cells presented higher levels of JNK phosphorylation. Furthermore, significant alterations in the DNA damage response, redox balance, and mitochondrial membrane potential were shown in NEK6-KO cells. For this reason, we hypothesized that the loss of NEK6 expression could lead to cellular apoptosis. Therefore, WT and NEK6-KO cells were stained with annexin and propidium iodide and analyzed in flow cytometry, and the parameters such as early and late apoptosis and live cells were analyzed (Figure 5). The 83.7, 56.3, and 56.5 NEK6-KO cell lines showed higher levels of early and late apoptosis and reduced live cells (Figure 5A–D). Bcl-2, which plays an antiapoptotic role by regulating mitochondrial outer membrane permeabilization, was drastically reduced in the NEK6-KO cell lines (Figure 5E,F). The results suggest that elevated ROS levels and subsequent events in NEK6-KO cells may lead to cell death.

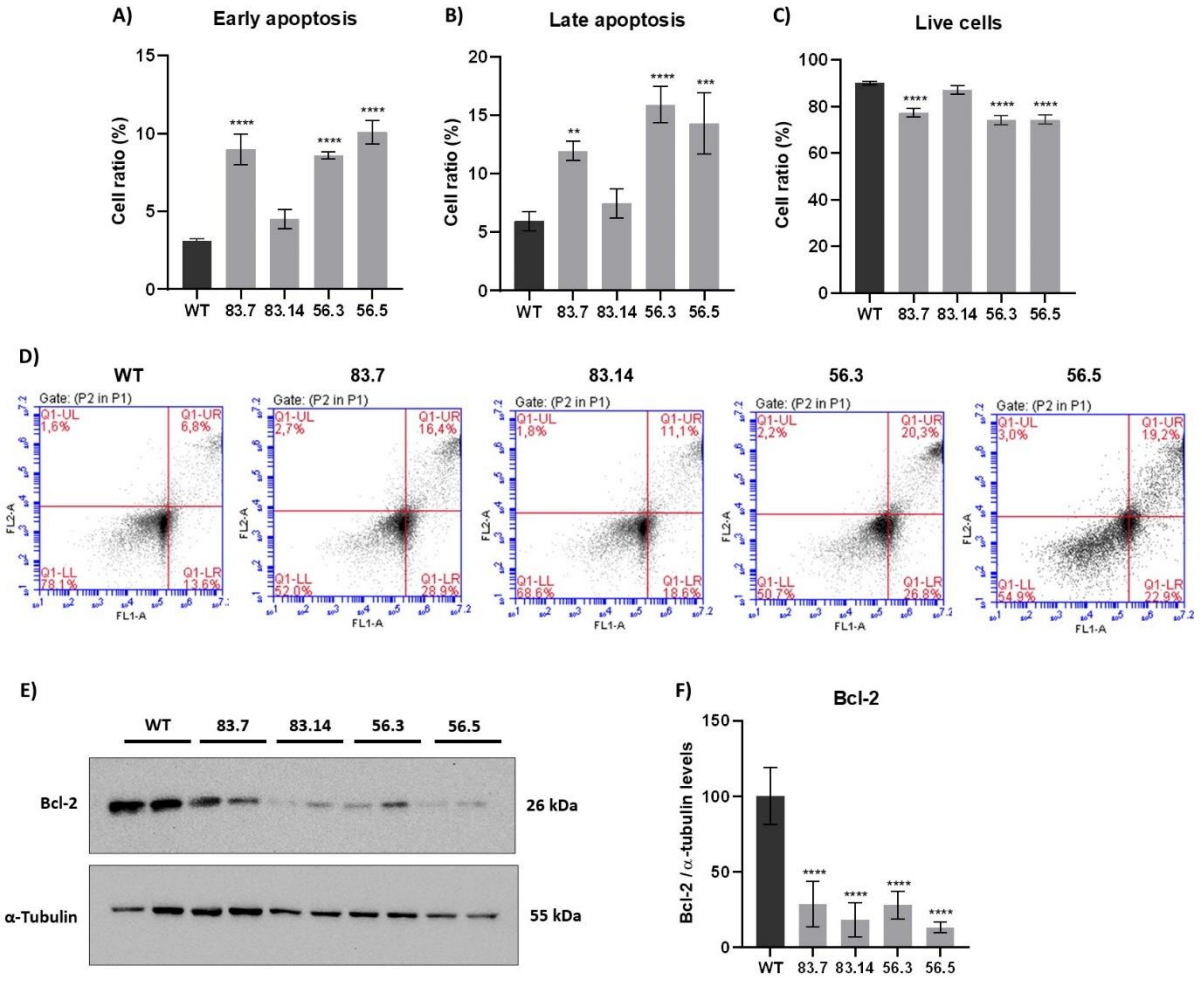

Figure 5. NEK6-KO induces cell death. (**A**) WT and NEK6-KO cells were stained with annexin V and propidium iodide to assess cell death by flow cytometry. NEK6 knockout cells (83.7, 56.3, and 56.5) showed a significant induction of early and (**B**) late cell death, (**C**) with the consequent reduction of live cells. (**D**) Representation of plots obtained by the flow cytometry analysis. (**E**) Bcl-2 expression was analyzed in NEK6-KO cell lines by Western blotting. (**F**) Statistical analysis of Bcl-2 expression. Bcl-2 expression was drastically reduced in NEK6-KO cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3.6. Targeted Deletion of NEK6 Sensitizes DU-145 to Cisplatin through Impairment of Antioxidant Defenses and Increase of DNA Damage

Cancer cells usually preserve a high basal level of ROS and are vulnerable to further increased ROS levels, exceeding a certain defensive threshold. Accordingly, ROS modulation has arisen as an anticancer strategy with the synthesis of various ROS-inducing or -responsive agents that target cancer cells [25,51]. It is known that exposure to cisplatin increases intracellular ROS and DNA damage, leading to apoptosis [52,53]. Considering that NEK6-KO cells have elevated basal levels of ROS, we treated these cells with cisplatin, an agent that induces ROS and DNA damage (Figure 6). We observed that the antioxidant defenses (SOD1, SOD2, and PRDX3); apoptosis marker (Bcl-xL); and DNA damage response (γH2AX) were altered in WT and NEK6-KO cisplatin-treated cells. We further identified that WT cells treated with cisplatin reduce SOD1 and PRDX3 expression. However, the NEK6-KO cells had a

pronounced reduction in SOD1 and PRDX3 expression in the presence of cisplatin compared to the WT cells. Additionally, WT cells treated with cisplatin increased the SOD2 expression, while NEK6-KO had the opposite effect, diminishing the SOD2 expression. Additionally, we also observed that cisplatin treatment in the NEK6-KO cells strongly reduced Bcl-xL expression and increased γH2AX in comparison to the WT cells. These results indicate that the lack of NEK6 sensitizes DU-145 cells to cisplatin through a significant reduction in antioxidant defenses, the induction of DNA damage, and apoptosis.

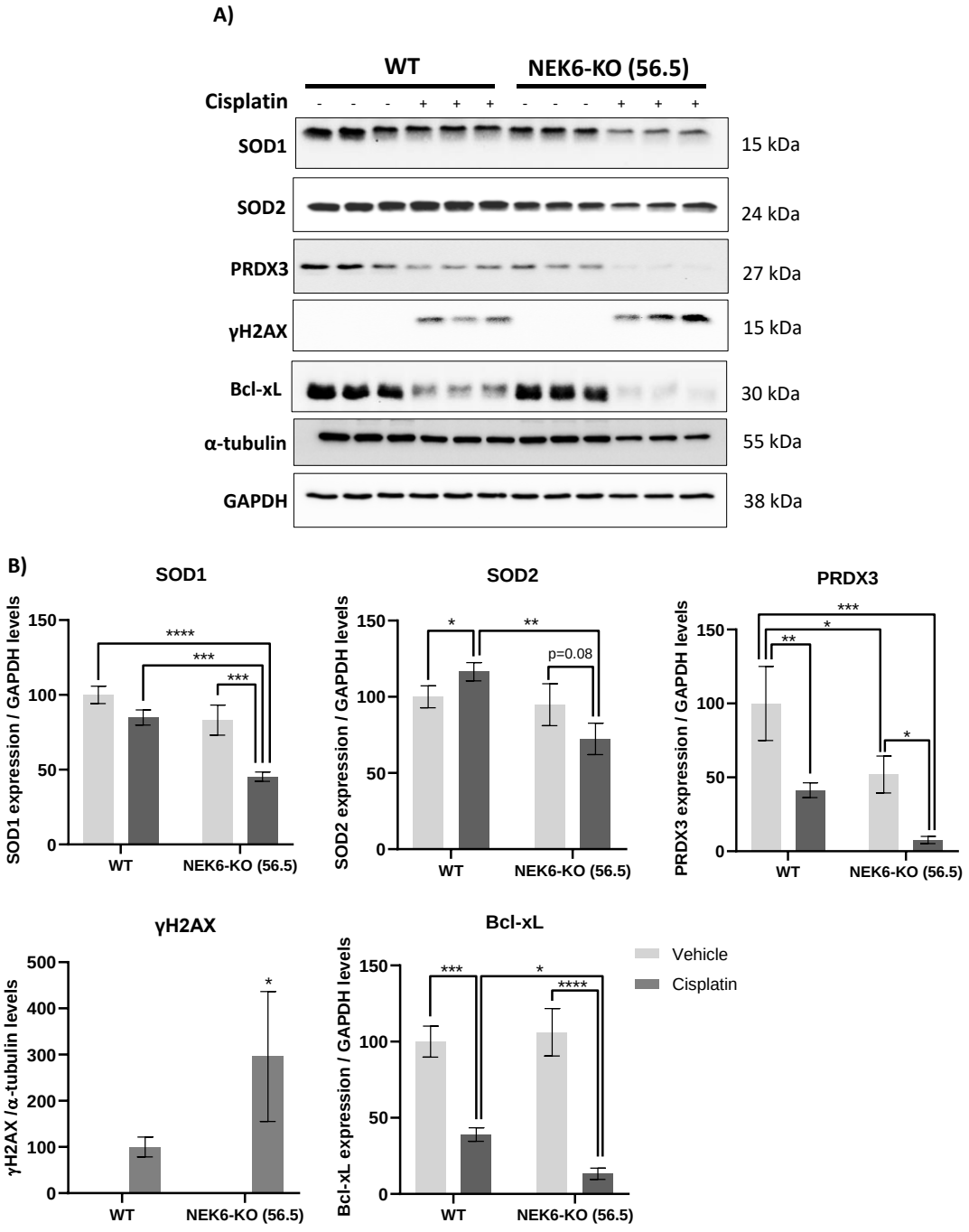

Figure 6. NEK6 depletion sensitizes DU-145 to cisplatin through the impairment of antioxidant defenses and increase of DNA damage. (**A**) WT and NEK6-KO

(56.5) cells were treated with cisplatin 30 μM for 24 hours. SOD1, SOD2, PRDX3, Bcl-xL expression, and γH2AX were evaluated by Western blotting. (**B**) Western blotting quantification was performed using ImageJ. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3.7. NEK6 May Be Involved in the NF-κB2 Translocation to the Nucleus

It is well known that NF-κB promotes cellular survival [54]. One important mechanism by which NF-κB regulates survival pathways is increasing the expression of antioxidant proteins in response to the ROS levels. Therefore, NF-κB attenuates the ROS levels, protecting cells from ROS-induced death [55]. SOD2 is a well-known NF-κB transcription target gene [56–58]. Considering that NEK6-KO cells showed increased ROS, lower levels of SOD2 expression at the mRNA and protein levels and NEK6 overexpression elevated the SOD2 expression, we hypothesized that NF-κB localization and activity could be related to NEK6 expression. To validate this hypothesis, we performed cellular fractionation, isolating the nucleus and cytosol from WT, 83.14, and 56.5 NEK6-KO cells (Figure 7A). We observed that the p52 NF-κB2 subunit was more localized to the nucleus of WT cells than NEK6-KO cells, suggesting that the transcriptional activity of NF-κB2 may be intensified in the presence of NEK6 expression. These data reinforce the hypothesis that NEK6 knockout cells may have a deficiency in the activation of NF-κB2, which could be causing lower levels of SOD2. We also overexpressed NEK6 using GFP-NEK6 plasmid in NEK6-KO (56.5) cells and investigated whether NEK6 could be involved in NF-κB2 nuclear translocation by immunofluorescence (Figure 7B). These data suggest that NEK6 mediates the nuclear translocation of NF-κB2. However, further experiments need to be done to investigate this axis.

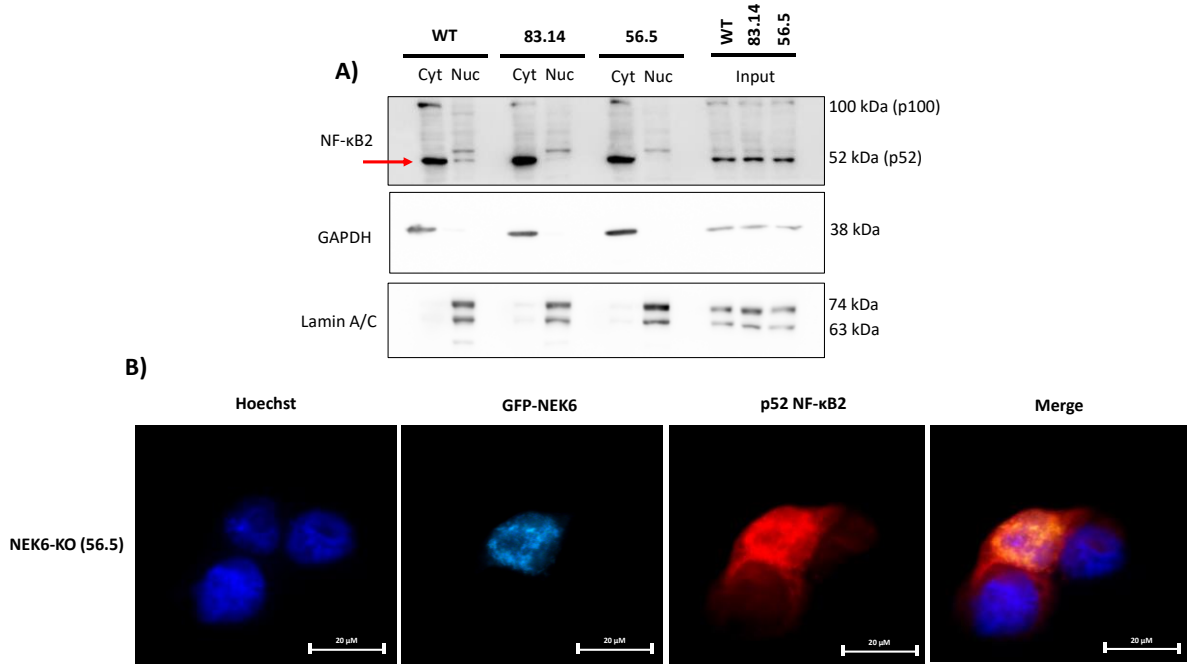

Figure 7. NEK6 is involved in the nuclear translocation of NF-κB2. (**A**) Nuclear (Nuc) and cytoplasmic (Cyt) fractions were obtained from WT and NEK6-KO (83.14 and 56.5) cells. GAPDH and Lamin A/C were used as the cytoplasmic and nuclear control, respectively. (**B**) NEK6-KO (56.5) cells were transfected with GFP-NEK6, and an immunofluorescence assay was performed to stain NF-κB2 in red. Hoechst 33342 was used for staining the nuclei.

4. Discussion

This paper shows that NEK6 is involved in CRPC by regulating the redox balance and DNA damage response. The NEK6 lack in DU-145 prostate cancer cells reduced the proliferation, viability, and mitochondrial membrane potential and induced apoptosis. The literature already emphasizes that the family of NEKs should be explored as interesting targets in cancer treatment [11,19]. The inhibition of NEK6 has been seen as an executable target in cancer, and inhibitors of this kinase have already been explored in other types of cancer [21,59]. For this reason, it is essential to understand the survival pathways that NEK6 regulates in CRPC, because NEK6 inhibitors may be used as a cancer therapy.

Oxidative stress may cause aggressiveness in most cancer types, including prostate cancer. A moderate increase in ROS induces cell proliferation, whereas excessive amounts of ROS promote cell death [25,60]. Cancer cells react to an increase in the ROS levels and survive in this oxidative stress ambient by inducing the transcription of antioxidant enzymes. Then, it is essential to understand these pathways for efficiently elaborate therapies that modify the ROS levels. ROS inductors seem to have the ability to induce apoptosis and inhibit tumor growth, cell migration, and invasive properties in several cancers [60,61]. Here, we showed that a NEK6 lack reduces the expression of SOD2 at the mRNA level and also SOD1, SOD2, and PRDX3 at the protein level. Additionally, NEK6 overexpression in DU-145 cells increases the same antioxidant proteins. Along with that, the lack of NEK6 generates more ROS in DU-145 cells. These data suggest that NEK6 regulates the redox balance through the modulation of antioxidant proteins. Additionally, SOD1, SOD2, and PRDX3 have extensive literature showing their pro-oncogenic roles and their involvement with chemotherapy resistance through oxidative stress [62,63,64,65,66,67,68,69,70,71]. The modulation of these antioxidant proteins reveals a new way for NEK6 regulating cell survival in CRPC.

SOD1 is an essential antioxidant enzyme that is widely distributed in the cell. Its main function is to catalyze the dismutation of O2− into H2O2, protecting cells from oxidative stress [64]. A study has shown that a specific SOD1 inhibitor repressed cancer cell growth and promoted cancer cell cycle arrest and apoptosis by mediating the ROS changes in several cancers [62]. SOD1 has also been linked to chemotherapy resistance [63,65]. SOD1 was identified as overexpressed in prostate cancer xenograft animals resistant to mitoxantrone (MTX), an antineoplastic agent used in CRPC [63]. For future studies, we suggest that an eventual combination of NEK6 inhibitors with MTX may be an interesting strategy for reducing the resistance of cancer cells to the chemotherapeutic agent.

SOD2 is an enzyme that catalyzes the dismutation of O2− into H2O2 in the mitochondria, causing a reduction of ROS. The role of SOD2 in cancer is controversial. Initially, SOD2 was considered a tumor suppressor gene based on its low levels in several types of cancer [72]. However, more recent studies revealed that low levels of SOD2 are an early event of tumors, and higher levels of SOD2 are associated with

tumor progression and metastasis [73]. The mRNA and protein levels of SOD2 were found elevated in samples from patients with middle stage prostate cancer [73]. SOD2 has recently been shown to increase the GLUT-1 and glucose uptake, which is essential for prostate cancer cell survival [74]. Several transcription factors are shown to regulate SOD2 expression, such as Nuclear Factor-Kappa B (NF-κB) and Nuclear factor erythroid 2-related factor 2 (Nrf2), among others [75,76]. Since the absence of NEK6 has modulated the SOD2 expression at the mRNA and protein levels, we evaluated the nuclear content of p52 NF-κB2 in NEK6-overexpressing cells. We observed that NEK6 regulated the nuclear localization of p52 NF-κB2. We suggest NEK6 may also regulate survival pathways and SOD2 expression by modulating NF-κB signaling.

PRDX3 is a mitochondrial peroxidase, which plays a role in cell protection against oxidative stress by detoxifying peroxides [77]. PRDX3 is overexpressed in several types of cancer, including prostate cancer, protecting cells against apoptosis [68,69,70,71]. Additionally, PRDX3 has been found overexpressed in castration-resistant prostate cancer cells, which culminates in promoting cell survival by protecting them from oxidative stress [71]. For this reason, PRDX3 is a potential target for CRPC. A study by our group showed that NEK6 interacts with PRDX3 [40]. NEK6 may regulate the expression of PRDX3 through this previously established interaction.

ROS promote the phosphorylation and activation of JNK and DNA damage response, leading to apoptosis [48,78]. The phosphorylation and activation of ATM and JNK mediate the formation of yH2AX foci [79]. NEK6 knockout induces ROS generation, activates JNK, a stress-sensing kinase, and induces DNA damage. Considering the cellular effects of a NEK6 lack on DNA damage and apoptosis, we suggest that future studies can explore NEK6 inhibitors as potential inducers of synthetic lethality in prostate cancer.

Bcl-2 is located in the mitochondrial outer membrane (MOM) and plays important antiapoptotic roles [80]. The permeabilization of MOM releases proapoptotic factors such as the apoptosis-inducing factor (AIF) and cytochrome C from the mitochondria. AIF enters the nucleus and generates extensive DNA fragmentation, while cytochrome C in the cytosol can initiate the activation cascade of caspases. The antiapoptotic Bcl-2 contributes to MOM integrity and prevents the release of these proapoptotic factors from the mitochondria [81]. Additionally, Bcl-2 is critical for the survival of androgen-independent prostate cancer cells and also required for the progression of prostate cancer cells from an androgen-dependent to an androgen-independent growth stage [82]. Thus, we evaluated apoptosis and Bcl-2 expression in the WT and NEK6-KO cell lines. We revealed that NEK6-KO reduces Bcl-2 protein expression and increases apoptosis in DU-145 cells. We also found that NEK6-KO cells showed an increase in mitochondrial membrane depolarization, which may be related to a reduction in the Bcl-2 levels and induction of apoptosis. These results revealed novel cell survival mechanisms regulated by NEK6 in a cellular model of CRPC.

By exploring the modulation of antioxidant defenses in WT and NEK6-KO cells, we observed that SOD1, SOD2, and PRDX3 expression

were significantly reduced in NEK6-KO cells treated with cisplatin when compared to WT cells, which may explain why NEK6-KO cells have a higher sensitivity to this drug. The increase in SOD2 expression may be a mechanism that triggers a resistance to chemotherapy [66,67]. The TNF-α-mediated upregulation of SOD2 is involved in cisplatin resistance in esophageal cancer. The upregulation of SOD2 by TNF-α was inhibited by blocking the NF-κB pathway, suggesting that SOD2 via the NF-κB signaling pathway contributes to the proliferation of esophageal cancer cells [67]. In our data, SOD2 expression was found elevated in WT cells treated with cisplatin, suggesting a resistance mechanism in WT cells. A reduction in SOD2 expression in NEK6-KO cisplatin-treated cells means that this possible resistance mechanism found in WT was eliminated in NEK6-KO cells. Data showing a lower expression of Bcl-xL in NEK6-KO cisplatin-treated cells highlight an induction of apoptosis in these cells compared to WT cells. Cisplatin treatment also increased γH2AX in NEK6-KO cells compared to WT, showing a NEK6 lack causes genomic instability and may be responsible for increased cell death in NEK6-KO cells. Thus, we suggest that a NEK6 lack sensitizes cells to cisplatin, since the treatment with cisplatin in these NEK6-deficient cells greatly diminished the antioxidant defenses (SOD1, SOD2, and PRDX3), which may culminate in excessive ROS levels and DNA damage. Targeting NEK6 in combination with other anticancer drugs that destabilize the redox balance may be an interesting strategy to increase the ROS levels above the tolerable levels for cells. Another point is that, in Figure 2F, we observed a decrease in the mitochondrial membrane potential in NEK6-KO cells, indicating a reduction in mitochondrial activity. Moreover, cisplatin impairs the electron transport chain function, increases the intracellular ROS levels, and affects the mitochondrial viability [83]. Then, cisplatin may be acting in synergism with the effects in mitochondria mediated by the NEK6 lack, sensitizing these cells to cisplatin.

We performed our experiments using only one cell line, which may be considered a limitation of our study. We believe that the role of NEK6 in other prostate cancer cell lines should be further investigated, but our present data point out an important role of NEK6 in cisplatin sensitivity and as a co-adjuvant therapy for prostate cancer treatment.

5. Conclusions

This study showed that NEK6 regulates the redox balance and DNA damage response in a model of a castration-resistant prostate cancer cell line. The regulation of antioxidant defenses by NEK6 may be interesting in strategies focused on exacerbating the ROS levels and generating DNA damage. A NEK6 lack reduced proliferation, viability, cell migration, and the mitochondrial potential membrane, while elevating the ROS levels and DNA damage-induced cell death. A NEK6 lack sensitizes DU-145 cells to cisplatin. These data revealed that NEK6 may be an important therapeutic target in CRPC. Exploring the effects of NEK6 inhibitors on CRPC, alone and together with other chemotherapeutic agents, may be relevant for a new treatment approach. Future studies should investigate the regulation of NF-κB2 by NEK6 and explore whether it is mediated by physical interactions, for example, phosphorylation, or indirectly by other interactors. The involvement of NEK6 in the mitochondrial dynamics can also be investigated, as we have seen that NEK6 alters the expression of several mitochondrial proteins.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1: Figure S1. Detection of up to four indels in the NEK6-KO cell lines by Sanger-type sequencing. Figure S2. Knockout of NEK6 alters NEK2, NEK7, NEK9, and NEK11 expression.

Author Contributions: I.C.B.P. designed the study, conducted the experiments, and wrote the manuscript. F.L.B., I.R.e.S., and L.K.I. designed the study. M.B.S., M.C.S.M., M.M.G., and L.G.S.d.S. assisted in the experiments. R.M.N.B. and F.M.S. revised the manuscript, provided critical infrastructure, obtained funding, and co-supervised the study. J.K. conceived the study, obtained the main funding, and supervised the project. All authors have read and agreed to the published version of the manuscript.

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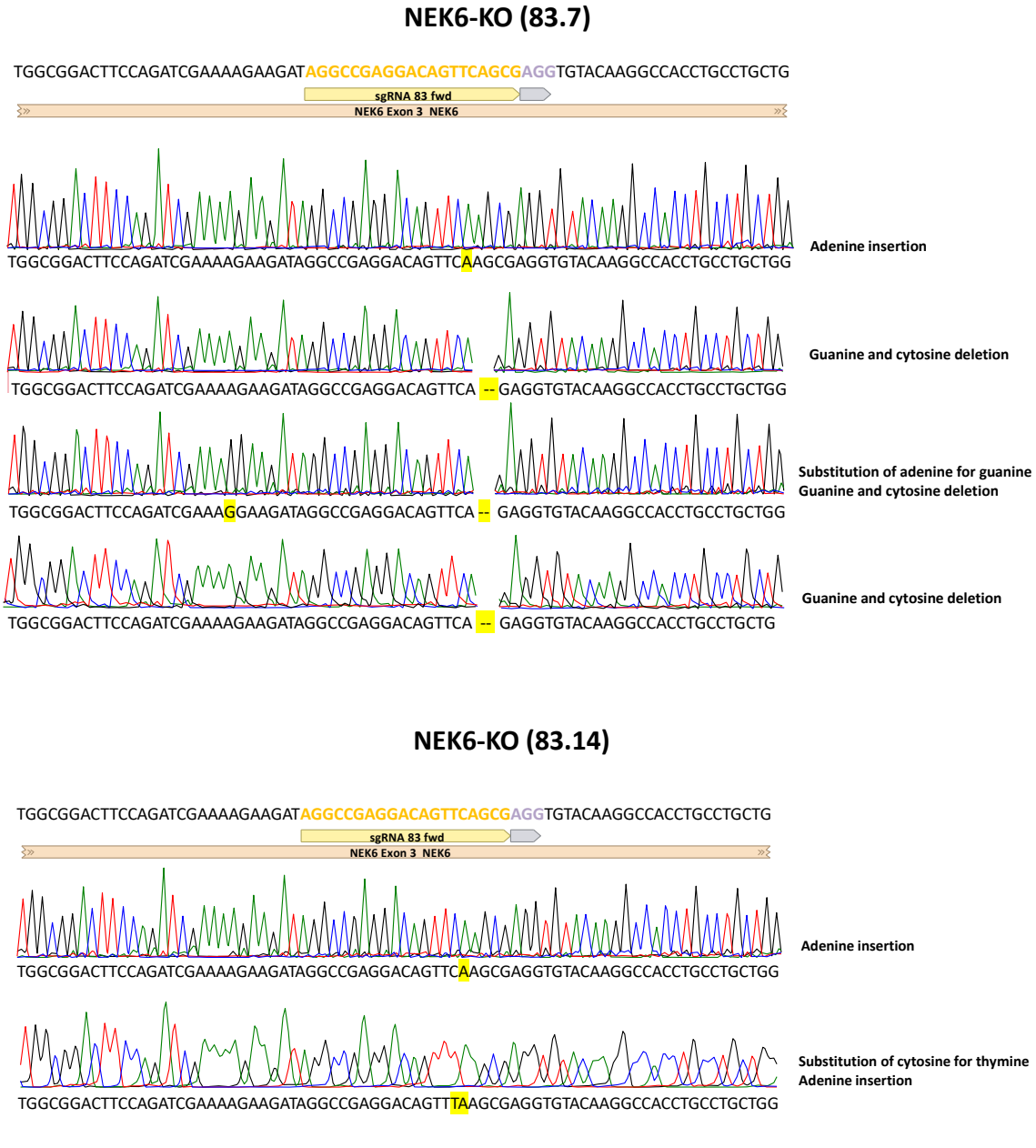
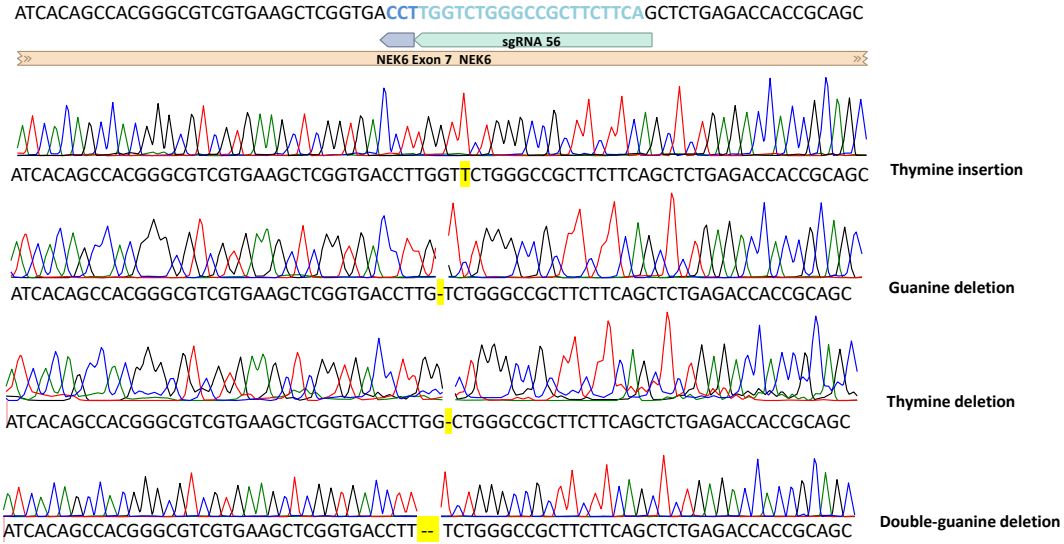
Supplementary files

Supplementary figure 1 (Figure S1). Detection of up to four indels in NEK6-KO cell lines by Sanger-type sequencing.



NEK6-KO (56.3)



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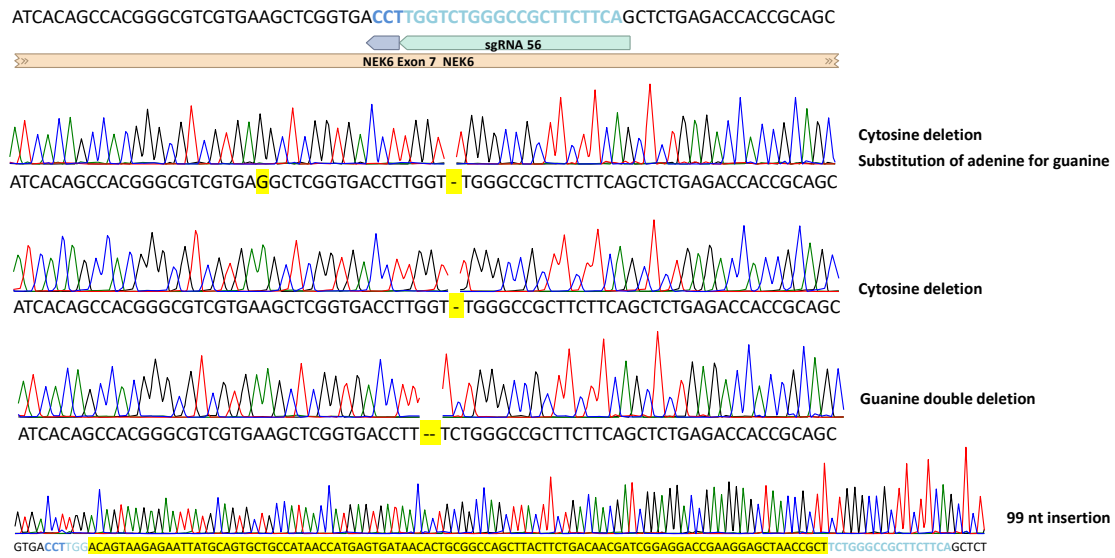
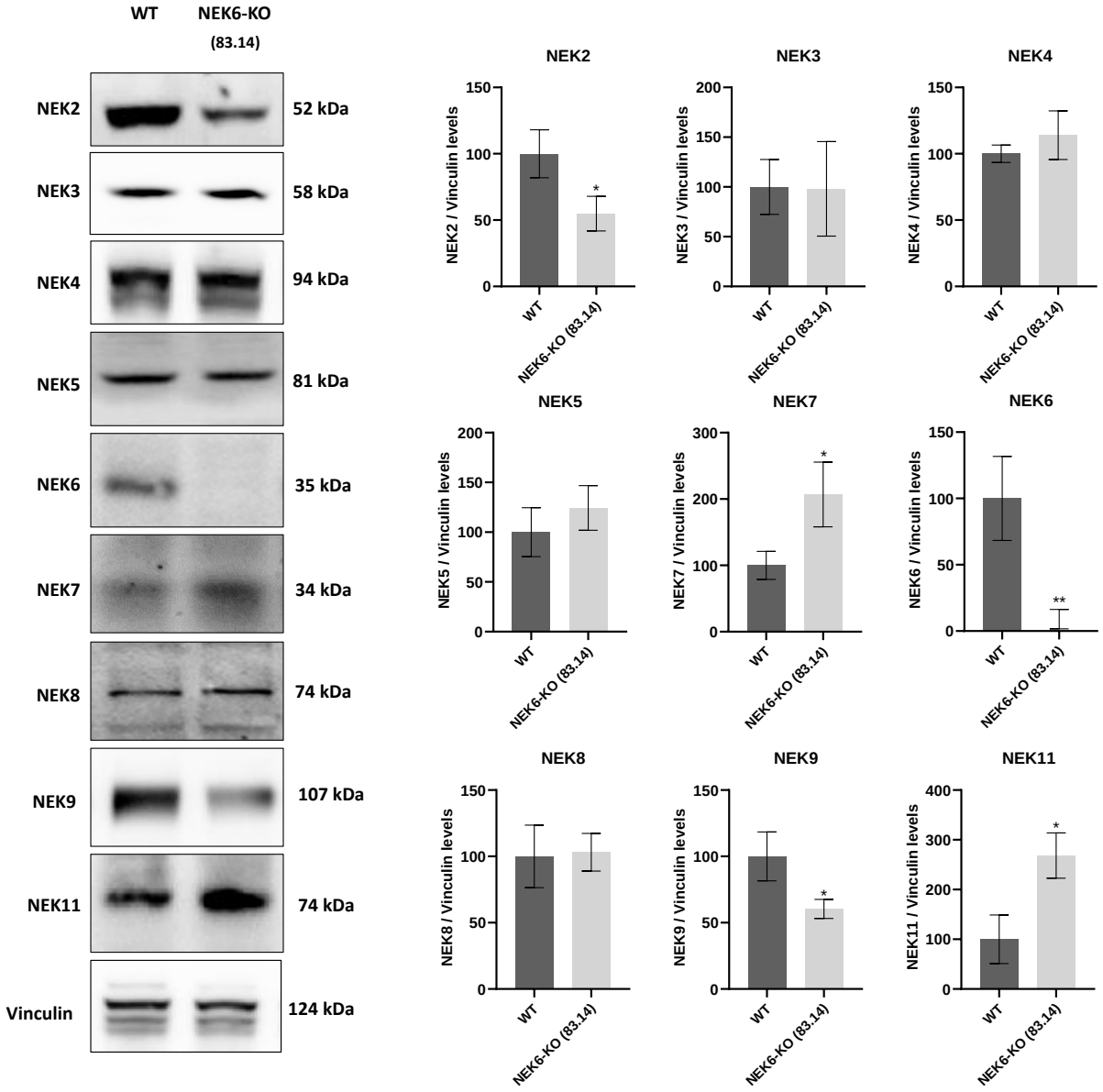


Supplementary figure 2 (Figure S2). Knockout of NEK6 alters NEK2, NEK7, NEK9, and NEK11 expression.



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3.2.1 Dados complementares e não publicados referentes ao artigo anterior

Material and methods

NEK6-I treatment followed by scratch and western blotting.

NEK6-I or (MolPort-002-933-483) ((5Z)-2-hydroxy-4-methyl-6-oxo-5-[(5-phenylfuran-2-yl)methylidene]-5,6-dihydropyridine-3-carbonitrile) was purchased from MolPort (Molport-002-933-483). DU-145 cells were seeded at a density of 2×10^5 cells/well in 24-well plates, incubated until confluence, and cultured in RPMI 1640 containing 1% FBS treated with 5 µg/mL mitomycin-C (Sigma, 10107409001) for 2 h to inhibit proliferation. After mitomycin incubation, RPMI 1640 media containing 1% FBS and NEK6-I at 30 µM was added to the cells for 48 h. The images were captured at 0, 24, and 48 h. The scratch area was analyzed under a light microscope (Optika Italy) and using Optika Proview Software. To analyze protein expression, we treated the cells with NEK6-I at 30 µM for 48 hours and collected the protein extract for western blotting.

Cisplatin and NAC treatment

DU-145 cells were plated in 6-well plates (2×10^5 cells/well) and at 96 well-plates (8×10^3 cells/well). After 24 hours cisplatin (Calbiochem, CAS 15663-27-1,) was used at dilutions ranging from 15 µM to 75 µM 24 hours to analyze NEK6 expression and cell viability by MTT assay, respectively. To analyze the impact of ROS in cisplatin treatment, we used NAC (Sigma, #A7250) with 10 mM for 24 hours and cisplatin at 30 µM for 24 hours. MTT assay was performed to evaluate cell viability.

Results

NEK6 regulates cell migration in DU-145 cells.

A recent NEK6 inhibitor (NEK6-I) was identified by De Donato and colleagues (Donato, De *et al.*, 2018). This compound was able to inhibit the proliferation of ovarian, lung, and colon cell lines with IC_{50} values below 100 µM. The inhibitor alters NEK6 activity, but not its expression. Another interesting point is that the inhibitor does not interfere with NEK7 activity, the member of the NEKs family with the greatest structural similarity. We investigated the role of this NEK6 inhibitor in cell migration. Then, we treated the cells with mitomycin-C to not influence cell proliferation, followed by 30 µM of NEK6-I. We found that NEK6-I significantly reduced DU-145 cell migration at 48

hours (Figure 1A). NEK6-I also reduced the expression of SOD1, and SOD2 and increased the phosphorylation of H2AX (Figure 1B). Thus, NEK6 is involved in cell migration, which may be through the regulation of antioxidant defenses and DNA damage pathways.

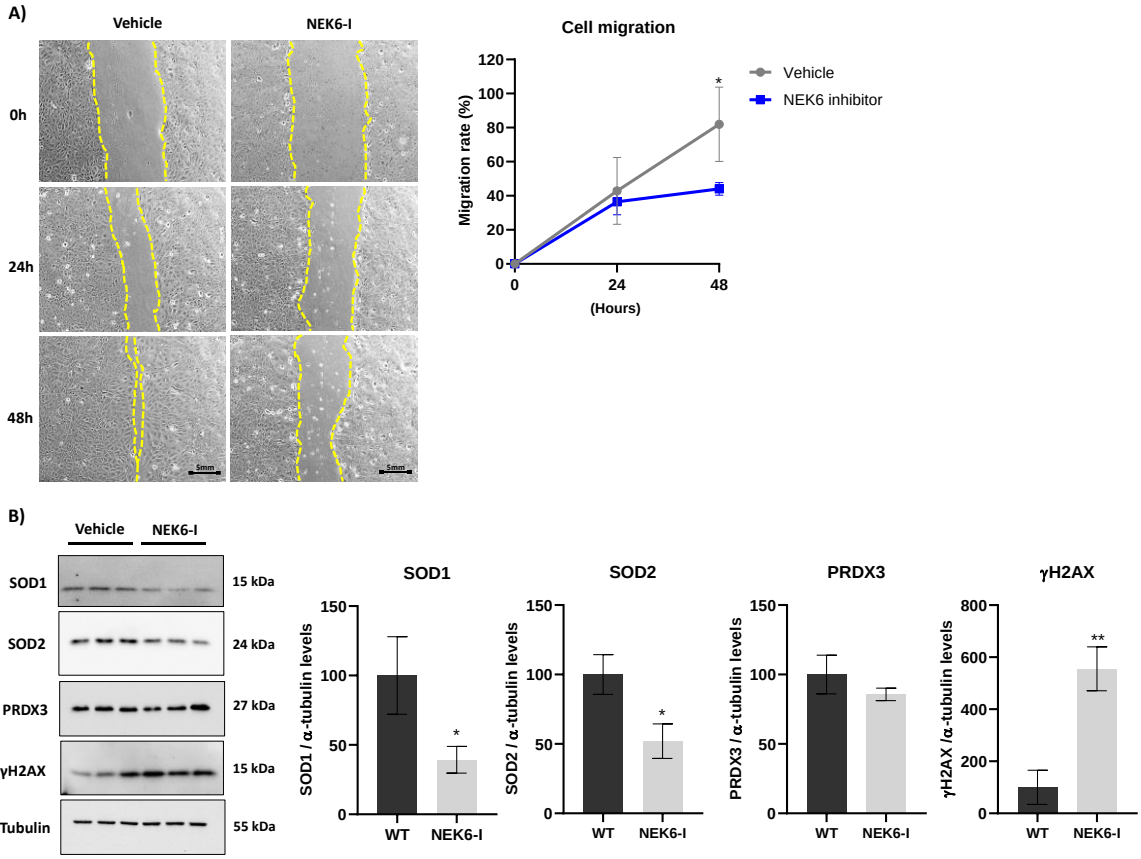

Figure 1. NEK6 is involved in cell migration in DU-145 cells. (A) DU-145 WT cells were treated with NEK6-I (30 µM) for 48 hours. Wound-healing assay was performed and images were registered at 0, 24, and 48 hours. Scratch areas were analyzed by ImageJ. **(C)** DU-145 WT cells were treated with 30 µM NEK6-I for 48 hours. SOD1, SOD2, PRDX3 expression, and γH2AX were analyzed by western blotting.

NEK6 is involved in the cisplatin response.

Cancer cells usually preserve a high basal level of ROS and are vulnerable to further increased ROS levels exceeding a certain defensive threshold. Accordingly, ROS modulation has arisen as an anticancer strategy with the synthesis of various ROS-inducing or responsive agents that target cancer cells (Ndombera, 2017; Perillo *et al.*, 2020). It is known that exposure to cisplatin increases intracellular ROS and DNA damage, leading to apoptosis (Marengo *et al.*, 2011; Zuliani *et al.*, 2005). Considering NEK6-KO cells displayed increased ROS levels and DNA damage, we challenged DU-145 WT cells with cisplatin using several concentrations (15-75 µM) for 24 hours.

Cisplatin reduced NEK6 expression in a dose-dependent manner (Figure 2A). After that, WT and NEK6-KO cells (83.14 and 56.5) were treated with cisplatin at the same conditions described in Figure 2A. We demonstrated that NEK6-KO cells were more sensitive to cisplatin than WT cells (Figure 2B). Then, we investigated whether cisplatin reduced cell viability through oxidative stress. DU-145 WT and NEK6-KO cells (56.5) were treated with 30 µM of cisplatin and 10 mM of NAC for 24 hours. We observed that NAC recovered cell viability from cisplatin-treated cells, and it was more pronounced in 56.6 NEK6-KO cells (Figure 2C). To assess whether H2AX phosphorylation could be in part mediated by ROS, we treated DU-145 WT and NEK6-KO cells with 10 mM of NAC for 24 hours and evaluated yH2AX phosphorylation (Figure 2D). We observed a reduction in H2AX phosphorylation in 83.14, 56.3, and, 56.5 NEK6-KO cells treated with NAC when compared to non-treated cells.

The reactive oxygen species generated in cells lacking NEK6 may be related to the DNA damage observed in these cells. Therefore, NEK6 knockout cells may respond more to cisplatin both by exceeding acceptable levels of ROS, but also by, together with cisplatin, causing greater genomic instability, leading to cell death. Therefore, we suggest that NEK6 is involved in the cellular response to cisplatin, which could be through the regulation of oxidative stress and DNA damage.

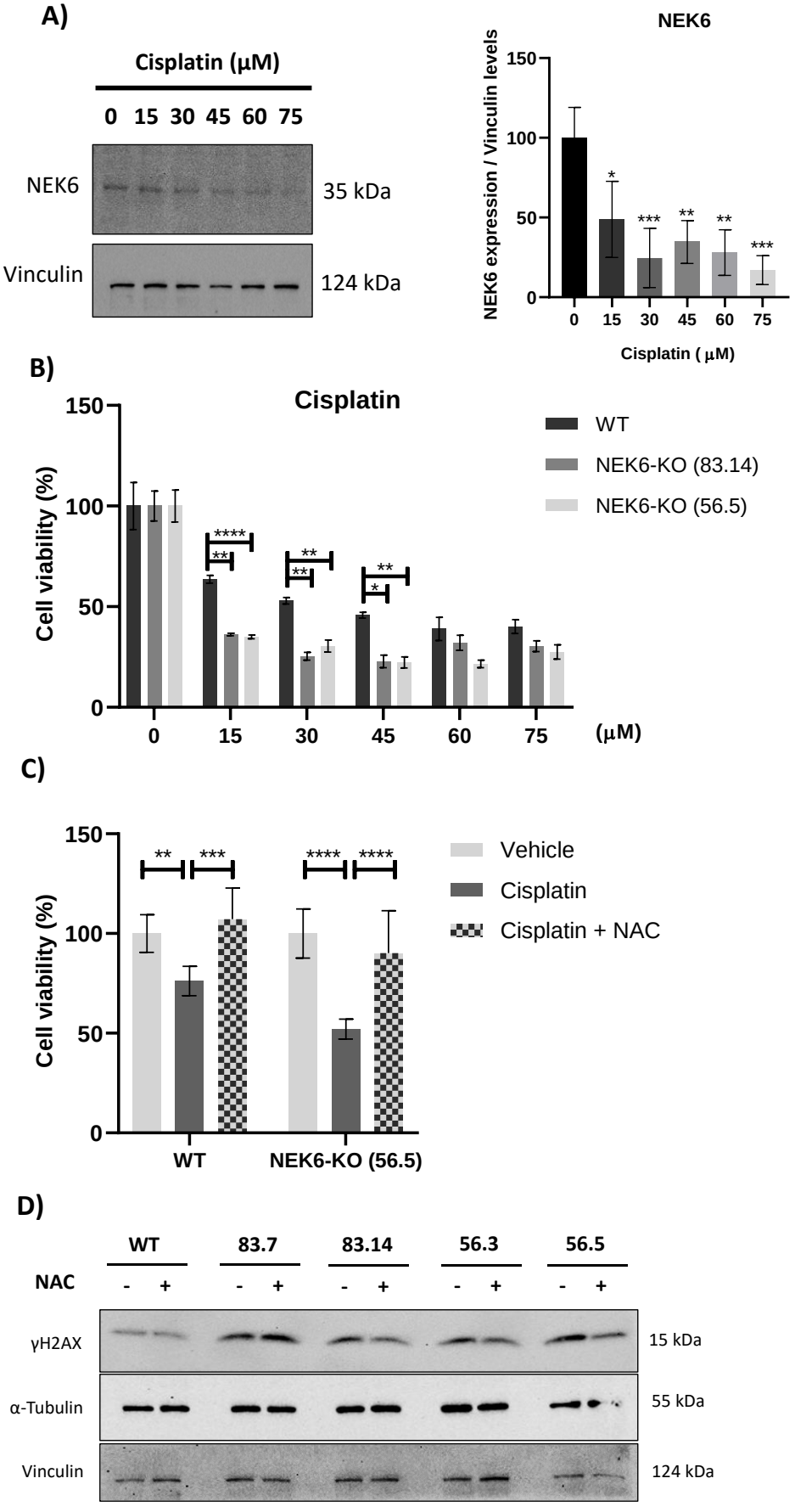
A)
Cisplatin (μM)
0 15 30 45 60 75
NEK6
35 kDa
Vinculin
124 kDa
NEK6
NEK6 expression / Vinculin levels
Cisplatin (μM)
B)
Cisplatin
WT
NEK6-KO (83.14)
NEK6-KO (56.5)
Cell viability (%)
(μM)
C)
Vehicle
Cisplatin
Cisplatin + NAC
Cell viability (%)
WT
NEK6-KO (56.5)
D)
WT
83.7
83.14
56.3
56.5
NAC
γH2AX
α-Tubulin
Vinculin
15 kDa
55 kDa
124 kDa

Figure 2. Cisplatin reduces NEK6 expression and NEK6-KO sensitizes DU-145 cells to cisplatin. (A) DU-145 WT cells were treated with several concentrations of cisplatin for 24 hours and the NEK6 expression was evaluated by western blotting. **(B)** WT and NEK6-KO cells (83.14 and 56.5) were treated with cisplatin in the same condition of figure 2A. **(C)** WT and NEK6-KO (56.5) were submitted to cisplatin (30 µM) and NAC (10 mM) treatments for 24 hours. **D)** DU-145 WT and NEK6-KO cells were treated with NAC and γH2AX were evaluated by western blotting.

4. ARTIGOS EM ELABORAÇÃO

4.1. Targeting NEK6 promotes docetaxel-induced chemosensitivity in prostate cancer cells

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Abstract

Studies have shown that NEK6 mediates the development of castration-resistant prostate cancer (CRPC). In a previous study, using CRISPR/Cas9 system, we showed that NEK6 knockout reduced antioxidant defenses, increased Reactive Oxygen Species and DNA damage, and also intensified cisplatin effects in prostate cancer cells. However, the pathways regulated by NEK6 in CRPC and also its relevance in chemotherapy strategies need to be further elucidated. The use of NEK6 inhibitors is an innovative and recently proposed strategy for other types of cancer. Here, we used a NEK6 inhibitor to evaluate the chemosensitivity induced by co-treatment with docetaxel, the main chemotherapeutic agent in CRPC. We also used NEK6 knockout in DU-145 and PC-3 cells to evaluate the chemosensitivity to docetaxel. We observed that compound 8 sensitizes DU-145 cells to docetaxel treatment, induced significant markers of apoptosis (PARP1 cleavage, BAK), reduced survival pathways (phosphorylated p65), and cell migration (TJP1). The NEK6 inhibitor alone was able to significantly reduces Bcl-xL, phosphorylated Bad, and TJP1 expressions. Also, the NEK6 inhibitor displayed an attractive IC_{50} in DU-145 and PC-3 prostate cancer cell lines, which may be an interesting compound to be explored in prostate cancer. Co-treatment with docetaxel and NEK6 inhibitor significantly reduced colony formation and cell migration. NEK6 knockout in DU-145 and PC-3 cells sensitizes cells to docetaxel treatment. Overall, NEK6 may be a powerful target in prostate cancer cells.

Key-words: NEK6, CRPC, docetaxel, NEK6 inhibitor, chemosensitivity

Introduction

Prostate cancer is the second most common type of cancer in men worldwide [1]. Although commonly employed therapies such as surgery, radiotherapy, and hormone deprivation have increased patient survival, some individuals develop a condition called castration-resistant prostate cancer (CRPC), in which cancer cells continue to grow even in the absence or low levels of testosterone [2]. Patients undergoing CRPC treatment have a very significant reduction in survival and are generally associated with an advanced stage of the disease and metastasis [3]. Docetaxel is a chemotherapeutic approved by the FDA in 2014 for the treatment of metastatic CRPC [4]. Half of the cases respond to docetaxel, but unfortunately, develop resistance later on [5]. Therefore, finding a drug that improves docetaxel response and prevents the development of resistance and metastasis would be of extreme interest for the treatment of individuals with CRPC.

NIMA (Never In Mitosis, gene A)-related kinase-6 (NEK6) is a serine/threonine protein kinase that is part of the Never In Mitosis A Kinases (NIMA) family [6]. NEK kinases are less explored kinases involved in the regulation of the cell cycle [7], primary cilia [8–10], mitochondrial activity [11–13], and DNA damage [13]. NEKs are considered biomarkers of several types of cancer, such as breast [14], liver [15], colorectal [16], prostate [13,17], lung [18], ovarian [19], pancreatic [20], thyroid [21], and gastric [22].

The high-throughput screening starting from 601 kinases, showed that NEK6 is the main kinase responsible for castration resistance in prostate cancer [17]. A study carried out by our group showed that NEK6 knockout reduced proliferation, viability, and mitochondrial activity, in addition to reducing the expression of antioxidant defenses and increasing the Reactive Oxygen Species (ROS) and DNA damage in prostate cancer cells [13]. NEK6 inhibitors have been promisingly investigated [23]. (5Z)-2-hydroxy-4-methyl-6-oxo-5-[(5-phenylfuran-2yl) methylidene]-5,6-dihydropyridine-3-carbonitrile, also named compound 8, was identified as a compound able to inhibit NEK6, displaying antiproliferative activity against a panel of human cancer cell lines [24]. Besides, compound 8 displayed a synergistic effect with cisplatin and paclitaxel in an ovarian cancer cell line [24].

The present study aimed to evaluate the relevance of NEK6 in DU-145 and PC-3 prostate cancer cells and mainly how cancer cells behave with their inhibition or depletion plus docetaxel treatment. For this purpose, we investigated whether the

NEK6 inhibitor (compound 8) further sensitizes prostate cancer cells to docetaxel. Also, we knocked out the NEK6 gene in DU-145 and PC-3 cells to evaluate chemosensitivity to docetaxel. Alterations in cell viability, colony formation, and cell migration, as well as modulation in apoptosis, survival, and cell migration pathways, were assessed in the absence of NEK6 expression or inhibition.

Material and methods

Cell culture

DU-145 and PC-3 cell lines were cultivated in RPMI 1640 (Thermo Scientific, #11875093) medium supplemented with 10% fetal bovine serum (FBS, #12657029) and 1% penicillin/streptomycin (Gibco, #15140-122). Cells were cultured at 37 °C in an incubator containing 5% carbon dioxide. NEK6-KO clones in DU-145 cells were characterized as described in [13]. NEK6-KO clones in PC-3 cells were generated using a previous study of Pavan et al. (2023) and characterized by western blotting and in silico by Synthego's ICE analysis (Supplementary Figure 1). Cells were used for experiments within 10-25 passages from thawing.

TMNplot and GEPIA analysis

TMNplot is an integrated database containing a set of available data at the transcriptome level of normal and tumor cells (www.tnmplot.com/analysis/) [25]. Transcriptional expression of NEK6 from normal and tumor tissues was observed by TMNplot. Fold-change was obtained by its mean (fold-change mean). The Gene Expression Profiling Interactive Analysis (GEPIA) database was used to compare survival overall in prostate adenocarcinoma patients with lower and higher NEK6 expression (www.gepia.cancer-pku.cn/) [26]. GAPDH was used as an expression normalizer. It was considered as statistical significant differences in results with $p<=0.05$.

Cell viability assay

To assess the viability of docetaxel-treated cells, we used the MTT assay. WT and NEK6-KO cells were plated in 96-well plates (1×10^4 cells/well) and cultured overnight. The cells were treated with concentrations of 10, 20, 40, 80, and 160 nM of docetaxel for 48 hours, and 10 µL of MTT stock solution (12 mM) was added to the cells. After 2 hours, a solution of isopropanol and 1N HCl in a 25:1 ratio were added to

cells and the formazan crystals were solubilized for 15 min at 37 °C. The absorbance was measured at 570 nm.

Determination of IC_{50} value

NEK6 inhibitor ((5Z)-2-hydroxy-4-methyl-6-oxo-5-[(5-phenylfuran-2-yl)methylidene]-5,6-dihydropyridine-3-carbonitrile) (MolPort-002-933-483), called NEK6-I in our study, were tested at 15, 30, 45, 60, 75, and 90 µM for 48 hours. Cell viability was measured by MTT assay. X Values were transformed using X=Log(X) and further, nonlinear regression was performed. IC_{50} values were obtained in Prisma 8.0 software. The experiment was performed three times independently.

Clonogenic assay

Wild-type DU-145 and PC-3 cells were treated in 6 well-plates with docetaxel (5 nM), NEK6-I (DU-145: 37.46 µM and PC-3: 48.2 µM), or a combination of docetaxel and NEK6-I (DU-145 5 nM docetaxel + 37.46 µM NEK6-I; PC-3 5 nM docetaxel + 48.2 µM NEK6-I). Following 48 hours of incubation, the cells were trypsinized, and 5×10^2 cells were plated in 6-well plates. Dishes were cultured in the incubator for 8 days at 37 °C and then stained with violet crystal solution (0.05% violet crystal w/v, 1% formaldehyde, 1% PBS, 1% methanol, and deionized water) for 20 minutes at room temperature. The number of colonies was quantified using ImageJ software. Demonstrative images of the colonies were obtained under an optical microscope (Optika Italy) and using Optika Proview software. The assays were repeated three times for each cell line.

NEK6-I and docetaxel co-treatment

DU-145 cells were treated with 5 nM docetaxel and 30 µM NEK6 inhibitor. In the co-treatment, these same concentrations were maintained. Treatments lasted 48 hours, followed by MTT, western blotting, or wound healing, which are better described in the specific topics of each method. The control was treated with a DMSO vehicle.

Western blotting

Using a cell lysis buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease, and phosphatase inhibitor cocktail), the proteins were collected from DU-145 cells. The proteins were quantified using Pierce™ BCA Protein Assay

Kits (Thermo Scientific™, #23225). A total of 30 µg of total protein were applied in SDS-PAGE. The proteins were transferred to 0.45-µm nitrocellulose membranes (Bio-Rad Laboratories, Inc.) and incubated for 1 hour at room temperature with milk dissolved in TBS-Tween-20 (TBS-T; 50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% Tween-20). The membranes were incubated with primary antibodies overnight at 4 °C. Further, the membranes were washed three times with TBS-Tween-20, followed by incubation with secondary antibodies for 1 h at room temperature. The membranes were washed three times with TBS-Tween-20. Protein bands were visualized using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.) in ChemiDoc Imaging System (Bio-Rad Laboratories, Inc.) and densitometry was performed using ImageJ software v1.53.

Primary antibodies PARP1 (Cell signaling, #9542), Bcl-xL (Cell signaling, #2764P), BAK (Cell signaling, #6947P), p-BAD (Cell signaling, #5284), p-p65 (Cell signaling, #3031), anti-vinculin (Abcam, Ab18058), anti-β-actin (Cell signaling, #4970). Primary antibodies were used 1:2000. Secondary antibodies: HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich; AP308P; 1:2000), goat anti-rabbit IgG (Sigma-Aldrich; AP307P; 1:5000), and goat (Sigma-Aldrich, A5420).

Wound healing

DU-145 and PC-3 cells were seeded at a density of 2×10^5 cells/well in 24-well plates, incubated until confluence, and cultured in RPMI 1640 containing 1% FBS treated with 5 µg/mL mitomycin-C (Sigma, 10107409001) for 2 h to inhibit proliferation. After mitomycin incubation, RPMI 1640 media containing 1% FBS and docetaxel, NEK6-I, and co-treatment with docetaxel and NEK6-I was added to cells. The wounded area was made with a p200 tip. The images were captured at 0 and 24 hours after treatment. To analyze cell migration in WT and NEK6-KO DU-145 cells, the same protocol was performed and the images were captured at 0, 24, and 48 h. The scratch area was analyzed under a light microscope (Optika Italy) and using Optika Proview Software.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 8.01 software (https://www.graphpad.com/). The data were expressed as means and SD, and the mean difference was evaluated through either Student's unpaired t-test or One-way

ANOVA, followed by Tukey's or Dunnett's post-test, with statistical significance denoted as *p<0.05, **p<0.01, ***p<0.001, or ****p<0.0001.

Results

Using TMNplot, we evaluated the expression of NEK6 in normal tissue obtained from non-cancerous patients and pediatric tissues and compared it with prostate cancer tissue from patients (Figure 1A). This analysis shows an increase of 1.5-fold in NEK6 expression in samples from patients with prostate cancer. A representative violin plot was also demonstrated (Figure 1B). We performed this same analysis by comparing NEK6 expression in prostate cancer tissue samples from patients with their adjacent noncancerous tissues (Figure 1C). NEK6 expression has an increase of 1.25-fold in prostate cancerous tissues. Another representative violin plot was also demonstrated (Figure 1D). Using GEPIA, we analyzed whether NEK6 expression in prostate adenocarcinoma was associated with survival in these patients (Figure 1E). Survival analysis revealed that patients with greater expression of NEK6 have lower overall survival. We concluded that elevated NEK6 expression has clinical relevance in the context of prostate adenocarcinoma.

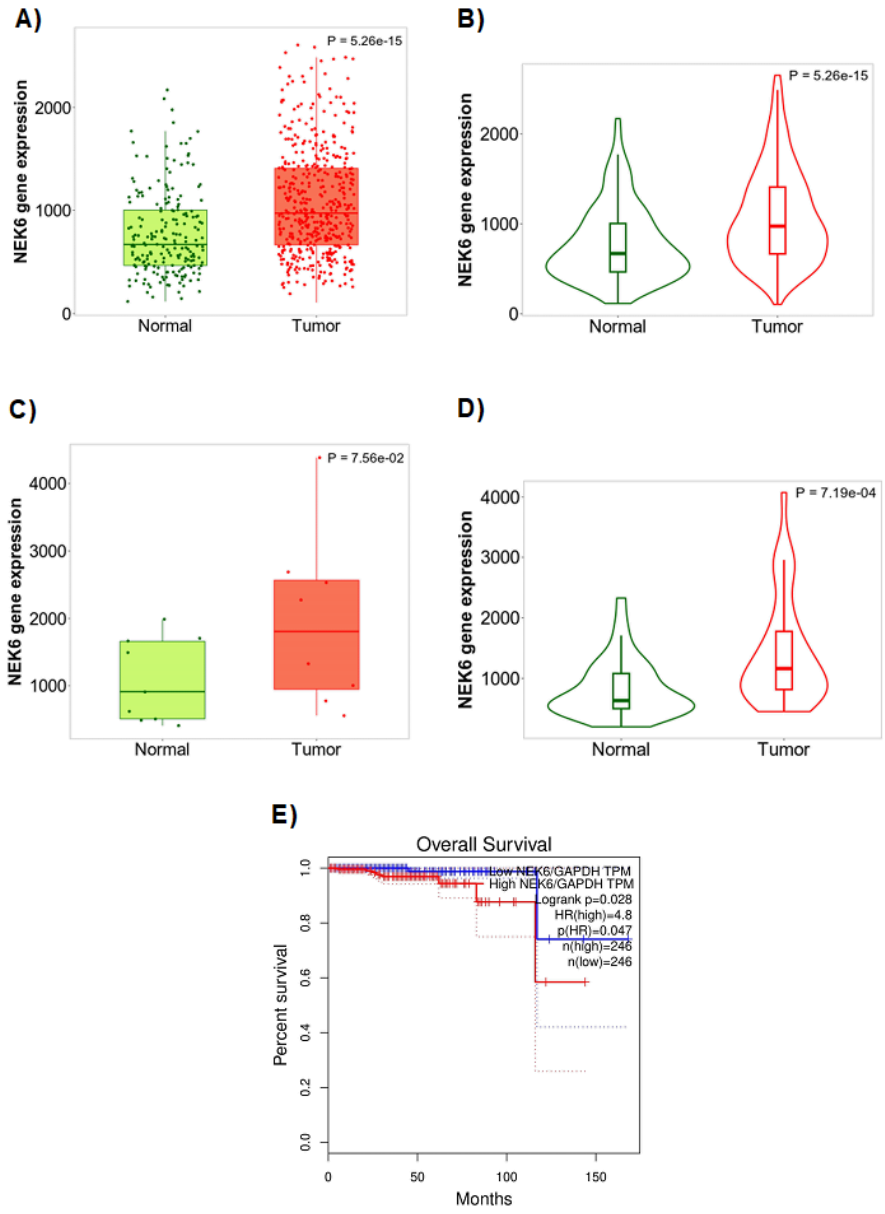


Figure 1. NEK6 is up-regulated in prostate cancer and is correlated with poor survival. Analysis of NEK6 expression by RNA-seq data in non-cancerous patients and pediatric tissues and adenocarcinoma prostate tissues (Figure 1A and 1B). NEK6 expression in prostate cancer tissue and normal adjacent tissue from the same patient (Figures 1C and 1D). Overall survival analysis was also examined in prostate adenocarcinoma samples expressing low and high NEK6 (Figure 1E).

We generated NEK6 knockout clones in DU-145 cells and validated them in a previous paper [13]. We questioned whether NEK6 would have relevance in response to docetaxel treatment. Then, we challenge the NEK6-KO (83.14 and 56.5) in DU-145 cells and NEK6-KO (83.4 and 56.1) in PC-3 cells with increasing concentrations of docetaxel and evaluated cell viability (Figure 2A and Figure 2B, respectively). At several concentrations, NEK6-KO cells from DU-145 and PC-3 were shown to be more

sensitive to docetaxel.

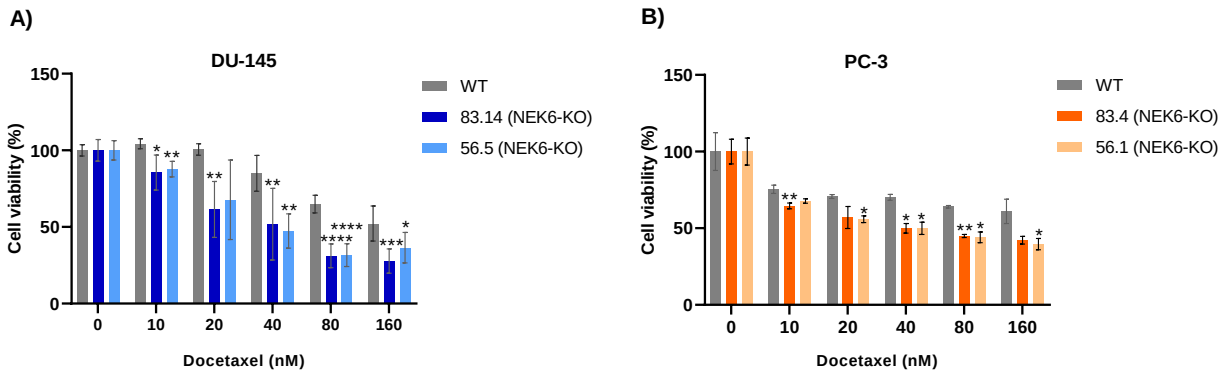


Figure 2. Depletion of NEK6 by CRISPR-Cas9 enhances the chemosensitivity of docetaxel in DU-145 and PC-3 cells. NEK6 knockout in DU-145 (83.14 and 56.4) and PC-3 (83.4 and 56.1) were treated with 0, 10, 20, 40, 80, and 160 nM of docetaxel for 48 hours (Figures 2A and 2B, respectively) and the cell viability was assessed by MTT assay. Cell viability was normalized to percentage. All experiments were conducted independently three times. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$, and **** $p < 0.0001$.

A selective NEK6 inhibitor was recently identified by Donato and colleagues [24]. This compound was able to inhibit the proliferation of ovarian, lung, and colon cell lines with an IC_{50} value below 100 µM. The inhibitor alters NEK6 activity, but not its expression. Another interesting point is that the inhibitor does not interfere with NEK7, the member of the NEKs family with the greatest structural similarity compared to NEK6 [24]. Thus, we treated DU-145 and PC-3 cells with NEK6 inhibitor (NEK6-I) at different concentrations (0, 15, 30, 45, 60, 75, and 90 µM) for 48 hours and evaluated cell viability. Results showed an IC_{50} value of 37.46 µM for NEK6-I in DU-145 cells (Figure 3A) and 48.20 µM in PC-3 cells (Figure 3B). Considering that the NEK6 inhibitor may interfere with cell viability in prostate cancer cells, then we evaluated the clonogenic ability of DU-145 and PC-3 cells treated with docetaxel, NEK6-I, and both together (Figure 3C). NEK6-I alone was able to significantly reduce colony formation in DU-145 cells, but not in PC-3 cells. However, when we treated DU-145 and PC-3 cells with docetaxel plus NEK6-I, both cells showed a reduction in clonogenic capacity when compared to only docetaxel treatment. This suggests that somehow NEK6 inhibition may increase the chemosensitivity of prostate cancer cells to docetaxel.

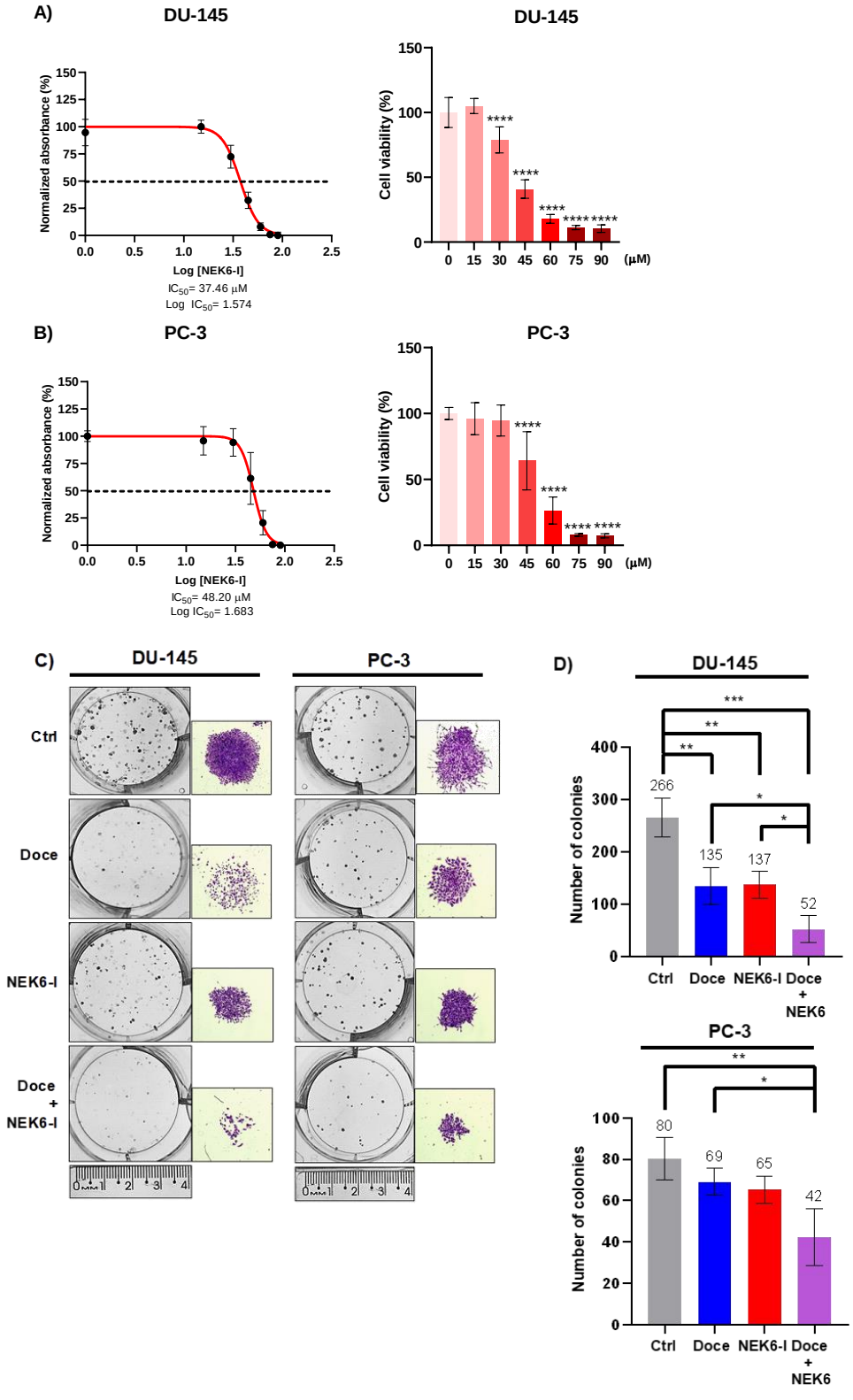


Figure 3. NEK6 inhibitor sensitizes DU-145 and PC-3 cells to docetaxel. DU-145 and PC-3 cells were treated with increasing concentrations of the NEK6 inhibitor for 48 hours and the cell viability was assessed by MTT assay (Figure 3A and 3B, respectively). Clonogenic assay was performed using cells treated with docetaxel, NEK6 inhibitor, and both compounds for 48 hours. The number of colonies was

counted by ImageJ software. All experiments were conducted independently three times. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$, and **** $p < 0.0001$.

We further investigated the increased docetaxel sensitivity of DU-145 cells treated with a NEK6 inhibitor. Then, first, we evaluated the viability of DU-145 cells treated with docetaxel, NEK6 inhibitor, and both compounds together (Figure 4A). We found that cells treated with docetaxel and inhibitor had reduced cell viability compared to the control. However, when docetaxel was co-treated with a NEK6 inhibitor, DU-145 cells had a more intense reduction in cell viability. After that, we explored the expression of proteins related to cell apoptosis, such as PARP1, BAK, Bcl-xL, BAD phosphorylated, to cell survival, such as phosphorylated p65 (NF-κB), and modulators of cell migration, such as TJP1. NEK6 expression was also evaluated (Figure 4B). The combination of docetaxel and NEK6 inhibitor increases PARP1 protein cleavage and BAK expression compared to docetaxel alone, suggesting an enhancement in the apoptotic process. We also observed that the NEK6 inhibitor reduces Bcl-xL expression and BAD phosphorylation, revealing a new mechanism of action. p65 phosphorylation (NF-κB) is markedly reduced in NEK6 inhibitor treatment. Furthermore, the co-treatment potentiated the reduction in p65 phosphorylation when compared with docetaxel alone. TJP1 was significantly reduced in co-treatment and also with NEK6 inhibitor treatment. The NEK6 inhibitor reduced the expression of NEK6 in DU-145, which is an unexpected fact since the study that characterized this inhibitor revealed that there was a reduction only in its activity [24].

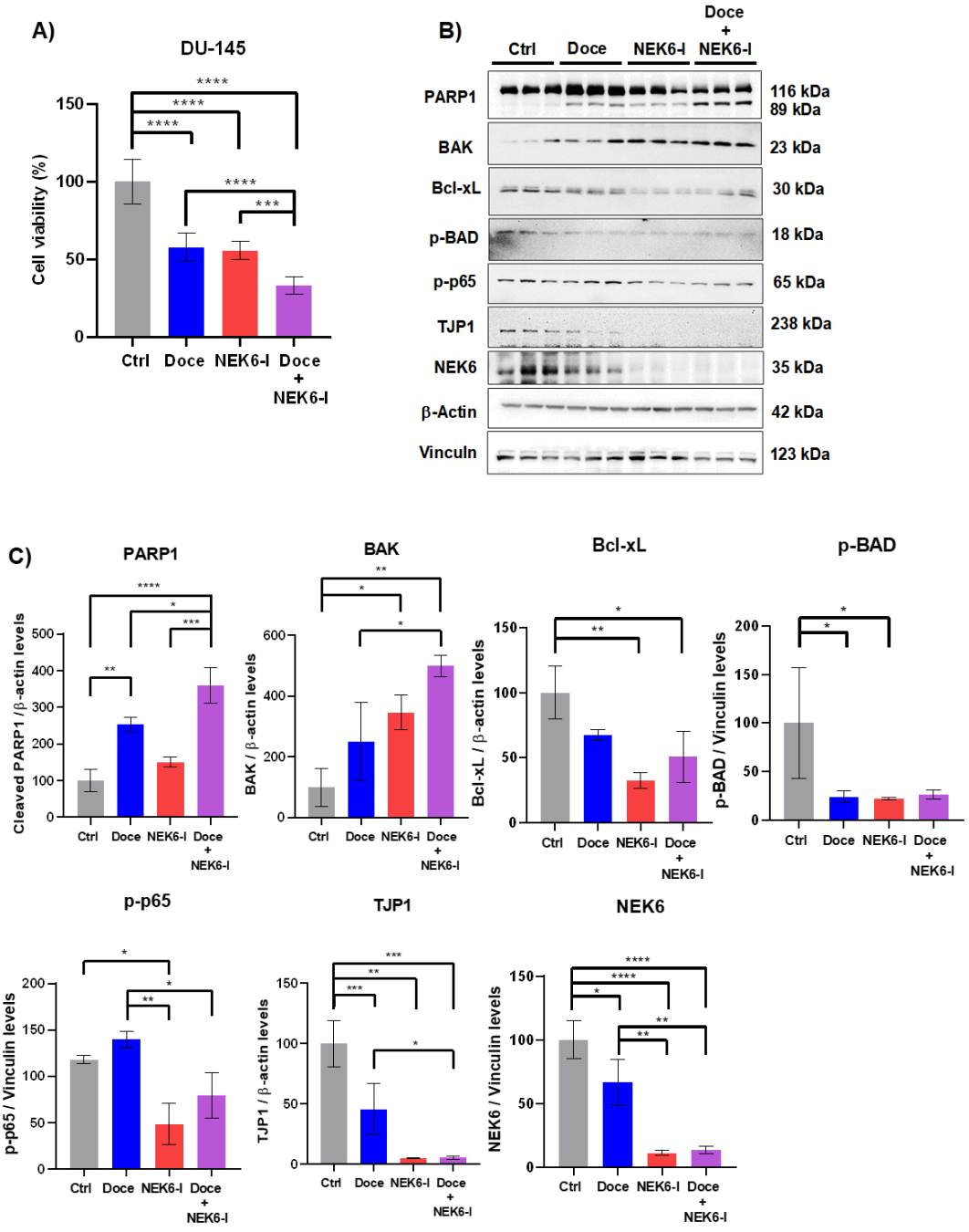


Figure 4. The NEK6 inhibitor enhances the effect of docetaxel through modulation of apoptotic, survival, and cell migration signaling in DU-145 cells. Cells were treated with docetaxel (5 nM), NEK6 inhibitor (30 µM), and co-treatment with both (5 nM docetaxel and 30 µM NEK6-I). MTT assay was performed to analyze cell viability (Figure 4A). Western blotting was performed to analyze PARP1, BAK, Bcl-xL, pBAD, p-65 NF-κB, TJP1, and NEK6 expression. All experiments were conducted independently three times. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$, and **** $p < 0.0001$.

As seen previously, NEK6 inhibition decreases the expression of TJP1, a protein related to cell adhesion and migration, we investigated whether cell migration

is indeed compromised in DU-145 and PC-3 cells treated with the NEK6 inhibitor and/or NEK6 inhibitor plus docetaxel. The migration rate of DU-145 cells submitted to co-treatment with docetaxel and NEK6-I was lower compared to these cells treated with docetaxel alone (Figure 5A). We observed a reduction in migration in PC-3 cells treated with docetaxel, NEK6 inhibition, and co-treatment when compared to control cells (Figure 5B). Also, it was observed a significant reduction in cell migration in the docetaxel and NEK6-I group when compared to cells treated with docetaxel alone (Figure 5B). We also checked cell migration in DU-145 knocked out to the NEK6 gene, we found that NEK6-KO cells have a lower cellular migration in these cells compared to NEK6-expressing cells. Therefore, we suggest that NEK6 inhibition may reduce cell migration in prostate cancer cells and enhance the anti-metastatic effect of docetaxel, thus contributing to its therapeutic effects in cancer.

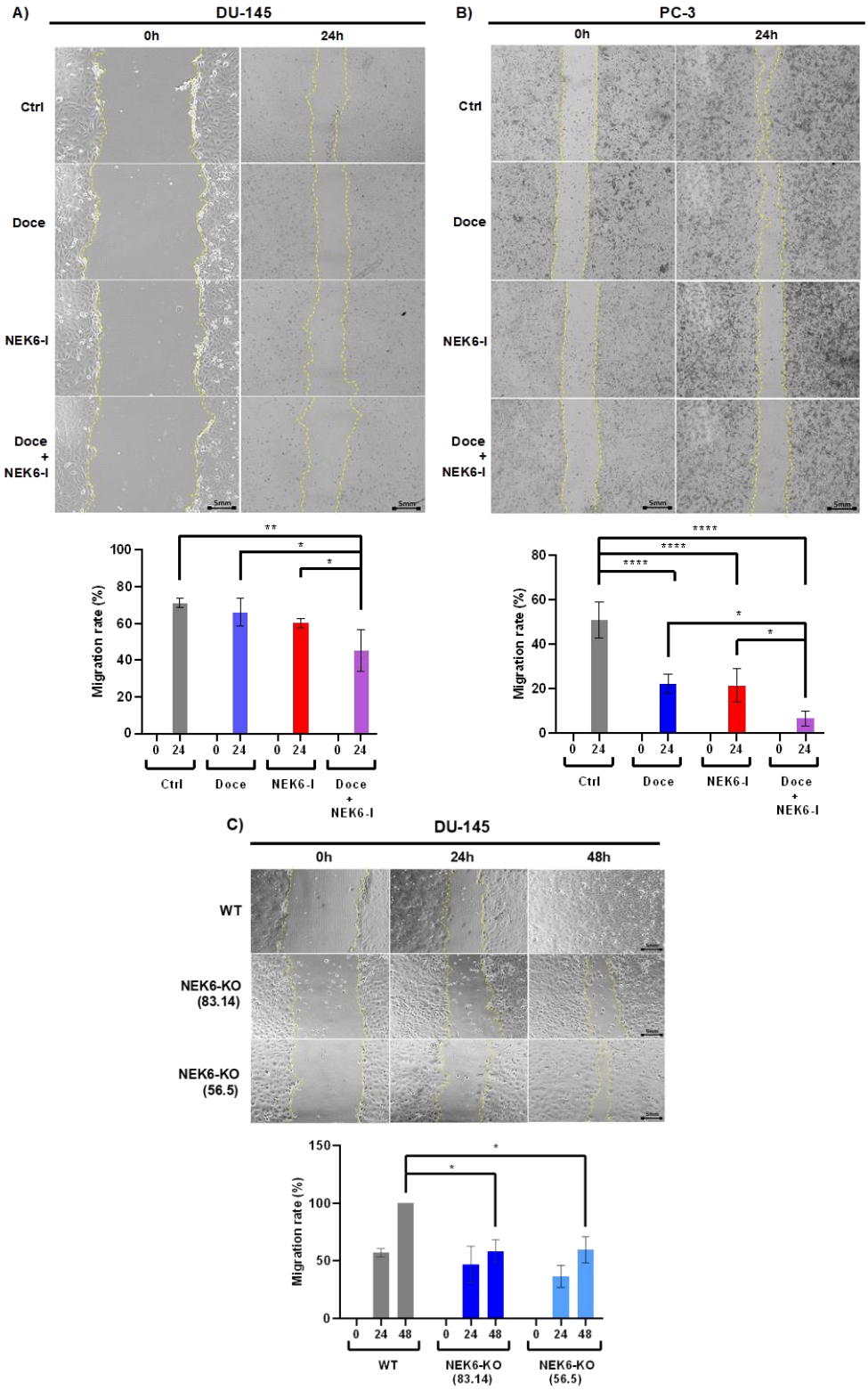


Figure 5. NEK6 inhibition reduces cell migration in DU-145 and PC-3 cells. DU-145 and PC-3 cells were treated with 5 nM of docetaxel, and 30 µM of NEK6 inhibitor and co-treated with 5 nM of docetaxel and 30 µM of NEK6 inhibitor (Figure 5A and 5B, respectively). WT and NEK6-KO cells (83.14 and 56.5) were scratched and the cell migration area was measured by ImageJ software. All experiments were conducted independently three times. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$, and **** $p < 0.0001$.

Discussion

Abnormal expression of NEK6 has been linked to various types of cancer [19,24,27,28], including prostate cancer [29]. In prostate cancer, NEK6 is overexpressed in both cancer tissues and cell lines [29]. Some recent studies have been encouraging targeting the NEK6 protein as a potential treatment for cancer [23,24].

Our research group established that NEK6 regulates redox balance and DNA damage response in DU-145 prostate cancer cells [13]. Other important findings of the study revealed that NEK6 knockout can reduce antioxidant defenses, such as SOD1, SOD2, and PRDX3, increase the amount of intracellular ROS and generate DNA damage. Furthermore, the NEK6 knockout alters the permeability of the mitochondrial membrane, induces cell death, and intensifies the effect of cisplatin, which is also used in prostate cancer chemotherapy. We also observed that SOD2 expression is increased in treatment with cisplatin, which some studies show is a potential mechanism of chemotherapy resistance. However, NEK6 knockout cells treated with cisplatin lose this characteristic, having a significant reduction of SOD2. We, therefore, suggest that the knockout of NEK6 could alter cellular mechanisms to reduce cisplatin resistance [13].

NEK6 has been linked to cisplatin and paclitaxel resistance in ovarian cancer [19,24]. Chemotherapy based on docetaxel is the main strategy for the treatment of castration-resistant prostate cancer patients. However, it is a palliative treatment, and it is usually associated with resistance and metastasis. In this paper, we report that NEK6 is overexpressed in prostate cancer samples and is also related to poor overall survival (Figure 1). Assuming that there are no studies evaluating the relationship between NEK6 and docetaxel chemotherapy, this study intended to evaluate the role of NEK6 in influencing the response of prostate cancer cells to docetaxel, as well as discover mechanisms NEK6 may contribute to chemoresistance.

As a first approach, we knocked out the NEK6 gene in DU-145 and PC-3 cells and challenged with increasing concentrations of docetaxel. it was found that NEK6 depletion sensitized DU-145 and PC-3 cells to cytotoxicity induced by docetaxel (Figure 2). We suggest that the mechanisms mediated by NEK6 in the cell, either by alteration in expression, or interaction with other proteins, may contribute to docetaxel's anti-cancer effects, and may even be a target of docetaxel.

As a next step, we would like to know whether the promising compound

characterized as a NEK6 inhibitor (named Compound 8) by Donato and colleagues [24] would have a cytotoxic effect on prostate cancer cells. We found that the IC_{50} of the compound for DU-145 was 37.45 µM and for PC-3 was 48.2 µM. The cytotoxicity effect of compound 8 against a panel of cancer cells was determined by Donato, overall the compound had an IC_{50} of less than 100 µM against a panel of cancer cells such as ovary, breast, lung, and colon. These results are very interesting because are the first report indicating the effect of this compound on prostate cancer cells. However, it is still a compound that needs further investigation of its molecular effects, mainly in the prostate cancer cell line.

We initiated a further investigation of this NEK6-inhibiting compound in prostate cancer by clonogenic assay on DU-145 and PC-3 cells. Furthermore, we explored its effect in combination with docetaxel (Figures 3C and 3D). NEK6-I alone was able to reduce the number of colonies in DU-145 cells, as well as docetaxel. When co-treatment with docetaxel and inhibitor was performed, the effect was even more pronounced. In PC-3 cells, however, there was a reduction in the number of colonies with the inhibitor alone, but it was not statistically significant. However, treatment with docetaxel and NEK6-I was able to strongly reduce clonogenic capacity when compared with docetaxel treatment alone. These results are in agreement with the results shown in Figure 2, which showed that NEK6 lacks sensitized cells to docetaxel. One of the mechanisms by which increased cytotoxicity may occur in the combination of these compounds may be due to the exaggerated increase in ROS, as we already know that NEK6 modulates antioxidant proteins and consequently increases oxidative stress. Docetaxel is also able to increase ROS levels [30,31]. Thus, the exaggerated increase in ROS makes cells more sensitive to cell death. However, many other mechanisms may be involved in the inhibition of NEK6, which contribute to the increased sensitivity to docetaxel.

We performed an MTT assay to investigate the cell viability after treatment with docetaxel and NEK6 inhibitor alone and also in a co-treatment (Figure 4). We found that the combination of these drugs was able to significantly reduce the viability of DU-145 cells. To deepen the knowledge underlying the role of NEK6 and the chemotherapeutic effect of docetaxel in prostate cancer progression, apoptotic, survival, and cell adhesion and migration signaling were explored (Figure 4).

The poly (ADP-ribose) polymerase 1 (PARP1) binds tightly to DNA strand breaks, performed the auto–poly ADP-ribosylation, and allows for repair enzyme

access to the damaged DNA [32]. Cleavage of PARP1 and release of an 89 kDa fragment is associated with stimulation of cell death [33]. PARP1 is cleaved by caspase 3 and 7 at the beginning of cell apoptosis, limiting its action in DNA repair [33]. PARP1 is also cleaved by cathepsins, inducing autophagic and necrotic cell death pathways [34,35]. In our data, the treatment of docetaxel in DU-145 cells induced PARP1 cleavage, however, the combination of docetaxel and the NEK6 inhibitor was much more effective in cleaving PARP-1, and consequently, inducing cell death. Other proteins related to the apoptotic pathway were evaluated, such as BAK, Bcl-xL, and phosphorylated BAD.

BAK mediates mitochondrial outer-membrane permeabilization (MOMP) through oligomerization in the mitochondrial membrane [36]. Chemotherapy agents trigger the activation of BAK and BAX, which integrate apoptotic versus survival signals to determine cell fate [37]. Once an apoptotic 'threshold' has been crossed and enough BAK/BAX molecules have been activated, a cell becomes committed to dying by apoptosis [37]. The NEK6-I treatment increased BAK expression, while docetaxel did not significantly increase. Our results revealed that BAK expression was higher in co-treatment with docetaxel and NEK6-I when compared to docetaxel only. We also observed Bcl-xL, an anti-apoptotic protein, was reduced in NEK6-I treatment. However, no differences were identified in co-treatment. Bcl-xL expression is highly present in samples from patients with CRPC. It is known that Bcl-xL can interact with Bak, repressing its pro-apoptotic activity, and contributing to castration resistance and prostate cancer progression [38]. Phosphorylation of Bad at S112 by PKAc also causes dissociation of BAD and Bcl-xL at the mitochondrial outer membrane. BAD binds to 14-3-3 in the cytoplasm, promoting apoptosis [39]. We also observed that docetaxel and NEK6-I reduced BAD phosphorylation, which maintained the same level of expression in co-treatment. Gathering together this information, changes in the apoptotic pathway were observed with NEK6 inhibitor treatment and/or co-treatment of docetaxel with a NEK6 inhibitor, suggesting that increased chemosensitivity to docetaxel in DU-145 occurs together with NEK6 inhibition. Or simply the presence of the inhibitor is capable of stimulating apoptotic pathways.

It is well established that activation of NF-κB p65 is associated with castration-resistant prostate tumors and the progression of prostate cancer [40]. NFκB contributes to androgen-independent prostate cancer cell growth in the absence of androgen signaling [40]. Our data showed that NEK6-I treatment reduces p65

phosphorylation, as well as in co-treatment with docetaxel and NEK6-I compared to docetaxel alone.

TJP1 (Tight junction protein 1), also known as ZO-1, is an important structural protein that is involved in the formation and stability of tight junctions between epithelial cells. Tight junctions are important structures for maintaining the integrity of the epithelial barrier and controlling the traffic of molecules and cells across it. The involvement of TJP1 with cell migration is controversial. ZO-1 expression is associated with metastatic features [41–45], but other studies showed that decreased expression of ZO-1 is associated with tumor metastases [46,47]. We analyzed TJP1/ZO1 expression and observed that there is a significant reduction in the expression of this protein in treatment with docetaxel and NEK6-I alone. However, co-treatment further reduced the expression of this protein. Therefore, we suggest that the NEK6 inhibitor alters cell migration, but this is also observed more markedly in co-treatment with docetaxel.

Therefore, we analyzed cell migration through the wound healing assay (figure 5), we observed that co-treatment of docetaxel with NEK6-I reduced cell migration when compared with docetaxel alone in DU-145 cells. In PC-3 cells, the effect was more pronounced, since the NEK6 inhibitor itself was able to reduce migration, but in action with docetaxel, cell migration was even more reduced. NEK6 depletion by CRISPR/Cas9 also reduces cell migration in DU-145 cells. We suggest that the reduction in migration may be associated with the reduction in TJP1 in treatments alone and in combination.

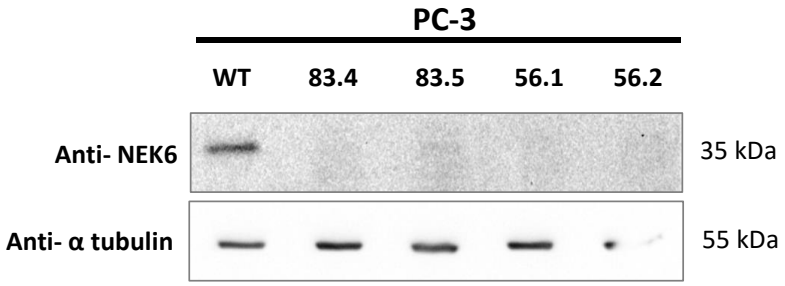
Conclusion

Overall, NEK6 plays an important role in the development and progression of prostate cancer, and targeting this protein may provide a promising therapeutic approach for the treatment of this disease. Inhibition of NEK6 may sensitize the chemotherapy effects of docetaxel, inducing apoptosis, cell migration, and reducing survival pathways. Targeting NEK6 has emerged as a potential therapeutic strategy for prostate cancer treatment.

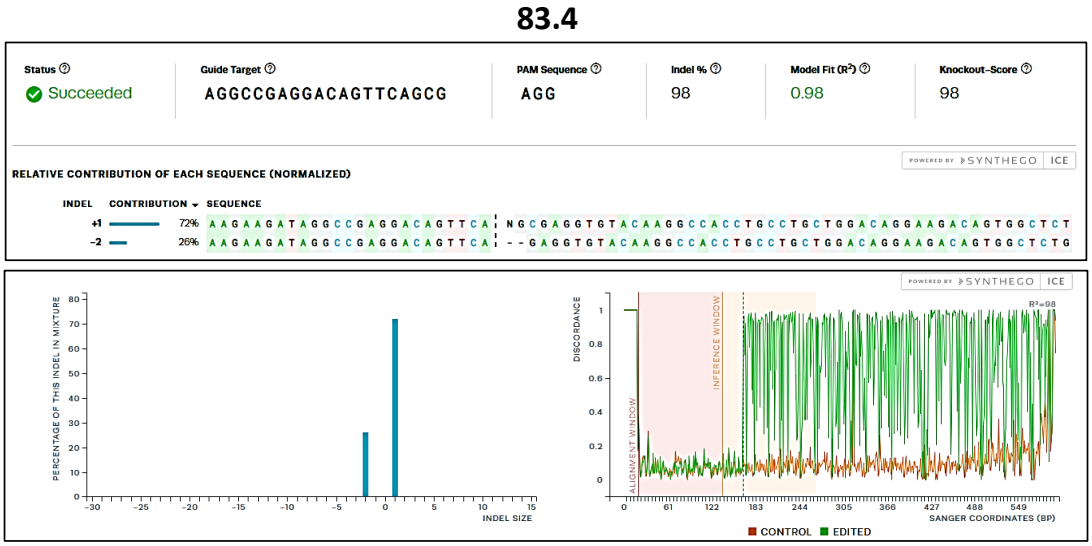
Supplementary files

Supplementary figure 1 (Figure S1). In figure S1-A, we screened some clones of NEK6 knockouts in PC-3 cells. In Figures S1-B and S1-C, we performed the in silico analysis of the indels generated in knockouts 83.4 and 56.1 using the ICE analysis of the Synthego software. Note: Clone 56.1 showed 4 indels. Since chromosome 9 (NEK6 location) of PC-3 is diploid, then we should only find 2 indels. We suggested clone 56.1 is a mixture of 2 clones. However, both knockouts have a high knockout score and NEK6 protein depletion as observed in Figure S1-A.

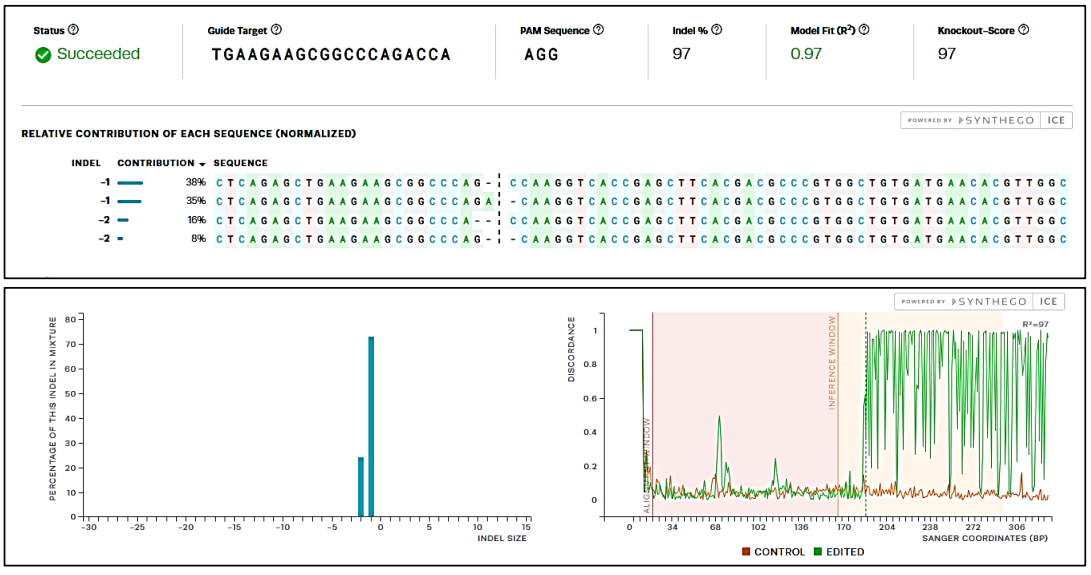
A)



B)



C)



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4.2. The NEK6-Hexokinase 2 axis is a survival mechanism modulated by resveratrol

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Abstract

Resveratrol has been extensively studied for its potential in treating advanced prostate cancer, as it can modify important survival pathways and increase the susceptibility of cancer cells to other chemotherapy drugs. NIMA-related Kinase-6 (NEK6) has been shown by several studies to be an essential protein kinase for the development of advanced prostate cancer, as well as castration resistance and chemoresistance. We found that resveratrol reduced NEK6 expression in a dose-dependent manner, as well as the hexokinase 2 (HK2) expression in DU-145 prostate cancer cells. In NEK6-deficient cells, HK2 expression was not modulated by resveratrol, indicating that the effect of resveratrol on HK2 may be mediated by NEK6. We used NEK6-deficient DU-145 cells and found that they are more sensitive to cell death mediated by resveratrol treatment. We also showed that NEK6-deficient cells had a lower expression of HK2. Congruently, overexpression of NEK6 increased HK2 expression, thereby revealing the regulation of HK2 by NEK6. We also showed that NEK6-deficient cells have reduced lactate production, which may indicate a negative modulation of the Warburg effect. Considering these presented data, we identified a potential survival mechanism involving NEK6 and HK2, which is involved in metabolism and is modulated by the natural anti-cancer agent, resveratrol. We suggest that the combination of resveratrol with other chemotherapeutic agents in cancers in which NEK6 plays a relevant role, such as Castration-Resistant Prostate Cancer (CRPC), should be further explored.

Key-words: NEK6, resveratrol, hexokinase-2, CRPC.

Introduction

Prostate Cancer (PCa) is characterized by a high incidence rate among men worldwide [1]. The main treatment for PCa is androgen deprivation. However, many of these people progress to a resistance stage, called Castration-Resistant Prostate Cancer (CRPC). This stage is usually associated with metastasis and reduced life expectancy of patients [2].

NIMA-related kinases (NEKs) belong to a protein family of 11 serine/threonine kinases. They are involved in cell cycle regulation [3], primary cilium function [4–6], DNA damage response [7], and mitochondrial metabolism [8,9]. The NIMA-related Kinase-6 (NEK6) participates in mitotic spindle kinetochore fiber formation, metaphase-anaphase transition, cytokinesis, and checkpoint [10]. NEK6 is considered a biomarker in several types of cancer, such as non-small-cell lung carcinoma (NSCLC) [11], breast [12], colorectal [13], liver [14], and gastric cancer [15]. Furthermore, NEK6 is associated with resistance to cancer chemotherapy, such as paclitaxel and cisplatin in ovarian prostate cancer [16]. A high throughput screen identified that NEK6 is a central kinase in androgen-independent tumor growth in CRPC. Besides, NEK6 is highly expressed in human prostate cancer samples and cancer cell lines [17]. In resume, NEK6 contributes to the survival and growth of prostate cancer, however, the mechanisms are not elucidated.

Resveratrol (trans-3,5,4-trihydroxystilbene) is a natural polyphenol, which reduces tumor growth in cancer, such as neuroblastoma, colon, prostate, liver, and breast [18–20]. Resveratrol acts in the three stages of carcinogenesis (initiation, promotion, and progression) by modulating signal transduction pathways that control cell division and growth, apoptosis, inflammation, angiogenesis, and metastasis [19]. Accumulating data have shown the anti-cancer properties of resveratrol. Resveratrol reduces cell viability, migration, and invasiveness of prostate cancer cells [21], and also generates few adverse effects, which turns resveratrol into an interesting strategy for cancer therapy [22]. Besides, resveratrol also exhibits a regulation in the Tumor Microenvironment (TME), acting as a tumor-suppressive nutraceutical [23]. Resveratrol also presented an accentuated anti-tumor effect in lung and hepatocellular cancer through the downregulation of the glycolytic enzyme, Hexokinase 2 (HK2), and consequently, glycolysis [24,25].

Most cancer cells preferentially utilize glycolysis, even in the presence of oxygen, as an energy source. It is named “aerobic glycolysis” or the Warburg effect

[26]. The overproduction of lactate, as a result of the hyperactivation of glycolysis, provides a propitious TME to promote metastasis and drug resistance [27]. HK2 is a prostate cancer marker and is positively correlated with Gleason score, cell growth, and metastasis. The overexpression of HK2 increases the rate of glycolysis and is extremely necessary for the occurrence of tumors [28].

Resveratrol is already widely studied in advanced prostate cancer due to its ability to modulate essential survival pathways and sensitize cancer cells when treated with other chemotherapy drugs [29,30]. We provided in this manuscript that NEK6 is a new regulatory target of resveratrol, as well as hexokinase 2 in DU-145 cells. NEK6-deficient cells are more sensitive to resveratrol, which may reveal that targeting NEK6 is important to the anti-cancer effects mediated by resveratrol and/or synergy between these two interventions. NEK6 overexpression increased HK2 expression, and its knockout reduced HK2 expression, concomitantly with reduced lactate production. We suggested that there is a regulatory axis between NEK6 and HK2, which is modulated by resveratrol in DU-145 cells.

Material and Methods

Cell culture

The RPMI 1640 (Thermo Scientific, #11875093) medium, which contained 10% fetal bovine serum (FBS, #12657029) and 1% penicillin/streptomycin (Gibco, #15140-122), was used to cultivate DU-145 cell line. The cells were cultured at 37 °C in an incubator with 5% carbon dioxide. NEK6-KOs in DU-145 cells were characterized according to [31]. The cells utilized in the experiments were between 10-25 passages from the time of thawing.

Cell treatment

Resveratrol (#R0071-1G), 99% purity, was kindly donated by Dr. Edson Antunes of University of Campinas (UNICAMP). To evaluate the NEK6 expression after resveratrol treatment, DU-145 WT cells were seeded into 6-well plates at a density of 2×10^5 cells/well. After 24 hours, cells were treated with 50, 100, and 200 µM of resveratrol for 24 hours. The protein extract was collected for western blotting assay. To evaluate the HK2 expression, WT, and NEK6-KO cells were seeded into 6-well plates at a density of 2×10^5 cells/well. After 48 hours, the protein extract was collected for western blotting assay. We also plated WT and NEK6-KO cells (56.5) into 6-well

plates at a density of 2×10^5 cells/well. After 24 hours, we treated the cells with 100 and 200 µM of resveratrol. The protein extract was collected to analyze NEK6 and HK2 expression.

MTT assay

WT and NEK6-KO cells were seeded at a density of 8×10^3 cells per well in 96-well plates in triplicate to assess cell viability. Once the cells reached confluence after three days, resveratrol treatment was performed at increasing concentrations of resveratrol (10, 20, 50, 100, and 200 µM) in medium for 24 hours. Cell viability was evaluated by adding 10 µL of 12 mM MTT (Invitrogen™, M6494) to each well and then incubating for 2 hours at 37 ºC. After that, formazan crystals were solubilized in an HCl and isopropanol solution at 37 ºC for 15 minutes, and the optical density was measured at 570 nm.

NEK6 overexpression

WT and NEK6-KO cells were seeded into 6-well plates at a density of 2×10^5 cells/well, and when they reached 80% confluency, Lipofectamine 3000 (Invitrogen™, L3000001) was used to transfect the cells according to the manufacturer's instructions. To overexpress NEK6 in the cells, 2500 ng of pFLAG-NEK6 plasmid was used, and an equivalent amount of pFLAG-Ø, an empty vector, was used as the assay control. After 48 hours the protein extract was collected for western blotting analysis.

Apoptosis assay

The protocol was performed following the manufacturer's instructions ®Sigma (FitC Annexin V Apoptosis Detection). WT and NEK6-KO (83.14) cells were plated at a density of 2×10^5 cells/well in 6-well plates and kept for 24 hours at 37 ºC in the incubator. Treatment with resveratrol was made at 100 µM for 24 hours. The next day, cells were washed with 1X PBS, trypsinized, and counted to collect 1×10^6 cells for the experiment. Cells were centrifuged for 5 minutes, washed with 1X PBS again, centrifuged to remove 1X PBS, and resuspended in 100 µL 1X Binding buffer. 3 µL of annexin V and 3 µL of propidium iodide (PI) were added. Vortex cells lightly and incubate for 15 minutes at room temperature in the dark. An additional 100 µL of 1X Binding buffer was added to each sample and waste within 1 hour. A control was performed with annexin, another with PI only, and another in the absence of both

reagents.

Western blotting

Cell lysis buffer (50 mM Tris HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100) containing Roche protease inhibitors was used to extract protein samples. After incubation on ice for 15 minutes and centrifugation at 12,000g for 10 minutes, the supernatants of WT and KO DU-145 cells were collected and protein samples were quantified using the bicinchoninic acid method (BCA; Thermo Scientific). The proteins were denatured with laemmli (SDS) at 95 ºC for 10 minutes, an SDS page was performed and the proteins were transferred to a Nitrocellulose (GE) membrane. The membrane was blocked with a solution of 0.1% Tween 20 and 5% skimmed milk powder in TBS for approximately 16 hours at 4 ºC, then incubated with primary antibodies diluted in TBS-T containing 3% albumin for 16 hours at 4 ºC with agitation. The membrane was washed 3 times with washing solution (0.1% Tween 20 in TBS) at room temperature for 10 minutes each and incubated with a secondary antibody against mouse, rabbit, or goat IgG conjugated with peroxidase (Amersham) diluted 1: 1000 in blocking solution for 1 hour at room temperature. The membrane was washed again 3 times with washing solution (0.1% Tween 20 in TBS) at room temperature and incubated with the ECL Plus Western Blotting Detection System (GE Healthcare) for 2 minutes. Finally, the membrane was revealed in a G:Box using Genesys software. The antibodies used were the following: NEK6 (Santa Cruz, sc-50752), HK2 (Cell signaling, #2867), α-tubulin (Calbiochem, CP06), and Vinculin (Abcam, Ab18058).

Measurement of lactate and glucose

To quantify lactate, we used the Lactate Assay Kit (Abcam, ab65330). The cells were plated at the density of 8×10^3 cells in 96 well. The cells were resuspended in the Lactate Assay Buffer, followed by centrifugation. The supernatants were collected. The absorbance is read at 570 nm. The amount of lactate in the sample is determined by comparing the signal generated by the sample to a known lactate concentration standard. To quantify glucose, we followed the Glucose GOD-PAP kit (LaborLab, #1770130) instructions. We plated 8×10^3 cells in 96 well plates using a culture medium without phenol red. We added reagent A, which has a solution containing glucose oxidase (GOD), peroxidase (POD), 4-aminophenazone (4-AF), phosphate buffer pH 7.0, and 4-hydroxybenzoate, in the supernatant. This reaction emits a red color at 37

ºC after 5 minutes. Quantification of absorbance is performed at 505 nm. The amount of glucose in the sample is determined by comparing the signal generated by the sample to a known glucose concentration standard.

Statistical and biostatistical analysis

Statistical analysis was conducted using GraphPad Prism 8.01 software (https://www.graphpad.com/). The data were expressed as means and SD, and the mean difference was evaluated through either Student's unpaired t-test or One-way ANOVA, followed by Tukey's or Dunnett's post-test, with statistical significance denoted as *p<0.05, **p<0.01, ***p<0.001, or ****p<0.0001. The correlation between gene expressions in prostate adenocarcinoma (PRAD) was analyzed using the GEPIA platform. The strength of the correlation between two genes was quantified using Spearman’s correlation coefficient. The degree of correlation was categorized based on Schober and colleagues' classification [32].

Results

Reduction in NEK6 expression may mediate the pro-cell death effects of resveratrol in DU-145 cells.

We treated DU-145 WT cells with resveratrol at concentrations of 50, 100, and 200 µM for 24 hours. It was observed that resveratrol modulated the expression of NEK6 in a dose-dependent manner (Figure 1A). We evaluated the viability of WT and NEK6-KO cells (83.14 and 56.5) after treatment with resveratrol at various concentrations (10, 20, 50, 100, and 200 µM) for 24 hours. NEK6 lack sensitized the DU-145 cells to resveratrol at 50, 100, and 200 uM considering NEK6-KO (83.14) and at 100 and 200 µM in NEK6-KO 56.5 (Figure 1B). Further, we stained cells with Annexin V and propidium iodide and assessed cell death after 100 µM resveratrol treatment for 24 hours in NEK6-KO 83.14 cells. We observed that resveratrol induced more pronounced late cell death in NEK6-KO than in WT cells (Figure 1C).

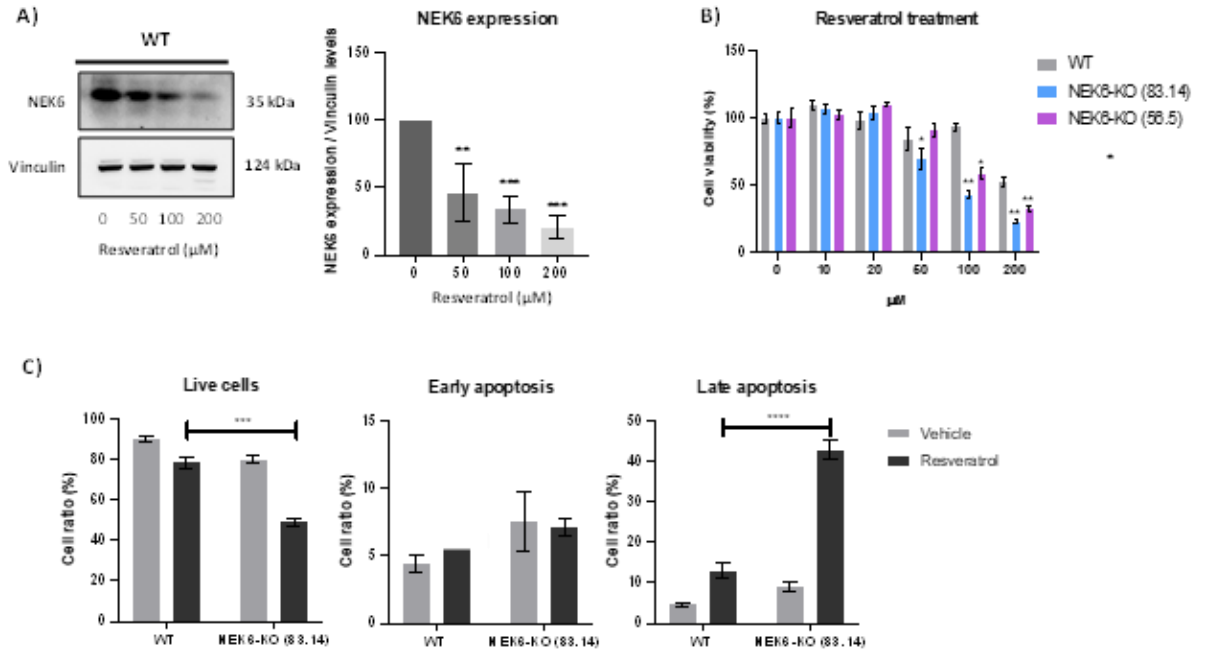

Figure 1. Resveratrol down-regulated NEK6 expression and its lack sensitizes DU-145 cells to resveratrol. DU-145 cells were treated with increasing doses of resveratrol (50, 100, and 200 µM) for 24 hours. NEK6 expression was assessed by western blotting (Figure 1A). NEK6 knockout cells (83.14 and 56.5) were treated with increasing doses of resveratrol (10, 20, 50, 100, and 200 µM) for 24 hours (Figure 1B). DU-145 WT and NEK6-KO (83.14) cells were treated with 100 µM of resveratrol for 24 hours and stained with FIT-C annexin V and propidium iodide. The evaluation of apoptosis was measured by flow cytometry (Figure 1C). All experiments were conducted independently three times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

NEK6 modulates HK2 expression and lactate production.

The GEPIA platform was also used to measure the correlation between the NEK6 and HK2 gene expression in PRAD samples. Spearman's correlation coefficient (0.49) showed a moderated and positive correlation between the NEK6 and HK2 genes (Figure 2A). To confirm these data, we overexpressed NEK6-FLAG in DU-145 cells and analyzed HK2 protein expression. As a result, HK2 expression was significantly increased in DU-145 cells overexpressing NEK6 (Figure 2B). We also proved the correlation of these genes by evaluating HK2 expression in NEK6-KO cells. Congruently, HK2 is lower expressed in NEK6-KO when compared to WT cells (Figure 2C). Whereas NEK6 knockout cells had a reduction in HK2 expression, we investigated whether there was also a reduction in lactate expression (Figure 2D). We observed a lactate reduction in NEK6-deficient cells. However, when analyzing the amount of glucose present in the cell culture medium, WT, and NEK6-KO, we did not observe

changes in this aspect (Figure 2E). Then, we suggested that NEK6 lack diminished HK2 expression, and consequently, lactate production, without changing the amount of glucose.

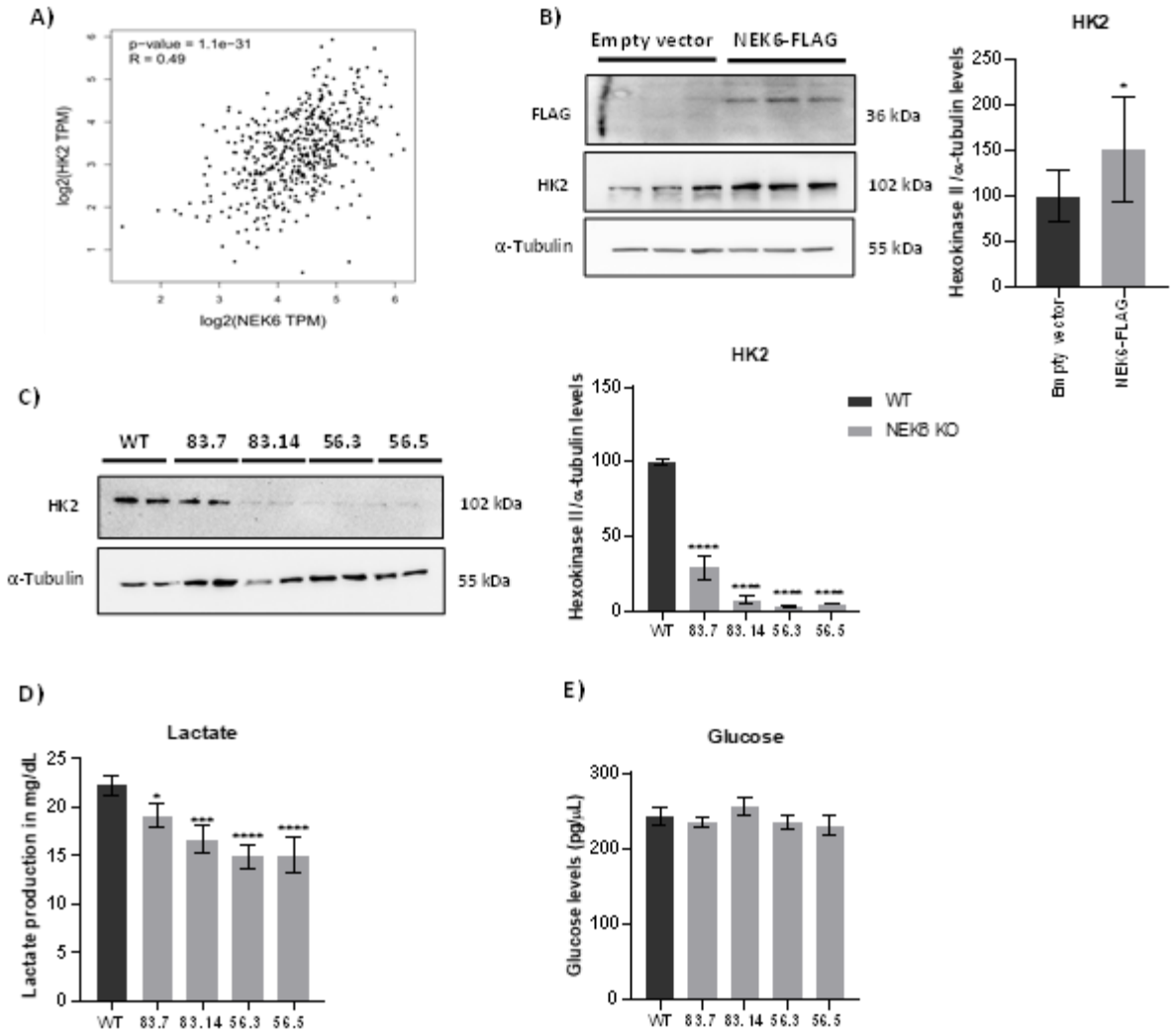

Figure 2. NEK6 mediates hexokinase-2 expression and lactate production. Correlation between NEK6 and HK2 expression was performed by the GEPIA platform (Figure 2A). Overexpression of an empty vector and NEK6-FLAG was performed in WT DU-145 cells. FLAG and HK2 expressions were analyzed by western blotting (Figure 2B). HK2 expression was evaluated in DU-145 NEK6-KO cells by western blotting (Figure 2C). Lactate and glucose levels were quantified in WT and NEK6-KO cells, respectively (Figures 2D and 2E). All experiments were conducted independently three times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Resveratrol modulates NEK6 and HK2 expression in DU-145 cells.

Since resveratrol reduced NEK6 expression (Figure 1A), we challenged WT and NEK6-KO (56.5) cells with resveratrol (100 and 200 µM) for 24 hours and checked if HK2 was also altered. We showed that HK2 expression is reduced in resveratrol

treatment, as well as NEK6, in WT cells (Figure 3). However, there is no modulation of HK2 expression in NEK6-deficient cells. These data may suggest that resveratrol modulates HK2 in a NEK6-dependent manner, providing a possible new mechanism of action of resveratrol.

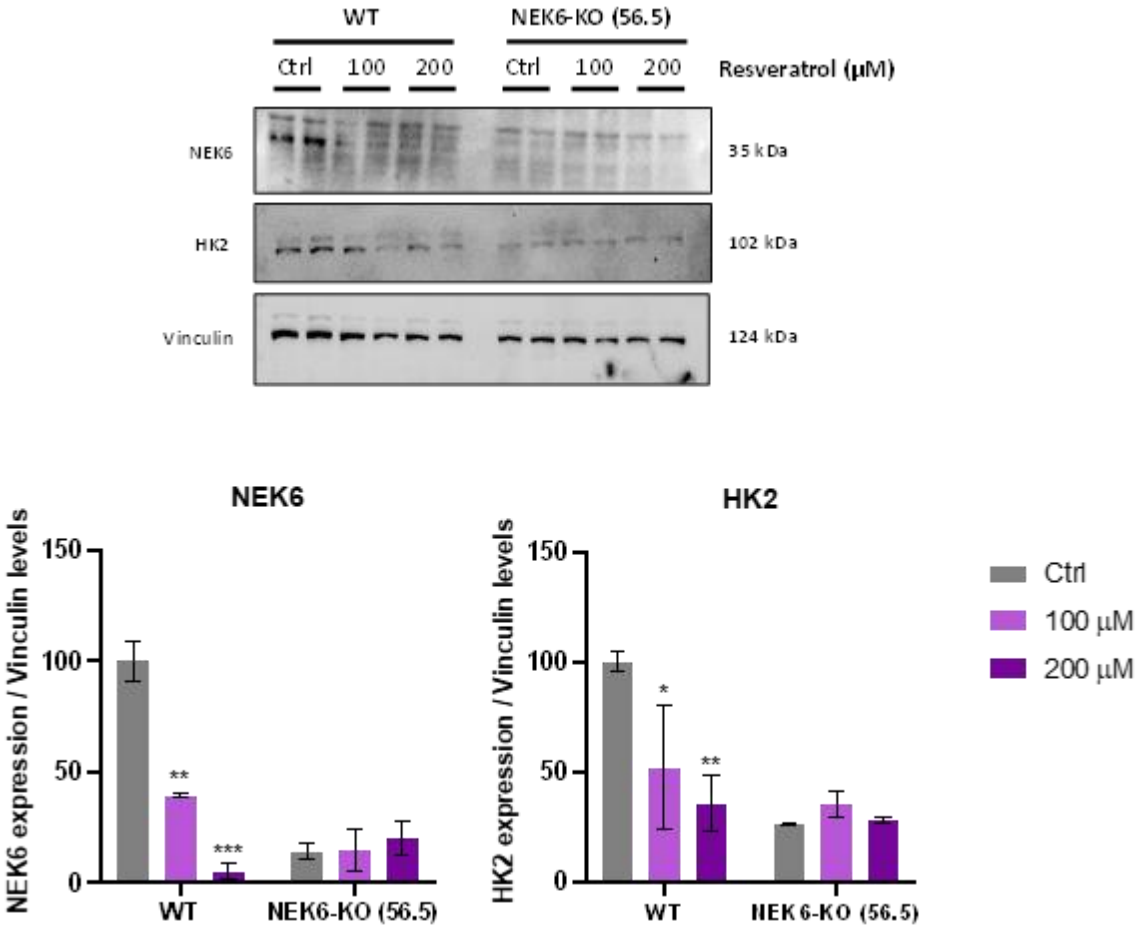

Figure 3. Resveratrol modulates NEK6 and HK2 expression in DU-145 cells. WT and NEK6-KO (56.5) were treated with resveratrol (100 and 200 μM) for 24 hours and NEK6 and HK2 expressions were measured by western blotting. The experiment was conducted independently three times. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Discussion

NEK6 has been explored as a therapeutic target in cancer [33]. However, few compounds are known to inhibit its expression and/or activity [34–36]. In this manuscript, we showed that resveratrol can inhibit NEK6 expression in a dose-dependent manner in DU-145 cells. We also showed that NEK6 lack sensitizes cancer cells to resveratrol treatment, inducing cell death.

Resveratrol has been shown to inhibit cell survival pathways, being able to

directly activate the apoptosis pathway and block anti-apoptotic mechanisms, sensitizing cancer to cell death [37]. In this manner, inhibition of NEK6 by resveratrol may be a resveratrol-mediated survival reduction mechanism. We suggest that when we abolish the total expression of NEK6 by CRISPR/Cas9, synergism and stronger activation of apoptotic pathways may occur, which leads to greater sensitization of cancer cells to death. This also indicates that NEK6 may play an important role in the anti-cancer effects that resveratrol plays on tumor cells.

The phosphorylation of glucose, a crucial step in the processing of glucose, is carried out by HK2 [38]. The metabolic phenotype of cells, supporting cancerous growth, is altered by the expression of HK2 [24]. HK2 is exclusively expressed in human prostate cancer tissue compared with normal prostate tissue, and its expression is particularly elevated in human prostate cancer that has PTEN/p53 mutations, which is common in lethal CRPC [39]. In xenograft mouse models of prostate cancer, genetic research indicates that the growth of tumors driven by Pten/p53 deficiency requires HK2-mediated aerobic glycolysis, which is commonly referred to as the Warburg effect [39]. The TP53 gene in DU145 cells has undergone two distinct point mutations (Phe223Leu and Val274Phe) located on separate alleles, resulting in a nonfunctional protein [40]. Therefore, HK2, as well as the Warburg effect, are relevant points to be evaluated in the cell model chosen in our study, DU-145.
Here, we provide evidence that the knockout of NEK6 reduces HK2 expression in DU-145 cells. Congruently, NEK6 overexpression increases HK2 expression. Bioinformatics data from prostate cancer samples show that there is a moderately positive correlation between these two genes in PRAD. Therefore, we have evidence for the first time that there is an axis involving NEK6 and hexokinase 2, both survival markers in CRPC.

In cancer cells, HK2 is often upregulated and contributes to the Warburg effect, which is the phenomenon where cancer cells preferentially utilize glycolysis over oxidative phosphorylation, even in the presence of oxygen [41]. One of the consequences of the Warburg effect is increased lactate production, which promotes tumor growth and survival [42]. HK2-induced proliferation, resistance to apoptosis, and lactate production [43]. Our data showed that NEK6-deficient cells showed a reduction in lactate production, which may be related to a reduction in HK2. In our previous study already published, we demonstrated that NEK6-KO in DU-145 reduced the clonogenic capacity, proliferation, cell viability, and mitochondrial membrane permeability, and

increased cell death [31]. The modulation of HK2 by NEK6 may be related to these functional alterations observed in prostate cancer cells. We did not observe changes in the amount of glucose in the supernatant of WT and NEK6-deficient cells. Another study revealed that deleting HK2 systematically in genetic mouse models hinders the advancement of tumors but does not affect regular glucose maintenance in vivo. This suggests that HK2 could be a targeted therapy for cancer without any harmful physiological effects [44].

Studies carried out in other types of cancer, such as hepatocellular carcinoma and non-small cell lung cancer showed that resveratrol inhibits HK2 and reduced tumor growth in vivo [24,45]. Furthermore, resveratrol inhibited HK2 in endothelial cells, causing a decrease in angiogenesis [46]. According to the study, the resveratrol treatment in aerobic glycolytic cells caused the activation and oligomerization of Bax on mitochondria may have been facilitated through the downregulation of HK2 [24]. Several types of cancer have been demonstrated to have a connection between mitochondria-bound-HK2 and apoptosis, as well as the evasion of mitochondrial cell death [47]. An interesting point to be evaluated is checking if NEK6 is involved in HK2 translocation to the mitochondria since we had already proved that NEK6-KO altered the permeability of the mitochondria membrane and also regulated some mitochondrial proteins expression [31].

In this manuscript, we demonstrated that resveratrol inhibits NEK6 expression in a dose-dependent manner in DU-145 prostate cancer cells, indicating that NEK6 could also be a protein involved in cell survival pathways. Studies already showed how resveratrol inhibits cell survival signaling pathways, including apoptosis inhibitor proteins (IAPs), such as survivin [48] Bcl-xL and Mcl-1 [49], via NF-kappaB via SIRT1 [50], via PI3K/AKT and WNT/beta-catenin and MAPK [51]. We observed that resveratrol is also able to reduce HK2 expression. The downregulation of HK2 by resveratrol may be through the downregulation of NEK6, configuring a new mechanism of action for resveratrol. One of the antitumor functions of resveratrol already described is the reduction of lactate levels, which contributes to the reversal of the Warburg effect, modification of the tumor microenvironment, and reduction of proliferation [52,53]. NEK6 knockout cells showed a reduction in lactate levels. Since resveratrol is already known to reduce lactate, then we hypothesized that this mechanism is mediated by the NEK6-HK2 axis. Future studies are needed to better understand the relationship between NEK6 and the Warburg effect.

Conclusion

Due to its capacity to alter crucial survival pathways and enhance the sensitivity of cancer cells to other chemotherapy medications, resveratrol has already been extensively investigated for its effectiveness in treating advanced prostate cancer. NEK6 and HK2 were shown to be down-regulated targets after resveratrol treatment in DU-145 cells. Furthermore, we identified that NEK6 modulates HK2 expression, thus suggesting that the NEK6-HK2 axis may be a new mechanism for reducing cell survival of resveratrol. Cells lacking NEK6 exhibit increased sensitivity to resveratrol, potentially indicating a synergistic effect between these interventions and the relevance of NEK6 in the anti-cancer effect of resveratrol. Decreased lactate production in NEK6-KO cells indicates that this mechanism may be related to HK2 modulation. Therefore, we suggest that NEK6 inhibition by resveratrol may be used in favor of combining resveratrol with other chemotherapeutics in certain cancers where NEK6 is relevant, such as CRPC. Further research is necessary to gain a more comprehensive understanding of how NEK6 is linked to the Warburg effect.

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5. DISCUSSÃO GERAL

O nosso estudo mostrou que a NEK6 está envolvida no câncer de próstata resistente à castração, regulando o equilíbrio redox e a resposta ao dano no DNA (Pavan *et al.*, 2023). A falta de NEK6 em células DU-145 de câncer de próstata reduziu a proliferação, viabilidade e potencial de membrana mitocondrial, além da indução à apoptose celular. A literatura existente já enfatiza que a família das NEKs deve ser explorada como alvos estratégicos no tratamento do câncer (Oliveira, de *et al.*, 2020; Panchal e Evan Prince, 2022). A inibição de NEK6 tem sido vista como um potencial alvo no câncer, e os inibidores dessa quinase já estão sendo investigados em alguns tipos de câncer (Panchal, Mohanty e Prince, 2022; Rojo *et al.*, 2004). Por esse motivo, é essencial entender as vias de sinalização e mecanismos de sobrevivência que a NEK6 está envolvida no câncer de próstata resistente à castração, bem como explorar os inibidores dessa quinase e as possibilidades deles terem um futuro terapêutico.

O estresse oxidativo pode causar agressividade na maioria dos tipos de câncer, incluindo câncer de próstata (Mondal *et al.*, 2021). Um aumento moderado de ROS induz a proliferação celular, enquanto quantidades excessivas de ROS promovem a morte celular (Donato, De *et al.*, 2018; Perillo *et al.*, 2020). As células cancerígenas reagem a um aumento nos níveis de ROS e sobrevivem neste ambiente de estresse oxidativo através da indução da transcrição de enzimas antioxidantes. É essencial entender essas vias para elaborar terapias eficientes que modifiquem os níveis de ROS. Os indutores de ROS parecem ter a capacidade de induzir apoptose e inibir o crescimento do tumor, migração celular e características invasivas em vários tipos de câncer (Das, Suman e Damodaran, 2014; Donato, De *et al.*, 2018). Nossos dados mostram que a falta de NEK6 reduz a expressão de SOD2 no nível do RNA mensageiro e também SOD1, SOD2 e PRDX3 no nível protéico. Além disso, a superexpressão de NEK6 em células DU-145 aumenta as mesmas proteínas antioxidantes. Adicionalmente, a depleção de NEK6 gera mais ROS nas células. Esses dados sugerem que NEK6 regula o balanço redox através da modulação de proteínas antioxidantes. Além disso, SOD1, SOD2 e PRDX3 têm extensa literatura mostrando seus papéis pró-oncogênicos e envolvimento com a resistência à quimioterapia através do estresse oxidativo (Kim *et al.*, 2010; Li *et al.*, 2019; Ma *et al.*, 2021; Papa, Manfredi e Germain, 2014; Ramasamy *et al.*, 2020; Sun, Dong e Wu, 2017; Ummanni *et al.*, 2012; Zhu *et al.*, 2019; Zuo *et al.*, 2019). A modulação dessas proteínas antioxidantes revela um novo mecanismo de NEK6 regular a sobrevivência

celular em CPRC.

SOD1 é uma enzima antioxidante essencial amplamente distribuída na célula. Sua principal função é catalisar a dismutação de O^{2-} em H_2O_2, protegendo as células do estresse oxidativo (Das, Suman e Damodaran, 2014). Um estudo mostrou que um inibidor específico de SOD1 reprimiu o crescimento de células cancerígenas e promoveu a parada do ciclo celular e a indução à apoptose, através do aumento de ROS em vários tipos de câncer (Li *et al.*, 2019). A SOD1 também foi associada à resistência à quimioterapia (Kim *et al.*, 2010; Zhu *et al.*, 2019). A SOD1 foi identificada como superexpressa em animais com xenoenxertos de câncer de próstata resistentes à mitoxantrona (MTX), um agente antineoplásico usado em CPRC (Zhu *et al.*, 2019). Para estudos futuros, sugerimos que uma eventual combinação de inibidores de NEK6 com MTX pode ser uma estratégia interessante para reduzir a resistência das células cancerígenas ao agente quimioterápico.

SOD2 é uma enzima que catalisa a dismutação de O^{2-} em H_2O_2 na mitocôndria, causando uma redução de ROS. O papel da SOD2 no câncer é controverso. Inicialmente, SOD2 foi considerado um gene supressor de tumor com base em seus baixos níveis em vários tipos de câncer (Kim *et al.*, 2017). No entanto, estudos mais recentes revelaram que baixos níveis de SOD2 são um evento precoce de tumores, e níveis mais altos de SOD2 estão associados à progressão do tumor e à metástase (Kim *et al.*, 2017). Os níveis de RNA mensageiro e proteína de SOD2 foram encontrados elevados em amostras de pacientes com câncer de próstata em estágio intermediário (Miar *et al.*, 2015). Recentemente, foi demonstrado que SOD2 aumenta a captação de GLUT-1 e glicose, o que é essencial para a sobrevivência das células do câncer de próstata (Quiros-Gonzalez *et al.*, 2022). Vários fatores de transcrição são mostrados para regular a expressão de SOD2, como o Fator Nuclear-Kappa B (NF-κB) e o fator nuclear eritroide 2 relacionado ao fator 2 (Nrf2), entre outros (Das, Lewis-Molock e White, 1995; Zhang *et al.*, 2022). Uma vez que a ausência de NEK6 modulou a expressão de SOD2 nos níveis de mRNA e proteína, avaliamos o conteúdo nuclear de p52 NF-κB2 em células com superexpressão de NEK6. Observamos que NEK6 regula a localização nuclear de p52 NF-κB2. Sugerimos que NEK6 também pode regular as vias de sobrevivência e a expressão de SOD2, através da sinalização direta ou indireta de NF-κB.

PRDX3 é uma peroxidase mitocondrial, que desempenha um papel na proteção celular contra o estresse oxidativo por peróxidos desintoxicantes (Cao, Bhella e

Lindsay, 2007). O PRDX3 é superexpresso em vários tipos de câncer, incluindo câncer de próstata, protegendo as células contra a apoptose (Byun *et al.*, 2018; Ramasamy *et al.*, 2020; Ummanni *et al.*, 2012). Além disso, o PRDX3 foi encontrado superexpressa em células de câncer de próstata resistentes à castração, o que culmina na promoção da sobrevivência celular, protegendo-as do estresse oxidativo (Whitaker *et al.*, 2013). Por esta razão, PRDX3 é um alvo potencial para CPRC. Um estudo do nosso grupo mostrou que NEK6 interage com PRDX3 (Vaz Meirelles *et al.*, 2010). É possível que NEK6 possa regular a expressão de PRDX3 por meio dessa interação previamente estabelecida, entretanto estudos precisam ser feitos para validar esse mecanismo.

As ROS promovem a fosforilação e ativação da resposta ao dano de JNK e DNA, levando à apoptose (Shi *et al.*, 2014; Simon, Haj-Yehia e Levi-Schaffer, 2000). A fosforilação e ativação de ATM e JNK medeiam a formação de focos yH2AX (Feraudy, de *et al.*, 2010). O nocaute de NEK6 induz a geração de ROS, ativa JNK, uma quinase sensível ao estresse e induz danos ao DNA. Considerando os efeitos celulares da falta de NEK6 no dano ao DNA e na apoptose, sugerimos que estudos futuros possam explorar os inibidores de NEK6 como potenciais indutores de letalidade sintética no câncer de próstata.

A Bcl-2 está localizada na membrana externa mitocondrial (MOM) e desempenha importantes funções antiapoptóticas (Wang *et al.*, 2011). A permeabilização de MOM libera fatores pró-apoptóticos, como o fator indutor de apoptose (AIF) e o citocromo C da mitocôndria. AIF entra no núcleo e gera extensa fragmentação do DNA, enquanto o citocromo C no citosol pode iniciar a cascata de ativação de caspases. A Bcl-2 é antiapoptótica e contribui para a integridade do MOM, impedindo a liberação desses fatores pró-apoptóticos da mitocôndria (Kowaltowski, Vercesi e Fiskum, 2000). Além disso, a Bcl-2 é crítica para a sobrevivência das células de câncer de próstata independentes de andrógenos e também necessário para a progressão das células de câncer de próstata de um estágio de crescimento dependente de andrógeno para um independente de andrógeno (Lin *et al.*, 2007). Assim, avaliamos a apoptose e a expressão de Bcl-2 nas linhagens celulares WT e NEK6-KO. Nós identificamos que as células NEK6-KO tem uma expressão reduzida da proteína Bcl-2 e aumento da apoptose. Também descobrimos que as células NEK6-KO apresentaram um aumento na despolarização da membrana mitocondrial, o que pode estar relacionado a uma redução nos níveis de Bcl-2 e indução de

apoptose. Esses resultados revelaram novos mecanismos de sobrevivência celular regulados por NEK6 em um modelo celular de CPRC.

Ao explorar a modulação das defesas antioxidantes em células WT e NEK6-KO, observamos que a expressão de SOD1, SOD2 e PRDX3 foi significativamente reduzida em células NEK6-KO tratadas com cisplatina quando comparadas com células WT, o que pode explicar parcialmente o porquê das células NEK6-KO terem uma maior sensibilidade a este quimioterápico. O aumento na expressão de SOD2 pode ser um mecanismo que desencadeia uma resistência à quimioterapia (Ma *et al.*, 2021; Zuo *et al.*, 2019). A regulação positiva de SOD2 mediada por TNF-α está envolvida na resistência à cisplatina no câncer de esôfago. A regulação positiva de SOD2 por TNF-α foi inibida pelo bloqueio da via NF-κB, sugerindo que SOD2 através da via de sinalização NF-κB contribui para a proliferação de células de câncer de esôfago (Zuo *et al.*, 2019). Em nossos dados, a expressão de SOD2 foi encontrada elevada em células WT tratadas com cisplatina, sugerindo um mecanismo de resistência em células WT. Uma redução na expressão de SOD2 em células NEK6-KO tratadas com cisplatina significa que esse possível mecanismo de resistência encontrado em WT foi eliminado em células NEK6-KO. Os dados mostram uma expressão mais baixa de Bcl-xL em células NEK6-KO tratadas com cisplatina indicam uma maior indução de apoptose nessas células em comparação com células WT. O tratamento com cisplatina também aumentou γH2AX em células NEK6-KO em comparação com WT, mostrando que a falta de NEK6 causa instabilidade genômica e pode ser responsável pelo aumento da morte celular em células NEK6-KO. Assim, sugerimos que a falta de NEK6 sensibiliza as células à cisplatina, uma vez que o tratamento com cisplatina nessas células deficientes em NEK6 diminuiu muito as defesas antioxidantes (SOD1, SOD2 e PRDX3), o que pode culminar em níveis excessivos de ROS e danos ao DNA. Inibir NEK6 em combinação com outras drogas anticancerígenas que desestabilizam o equilíbrio redox pode ser uma estratégia interessante para aumentar os níveis de ROS acima dos níveis toleráveis para as células, induzindo portanto a morte celular. Outro ponto é que observamos uma diminuição do potencial de membrana mitocondrial nas células NEK6-KO, indicando uma redução na atividade mitocondrial. Além disso, a cisplatina prejudica a função da cadeia de transporte de elétrons, aumenta os níveis intracelulares de ROS e afeta a viabilidade mitocondrial (Marullo *et al.*, 2013). Então, a cisplatina pode estar agindo em sinergismo com os efeitos nas mitocôndrias mediados pela falta de NEK6,

sensibilizando essas células à cisplatina.

Realizamos nossos experimentos utilizando apenas uma linhagem celular, o que pode ser considerado uma limitação do nosso estudo. Acreditamos que o papel da NEK6 em outras linhagens celulares de câncer de próstata deve ser mais investigado, mas nossos dados atuais apontam um papel importante da NEK6 na sensibilidade à cisplatina e como uma terapia coadjuvante para o tratamento do câncer de próstata.

A NEK6 foi associada à resistência à cisplatina e ao paclitaxel no câncer de ovário (Donato, De *et al.*, 2015, 2018). A quimioterapia baseada em docetaxel é a principal estratégia para o tratamento de pacientes com câncer de próstata resistente à castração. No entanto, é um tratamento paliativo e geralmente está associado a resistência e metástase. Nesse estudo, relatamos que a NEK6 é superexpressa em amostras de câncer de próstata e também está relacionada à baixa sobrevida global dos pacientes. Partindo do pressuposto de que não existem estudos avaliando a relação entre NEK6 e quimioterapia com docetaxel, este estudo pretendeu avaliar qual o efeito da inibição de NEK6 em conjunto com o tratamento com docetaxel nas células de câncer de próstata.

Como primeira abordagem, eliminamos o gene NEK6 em células DU-145 e PC-3 e desafiamos com concentrações crescentes de docetaxel. Verificou-se que a depleção de NEK6 sensibilizou as células DU-145 e PC-3 à citotoxicidade induzida por docetaxel. Sugerimos que os mecanismos mediados pela NEK6 na célula, seja por alteração na expressão ou por interação com outras proteínas, podem contribuir para os efeitos anticancerígenos do docetaxel, podendo até mesmo ser um alvo do docetaxel.

Como próximo passo, gostaríamos de saber se o promissor composto caracterizado como um inibidor de NEK6 (denominado Composto 8) pelo estudo de Donato (Donato, De *et al.*, 2018) teria um efeito citotóxico em células de câncer de próstata. Descobrimos que o IC_{50} do composto para DU-145 foi de 37,45 µM e para PC-3 foi de 48,2 µM. O efeito de citotoxicidade do composto 8 contra um painel de células cancerígenas foi determinado por Donato, em geral, o composto tinha um IC_{50} inferior a 100 µM contra um painel de células cancerígenas, como ovário, mama, pulmão e cólon. Os resultados obtidos no nosso estudo são relevantes, porque é o primeiro relato indicando o efeito desse composto nas células cancerígenas da próstata. De forma geral, ainda é um composto que necessita de maiores

investigações sobre seus efeitos moleculares, principalmente no que se diz respeito ao câncer de próstata.

Iniciamos uma investigação deste composto inibidor de NEK6 no câncer de próstata por ensaio clonogênico em células DU-145 e PC-3. Além disso, exploramos seu efeito em combinação com docetaxel. O NEK6-I sozinho foi capaz de reduzir o número de colônias nas células DU-145, assim como o docetaxel. Quando o co-tratamento com docetaxel e inibidor foi realizado, o efeito foi ainda mais pronunciado. Nas células PC-3, porém, houve redução no número de colônias apenas com o inibidor, mas sem significância estatística. No entanto, o tratamento com docetaxel e NEK6-I foi capaz de reduzir fortemente a capacidade clonogênica quando comparado ao tratamento com docetaxel sozinho em células PC-3. Esses dados estão de acordo com os resultados que mostraram que a depleção de NEK6 por CRISPR-Cas9 sensibilizam as células ao docetaxel. Um dos mecanismos pelos quais pode ocorrer o aumento da citotoxicidade na associação desses compostos pode ser devido ao aumento exacerbado de ROS, pois já sabemos que a NEK6 modula as proteínas antioxidantes e, consequentemente, aumenta o estresse oxidativo. O docetaxel também é capaz de aumentar os níveis de ROS (Swetha *et al.*, 2020; Zhang *et al.*, 2018). Assim, o aumento excessivo de ROS torna as células mais sensíveis à morte celular. No entanto, muitos outros mecanismos podem estar envolvidos na inibição da NEK6, que contribuem para o aumento da sensibilidade ao docetaxel.

Realizamos um ensaio de MTT para investigar a viabilidade celular após o tratamento com docetaxel e inibidor de NEK6 individualmente e também em co-tratamento. Descobrimos que a combinação dessas drogas foi capaz de reduzir significativamente a viabilidade das células DU-145. Para aprofundar o conhecimento subjacente ao papel da NEK6 e do efeito quimioterapêutico do docetaxel na progressão do câncer de próstata, a sinalização apoptótica, de sobrevivência e de migração celular foram exploradas.

A poli (ADP-ribose) polimerase 1 (PARP1) liga-se firmemente às quebras da fita de DNA, realiza a auto-poli ADP-ribosilação e permite o acesso da enzima de reparo ao DNA danificado (Zhang *et al.*, 2020). A clivagem de PARP1 e a liberação de um fragmento de 89 kDa estão associadas à estimulação da morte celular (Kaufmann *et al.*, 1993). A PARP1 é clivada pelas caspases 3 e 7 no início da apoptose celular, limitando sua ação no reparo do DNA (Kaufmann *et al.*, 1993). PARP1 também é clivado por catepsinas, induzindo vias de morte celular autofágica

e necrótica (Chaitanya e Babu, 2009; Gobeil *et al.*, 2001). Em nossos dados, o tratamento com docetaxel em células DU-145 induziu a clivagem de PARP1, no entanto, a combinação de docetaxel e o inibidor de NEK6 foi muito mais eficaz na clivagem de PARP-1 e, conseqüentemente, na indução da morte celular. Outras proteínas relacionadas à via apoptótica foram avaliadas, como BAK, Bcl-xL e BAD fosforilada.

BAK medeia a permeabilização da membrana externa mitocondrial (MOMP) através da oligomerização na membrana mitocondrial (Moldoveanu *et al.*, 2006). Agentes quimioterápicos desencadeiam a ativação de BAK e BAX, que integram sinais apoptóticos versus de sobrevivência para determinar o destino celular (Fox, 2015). Uma vez que um 'limiar' apoptótico foi ultrapassado e há moléculas BAK/BAX suficientemente ativadas, a célula torna-se comprometida a morrer por apoptose (Fox, 2015). O tratamento com NEK6-I aumentou a expressão de BAK, enquanto o docetaxel não aumentou significativamente. Nossos resultados revelaram que a expressão de BAK foi maior no co-tratamento com docetaxel e NEK6-I quando comparado apenas com docetaxel. Também observamos que Bcl-xL, uma proteína anti-apoptótica, foi reduzida no tratamento com NEK6-I. No entanto, não foram identificadas diferenças no co-tratamento. A expressão de Bcl-xL está altamente presente em amostras de pacientes com CRPC. Sabe-se que Bcl-xL pode interagir com Bak, reprimindo sua atividade pró-apoptótica e contribuindo para a resistência à castração e progressão do câncer de próstata (Castilla *et al.*, 2006). A fosforilação de Bad em S112 por PKAc também causa a dissociação de BAD e Bcl-xL na membrana mitocondrial externa. O BAD liga-se ao 14-3-3 no citoplasma, promovendo a apoptose (Downward, 1999). Também observamos que docetaxel e NEK6-I reduziram a fosforilação de BAD, que manteve o mesmo nível de expressão no co-tratamento. Reunindo essas informações, foram observadas alterações na via apoptótica com tratamento com inibidor de NEK6 e/ou co-tratamento com docetaxel com o inibidor de NEK6, sugerindo que o aumento da quimiossensibilidade ao docetaxel em DU-145 ocorre junto com a inibição de NEK6. Ou simplesmente a presença do inibidor é capaz de estimular vias apoptóticas.

Está bem estabelecido que a ativação de NF-κB p65 está associada a tumores de próstata resistentes à castração e à progressão do câncer de próstata (McCall *et al.*, 2012). O NF-κB contribui para o crescimento de células de câncer de próstata independentes de andrógenos na ausência de sinalização de andrógenos (McCall *et*

al., 2012). Nossos dados mostraram que o tratamento com NEK6-I reduz a fosforilação de p65, bem como em co-tratamento com docetaxel e NEK6-I em comparação com docetaxel sozinho.

TJP1 (Tight Junction Protein 1), também conhecida como ZO-1, é uma importante proteína estrutural que está envolvida na formação e estabilidade de junções entre as células epiteliais. Junções apertadas são estruturas importantes para manter a integridade da barreira epitelial e controlar o tráfego de moléculas e células através dela. O envolvimento de TJP1 com a migração celular é controverso. A expressão de ZO-1 está associada a características metastáticas (Huo *et al.*, 2011; Kim *et al.*, 2021; Lee *et al.*, 2022; Polette *et al.*, 2005; Tan *et al.*, 2005), mas outros estudos mostraram que a diminuição da expressão de ZO-1 está associada a metástases tumorais (Martin e Jiang, 2009; Zhang *et al.*, 2019). Analisamos a expressão de TJP1/ZO1 e observamos que há uma redução significativa na expressão dessa proteína no tratamento com docetaxel e NEK6-I isoladamente. No entanto, o co-tratamento reduziu ainda mais a expressão dessa proteína. Portanto, sugerimos que o inibidor de NEK6 altera a migração celular, mas isso também é observado de forma mais acentuada no co-tratamento com docetaxel.

Portanto, analisamos a migração celular através do ensaio de scratch, observamos que o co-tratamento de docetaxel com NEK6-I reduziu a migração celular quando comparado com docetaxel sozinho em células DU-145. Nas células PC-3, o efeito foi mais pronunciado, pois o próprio inibidor de NEK6 foi capaz de reduzir a migração, mas na ação com docetaxel, a migração celular foi ainda mais reduzida. A depleção de NEK6 por CRISPR/Cas9 também reduz a migração celular em células DU-145. Sugerimos que a redução da migração pode estar associada à redução do TJP1 nos tratamentos isolados e em combinação. O inibidor de NEK6 reduziu a expressão de NEK6 em DU-145, o que é um fato inesperado, pois o estudo que caracterizou esse inibidor revelou que houve redução apenas em sua atividade (Donato, De *et al.*, 2018).

Também evidenciamos o papel do resveratrol na redução da expressão de NEK6. O resveratrol é amplamente reconhecido como um agente quimiopreventivo e demonstrou potencializar a atividade antitumoral dos quimioterápicos convencionais em vários tumores, incluindo o câncer de próstata (Zaffaroni e Beretta, 2020). Vários estudos vem sendo feitos no sentido de combinação de drogas com o resveratrol, bem como abordagens de nanomedicina para melhorar a atividade e entrega do resveratrol

(Zaffaroni e Beretta, 2020). Em nossos dados, mostramos que a depleção de NEK6 por CRISPR-Cas9 sensibiliza as células de câncer de próstata DU-145 ao tratamento com resveratrol, induzindo a morte celular. Sugerimos, então, que quando abolimos a expressão total de NEK6 pode ocorrer sinergismo e ativação intensa das vias apoptóticas, o que leva a uma maior sensibilização das células cancerígenas. Isso também indica que a modulação de NEK6 pode desempenhar um papel importante nos efeitos anti-cancerígenos que o resveratrol desempenha nas células tumorais.

Nós demonstramos que o resveratrol inibe a expressão de NEK6 de maneira dose-dependente em células de câncer de próstata DU-145, indicando que NEK6 também pode ser uma proteína envolvida nas vias de sobrevivência celular. Estudos já mostraram como o resveratrol inibe as vias de sinalização de sobrevivência celular, incluindo proteínas inibidoras de apoptose (IAPs), como survivina (Altieri, 2003), Bcl-xL e Mcl-1 (Jazirehi e Bonavida, 2004), via NF-kappaB via SIRT1 (Yeung *et al.*, 2004), via PI3K/AKT e WNT/beta-catenina e MAPK (Liu *et al.*, 2014).

A fosforilação da glicose, uma etapa crucial no processamento da glicose, é realizada pela HK2 (Deeb, Malkki e Laakso, 1993). O fenótipo metabólico das células, suportando o crescimento canceroso, é alterado pela expressão de HK2 (Dai *et al.*, 2015). A HK2 é expressa exclusivamente no tecido do câncer de próstata humano em comparação com o tecido da próstata normal, e sua expressão é particularmente elevada no câncer de próstata humano que possui mutações PTEN/p53, o que é comum no CRPC letal (Wang *et al.*, 2014). Em modelos de xenoenxerto de camundongos de câncer de próstata, a pesquisa genética indica que o crescimento de tumores impulsionados pela deficiência de Pten/p53 requer glicólise aeróbica mediada por HK2, que é comumente referido como efeito Warburg (Wang *et al.*, 2014). O gene *TP53* nas células DU145 sofreu duas mutações pontuais distintas (Phe223Leu e Val274Phe) localizadas em alelos separados, resultando em uma proteína não funcional (Chappell *et al.*, 2012). Portanto, HK2, assim como o efeito Warburg, são pontos relevantes a serem avaliados no modelo celular escolhido em nosso estudo, DU-145.

Aqui, fornecemos evidências de que o nocaute de NEK6 reduz a expressão de HK2 em células DU-145. Congruentemente, a superexpressão de NEK6 aumenta a expressão de HK2. Dados de bioinformática de amostras de câncer de próstata mostram que existe uma correlação moderadamente positiva entre esses dois genes no PRAD. Portanto, temos evidências pela primeira vez de que existe um eixo

envolvendo NEK6 e hexoquinase 2, ambos envolvidos no CRPC.

Nas células cancerígenas, HK2 é frequentemente regulado positivamente e contribui para o efeito Warburg, que é o fenômeno em que as células cancerígenas utilizam preferencialmente a glicólise sobre a fosforilação oxidativa, mesmo na presença de oxigênio (Sun *et al.*, 2021). Uma das consequências do efeito Warburg é o aumento da produção de lactato, que promove o crescimento e a sobrevivência do tumor (Anderson *et al.*, 2017). Nossos dados mostraram que as células deficientes em NEK6 apresentaram uma redução na produção de lactato, o que pode estar relacionado a uma redução na HK2. Em nosso estudo anterior já publicado, demonstramos que NEK6-KO em DU-145 reduziu a capacidade clonogênica, proliferação, viabilidade celular e permeabilidade da membrana mitocondrial e aumentou a morte celular (Pavan *et al.*, 2023). A modulação de HK2 por NEK6 pode estar relacionada a essas alterações funcionais observadas em células de câncer de próstata. Não observamos alterações na quantidade de glicose no sobrenadante de células WT e deficientes em NEK6. Outro estudo revelou que a exclusão sistemática de HK2 em modelos genéticos de camundongos impede o avanço de tumores, mas não afeta a manutenção regular da glicose *in vivo*. Isso sugere que o HK2 pode ser uma terapia direcionada para o câncer sem quaisquer efeitos fisiológicos prejudiciais (Deng e Lu, 2015). Uma das funções antitumorais do resveratrol já descritas é a redução dos níveis de lactato, o que contribui para a reversão do efeito Warburg, modificação do microambiente tumoral e redução da proliferação (Chen *et al.*, 2022; Saunier *et al.*, 2017). Nós observamos que os nocautes de NEK6 apresentaram uma redução na produção de lactato, o que pode ser devido a diminuição da expressão de HK2.

Outro ponto a ser dado atenção é que o aumento da expressão de HK2 aumenta os intermediários da via glicolítica, que podem ser utilizados em outras vias anabólicas, como a via das pentoses fosfato, suprindo a demanda de nucleotídeos utilizados pelas células cancerosas (Jin e Zhou, 2019). Sendo assim, avaliar a via das pentoses fosfato pode ser interessante no contexto da modulação de NEK6.

De maneira geral, sugerimos que a inibição da NEK6 pelo resveratrol pode ser usada em favor da combinação do resveratrol com outros quimioterápicos em certos tipos de câncer em que a NEK6 é relevante, como CPRC. Mais pesquisas são necessárias para obter uma compreensão mais abrangente de como o NEK6 está ligado ao efeito Warburg.

6. CONCLUSÃO GERAL

Há relevância da proteína NEK6 no modelo de câncer de próstata está evidente pelos resultados apresentados, bem como em alguns poucos estudos presentes na literatura. A busca por um inibidor de NEK6 é um assunto abordado por alguns pesquisadores, a sua validação em células de câncer de próstata torna-se o primeiro passo para que seja possível explorar seus mecanismos e possível ação anti-cancer nesse modelo. Mostramos que o nocaute de NEK6 modula vias de sinalização de estresse oxidativo, dano no DNA, vias apoptopticas, de sobrevivência, como o NF-κB, e migração celular, além de estar associado com intensificar os efeitos da cisplatina e docetaxel, principais quimioterápicos usados em CPRC. O resveratrol, composto com características anti-tumorais bem descritas, principalmente no câncer de próstata mostrou inibir a proteína NEK6 e HK2, um eixo de sinalização que pode ser melhor explorado em estudos futuros. Dessa forma, esse estudo contribui para o melhor entendimento das funções de NEK6 no contexto do câncer de próstata avançado.

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ANEXOS

- **Certificado de apresentação de poster em eventos internacionais**

Cell Symposia

Gene- and Cell-Based Therapies: CRISPR, Stem Cells, and Beyond

March 2 – 4, 2020 — San Francisco, CA, USA

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65th Brazilian Congress of Genetics
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CERTIFICADO

Certificate of poster presentation

This hereby certifies that

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presented the poster:

THE ROLE OF NEK6 PROTEIN IN PROGRESSION OF CASTRATION-RESISTANT PROSTATE CANCER (CRPC) USING THE CRISPR/CAS9 GENE EDITING SYSTEM.

Autores: Pavan, I.C.B ; Issayama, L.K ; Silva, F.R ; Morelli, A.P ; Pereira, N.F.P ; Silva, L.G.S ; Simabuco, F.M.S ; Kobarg. J

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Márcio de Castro Silva Filho
Presidente da SBG

SBG SOCIEDADE BRASILEIRA DE GENÉTICA

Antonio Mateo Solé Cava
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Isadora Carolina Betim Pavan

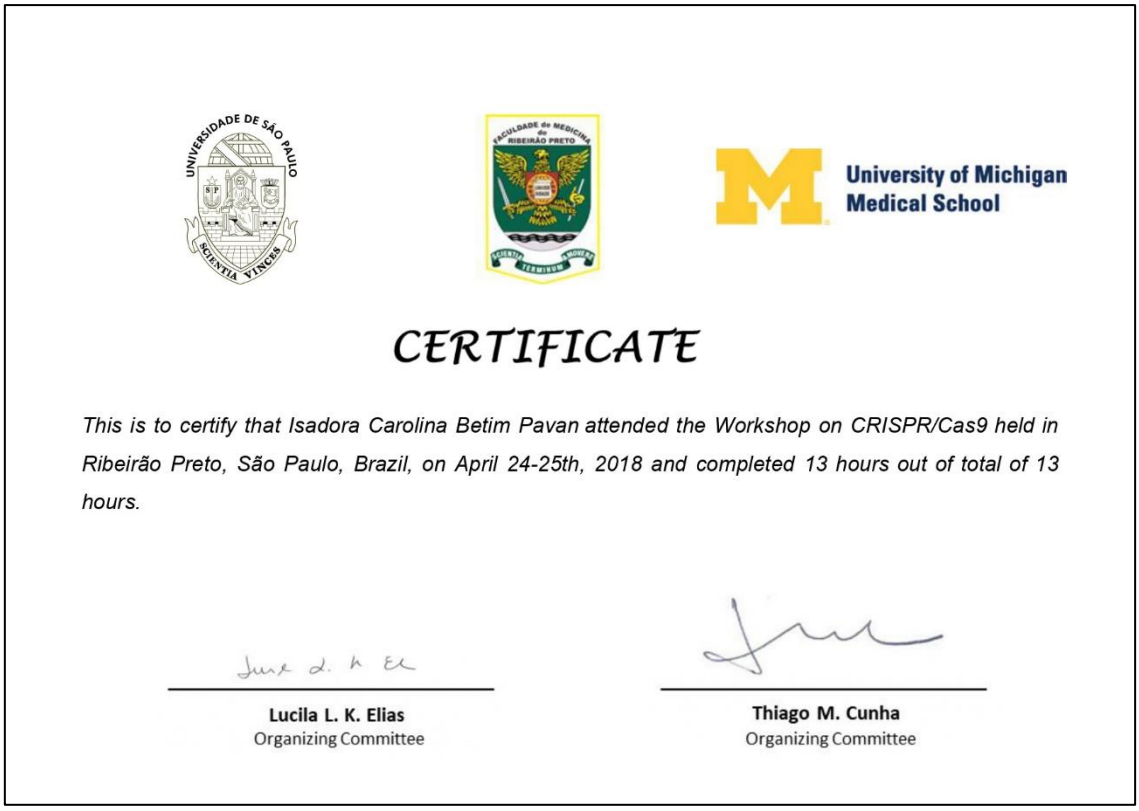
participou como **PALESTRANTE** da **I Jornada GEMOCA Online**, realizada no período de 20 a 24 de julho de 2020, na plataforma Even3, com a palestra "CRISPR-Cas9: uma ferramenta de edição gênica", no modo On-Demand (Gravado).

Campinas, 24 de Julho de 2020

Profª. Dra. Laura Sterian Ward
Coordenadora do Laboratório de Genética Molecular do Câncer

Profª. Dra. Natássia Elena Bufalo
Presidente da I Jornada GEMOCA Online

- **Certificado de participação em cursos**



University of Michigan
Medical School

CERTIFICATE

This is to certify that Isadora Carolina Betim Pavan attended the Workshop on CRISPR/Cas9 held in Ribeirão Preto, São Paulo, Brazil, on April 24-25th, 2018 and completed 13 hours out of total of 13 hours.

Lucila L. K. Elias
Organizing Committee

Thiago M. Cunha
Organizing Committee

GENÉTICA 2019
17 a 20 de setembro de 2019
Hotel Monte Real Resort - Águas de Lindóia - SP
65th Brazilian Congress of Genetics

CERTIFICADO

Certificate of Completion

This hereby certifies that

Isadora Carolina Betim Pavan

attended the course:

"6 - Proteômica e sistemas biológicos no estudo de doenças humanas"

during the

Genética 2019,
in Hotel Monte Real Resort, Águas de Lindóia, SP

Márcio de Castro Silva Filho
Presidente da SBG

SBG SOCIEDADE BRASILEIRA DE GENÉTICA

Antonio Mateo Solé Cava
Primeiro Secretário da SBG

- **Certificado de organização de eventos**

UNICAMP ProEC Pró-Reitoria de Extensão e Cultura ESCOLA DE EXTENSÃO

DECLARAÇÃO

Declaramos para os devidos fins que o(a) Sr(a): ISADORA CAROLINA BETIM PAVAN, ministrou aulas no Curso de Difusão Científica abaixo especificado:

Curso: APLICAÇÕES PRÁTICAS DA TECNOLOGIA CRISPR/CAS9
Modalidade Extensão Universitária
Processo n°: 01-P-24936/2018
Carga Horária Total do Curso: 40 horas
Período: 15/07/2019 à 19/07/2019

Carga Horária Ministrada pelo(a) professor(a): 0020:00 horas

Cidade Universitária 'Zeferino Vaz', 28/08/2019

PROF. DR. EDUARDO GALEMBECK
DIRETOR DA ESCOLA DE EXTENSÃO
DIRETORIA DE EXTENSÃO
PROEC/UNICAMP

EXTECAMP - Escola de Extensão da Unicamp
Rua Saturnino de Brito, nº 323, 2º andar. Cidade Universitária - Campinas - SP.
www.extecamp.unicamp.br | +55 19 35214647 - 35214646

- **Certificado de participação em bancas**

Universidade Federal de São Paulo
Campus Baixada Santista
UNIFESP
Curso de Nutrição

D E C L A R A Ç Ã O

Declaro, para os devidos fins, que o (a) **Ms. Isadora Carolina Betim Pavan** participou como Membro Titular da Banca do Trabalho de Conclusão de Curso - TCC intitulado ***"Avaliação do potencial pró-inflamatório induzido por Lipopolissacarídeo, combinado ou não com o Ácido Graxo Saturado Palmitato, em linhagem celular de micróglias: estudo in vitro da relação obesidade, inflamação e sistema nervoso central"***, apresentado pelo (a) aluno (a) **VALKIRIA GUILHERME ASSIS DA SILVA**, do curso de Nutrição da Universidade Federal de São Paulo. Declaro, ainda, que a Banca foi constituída pelos seguintes membros: Prof. Dr. Cristiano Mendes da Silva (presidente/orientador) e Nutr. Ms. Clarissa Tavares Dias.

Santos, 25 de fevereiro de 2021.

Profa. Dra. Claudia Alves Pereira
Universidade Federal de São Paulo
Coordenadora do Módulo Trabalho de Conclusão de Curso II
Curso de Nutrição

Rua Silva Jardim, 136 - Vila Mathias - Santos/SP - CEP: 11015-020

Autor: Caio Felipe Romeiro

Título: O papel da cafeína em cultura de células neuronais no contexto da Doença de Alzheimer

Natureza: Trabalho de Conclusão de Curso em Nutrição

Instituição: Faculdade de Ciências Aplicadas, Universidade Estadual de Campinas

Aprovado em: 10/12/2021.

BANCA EXAMINADORA

Profa. Dra. Rosângela Maria Neves Bezerra – Presidente
Faculdade de Ciências Aplicadas (FCA/UNICAMP)

Prof. Dr. Fernando Moreira Simabuco – Coorientador
Faculdade de Ciências Aplicadas (FCA/UNICAMP)

Isadora Carolina Betim Pavan

Msa. Isadora Carolina Betim Pavan – Avaliadora
Faculdade de Ciências Aplicadas (FCA/UNICAMP)

Este exemplar corresponde à versão final da monografia aprovada.

Profa. Dra. Rosângela Maria Neves Bezerra
Faculdade de Ciências Aplicadas (FCA/UNICAMP)