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Poszukiwanie racjonalnych strategii przełamywania chemiooporności potrójnie ujemnego raka piersi - kinaza MLK4 jako nowy cel terapeutyczny

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1.

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2.

Mehlich D., Łomiak M., Sobiborowicz A., Mazan A., Dymerska D., Szewczyk Ł.M., Mehlich A., Borowiec A., Prełowska M.K., Górczynski A., Jabłoński P., Iżycka-Świeszewska E., Nowis D., Marusiak A.A. „**MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance”** Cell Death and Disease, 2021, Nov 27;12(12):1–13. DOI: 10.1038/s41419-021-04405-0

Spis treści

Wykaz stosowanych skrótów

ADC - koniugaty przeciwciał z lekami (*Antibody-drug conjugate)*

AKT - kinaza białkowa B (*Protein kinase B*)

ATM - jedna z kinaz serynowo-treoninowych, dla której gen jest zmutowany w ataksji-teleangiektazji (*Ataxia-telangiectasia mutated kinase*)

CDK4/6 - kinaza zależna od cyklin 4/6 (*Cyclin-dependent kinase 4/6)*

CK1 - kinaza kazeinowa 1 (*Casein kinase 1*)

EGFR - receptor naskórkowego czynnika wzrostu (*Epidermal growth factor receptor*)

ERK - kinaza regulowana sygnałem zewnątrzkomórkowym (*Extracellular signal-regulated kinase*)

GEO NCBI - baza danych ekspresji genów Narodowego Centrum Biotechnologii, USA (*Gene Expression Omnibus National Center for Biotechnology Information*)

HER2 - Receptor ludzkiego naskórkowego czynnika wzrostu (*Human epidermal growth factor receptor)*

CXCL-1 - chemokina z motywem CXC, białko regulowane wzrostem alfa (*Chemokine (C-X-C motif) ligand 1, growth-regulated alpha protein*)

IL-6 - interleukina 6 (*Interleukin 6*)

IL-8 - interleukina 8 (*Interleukin 8*)

JNK - kinaza końca c-JUN (*c-JUN N-terminal kinase*)

MAPK - kinazy białkowe aktywowane mitogenami (*Mitogen activated protein kinase*)

MAP3K - kinazy kinaz kinaz białkowych aktywowanych mitogenami (*Mitogen activated protein kinase kinase kinase*)

MELK - matczyno-zarodkowa kinaza zamka leucynowego (*Maternal embryonic leucine zipper* kinase)

MLK4 - kinaza mieszanego pochodzenia 4 (*Mixed-lineage kinase 4*)

NEMO - kluczowy modulator czynnika NF-kappa-B (*NF-kappa-B essential modulator*)

NHEJ - niehomologiczne łączenie końców (*Non-homologous end joining*)

NF-κB - czynnik jądrowy aktywujący lekkie łańcuchy kappa w aktywowanych komórkach B (*Nuclear factor kappa-light-chain-enhancer of activated B cells*)

PARP - polimeraza poli (ADP-rybozy) (*Poly (adenosine diphosphate-ribose) polymerase*)

PIM1 - kinaza zawierająca miejsce integracji prowirusa mysiej białaczki Moloney 1 (*Proviral integration site for Moloney murine leukemia virus-1 kinase*)

PI3K - kinaza 3 fosfatydyloinozytolu (*Phosphoinositide 3 kinase*)

TNBC - potrójnie ujemny rak piersi (*Triple-negative breast cancer)*

VEGFR - receptor czynnika wzrostu śródbłonka naczyniowego (*Vascular endothelial growth factor receptor*)

Streszczenie w języku polskim

Rak piersi jest najczęściej diagnozowanym nowotworem złośliwym i obok raka płuca stanowi główną przyczynę zgonów nowotworowych u kobiet w Polsce i na świecie. Podtyp potrójnie ujemny raka piersi (*ang. triple-negative breast cancer, TNBC*) charakteryzuje się brakiem obecności receptorów dla estrogenu i progesteronu oraz brakiem zwiększonej ekspresji receptora HER2. Na tle innych podtypów raka piersi, nowotwory potrójnie ujemne wyróżnia wysoka złośliwość, agresywny przebieg kliniczny choroby i niekorzystne rokowanie. Na przestrzeni ostatnich lat przeprowadzono szereg badań podstawowych i klinicznych, które pozwoliły na opracowanie nowych metod leczenia TNBC, do których należą między innymi immunoterapia, terapia z wykorzystaniem inhibitorów PARP oraz terapia z wykorzystaniem koniugatów związków cytotoksycznych i przeciwciał. Pomimo znacznego postępu badawczego, obecne możliwości leczenia celowanego TNBC pozostają ograniczone, a chemioterapia nadal jest najpowszechniej stosowaną metodą leczenia systemowego u chorych z tym nowotworem. Pierwotna i nabyta oporność komórek nowotworowych na chemioterapeutyki stanowi główną przeszkodę w uzyskaniu skuteczności klinicznej stosowanego leczenia. Istnieje zatem wyraźna potrzeba badań podstawowych, które pozwolą na lepsze poznanie mechanizmów leżących u podłoża chemiooporności i będą stanowiły pierwszy krok w kierunku opracowania nowych strategii terapeutycznych. Kinazy białkowe to enzymy, które poprzez reakcje fosforylacji (przenoszenia reszt kwasu fosforowego na substraty) koordynują aktywację wewnątrzkomórkowych szlaków przekazywania sygnału i tym samym mogą regulować wiele procesów biologicznych. Nadmierna aktywacja kinaz białkowych odgrywa istotną rolę w patogenezie i nabywaniu chemiooporności wielu nowotworów, w tym potrójnie ujemnych nowotworów piersi. Drobnocząsteczkowe inhibitory kinaz mogą być wykorzystane do przełamywania lekooporności komórek nowotworowych i poprawy wyników leczenia w terapii skojarzonej z chemioterapią. Mimo to w dotychczasowych badaniach klinicznych z udziałem chorych na TNBC, inhibitory kinaz wykazywały jedynie umiarkowaną skuteczność.

Pierwszy artykuł wchodzący w skład cyklu (Mehlich i Marusiak, Cancer Letters) to praca przeglądowa, w której przedstawiłem dotychczasowe badania kliniczne i przedkliniczne opisujące skuteczność terapeutyczną drobnocząsteczkowych inhibitorów kinaz w leczeniu TNBC. W artykule tym opisałem heterogenność w obrębie potrójnie ujemnych nowotworów piersi, która może w istotnym stopniu ograniczać skuteczność terapii celowanych. Ponadto,

przeprowadzona przeze mnie analiza wykazała, że dotychczasowe badania przedkliniczne i kliniczne skupiały się na terapiach ukierunkowanych na względnie niewielką część najlepiej poznanych kinaz. W oparciu o dotychczas zgromadzone dane uzasadnione wydaje się zatem poszukiwanie nowych kinaz, które mogą stanowić potencjalny cel terapii TNBC.

W drugim artykule wchodzącym w skład niniejszego cyklu (Mehlich i wsp., Cell Death and Disease), przedstawiłem oryginalne badania, które pozwoliły na kompleksowe scharakteryzowanie roli kinazy białkowej MLK4 w regulacji chemiooporności TNBC. Kinaza MLK4 należy do grupy kinaz MAP3K. Analiza danych uzyskanych metodą sekwencjonowania następnej generacji, wykazała, że gen kodujący kinazę MLK4 (*MAP3K21/KIAA1804*) ulega amplifikacji i zwiększonej ekspresji w ponad 20% przypadków raka piersi i ponad 50% przypadków raków o podtypie potrójnie ujemnym. Badania przeprowadzone wcześniej przez nasz zespół wykazały, że kinaza ta promuje proliferację i inwazyjny wzrost komórek raka piersi w modelach *in vitro* oraz *in vivo*. W ramach realizacji mojej pracy doktorskiej, przeprowadziłem analizę danych transkryptomicznych zgromadzonych w bazie GEO NCBI, która wykazała, że zwiększona ekspresja kinazy MLK4 w bioptatach pobranych od chorych z rakami potrójnie ujemnymi poddawanych chemioterapii przedoperacyjnej wiąże się z gorszą odpowiedzią na leczenie i niekorzystnym rokowaniem. W kolejnych doświadczeniach zaobserwowałem, że kinaza MLK4 promuje chemiooporność ludzkich linii komórek potrójnie ujemnego raka piersi w modelach *in vitro* oraz w mysim modelu ksenoprzeszczepienia *in vivo*. Zarówno wyciszenie ekspresji genu dla MLK4 jak i zablokowanie funkcji katalitycznej tej kinazy przy pomocy drobnocząsteczkowych inhibitorów uwrażliwiało komórki nowotworowe na klinicznie stosowane chemioterapeutyki. Ponadto, zahamowanie aktywności MLK4 nie nasilało cytotoksycznego działania chemioterapii względem kontrolnych komórek nienowotworowych. Wykorzystując metody cytometrii przepływowej i mikroskopii konfokalnej zaobserwowałem, że w komórkach z wyciszoną ekspresją genu dla MLK4 dochodzi do nasilonej indukcji apoptozy oraz akumulacji uszkodzeń DNA. Aby lepiej zrozumieć, w jaki sposób kinaza MLK4 promuje oporność na chemioterapię, przeprowadziłem kolejne eksperymenty z wykorzystaniem metod proteomicznych, testów reporterowych oraz modeli komórkowych z delecją genu *MLK4* wprowadzoną metodą CRISPR/Cas9. Doświadczenia te wykazały, że kinaza MLK4 poprzez aktywację kinazy ATM reguluje proces naprawy podwójnych pęknięć nici DNA indukowanych przez chemioterapeutyki. Następnie przeprowadziłem analizę profilu ekspresji genów w inkubowanych z chemioterapeutykami komórkach z wyciszoną i zachowaną ekspresją genu dla MLK4, wykorzystując metodę RNA-seq. Analiza ta wykazała, że MLK4 odpowiada za indukcję szlaku przekazywania sygnału

ATM-NEMO i aktywację czynników transkrypcyjnych z grupy NF-κB, które promują przeżycie komórek nowotworowych w odpowiedzi na chemioterapię.

Podsumowując, kinazy białkowe mogą stanowić atrakcyjny cel w terapii i przełamywaniu chemiooporności TNBC. Kolejne badania przedkliniczne i kliniczne z wykorzystaniem drobnocząsteczkowych inhibitorów kinaz powinny uwzględniać heterogenność biologiczną i zróżnicowaną odpowiedź na leczenie w tej grupie nowotworów. Wysoce uzasadnione jest prowadzenie badań mających na celu poszukiwanie nowych celów terapeutycznych. Uzyskane przeze mnie wyniki wskazują na to, że zahamowanie kinazy MLK4 może okazać się skuteczną strategią przełamywania chemiooporności u dużej części chorych z rakami potrójnie ujemnymi. Drobnocząsteczkowe inhibitory kinazy MLK4 mogą zatem pozwolić na opracowanie nowych, skuteczniejszych niż dotychczasowe, terapii skojarzonych w leczeniu tych nowotworów. Ponadto, uzyskane przeze mnie wyniki opisują niekanoniczne i do tej pory nieznane funkcje kinazy MLK4 w regulacji procesu naprawy DNA.

Streszczenie w języku angielskim

Title: Rational strategies to overcome triple-negative breast cancer chemoresistance - MLK4 kinase as a novel therapeutic target

Breast cancer is the most often diagnosed cancer and the second leading cause of cancer-related deaths in women in Poland and worldwide. Triple-negative breast cancer (TNBC) is a subtype of breast cancer defined by the absence of estrogen receptor, progesterone receptor, and HER2 expression. TNBC is characterized by aggressive clinical behavior, rapidly progressive disease course, and poor prognosis. Over recent decades, many preclinical and clinical studies have been performed to better understand the biology of TNBC and identify more efficient therapeutic strategies. Novel therapies for TNBC have been developed, including immunotherapies, PARP inhibitors, and antibody-drug conjugates. Despite these major advances, treatment of TNBC is still an unmet need. Due to the lack of routine targeted therapies, non-selective chemotherapy remains the most used therapeutic option for TNBC patients. Nevertheless, both intrinsic and acquired chemoresistance limits the clinical efficacy of the current therapies, leading to high relapse rates and poor prognosis. Therefore, there is a need for basic science research aiming to identify the molecular mechanisms of chemoresistance, which may translate into novel treatment strategies. Protein kinases are enzymes that alter the activity of their substrates through phosphorylation (transferring of the phosphate molecule). They play a crucial role in most signaling cascades, thereby regulating numerous processes within the cell. Aberrant activation of various protein kinases has been implicated in the pathogenesis of different types of human cancers, including TNBC. Small molecule inhibitors targeting protein kinases have been tested in clinical trials in patients with TNBC. However, most of the trials evaluating kinase inhibitors, either in monotherapy or in combination with chemotherapy, demonstrated only limited clinical efficacy of these agents.

The first publication included in this series is a review article describing the past and ongoing preclinical and clinical studies that evaluated the therapeutic efficacy of small-molecule kinase inhibitors in TNBC therapy. In this article, I described that TNBC heterogeneity significantly affects tumor response to targeted therapies. Furthermore, by performing a comprehensive literature review and analyzing ongoing studies, I demonstrated that the majority of the clinical trials focused only on a small subset of well-characterized kinases. Considering these observations, identifying novel druggable kinases will likely expand the therapeutic landscape of TNBC and provide new therapeutic solutions for TNBC patients.

In the second article included in this series, I described the original studies that characterized the role of MLK4 kinase in TNBC chemoresistance. Mixed-Lineage Kinase 4

(MLK4) is a member of the MAP3K family of serine/threonine kinases. High-throughput sequencing data indicated that the MLK4 gene (*MAP3K21/KIAA1804*) is amplified and/or overexpressed in 20% of breast cancer cases and over 50% of TNBC cases. Our previous studies demonstrated that MLK4 promotes breast cancer cell lines proliferation and aggressive growth *in vitro* and *in vivo*. Here, I aimed to investigate the role of MLK4 kinase in TNBC chemoresistance. By analyzing gene expression database (GEO NCBI), I found that high MLK4 expression in tumor tissue is associated with poor survival of TNBC patients treated with neoadjuvant chemotherapy. Next, using TNBC cell line models, I demonstrated that MLK4 promotes chemoresistance and survival of human TNBC cells *in vitro* and in mouse xenograft models *in vivo.* MLK4 knock-down or inhibition sensitized TNBC cell lines to several clinically used chemotherapeutic agents. I observed that MLK4-deficient cells displayed enhanced apoptosis induction and persistent DNA damage accumulation upon treatment with chemotherapeutics using flow cytometry and confocal microscopy techniques. To further investigate the mechanisms of MLK4-dependent chemoresistance of TNBC cells, I used phosphoproteomic profiling, reporter assays, and CRISPR/Cas9 MLK4 knock-out cells. I found that MLK4 regulates DNA damage repair in response to genotoxic chemotherapy. Mechanistically, I demonstrated that loss of MLK4 impairs activation of the ATM kinase, which plays a major role in coordinating DNA damage response. Furthermore, I performed RNA-seq analysis of MLK4-depleted and control cells treated with chemotherapy, which indicated that MLK4 is required for DNA damage-induced activation of ATM-NEMO signaling axis and expression of several NF-κB dependent genes that facilitate TNBC chemoresistance.

In summary, protein kinases may serve as attractive therapeutic targets in the treatment of TNBC and overcoming tumor chemoresistance. Future preclinical and clinical studies investigating kinase inhibitors in TNBC should account for tumor heterogeneity that significantly affects the response to targeted therapies. Moreover, the identification of novel kinase targets is warranted, as it may significantly broaden the therapeutic landscape of TNBC. Here, I demonstrated for the first time that MLK4 confers chemoresistance in TNBC, and thus it may serve as a new therapeutic target in TNBC treatment. These results suggest that small-molecule MLK4 inhibitors combined with chemotherapy might be used as a rational treatment strategy for many TNBC patients. Moreover, I discovered novel and noncanonical functions of MLK4 kinase in regulating DNA damage response signaling.

Wstęp uzasadniający połączenie wskazanych publikacji w jeden cykl, jak i komentujący osiągniecie naukowe kandydata na tle dotychczasowego stanu wiedzy.

Przedstawiony cykl publikacji dotyczy nowych możliwości terapii celowanych w leczeniu potrójnie ujemnego raka piersi na tle dotychczasowego stanu wiedzy. W skład cyklu wchodzą dwie publikacje, których jestem pierwszym autorem (sumaryczny IF cyklu = 19,452; punktacja MEiN = 280):

1. Mehlich D, Marusiak AA. "Kinase inhibitors for precision therapy of triple-negative breast cancer: Progress, challenges, and new perspectives on targeting this heterogeneous disease", Cancer Letters;

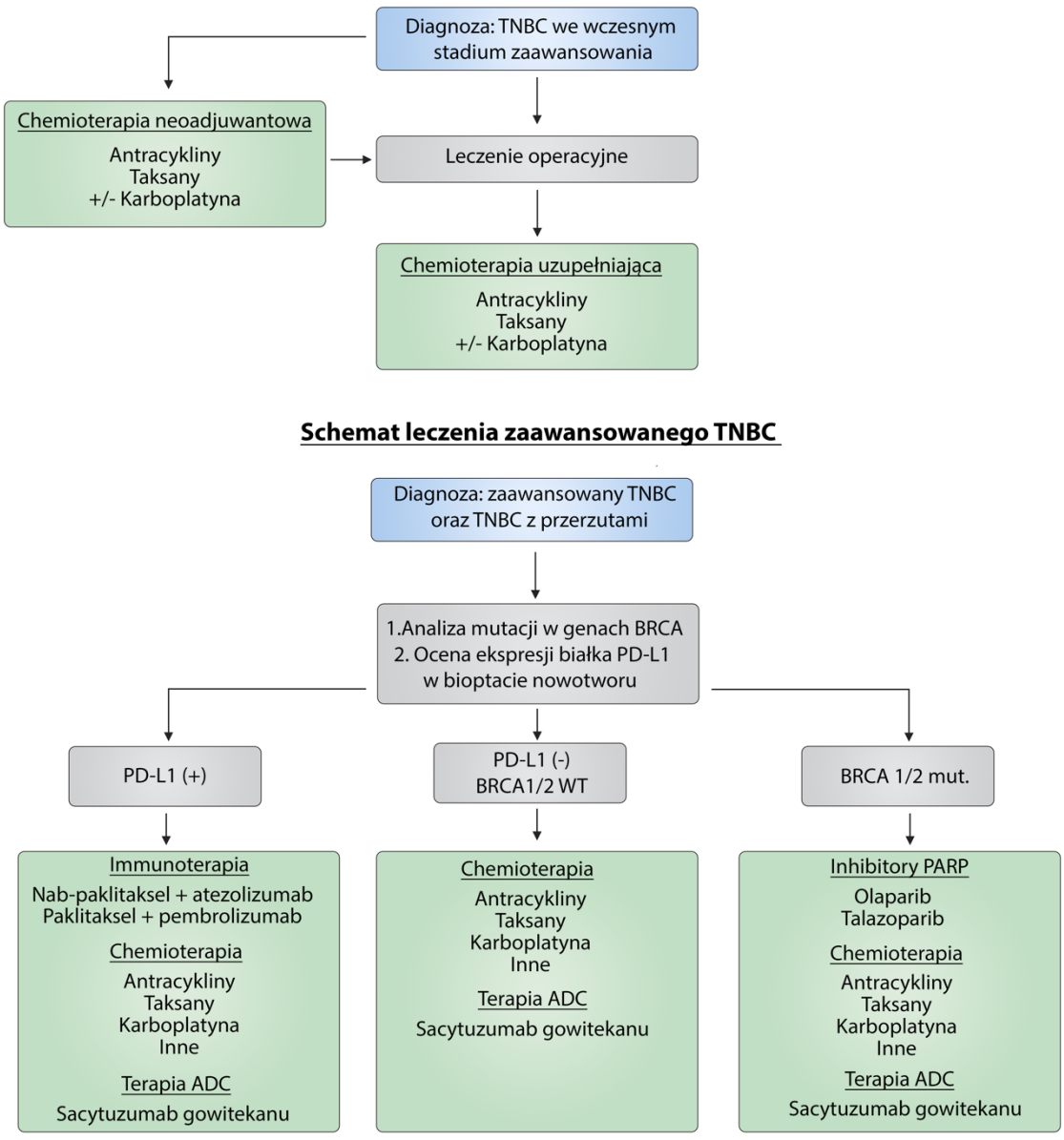
2. Mehlich D., Łomiak M., Sobiborowicz A., Mazan A., Dymerska D., Szewczyk Ł.M., Mehlich A., Borowiec A., Prełowska M.K., Górczynski A., Jabłoński P., Iżycka-Świeszewska E., Nowis D., Marusiak A.A. „MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance", Cell Death and Disease.

Rak piersi jest najczęściej diagnozowanym nowotworem złośliwym i po raku płuca stanowi główną przyczynę zgonów nowotworowych u kobiet w Polsce i na świecie [1]. Raki piersi stanowią bardzo heterogenną grupę nowotworów, różniących się od siebie zarówno cechami biologicznymi, rokowaniem, jak i odpowiedzią na leczenie. Współczesne leczenie raka piersi ma najczęściej charakter skojarzony, z uwzględnieniem metod leczenia miejscowego (chirurgia i radioterapia) i systemowego (chemioterapia, hormonoterapia, terapia celowana) [2]. Dla podejmowania decyzji terapeutycznych dotyczących leczenia systemowego istotne znaczenie ma określenie statusu receptora estrogenowego (*ang. estrogen receptor, ER*), progesteronowego (*ang. progesterone receptor, PR*), receptora ludzkiego czynnika wzrostu naskórka typu 2 (*ang. human epidermal growth factor 2, HER2*) oraz indeksu proliferacji Ki-67 w bioptacie nowotworu. Ponadto, na podstawie badań profilu ekspresji genów, zidentyfikowano zasadnicze podtypy molekularne raka piersi (luminalny A, luminalny B, wykazujący nadekspresję HER2 oraz bazalny) dla których opracowano zalecenia dotyczące leczenia systemowego [3,4]. Nowotwory wykazujące ekspresję receptorów dla hormonów steroidowych stanowią około 60-65% przypadków wszystkich złośliwych guzów piersi, a w klasyfikacji molekularnej odpowiadają one w większości podtypom luminalnym A i B. W leczeniu systemowym tych guzów stosuje się hormonoterapię oraz hormonoterapię w połączeniu z chemioterapią, a także inhibitory kinazy 4/6 zależnej od cyklin (*ang. cyclin-dependent kinase 4/6*). Z kolei w przypadku guzów wykazujących zwiększoną ekspresję receptora HER2, stanowiących około 15-20% nowotworów złośliwych piersi, stosowaną

metodą leczenia jest terapia celowana anty-HER2 w połączeniu z chemioterapią. Nowotwory niewykazujące ekspresji receptorów dla hormonów steroidowych ani zwiększonej ekspresji receptora HER2 klasyfikowane są jako raki potrójnie ujemne (*ang. triple negative breast cancer, TNBC*). Stanowią one ok. 15% wszystkich przypadków raka piersi i w 80% pokrywają się z podtypem bazalnym wg klasyfikacji molekularnej. Raki potrójnie ujemne występują częściej u młodych kobiet i charakteryzują się wysokim stopniem złośliwości, agresywnym przebiegiem klinicznym i gorszym rokowaniem w porównaniu do innych podtypów raka piersi. [5]. Z powodu braku punktu uchwytu dla leczenia celowanego, chemioterapia przedoperacyjna (neoadjuwantowa) i uzupełniająca przez dekady były jedynymi powszechnie stosowanymi metodami leczenia systemowego u chorych z rakami potrójnie ujemnymi [6]. Liczne badania przedkliniczne i kliniczne pozwoliły na opracowanie nowych metod leczenia potrójnie ujemnych nowotworów piersi, do których należą m.in. immunoterapia z wykorzystaniem inhibitorów punktów kontrolnych, terapie z wykorzystaniem inhibitorów PARP oraz terapia koniugatami przeciwciał z lekami o działaniu cytotoksycznym (ang. *antibody-drug conjugate, ADC*) [7–10]. Mimo to, jedynie niewielka część chorych z TNBC może być zakwalifikowana do leczenia z wykorzystaniem nowych leków, a chemioterapia nadal pozostaje podstawową metodą leczenia tych nowotworów (Ryc. 1).

Pierwotna oraz nabyta oporność na chemioterapię wiąże się z niekorzystnym rokowaniem chorych i obecnie stanowi jeden z najpoważniejszych problemów klinicznych [11,12]. Badania opublikowane na przestrzeni ostatnich lat pozwoliły na odkrycie mechanizmów związanych z pierwotną i nabytą opornością komórek nowotworowych na chemioterapię, do których należą m.in.: aktywacja transporterów usuwających leki z komórek nowotworowych, zahamowanie apoptozy poprzez uruchomienie antyapoptotycznych wewnątrzkomórkowych szlaków przekazywania sygnału, czy też aktywacja procesu naprawy DNA [13–15]. Ponadto, wykazano, że w odpowiedzi na chemioterapeutyki indukowana jest wzmożona sygnalizacja prozapalna w mikrośrodowisku nowotworu. W procesie tym biorą udział komórki podścieliska guza, komórki układu immunologicznego oraz komórki nowotworowe wydzielające cytokiny prozapalne takie jak: TNFα, CXCL2, IL-1, IL-6 czy IL-8. Cytokiny te mogą aktywować wewnątrzkomórkowe szlaki przekazywania sygnału promujące chemiooporność komórek nowotworowych i progresję nowotworu [14,16].

Nadmierna aktywacja kinaz białkowych odgrywa istotną rolę w indukowaniu mechanizmów związanych z chemiooopornością [17–19]. Drobnocząsteczkowe inhibitory kinaz mogą zatem potencjalnie uwrażliwiać komórki nowotworowe na cytotoksyczne działanie chemioterapii. Terapie skojarzone z wykorzystaniem chemioterapeutyków i inhibitorów kinaz



Rycina 1- Schematy leczenia TNBC we wczesnym stopniu zaawansowania i zaawansowanego TNBC. Zmodyfikowano w oparciu o Loibl i wsp. 2021. Wybór terapii systemowej w zaawansowanym TNBC zależny jest od obrazu klinicznego, poprzednio stosowanych terapii systemowych i indywidualnych preferencji chorego.

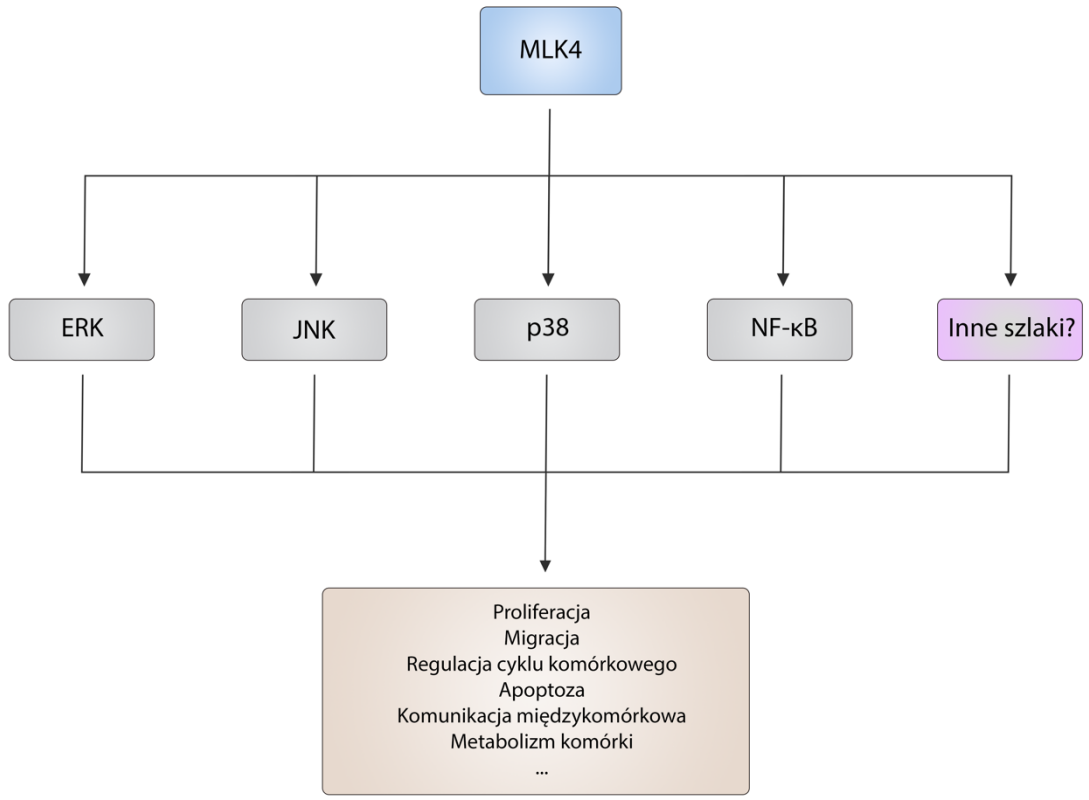
okazały się skuteczne i zostały włączone do schematów leczenia różnego rodzaju nowotworów, m.in. raka piersi HER2-dodatniego, raka płuca czy raka trzustki [20–23]. Terapie ukierunkowane na zahamowanie funkcji katalitycznej kinaz były również testowane u chorych z potrójnie ujemnymi rakami piersi, zarówno w monoterapii, jak i w skojarzeniu z chemioterapią. Dotychczasowe badania kliniczne nie potwierdziły jednak skuteczności inhibitorów kinaz w leczeniu TNBC. Prawdopodobną przyczyną niepowodzeń klinicznych jest

heterogenny charakter nowotworów potrójnie ujemnych. Wyodrębniono kilka podtypów TNBC o odmiennych cechach biologicznych. W klasyfikacji opracowanej przez Lehmann i wsp. w latach 2011-2016 wyróżniono cztery główne podtypy molekularne TNBC w oparciu o ich profil ekspresji genów: bazalny-1, bazalny-2, mezenchymalny oraz luminalny wykazujący ekspresję receptora androgenowego [24,25]. W najnowszych badaniach, heterogenność podtypów molekularnych TNBC potwierdzono wykonując analizy proteomiczne i fosfoproteomiczne [26–28]. Co istotne, wykazano, że poszczególne podtypy TNBC charakteryzują się odmiennym profilem aktywacji kinaz białkowych i odpowiedzią na terapie z wykorzystaniem inhibitorów kinaz [26]. Kolejną możliwą przyczyną ograniczonej skuteczności inhibitorów kinaz w leczeniu chorych na TNBC jest fakt, iż dotychczas testowane terapie ukierunkowane były jedynie na wąską grupę najlepiej poznanych kinaz [24,26,29–32]. Do leków najczęściej testowanych w badaniach klinicznych u chorych na TNBC należą inhibitory szlaku PI3K/Akt/mTOR. Inhibitory kinazy Akt - capivasertib oraz ipatasertib w kombinacji z paklitakselem wykazały obiecującą skuteczność w leczeniu TNBC w dwóch badaniach klinicznych II fazy (LOTUS i PAKT) [33,34]. Pomimo to, randomizowane badanie kliniczne III fazy przeprowadzone na grupie 255 chorych z TNBC (IPATunity130) nie potwierdziło skuteczności klinicznej kombinacji ipatasertibu i paklitakselu [35]. Kolejną grupą leków badanych u chorych na TNBC były inhibitory receptorowych kinaz tyrozynowych, do których należą m.in. sorafenib, sunitinib, i lapatinib. Badania kliniczne III fazy nie wykazały jednak skuteczności klinicznej kombinacji tych leków w połączeniu z cytotoksyczną chemioterapią [36–38]. Istnieje zatem potrzeba kolejnych badań, które pozwolą na kompleksową identyfikację mechanizmów leżących u podłoża chemiooporności oraz pozwolą na opracowanie nowych celów terapeutycznych i skutecznych terapii skojarzonych.

MLK4 (*ang. Mixed-lineage kinase 4*) należy do grupy kinaz MAP3K i rodziny kinaz MLK (*ang. Mixed-lineage kinases*). Kinazy te pośredniczą w przekazywaniu sygnałów zewnątrzkomórkowych i zaangażowane są w regulację różnorodnych procesów biologicznych, takich jak: proliferacja, różnicowanie, przeżycie i śmierć komórki [39]. MLK4 jest najpóźniej scharakteryzowaną i najmniej poznaną kinazą z grupy kinaz MLK. Gen kodujący kinazę MLK4 ulega amplifikacji i lub/zwiększonej ekspresji w komórkach nowotworowych ponad 50% przypadków raków potrójnie ujemnych [40,41]. Poprzednie prace wskazywały na zarówno proonkogenne jak i supresorowe funkcje MLK4 w różnego rodzaju nowotworach (Ryc. 2).

Badania przeprowadzone w modelach komórkowych raka jajnika wykazały, że kinaza MLK4 negatywnie reguluje szlak przekazywania sygnału związany z kinazami MAPK i hamuje inwazyjność komórek nowotworowych [42,43]. W przeciwieństwie do tych doniesień,

badania w komórkach czerniaka złośliwego i raka jelita grubego wykazały, że MLK4 fosforyluje kinazy MEK1/2 i MKK4/7, co przyczynia się do aktywacji kinaz MAPK – ERK1/2 oraz JNK1/2 [44–46]. Ponadto, prace opublikowane przez nasz zespół i innych badaczy wskazują na istotną rolę kinazy MLK4 w propagowaniu agresywnego wzrostu komórek nowotworowych poprzez aktywację czynnika transkrypcyjnego NF-κB [41,47]. Do tej pory nie opublikowano jednak badań wskazujących na udział kinazy MLK4 w regulacji mechanizmów oporności na chemioterapię. Przeprowadzona przeze mnie analiza danych transkryptomicznych wykazała, że zwiększona ekspresja mRNA dla kinazy MLK4 w nowotworze wiąże się z większą opornością guzów na chemioterapię neoadjuwantową. Celem kolejnych badań było scharakteryzowanie kinazy MLK4 jako potencjalnego celu w terapii chemiooopornych, potrójnie ujemnych raków piersi.



Rycina 2 - Rola kinazy MLK4 w regulacji wewnątrzkomórkowych szlaków przekazywania sygnału i procesów komórkowych. Opracowano na podstawie opublikowanych danych literaturowych.

W ramach niniejszego cyklu publikacji przedstawiłem obecny stan wiedzy na temat wykorzystania drobnocząsteczkowych inhibitorów kinaz w leczeniu TNBC oraz wskazałem nowe perspektywy i możliwości opracowania skutecznych terapii. Pierwszy artykuł wchodzący

w skład cyklu to praca przeglądowa, w której opisałem dotychczasowe badania kliniczne i przedkliniczne na temat skuteczności terapeutycznej drobnocząsteczkowych inhibitorów kinaz w terapii chorych z potrójnie ujemnym rakiem piersi [32]. Jest to pierwsza opublikowana praca, która kompleksowo systematyzuje obecny stan wiedzy na temat możliwości zastosowania inhibitorów kinaz w leczeniu TNBC. Jednocześnie praca ta wskazuje na kierunki przyszłych badań, które mogą pozwolić na opracowanie skuteczniejszych terapii skojarzonych z wykorzystaniem inhibitorów kinaz i przełamywanie lekooporności TNBC. Drugi artykuł wchodzący w skład cyklu przedstawia przeprowadzone przeze mnie badania, które pozwoliły na scharakteryzowanie roli kinazy białkowej MLK4 w regulacji chemiooporności TNBC [48]. W artykule tym wykazałem, że kinaza MLK4 promuje chemiooporność ludzkich linii TNBC w modelach *in vitro* oraz *in vivo*. Zbadałem, że wyciszenie ekspresji genu dla MLK4 w komórkach nowotworowych prowadziło do nasilonej indukcji apoptozy i akumulacji uszkodzeń DNA w komórkach inkubowanych z chemioterapeutykami. Podobnie, farmakologiczne zahamowanie aktywności kinazowej MLK4 nasilało cytotoksyczne działanie doksorubicyny na komórki TNBC. Zastosowana kombinacja leków wykazywała działanie synergistyczne wobec linii komórek nowotworowych, ale nie wobec kontrolnej nienowotworowej linii nabłonkowej. Ponadto, w komórkach pozbawionych aktywnej formy kinazy MLK4 stwierdziłem upośledzoną aktywację mechanizmów naprawy DNA i zmniejszoną aktywację szlaku sygnałowego ATM-NEMO-NF-κB. Uzyskane przeze mnie dane wskazują, że kinaza MLK4 może być nowym, atrakcyjnym celem terapii skojarzonych w leczeniu TNBC.

Podsumowując, publikacje uwzględnione w niniejszym cyklu opisują dotychczasowe terapie potrójnie ujemnego raka piersi z wykorzystaniem drobnocząsteczkowych inhibitorów kinaz oraz wskazują na nowe możliwości terapeutyczne. Przedstawione przeze mnie wyniki sugerują, że inhibitory kinazy MLK4 mogą w przyszłości poszerzyć arsenał leków stosowanych w terapii potrójnie ujemnego raka piersi. Zaprezentowane artykuły są spójnie tematycznie i stanowią istotny wkład w rozwój dyscypliny oraz cenne źródło wiedzy dla badaczy i lekarzy zainteresowanych onkologią molekularną i kliniczną.

Założenia i cel pracy

Z uwagi na ograniczone możliwości leczenia celowanego, chemioterapia pozostaje podstawową metodą w leczeniu TNBC. We wcześniejszych badaniach przedklinicznych wykazano, że kinazy białkowe odgrywają istotną rolę w patogenezie TNBC i nabywaniu oporności na chemioterapię, a drobnocząsteczkowe inhibitory kinaz były testowane w szeregu badań klinicznych u chorych na TNBC. Do tej pory nie zbadano roli kinazy MLK4 w procesach nabywania chemiooporności przez komórki nowotworowe, a także nie oceniono potencjału terapeutycznego inhibitorów MLK4 w leczeniu TNBC. Celem pierwszej publikacji przedstawionej w niniejszym cyklu był przegląd literatury i kompleksowa analiza skuteczności drobnocząsteczkowych inhibitorów kinaz w terapii nowotworów potrójnie ujemnych. Celem drugiej publikacji przedstawionej w niniejszym cyklu było zbadanie roli kinazy MLK4 w regulacji chemiooporności potrójnie ujemnego raka piersi i ocena skuteczności inhibitorów tej kinazy w modelach przedklinicznych TNBC.

Szczegółowe cele niniejszej pracy przedstawiono poniżej:

- Analiza wyników badań przedklinicznych i klinicznych opisujących skuteczność drobnocząsteczkowych inhibitorów kinaz w leczeniu TNBC
- Wskazanie nowych możliwych celów terapii z wykorzystaniem inhibitorów kinaz w leczeniu TNBC
- Zbadanie związku między ekspresją kinazy MLK4 w TNBC a odpowiedzią na chemioterapię neoadjuwantową
- Zbadanie wpływu wyciszenia ekspresji genu MLK4 na oporność komórek raka piersi na chemioterapię w modelach *in vitro* oraz *in vivo*
- Zbadanie synergistycznego działania inhibitorów kinaz MLK w skojarzeniu z chemioterapią wobec komórek raka piersi *in vitro*
- Zbadanie roli kinazy MLK4 w procesie nabywania chemiooporności z wykorzystaniem wysokoprzepustowych metod proteomicznych oraz sekwencjonowania RNA metodą następnej generacji
- Szczegółowe zbadanie roli kinazy MLK4 w regulacji procesu naprawy uszkodzeń DNA indukowanych przez chemioterapeutyki

- Szczegółowe zbadanie roli kinazy MLK4 w regulacji aktywacji czynników transkrypcyjnych NF-κB i sekrecji NF-κB -zależnych cytokin prozapalnych w odpowiedzi na chemioterapię.

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Kinase inhibitors for precision therapy of triple-negative breast cancer: Progress, challenges, and new perspectives on targeting this heterogeneous disease

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ABSTRACT

Triple-negative breast cancer (TNBC) is a highly heterogeneous disease that encompasses a subset of breast cancers that are defined by the absence of expression of the estrogen receptor, the progesterone receptor, and human epidermal growth factor receptor 2 (HER2, ERBB2). Among all breast cancer subtypes, TNBC is associated with the least favorable prognosis because of its aggressive clinical course and long-standing lack of effective targeted therapies. Recently, multi-omics profiling studies have provided unprecedented insights into the biological heterogeneity of TNBC, leading to the classification of these tumors into distinct molecular subtypes based on recurrent genetic aberrations, transcriptional patterns, and tumor microenvironment features. A significant number of kinase-driven molecular alterations have been identified across TNBC molecular subtypes, opening new possibilities for refining and broadening the current therapeutic landscape. Many small-molecule inhibitors of protein kinases have been tested in clinical trials in patients with TNBC, including drugs that target the PI3K/Akt/mTOR and MAPK signaling pathways, receptor tyrosine kinases, cyclin-dependent kinases, and DNA damage response signaling pathways. Although some of these agents had limited efficacy in an unselected population of TNBC patients, recent studies suggest that kinase inhibitors may provide significant clinical benefits in the framework of subtype-based and biomarker-guided therapeutic approaches. This review explores actionable therapeutic targets for TNBC molecular subtypes and describes recent clinical trials that investigated kinase inhibitors in the treatment of triple-negative breast tumors.

1. Introduction

Triple-negative breast cancer (TNBC) is a subtype of breast cancer (BC) that is defined by the absence of expression of the estrogen receptor (ER), the progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2, ERBB2). It accounts for 15–20% of all invasive breast tumors and is more prevalent in younger women of African and Hispanic descent and carriers of germline mutations of BC susceptibility genes [1]. TNBC is characterized by aggressive clinical behavior with high metastatic potential and a rapidly progressive disease course. Because of the lack of routine targeted therapies, nonselective chemotherapy has been the only available therapeutic option for TNBC patients. A high relapse rate and substantial toxicity limit the clinical efficacy of chemotherapy-based regimens, thereby contributing to the overall poor prognosis of TNBC patients [2].

Over recent decades, many preclinical and clinical studies have been performed to better understand the biology of TNBC and identify more efficient therapeutic strategies. Novel therapies for TNBC have been developed, dramatically changing clinical practice. Atezolizumab was the first immune-checkpoint inhibitor (ICI) that was approved by the United States Food and Drug Administration (FDA) for the treatment of metastatic TNBC with programmed death-ligand 1 (PD-L1) expression, bringing TNBC into the immunotherapy era. This FDA approval was based on results from the Phase III IMpassion 130 trial, which demonstrated improved progression-free survival (PFS) in patients who were

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List of abbreviations

ADC	antibody-drug conjugate
AR	androgen receptor
BL1	basal-like 1
BL2	basal-like 2
BLIS	basal-like and immune-suppressed
CSC	cancer stem cell
DDR	DNA damage response
ER	estrogen receptor
HER2	human epidermal growth factor receptor 2
HRR	homologous recombination repair
ICI	immune-checkpoint inhibitor
IM	immunomodulatory
LAR	luminal androgen receptor
M	mesenchymal
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MES	mesenchymal-like
mTOR	mammalian target of rapamycin
mTNBC	metastatic triple-negative breast cancer
NAC	neoadjuvant chemotherapy
ORR	objective response rate
OS	overall survival
pCR	pathological complete response
PDX	patient-derived xenograft
PFS	progression-free survival
PR	progesterone receptor
TNBC	triple-negative breast cancer

treated with a combination of atezolizumab and nab-paclitaxel *vs*. nab-paclitaxel alone [3]. Contrary to these findings, however, the Phase III IMpassion131 trial showed no benefit for either PFS or overall survival (OS) upon the addition of atezolizumab to paclitaxel in the same settings [4]. The negative results from this trial prompted withdrawal of the accelerated FDA approval by Roche/Genentech in the United States. A second ICI, the *anti*-PD-1 monoclonal antibody (mAb) pembrolizumab, was approved for the treatment of metastatic TNBC (mTNBC) based on reports from the KEYNOTE-355 trial. In this study, the addition of pembrolizumab to frontline chemotherapy resulted in superior PFS in patients with PD-L1-positive tumors [5]. More recently, pembrolizumab was also approved for high-risk early-stage TNBC when given in combination with chemotherapy, followed by single-agent use after surgery [6]. Inhibitors of poly (ADP-ribose) polymerase (PARP) represent another class of drugs that provide significant clinical benefits in TNBC therapy. Two PARP inhibitors, olaparib and talozaparib, were approved to treat advanced TNBC in patients with germline breast cancer 1/2 (BRCA1/2) mutations [7,8], and olaparib was granted approval for high-risk early-stage TNBC in combination with chemotherapy [9]. Finally, sacitizumab-govitecan, an antibody-drug conjugate (ADC) that is composed of the topoisomerase I inhibitor SN38 linked to a humanized mAb that targets trophoblast cell surface receptor 2 (TROP2), was approved for the treatment of unresectable locally advanced or metastatic TNBC [10].

Despite these major advances, the successful treatment of TNBC is still an unmet need. Currently, only approximately 40% of patients with mTNBC are eligible for treatment with ICIs. Furthermore, the benefits that are associated with immunotherapy are less pronounced in TNBC compared with many other solid tumors, and most patients who receive this treatment develop adaptive resistance [11]. Therapy with PARP inhibitors is limited to patients with germline BRCA1/2 mutations, which account for 10–30% of all TNBC cases. Nonetheless, approximately 50% of germline BRCA1/2 mutation carriers do not respond to PARP inhibitors in neoadjuvant settings, and the median response duration is only 6 months in advanced disease [12]. Thus, optimization of the currently available therapies and identification of novel molecular targets for the treatment TNBC is urgently needed.

Small-molecule inhibitors of protein kinases constitute a major part of drug discovery and development research in oncology. Many kinase-targeting agents have been tested in clinical trials in patients with TNBC. However, most past trials that evaluated kinase inhibitors and other targeted agents for the treatment of TNBC were performed in unselected patient populations, which may at least partially explain their limited efficacy. Nonetheless, recent studies provide unprecedented insights into the biological heterogeneity of triple-negative tumors, suggesting that the development of biomarker-guided and subtype-specific therapeutic strategies is essential for improving treatment outcomes. Identifying potentially druggable kinases across molecular subtypes of TNBC offers promising opportunities for refined and novel targeted therapies using small molecule inhibitors [13]. In this article, we review protein kinases that are implicated in the pathogenesis of TNBC and summarize the clinical development of kinase-targeting agents to treat triple-negative tumors.

2. Heterogeneity of TNBC and actionable targets across molecular subtypes

Significant efforts have been made in recent years to dissect the heterogeneity of TNBCs and classify these tumors into clinically relevant molecular subtypes (Fig. 1) [14–17]. In a landmark study, Lehmann and colleagues identified six distinct molecular subtypes of TNBC by analyzing gene expression profiles from bulk tumor tissues [14]. This initial classification was later refined by the same group, based on additional analyses that included the histopathological quantification and laser-capture microdissection of tumor specimens. Their findings revealed that transcripts from two previously described subtypes were contributed from tumor-infiltrating lymphocytes and tumor-associated stromal cells. The revised TNBC type 4 classification defined four major molecular subtypes: basal-like 1 (BL-1), basal-like 2 (BL-2), mesenchymal (M), and luminal androgen receptor (LAR) [15]. More recently, this classification was complemented by comprehensive analyses of genomic, epigenetic, proteomic, phospho-proteomic, metabolic, and tumor-microenvironmental landscapes of the established molecular subtypes, which enabled the identification of novel actionable targets for subtype-based precision treatment [18,19].

The BL-1 subtype is characterized by high mutational burden, deletions of DNA repair genes, and the homologous recombination repair (HRR) deficiency mutational signature. In agreement with these observations, BL-1 subtype tumors respond to DNA-damaging chemotherapies, as demonstrated by a relatively high pathological complete response (pCR) rate following neoadjuvant chemotherapy (NAC) compared with other subtypes [20]. Preclinical studies suggest that the BL-1 subtype may also have greater sensitivity to PARP inhibitors, DNA damage response signaling inhibitors, and cyclin-dependent kinase 1/2 (CDK1/2) inhibitors [19]. The BL-2 subtype is enriched in activating mitogen-activated protein kinase (MAPK) pathway mutations, which are generally rare in TNBC [21]. Furthermore, phospho-proteomic profiling indicated that the BL-2 subtype is characterized by the activation of the PI3K/Akt/mTOR and MAPK signaling, providing a rationale for targeting these pathways [19]. The M subtype of TNBC shows high mutational burden, genomic instability, and enrichment in growth factors, cell differentiation, and motility pathways. These tumors are also characterized by the absence of immune cell infiltration, low PD-L1 expression, and the transcriptional repression of antigen presentation genes, which

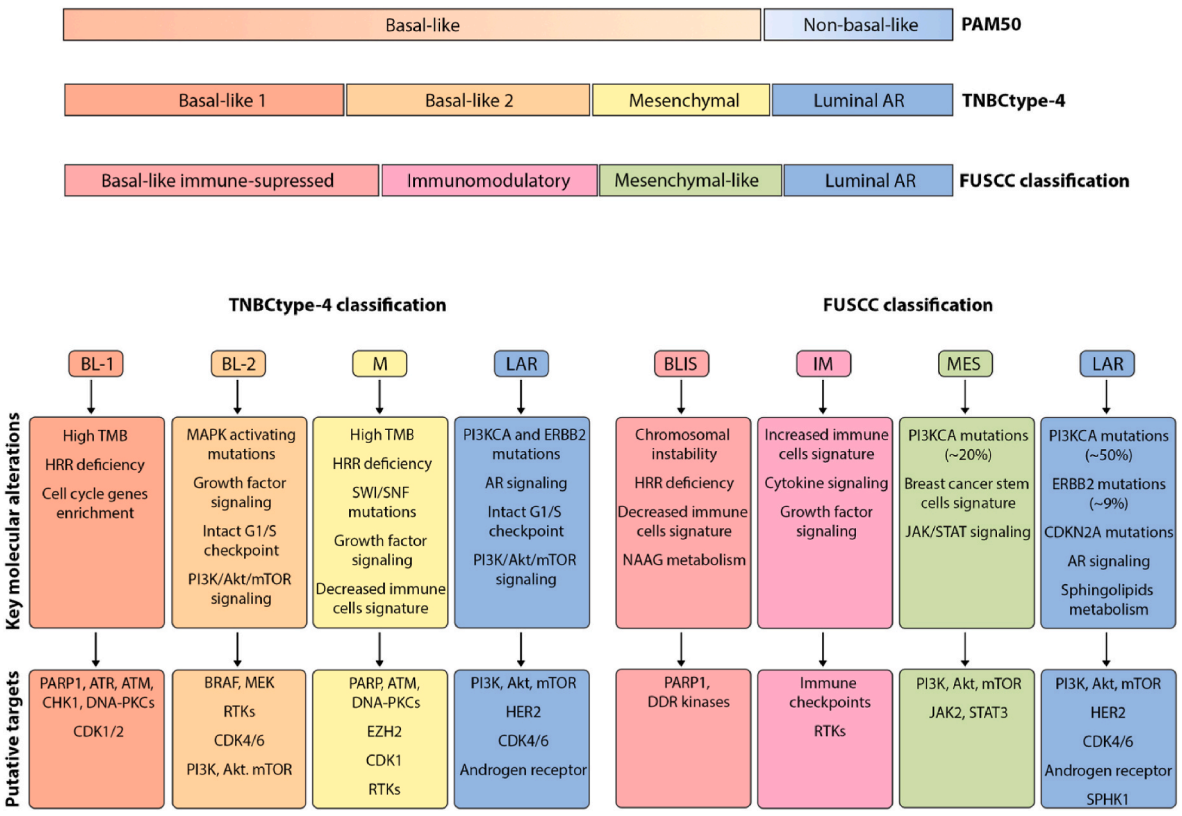

Fig. 1. Molecular heterogeneity of triple-negative breast cancer. The figure shows a schematic representation of TNBC molecular subtypes, according to TNBCtype-4 and Fudan University Shanghai Cancer Center (FUSCC) classifications. Key molecular alterations and putative actionable targets across molecular subtypes are indicated below. TMB, tumor mutational burden; HRR, homologous recombination repair, RTKs, receptor tyrosine kinases, AR, androgen receptor; NAAG, N-acetyl-aspartyl-glutamate; DDR, DNA damage response.

can be partially reversed by the pharmacological inhibition of an epigenetic modifier, polycomb repressor complex 2 (PRC2) [19]. The LAR subtype encompasses a unique subset of ER/PR-negative tumors, which are characterized by the expression of androgen receptor (AR) and a hormonally regulated transcriptional program [14,22]. Similar to BL-2 tumors, the LAR subtype shows the activation of PI3K/Akt/mTOR signaling, high retinoblastoma protein (Rb) phosphorylation, and low levels of E2F transcription factors [19]. Consequently, the LAR subtype shows a response to AR antagonists, PI3/Akt/mTOR inhibitors, and CDK4/6 inhibitors, as demonstrated by preclinical and early clinical studies [23–25]. Furthermore, genomic analyses revealed amplifications and fusions of fibroblast growth factor receptor 2 (FGFR2) that are specific to LAR tumors, suggesting the potential sensitivity of this subtype to FGFR2 inhibition [25].

Recently, based on genomic and transcriptomic analyses of a large cohort of East Asian patients, Jiang et al. validated and reinforced molecular subtypes of TNBC [22,26]. In addition to basal-like and immune-suppressed (BLIS), mesenchymal-like (MES), and LAR subtypes, the authors described the immunomodulatory (IM) subtype, which shows high immune cell signaling enrichment and a potential response to immunotherapy-based regimens [22].

Despite differences in methodologies and nomenclatures that are used by different subtyping studies, the molecular classifications of TNBC highly correlate with each other. Together, they provide a comprehensive picture of the heterogeneity of triple-negative tumors. Additional efforts to merge large-scale data and compare classification approaches may lead to a final consensus that could guide therapeutic decisions.

3. Clinical evidence from studies of kinase inhibitors in TNBC

The current landscape of targeted therapy for TNBC involves small-molecule kinase inhibitors of the PI3K/Akt/mTOR and MAPK pathways, receptor tyrosine kinases (RTKs), CDKs, and DNA damage response (DDR) signaling, many of which provide significant therapeutic benefits in other tumor types (Fig. 2, Supplementary Table S1) [27,28]. These agents target specific molecular alterations across different TNBC subtypes and are considered potential candidates for precision TNBC therapies based on molecular subtyping and biomarker analysis.

4. PI3K/Akt/mTOR inhibitors

The PI3K/Akt/mTOR signaling pathway plays an important role in the cellular response to growth factors, cytokines, and hormones, thereby regulating cell metabolism and proliferation and oncogenic transformation. Phosphoinositide 3 kinases (PI3Ks) are plasma membrane-associated lipid kinases that are directly stimulated by RTKs to transduce downstream signals. PI3Ks exist as heterodimers and are composed of regulatory subunits (e.g., p85a, p85b, or shorter variants in the PI3K Ia class and p101 in the PI3K Ib class) and catalytic subunits (e.g., p110α, p110β, and p110δ isoforms in the PI3K Ia class and p110γ isoform in the PI3K Ib class). Once activated, PI3K phosphorylates membrane phospholipids, leading to the recruitment and activation of a subset of targets, including Akt kinase, phosphoinositide-dependent kinase-1 (PDK1) and mTOR complex 2 (mTORC2). Akt kinases 1–3 constitute a major signaling node downstream of PI3K and phosphorylate hundreds of substrates to regulate cellular processes. The PI3K/Akt/mTOR signaling pathway is negatively regulated by many phosphatases, including phosphatase and tensin homolog (PTEN) and inositol

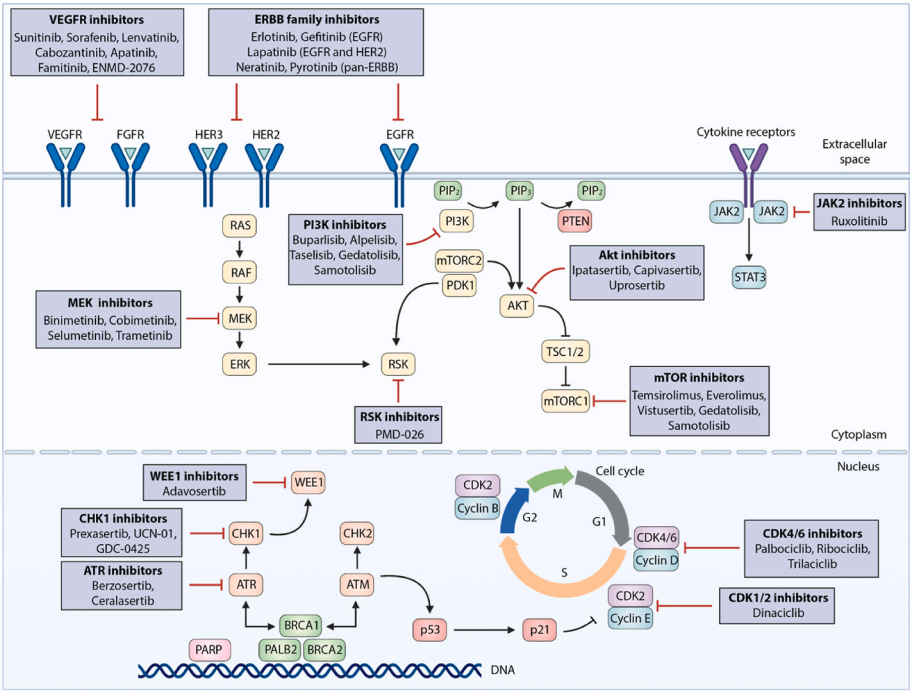

Fig. 2. – Kinase inhibitors under clinical development for the treatment of TNBC. Major kinase targets along with the drugs that have been tested in clinical trials are shown (grey boxes).

polyphosphate-4-phosphatase type II B (INPP4B) [29].

Multiple genomic alterations lead to hyperactivation of the PI3K/Akt/mTOR pathway in cancer, including activating mutations of *PIK3CA* (which encodes the p110α subunit), *AKT1*, or *MTOR* and inactivating mutations or a loss of *PTEN*, *PIK3R1*, or *INPP4B* [29,30]. Activating mutations of the *PI3KCA* and *AKT1* genes are enriched in the LAR subtype, with up to 50% of AR-positive tumors having *PI3KCA* mutations [17,18,31]. Although activating mutations are rare in basal tumors, they are characterized by frequent genomic deletions of *PTEN*, inactivating mutations of *PTEN*, and a loss of *PTEN* and *INBPP4B* expression, resulting in an enhancement of Akt signaling [18,32]. A high frequency of PI3K/Akt/mTOR pathway alterations was also reported in mesenchymal TNBCs [33]. Overall, different genomic alterations of the PI3K/Akt/mTOR signaling network are present in 25–50% of TNBCs and have been associated with greater sensitivity to the inhibition of this pathway in preclinical models [18,21,34], leading to the clinical development of PI3K/Akt/mTOR-targeting agents for TNBC therapy (Table 1).

Buparlisib (BKM120) is a pan-class I PI3K inhibitor that targets all four PI3K isoforms. The efficacy of buparlisib combined with fulvestrant was confirmed in patients with endocrine-resistant, hormone-positive BC in Phase III clinical trials (BELLE-2 and BELLE-3) [35,36]. However, treatment with buparlisib, either as monotherapy or in combination with paclitaxel, did not show significant clinical benefits in patients with advanced or metastatic TNBC [37,38]. In a randomized BELLE-4 trial, patients with locally advanced or metastatic HER2-negative tumors (25% represented by TNBC) were treated with paclitaxel plus either buparlisib or placebo, but the addition of buparlisib failed to improve PFS in the overall study and TNBC population.

Alpelisib is a selective PI3Kα inhibitor that targets both wildtype and mutated p110a, with similar potency (IC_{50} = 4.6, 4.0, and 4.8 nM for wildtype, E545K mutant, and H1047R mutant, respectively). The isoform-selective inhibition of PI3Kα is expected to reduce treatment-related toxicity and improve the therapeutic window compared with less specific pan-PI3K inhibitors [39]. Indeed, alpelisib showed encouraging efficacy along with a manageable toxicity profile in combination with nab-paclitaxel in patients with HER2-negative advanced-stage BC, including 13 patients with TNBC [40]. Currently, this combination is being tested in a Phase III EPIK-B3 trial in patients with metastatic TNBC and either *PIK3CA* mutations or PTEN loss (clinicaltrials.gov: NCT04251533). Based on preclinical studies that demonstrated reciprocal feedback between PI3K/Akt/mTOR pathway and AR signaling in prostate cancer [41], alpelisib is being tested in combination with the AR antagonist enzalutamide in patients with AR- and PTEN-positive metastatic breast cancer in a Phase I trial (NCT03207529). Another PI3Kα-specific inhibitor, taselisib, was evaluated *vs.* placebo in combination with enzalutamide in metastatic AR-positive BC patients, including the TNBC cohort. This Phase I/II study showed that TNBC patients with the LAR subtype who received taselisib plus enzalutamide trended toward a better clinical benefit rate compared with other subtypes. Because of the limited sample size, however, these results must be interpreted with caution [25].

PI3K inhibition leads to the downregulation of BRCA1/2 expression and homologous recombination deficiency in preclinical models, suggesting possible synergy between PI3K and PARP inhibitors [42]. Combinations of alpelisib plus olaparib and buparlisib plus olaparib were evaluated in Phase I trials in patients with TNBC and ovarian cancer, but the latter combination was associated with significant central nervous system toxicity [43,44]. In another Phase I trial, the PI3K and mTOR inhibitor gedatolisib was tested in combination with talazaparib in advanced triple-negative tumors or BRCA1/2-mutated, HER2-negative tumors (NCT03911973). Taselisib was also evaluated in a Phase I trial in combination with the CDK4/6 inhibitor palbociclib in patients with advanced solid tumors. Dual inhibitor therapy had clinical benefit in a subset of patients with PI3KCA-mutant TNBC, warranting further clinical studies to evaluate the efficacy of these nonchemotherapy-based approaches [45].

The novel, selective pan-Akt inhibitors ipatasertib and capivasertib were evaluated *vs.* placebo in combination with paclitaxel for the treatment of mTNBC in the Phase II LOTUS and PAKT trials. These studies reported improvements in PFS in patients with tumors that harbored genetic alterations of *PI3KCA*, *AKT*, and *PTEN* [46,47]. Furthermore, the Phase II FAIRLINE study that tested ipatasertib in addition to neoadjuvant chemotherapy revealed a numerically higher

Table 1
Clinical trials of PI3K/Akt/mTOR and MAPK inhibitors in TNBC. OS – overall survival, PFS – progression-free survival, CR- complete response, PR – partial response, SD – stable disease, CBR – clinical benefit rate, ORR – objective response rate, HR – hazard ratio, TTP – time to progression, BC – breast cancer, mBC – metastatic breast cancer, mTNBC – metastatic TNBC, AR – androgen receptor, NAC – neoadjuvant chemotherapy, DoR – duration of response.

PI3K/Akt/mTOR inhibitors

Trial (NCT identifier)	Phase	Drug	Kinase targets	Patients	TNBC patients (n)	Treatment	Results	Ref.
NCT01623349	I	Buparlisib Alpelisib	PI3K PI3Kα	Recurrent high-grade squamous ovarian cancer or recurrent TNBC or ovarian and BC with a germline BRCA mutation	13	Olaparib plus buparlisib Olaparib plus alpelisib	PR 28%, SD 44% in BC patients treated with buparlisib	[43, 44]
NCT01790932, NCT01629615	II	Buparlisib	PI3K	mTNBC	50	Buparlisib	CBR 12%, median PFS 1.8 mo [95% CI 1.6–2.3], median OS 11.2 mo [95% CI 6.2–25]	[37]
BELLE-4 (NCT01572727)	II/III	Buparlisib	PI3K	HER2- locally advanced or metastatic BC, including TNBC	127	Paclitaxel plus either buparlisib or placebo	Median PFS: 8.0 mo. vs. 9.2; HR 1.18 [95% CI 0.82–1.68]; Median PFS in TNBC population: 5.5 mo. vs. 9.3 mo; HR 1.86 [95% CI 0.91–3.79]	[38]
NCT03207529	I	Alpelisib	PI3Kα	AR+ and PTEN + metastatic breast cancer	Recruiting	Enzalutamide plus alpelisib	n/a	-
NCT2379247	I/II	Alpelisib	PI3Kα	HER2- mBC	43	Nab-paclitaxel plus alpelisib	ORR 52% [95% CI, 34%–70%], CR 7%, PR 52%	[40]
EPIK-B3 (NCT04251533)	III	Alpelisib	PI3Kα	Advanced TNBC either with a PIK3CA mutation or PTEN loss and without PIK3CA mutation	Recruiting	Nab-paclitaxel plus either alpelisib or placebo	n/a	-
TBCRC 032 (NCT02457910)	I/II	Taselisib	PI3Kα	AR + mTNBC	Phase I – 13 Phase II - 17	Enzaluatmide plus taselisib	CBR 35.7%; median PFS 3.4 mo (4.6 vs. 2.0 mo for LAR vs. non-LAR subtype)	[25]
NCT03911973	I	Gedatolisib	PI3K mTOR	Advanced TNBC or BRCA1/2-mutant HER2- BC	Recruiting	Talozaparib plus gedatolisib	n/a	-
LOTUS (NCT02162719)	II	Ipatasertib	Akt	Locally advanced or metastatic TNBC	124	Paclitaxel plus either ipatasertib or placebo	Median PFS: 6.2 mo. vs. 4.9 mo; HR 0.60 [95% CI 0.37–0.98], p = 0.037.	[46]
FAIRLANE (NCT02301988)	II	Ipatasertib	Akt	Early TNBC	151	Paclitaxel plus either ipatasertib or placebo	Overall pCR: 17% vs. 13%; PTEN-low pCR: 16%vs. 13%; PI3KCA/Akt1/PTEN-altered pCR: 18% vs. 12%	[48]
IPATunity130 (NCT03337724)	III	Ipatasertib	Akt	Locally advanced or metastatic TNBC	255	Paclitaxel plus either ipatasertib or placebo	Median PFS: 7.4 mo. vs. 6.1 mo; HR 1.02 [95% CI 0.71–1.45], p = 0.9237	[49]
NCT03800836	I	Ipatasertib	Akt	Locally advanced or metastatic TNBC	26	Paclitaxel/nab-paclitaxel plus ipatasertib plus atezolizumab	ORR 53%, median DoR 7.3 mo.	[52]
NCT03853707	I	Ipatasertib	Akt	mTNBC	23	Arm A: Ipatasertib plus paclitaxel and carboplatin Arm B: Ipatasertib plus carboplatin Arm C: Ipatasertib plus capecitabine and atezolizumab	Best overall responses A: PR 22% SD 44%, PD 33% B: PR 20%, SD 60%, PD 20% C: SD 75%	[51]
NCT04177108	III	Ipatasertib	Akt	Locally advanced or metastatic TNBC	242	Paclitaxel plus ipatasertib or placebo plus atezolizumab or placebo	n/a	-
BARBICAN (NCT03800836)	II	Ipatasertib	Akt	High-risk non-metastatic TNBC	144	NAC plus atezolizumab plus either ipatasertib or no ipatasertib	pCR 49.5% [95% CI 36.8%–61.8%] vs 48.5% [95% CI 36.2%–61.0%]	[53]
PAKT (NCT02423603)	II	Capivasertib	Akt	Locally advanced or metastatic TNBC	140	Paclitaxel plus either capivasertib or placebo	Median PFS: 5.9 mo. vs. 4.2 mo; HR 0.74 [95% CI 0.5–1.08], p = 0.06 [predefined significance level, 1-sided p = 0.10]; Median OS: 19.1 mo. vs. 12.6 mo; HR 0.61 [95% CI 0.37–0.99], p = 0.04	[47]
CAPItello-290 (NCT03997123)	III	Capivasertib	Akt	Locally advanced or metastatic TNBC	Recruiting	Paclitaxel plus either capivasertib or placebo	n/a	-
NCT00761644	I	Temsirolimus Everolimus	mTOR	Advanced, metaplastic TNBC	52	Liposomal doxorubicin plus bevacizumab plus	ORR 21%; CBR 40%	[33]

(continued on next page)

Table 1 (*continued*)

PI3K/Akt/mTOR inhibitors								
Trial (NCT identifier)	**Phase**	**Drug**	**Kinase targets**	**Patients**	**TNBC patients (n)**	**Treatment**	**Results**	**Ref.**
						temsirolimus or everolimus		
MAPK inhibitors								
Trial (NCT identifier)	**Phase**	**Drug**	**Kinase targets**	**Patients**	**TNBC patients (n)**	**Treatment**	**Results**	**Ref.**
COLET (NCT02322814)	II	Cobimetinib	MEK	Locally advanced or metastatic TNBC	90	Cohort I: Paclitaxel plus either cobimetinib or placebo Cohort II: Cobimetinib plus paclitaxel plus atezolizumab Cohort III: Cobimetinib plus nab-paclitaxel plus atezolizumab	Cohort I: Median PFS: 5.5 mo. vs. 3.8 mo; HR 0.75 [95% CI 0.43–1.24], p = 0.25; ORR 38.3% vs. 20.9 Cohort II: ORR 34.4% Cohort III: ORR 29.9%	[62]
NCT03106415	I/II	Binimetinib	MEK	Locally advanced or metastatic TNBC	Recruiting	Binimetinib plus pembrolizumab	n/a	-
InCITe (NCT03971409)	II	Binimetinib	MEK	Stage IV or unresectable TNBC	Recruiting	Avelumab plus binimetinib plus liposomal doxorubicin or avelumab plus sacituzumab govitecan	n/a	-
NCT04115306	I	PMD-026	RSK 1–4	Metastatic breast cancer and TNBC	Recruiting	PMD-026	n/a	-
Dual PI3K/Akt/mTOR and MAPK pathways inhibition strategies								
Trial (NCT identifier)	**Phase**	**Drug**	**Kinase targets**	**Patients**	**TNBC patients (n)**	**Treatment**	**Results**	**Ref.**
NCT01138085	I	Trametinib Uprosertib	MEK, AKT	Advanced solid tumors; unresectable or metastatic TNBC	20	Trametinib plus uprosertib	ORR 4.8% Median PFS in TNBC cohort: 54 days	[63]
TORCMEK (NCT02583542)	I/II	Selumetinib Vistusertib	MEK mTORC 1/2	Advanced solid tumors	N/A	Selumetinib plus vistusertib	n/a	-

pCR rate compared with placebo in patients with the aforementioned alterations [48]. However, the Phase III IPATunity130 trial showed that the combination of ipatasertib plus paclitaxel failed to improve PFS in patients with *PI3KCA*-, *AKT*-, or *PTEN*-altered advanced or metastatic TNBC [49]. A similar Phase III trial that is evaluating capivasertib plus paclitaxel in advanced or metastatic TNBC is ongoing (CAPItello-290 NCT03997123). PTEN loss and PI3K/Akt activation were postulated to be mechanisms that drive resistance to immunotherapy in TNBC [50]. Akt inhibitors are being exploited as potential strategies to enhance ICI efficacy in TNBC in clinical trials (NCT03800836, NCT03853707, NCT04177108) [51,52]. The report by Schmid et al. confirmed an objective response rate (ORR) in 54% of mTNBC patients who were treated with a triplet combination of ipatasertib, paclitaxel/nab-paclitaxel, and atezolizumab [52]. The same combination did not improve clinical outcomes in neoadjuvant settings, as shown by the results of the BARBICAN trial [53].

The allosteric mTOR inhibitors temsirolimus and everolimus in combination with bevacizumab and liposomal doxorubicin were tested in patients with metaplastic TNBC in a Phase I clinical trial. A metaplastic TNBC cohort was selected as a surrogate of the mesenchymal subtype and was characterized by a high frequency of PI3K pathway alterations, defined by a mutation in the pathway that was detected by sequencing or a loss of PTEN expression that was detected by immunohistochemistry. The study reported a promising ORR, which was positively correlated with PI3K pathway activation in tumor tissue [33]. However, this combination has not been tested in subsequent clinical trials.

Despite strong preclinical evidence and encouraging results from early-stage clinical studies, therapeutic targeting of the PI3K/Akt/mTOR pathway in TNBC did not show significant benefits in randomized Phase III clinical trials. Several factors might limit therapeutic outcomes of PI3K/Akt/mTOR inhibitors, including molecular heterogeneity that is associated with various genetic alterations within this pathway and feedback escape mechanisms that are initiated by PI3K inhibition and involve other signaling nodes, such as RTKs and MAPK pathways [34]. These observations provide a rationale for more rigorous patient selection and the development of combined therapeutic approaches, but potential high systemic toxicity that is associated with these treatments should also be considered.

Furthermore, the development of inhibitors that selectively target mutant over wildtype kinases represents a promising strategy to reduce treatment-related toxicity, and this approach can potentially be used to treat TNBC with *PI3KCA* mutations. In a recent study by Song et al., the authors reported that the PI3Kα inhibitors taselisib and involisib (GDC-0077) induced the degradation of p110a mutated protein in cancer cells and patient-derived xenografts (PDXs) [54]. Additionally, two novel PI3K mutant-specific allosteric inhibitors are under development (LOXO-783 from Petra Pharmaceuticals and RLY- 2608 from Relay Therapeutics). The effectiveness of therapies against mutated PI3K needs to be further investigated in TNBC models. It will be essential to determine whether treatments that target mutant kinases selectively improve the therapeutic index and promote favorable clinical outcomes.

5. MAPK inhibitors

MAPK pathways are activated by extracellular and intracellular stimuli to coordinate multiple cellular processes, such as proliferation, differentiation, transformation, survival, and apoptosis. MAPK signaling

cascades consist of at least three core kinases (MAP3K, MAP2K, and MAPK), sequentially phosphorylated to propagate signal transduction. The major MAPK pathways are named according to their MAPK components: extracellular signal-regulated kinases 1/2 (ERK1/2), ERK5, p38-mitogen activated protein kinases (p38), and c-JUN N-terminal kinase (JNK). Within the ERK1/2 pathway, the signal that is initiated by Rous sarcoma virus (RAS) family members is transmitted downstream through RAF, mitogen-activated protein kinase kinase (MEK), and ERK1/2. Following its phosphorylation, ERK1/2 translocate to the nucleus and regulate transcription factors to promote cell proliferation and survival [55]. The ERK1/2 pathway is frequently hyperactivated in human cancers, primarily through gain-of-function mutations of the *RAS* and *BRAF* genes. Consequently, small-molecule inhibitors that target BRAF and MEK are used in the treatment of different cancers, including melanoma, non-small-cell lung cancer, and thyroid cancer [56]. Although activating mutations in the RAS/RAF/MEK/ERK cascade are rare in BC, other alterations that lead to ERK1/2 hyperactivation were reported in TNBC, including the amplification of genes that encode MAPKs, overexpression of growth factor receptors, and loss of negative regulators of MAPK signaling, such as neurofibromin 1 (NF1) and dual specificity protein phosphatase 4 (DUSP4) [21,57–60]. Consistent with these observations, preclinical studies suggested that the inhibition of MAPK may preferentially suppress the growth, metastasis, and drug resistance of triple-negative tumors [61]. Following these observations, several MAPK-targeting compounds were evaluated in TNBC therapy (Table 1).

The FDA has approved four MEK inhibitors for clinical use to date: trametinib, binimetinib, selumetinib, and cobimetinib. Clinical trials of MEK inhibitors as single agents showed limited success in cancer therapy, indicating a rationale for developing combination therapies [56]. Notably, the MEK/ERK transcriptional signature is enriched in chemoresistant residual cells following NAC in TNBC, and MEK inhibitors exerted a synergistic effect against TNBC when combined with taxane chemotherapy [58]. Based on these observations, the combination of cobimetinib plus chemotherapy, with or without the *anti*-PDL1 mAb atezolizumab, was tested in a Phase II clinical trial (COLET). In this trial, the addition of cobimetinib did not lead to a significant increase in median PFS or ORR *vs.* paclitaxel monotherapy, albeit a trend toward a higher ORR was noted. Similarly, the combination of atezolizumab, cobimetinib, and taxane resulted in a modest ORR [62]. Other studies that are evaluating binimetinib combined with immunotherapy are currently underway (NCT03106415, NCT03971409).

As mentioned above, crosstalk between the MAPK and PI3K/Akt/mTOR signaling pathways may lead to the reciprocal compensatory activation of one pathway when the other pathway is inhibited [34]. Several clinical trials tested the effects of co-targeting both signaling cascades (Table 1). The combination of trametinib and Akt inhibitor uprosertib was evaluated in a Phase I dose-escalation trial in patients with solid tumors, including TNBC. This study found minimal clinical activity and significant toxicity upon combined treatment [63]. In another Phase I/II clinical trial, selumetinib was tested in combination with the mTORC1/2 inhibitor vistusertib in patients with advanced-stage cancers, including patients with TNBC (TORCMEK, NCT02583542). The other approach for co-targeting MAPK and PI3K/Akt/mTOR signaling is the inhibition of p90 ribosomal s6 kinase (RSK), a downstream effector of both pathways [64]. A first-in-human Phase I trial that is evaluating the RSK inhibitor PMD-026 is currently ongoing (NCT04115306). Additionally, binimetinib is being tested in combination with the CDK4/6 inhibitor palbociclib in a Phase I trial (PALBOBIN, NCT04494958).

Although MAPK inhibitors have not yet met clinical expectations in TNBC, the MAPK pathway remains an attractive target for precision therapies. Therefore, future studies that seek to facilitate the biomarker-guided selection of TNBC patients who may benefit from MAPK inhibitor treatment are warranted.

6. Receptor tyrosine kinase inhibitors

Receptor tyrosine kinases constitute a diverse subclass of proteins that share a similar structure, consisting of an extracellular ligand-binding domain, a single transmembrane helix, and an intracellular tyrosine kinase domain. A general mode of RTK activation involves their dimerization in the presence of ligand, which leads to activation of the kinase domain and receptor autophosphorylation [65]. Multiple prooncogenic signaling pathways are activated downstream of RTKs in TNBC cells. Therefore, monoclonal antibodies and small-molecule inhibitors that target various RTKs are being investigated as candidates for TNBC therapy. Because of the highly conserved kinase domains of RTKs, many inhibitors show relatively low substrate specificity and simultaneously inhibit multiple prooncogenic RTKs in cancer cells (Table 2).

Vascular endothelial growth factor receptors 1–3 (VEGFR1-3) are RTKs that play a critical role in angiogenesis. VEGFRs transduce downstream signals upon binding the major proangiogenic factors, vascular endothelial growth factors (VEGFs) [66]. Bevacizumab, a VEGF-A-targeting mAb, demonstrated efficacy in metastatic and early TNBC in several randomized clinical trials, but the results of subsequent studies were inconsistent. The currently available data do not unequivocally support the use of bevacizumab in TNBC therapy [67]. Sunitinib, a small-molecule inhibitor of VEGFR and related RTKs, initially demonstrated activity in patients with mTNBC in a Phase II open-label trial [68], but later studies that tested sunitinib either as monotherapy or in combination with chemotherapy were negative [69–71]. Sorafenib, a compound with a similar multikinase inhibition profile, significantly improved PFS in patients with HER2-negative advanced BC when added to capecitabine/gemcitabine chemotherapy in Phase II trials [72,73]. These results were not confirmed in the follow-up Phase III RESILIENCE trial, which showed no significant PFS improvement and a higher rate of treatment-related toxicity in the sorafenib plus capecitabine arm compared with the placebo plus capecitabine arm [74]. Cabozantinib, an inhibitor of VEGFR2 and MET, among other kinases, was evaluated as monotherapy in patients with mTNBC in a single-arm Phase II trial. This study showed a poor ORR, indicating the limited single-agent activity of cabozantinib [75]. Although the biomarker analysis in this study demonstrated systemic changes that were consistent with activation of the immune system in patients who were treated with cabozantinib, the following Phase II trial that tested the combination of cabozantinib and nivolumab also reported a poor response rate in TNBC patients [76]. In a recent Phase II study, monotherapy with ENMD-2076, a small-molecule multikinase inhibitor that targets VEGFRs, FGFRs, Fms-like tyrosine kinase 3 (FLT3) kinases, and cell division-regulating Aurora kinase A (AURKA), resulted in a partial response or clinical benefit that lasted more than 6 months in six of 36 patients with pretreated and metastatic TNBC. Patients with deleterious mutations of p53 trended toward a better response to treatment with ENMD-2076, warranting the further development of predictive biomarkers and mechanism-based combination strategies with this agent [77].

The VEGFR-targeting agents apatinib and famitinib were tested in a Phase Ib/II subtyping-based and genomic biomarker-guided umbrella trial (FUTURE) in patients with refractory mTNBC [22]. The *anti*-VEGFR therapy was associated with favorable outcomes in 23 patients with the BLIS subtype of TNBC (ORR = 26.1%). However, a relatively high rate of high-grade adverse effects was observed in this cohort. In a follow-up Phase II clinical trial (II FUTURE-C-PLUS), famitinib is currently being tested with nab-paclitaxel and the *anti*-PD1 mAb camrelizumab as a first-line treatment for patients with the IM subtype of TNBC. Notably, initial reports from this study indicate a highly encouraging ORR (39/48 patients, 81%); complete results are eagerly awaited [17,78]. Additional trials that tested combinations of antiangiogenic inhibitors and ICIs were also reported. A recent Phase II trial that evaluated the combination of camrelizumab with apatinib in patients with mTNBC reported a significantly higher ORR (43.3%) than previous studies that evaluated

Table 2
Major trials of small-molecule inhibitors targeting receptor tyrosine kinases in TNBC. OS – overall survival, PFS – progression-free survival, CR- complete response, PR – partial response, SD – stable disease, CBR – clinical benefit rate, ORR – objective response rate, HR – hazard ratio, BC – breast cancer, mBC – metastatic breast cancer, mTNBC – metastatic TNBC, NR – not reached, TTP – time-to-progression.

Trial (NCT identifier)	Phase	Drug	Kinase targets	Patients	TNBC patients (n)	Treatment	Results	Ref.
SUN1107	III	Sunitinib	VEGFR PDGFR c-KIT CSF1-R FLT-3	Locally advanced or metastatic HER2- BC	167	Sunitinib or capecitabine	Median PFS: 2.8 mo. vs. 4.2; HR 1.47 [95% CI 1.16–1.87]; Median OS: 15.3 mo. vs. 24.6 mo; HR 1.17 [95% CI 0.84–1.63]	[69]
NCT00393939	III	Sunitinib	VEGFR PDGFR c-KIT CSF1-R FLT-3	Locally advanced or metastatic HER2- BC	127	Docetaxel plus sunitinib or docetaxel alone	Median PFS: 8.6 mo. vs. 8.3; HR 0.92 [95% CI 0.72–1.19]; Median OS: 24.8 mo. vs. 25.5 mo; HR 1.21 [95% CI 0.91–1.60]	[71]
NCT00435409	III	Sunitinib	VEGFR PDGFR c-KIT CSF1-R FLT-3	Previously treated mBC	118	Capecitabine plus sunitinib or placebo	Median PFS: 5.5 mo. vs. 5.9; HR 1.22 [95% CI 0.95–1.58]; Median OS:16.4 mo. vs. 16.5 mo; HR 0.99 [95% CI 0.76–1.30]	[70]
EudraCT ID 2007-000290-32	II	Sorafenib	VEGFR PDGFR c-KIT, RET FLT-3 RAF family members	Locally advanced or metastatic HER2- BC	53	Capecitabine plus sorafenib or placebo	Median PFS: 6.4 mo. vs. 4.1; HR 0.58 [95% CI 0.41–0.81], p = 0.001; Median PFS in TNBC cohort: 4.3 vs. 2.5; HR 0.6 [95% CI 0.31–1.14] Median OS:22.2 mo. vs. 20.9 mo; HR 0.86 [95% CI 0.61–1.23]	[72]
NCT00493636	II	Sorafenib	VEGFR PDGFR c-KIT, RET FLT-3 RAF family members	Locally advanced or metastatic HER2- BC	50	Capecitabine/ gemcitabine plus sorafenib or placebo	Median PFS: 3.6 mo. vs. 2.7; HR 0.65 [95% CI 0.44–0.93], p = 0.02 Median OS: 13.4 mo. vs. 11.4 mo; HR 1.01 [95% CI 0.71–1.44]	[73]
RESILIENCE (NCT01234337)	III	Sorafenib	VEGFR PDGFR c-KIT, RET FLT-3 RAF family members	Locally advanced or metastatic HER2- BC	167	Capecitabine plus sorafenib or placebo	Median PFS: 5.5 mo. vs. 5.4; HR 0.973 [95% CI 0.0.779–1.21], p = 0.811 Median OS: 18.9 mo. vs. 20.3 mo; HR 1.195 [95% CI 0.943–1.513], p = 0.14	[74]
NCT01738438	II	Cabozantinib	MET VEGFR2 RET FLT-1/3/4 AXL Tie2	mTNBC	35	Cabozantinib	ORR 9% Median PFS 2.0 mo [95% CI 1.3–3.3]	[75]
NCT03316586	II	Cabozantinib	MET VEGFR2 RET FLT-1/3/4 AXL Tie2	mTNBC	18	Cabozantinib plus nivolumab	ORR 6%	[76]
NCT01639248	II	ENMD-2076	Aurora- A VEGFRs FGFRs PDGFRs FLT-3	Locally advanced or metastatic TNBC	41	ENMD-2076	CBR 16.7% [95% CI, 6–32.8%] in 6 mo.	[77]
FUTURE-SUPER (NCT 04395989)	II	Famitinib	VEGFR2, PDGFR c-KIT FLT-1/3	Immunomodulatory subtype locally advanced or metastatic TNBC	48	Famitinib plus camrelizumab plus nab-aclitaxel	ORR 81.3% (n = 39), [95% CI 70.2–92.3]	[78]
NCT03394287	II	Apatinib	VEGFR2 RET	Metastatic or unresectable TNBC	40	Camrelizumab plus apatinib (continuous dosing or intermittent dosing)	ORR 43.3% (n = 13) in continuous dosing cohort, PFS 3.7 mo in continuous apatinib cohort [95% CI 2–6.4] vs. 1.9 mo in intermittent apatinib cohort [95% CI 1.8–3.7]	[79]
LEAP-005 (NCT03797326)	II	Lenvatinib	VEGFRs FGFRs PDGFRs	Previously treated advanced TNBC	31	Lenvatinib plus pembrolizumab	ORR 29% Median PFS 4.0 mo [95% CI 2-NR]	[80]

(*continued on next page*)

Table 2 (*continued*)

Trial (NCT identifier)	Phase	Drug	Kinase targets	Patients	TNBC patients (n)	Treatment	Results	Ref.
EGF30001	III	Lapatinib	FLT4 KIT EGFR HER2	mBC	ER-, PR-, HER2- or unknown - 197	Paclitaxel plus lapatinib or paclitaxel plus placebo	Median TTP in HER2- cohort 25.1 vs. 24 mo.; HR 1.05 [95% CI 0.84–1.32]	[82]

apatinib or immunotherapy alone [79]. In another Phase II trial, the combination of a multikinase inhibitor levatinib plus pembrolizumab also showed promising antitumor activity (ORR = 29%) in patients with pretreated mTNBC [80]. Collectively, these findings suggest possible synergistic effects of RTK-targeting angiogenesis inhibitors and ICIs against triple-negative tumors and indicate the potential of RTKs inhibitors in the development of subtyping-based TNBC therapies.

The ERBB family of RTKs consists of four members: epidermal growth factor receptor (EGFR; also known as HER1/ERBB1), HER2 (ERBB2), HER3 (ERBB3), and HER4 (ERBB4). Overexpression of the *EGFR* (*HER1*, *ERBB1*) gene was observed in 30–60% of TNBC cases, and it correlated with a poor prognosis in TNBC patients [81]. Both EGFR-targeting mAbs and RTK inhibitors exerted a high antitumor effect in preclinical models of TNBC, but the results of subsequent clinical trials were disappointing [82–84]. Activating mutations of the *HER2* gene were found in approximately 3% of TNBC cases. Early-phase clinical trials with the irreversible pan-HER inhibitors neratinib and pyrotinib showed an encouraging response rate in HER2-mutated BC, including TNBC [22,85]. These observations warrant further studies in TNBC populations that are selected for HER2 mutation-directed therapies. In addition to small-molecule inhibitors and mAbs, there are promising ADCs in clinical development that target members of ERBB family receptors (recently reviewed in [86]). Based on the targeting capabilities of mAbs, these agents enable direct delivery of the cytotoxic payload to cancer cells. Moreover, the antibody component of ADCs can interfere with target function and dampen downstream signaling to inhibit tumor growth. Several trials that involve TNBC patients are ongoing, and ADCs may prove to be a novel powerful weapon against triple-negative breast tumors.

Multikinase inhibitors that target other RTKs, including TAM family kinases (TYRO3, AXL, MERTK), FGFRs, and platelet-derived growth factor receptors (PDGFRs), are also being tested in combination with chemotherapies and immunotherapies in ongoing clinical trials that involve TNBC patients (NCT04877821, NCT03992131, NCT04734262). Finally, a non-receptor tyrosine kinase, Janus kinase 2 (JAK2), which is involved in downstream signal transduction from cytokine receptors and multiple RTKs, was also pursued as a therapeutic target in TNBC [87]. Molecular subtyping data indicated that JAK2-STAT signaling is predominantly activated in the MES subtype, but the JAK1/2 inhibitor ruxolitinib had limited efficacy in patients with mTNBC [17]. Supporting the crucial role of this pathway in the maintenance of breast cancer stem cells (CSCs), MES tumors are enriched in CSC characteristics. Future studies should investigate whether JAK1/2 inhibitors play a role in targeting CSCs in the MES subtype of TNBC.

7. Cyclin-dependent kinase inhibitors

Cyclin-dependent kinases are serine/threonine protein kinases that are activated upon associations with their regulatory subunits, named cyclins, and phosphorylation by CDK-activating kinase (CAK). Cyclin-dependent kinases play a well-established role in regulating multiple cellular processes, including cell cycle progression, transcription, DNA damage repair, and apoptosis. CDK4/6-cyclin D and CDK2-cyclin E complexes regulate the transition from the G1 phase of the cell cycle to the S phase by phosphorylating Rb protein and derepressing E2F transcription factors. The dysregulation of CDK function drives the uncontrolled proliferation and survival of many human cancers. Therefore, CDKs have been extensively investigated as therapeutic targets. Recently, three CDK4/6 inhibitors (palbociclib, ribociclib, and abemaciclib) provided substantial improvements in treating ER-positive BC. They are now being used as upfront targeted therapy in combination with estrogen-blocking agents [88].

In contrast to hormone receptor-positive tumors, TNBC was initially found to be intrinsically resistant to CDK4/6 inhibitors [89]. Nevertheless, recent advances in our understanding of the biology of TNBC have shed new light on the use of CDK4/6 inhibitors in TNBC therapy, leading to several clinical trials with these agents (Table 3). Preclinical studies reported that the LAR subtype of TNBC is sensitive to CDK4/6 inhibition, similar to ER-positive models [90]. Consistent with these observations, a Phase I/II clinical trial that evaluated the combination of palbociclib with the antiandrogen agent bicalutamide in patients with AR-positive tumors met its primary endpoint (11 of 31 evaluable patients were progression-free at 6 months) [24]. A similar trial with ribociclib and bicalutamide is ongoing (NCT03090165). Moreover, other combination strategies that involve CDK4/6 inhibitors are currently evaluating the treatment of mTNBC, including palbociclib plus binimetininb and ribociclib plus the histone deactylase inhibitor belinostat (NCT04494958, NCT04315233).

Apart from already approved CDKs inhibitors, several new agents are being tested in clinical trials. In a randomized Phase II trial, the CDK4/6 inhibitor trilaciclib, which is known to protect hematopoietic stem cells from chemotherapy-induced exhaustion, was administered intravenously prior to gemcitabine and carboplatin *vs.* chemotherapy alone in patients with metastatic TNBC [91]. The primary objective of this study was to assess safety and tolerability of the combination therapy. The primary endpoints were the duration of severe neutropenia during cycle 1 and occurrence of severe neutropenia during the treatment period. Overall survival was included as a secondary endpoint. Although no significant differences were found in myelosuppression-related primary endpoints, the combination regimens were associated with significant improvements in OS and PFS [91,92]. Based on these findings, a randomized Phase III trial was recently announced to evaluate the combination of trilaciclib and gemcitabine chemotherapy (NCT04799249).

Dinaciclib is a potent small-molecule inhibitor that targets CDK1, CDK2, CDK5, and CDK9. Treatment with dinaciclib potentiated antitumor effects of *anti*-PD-1 therapy in syngeneic mouse models [93]. Currently, the combination of dinaciclib and pembrolizumab is being evaluated in a Phase I trial in patients with advanced TNBC. Initial reports from this study indicate an encouraging response that correlates with baseline Myc protein expression in tumor biopsies along with a manageable toxicity profile [94]. A previous Phase I trial that tested dinaciclib in addition to epirubicin in patients with metastatic BC reported significant toxicity that was associated with this combination treatment [95].

Collectively, recent findings provide a strong rationale for further clinical evaluations of CDK4/6 inhibitors for the treatment of TNBC, particularly the LAR subtype. The results of ongoing trials are expected to provide additional insights into the efficacy of CDK inhibitor-based regimens in TNBC patients. The identification of novel clinicopathological and molecular biomarkers is necessary to better stratify patients who are most likely to benefit from these therapies.

Table 3
Clinical trials of small-molecule inhibitors targeting cyclin-dependent kinases in TNBC. OS – overall survival, CR- complete response, PR – partial response, SD – stable disease, HR – hazard ratio, BC – breast cancer, mBC – metastatic breast cancer, mTNBC – metastatic TNBC, AR – androgen receptor; NR- not reached.

Trial (NCT identifier)	Phase	Drug	Kinase targets	Patients	TNBC patients (n)	Treatment	Results	Ref.
NCT02605486	I/II	Palbociclib	CDK4/6	AR + mTNBC	33	Palbociclib plus bicalutamide	11 patients were progression-free at 6 mo (10SD, 1PR)	[24]
NCT04494958	I/II	Palbociclib Binimetinib	CDK4/6 MEK	Advanced TNBC with ERK activation and/or CDK4/6	n/a	Palbociclib plus binimetinib	n/a	-
CHARGE (NCT04315233)	I	Ribociclib	CDK4/6	mTNBC	n/a	Ribociclib plus belinostat	n/a	-
NCT03090165	II	Ribociclib	CDK4/6	AR + advanced TNBC	n/a	Ribociclib plus bicalutamide	n/a	-
NCT02978716	II	Trilaciclib	CDK4/6	mTNBC	102	Gemcitabine plus carboplatin (days 1, 8) -group 1; or gemcitabine plus carboplatin plus trilaciclib (days 1,8) -group 2; or gemcitabine plus carboplatin plus trilaciclib (days 1, 2, 8, 9) - group 3	OS: group 1–12.6 mo, OS group 2-NR, OS group 3–17.8 mo, HR combined trilaciclib groups vs. chemotherapy only 0.37 [95% CI 0.2–0.6], $p < 0.0001$	[91, 92]
NCT04799249	III	Trilaciclib	CDK4/6	Locally advanced or metastatic TNBC	Recruiting	Trilaciclib or placebo administered prior to gemcitabine and carboplatin	n/a	-
NCT01624441	I	Dinaciclib	CDK1, CDK2, CDK5, CDK9 CDK12	mTNBC	9	Dinaciclib plus epirubicin	No responses	
NCT01676753	I	Dinaciclib	CDK1, CDK2, CDK5, CDK9 CDK12	Advanced TNBC	32	Dinaciclib plus pembrolizumab	CR 3.4%, PR 13.8% SD 20.6%	[94]

8. DNA damage response inhibitors

TNBC is characterized by a high frequency of alterations of DDR signaling pathways. Although specific DDR defects may promote oncogenic transformation, compensatory DNA repair systems are required for the survival of cancer cells and resistance to genotoxic therapies [96]. Deficiency of the HRR system is the most common aberration in BRCA1/2-mutated tumors and in a subset of tumors that do not carry these mutations but display the "BRCAness" phenotype [97]. A large body of preclinical and clinical evidence indicates that HRR-deficient cancers are intrinsically sensitive to inhibitors of the PARP enzyme, through mechanisms of synthetic lethality. Based on these findings, PARP inhibitors have been approved for the treatment of BRCA1/2-–associated advanced TNBC and high-risk early-stage tumors [7–9].

The clinical success of PARP inhibitors highlights the potential of DDR-targeting agents in TNBC therapy. Three PI3K-related kinases, namely ataxia-telangiectasia mutated (ATM), ATM- and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK), are the most upstream regulators of DDR signaling. These proteins are directly activated in response to DNA lesions and subsequently phosphorylate numerous substrates to coordinate DNA repair pathways, cell cycle arrest, and other cellular processes. Checkpoint kinase 1 (CHK1) is a conserved downstream target of ATR. Following its activation, CHK1 regulates the function of Wee1-like protein kinase (WEE1) kinase and CDC25 family phosphatases, leading to G2/M checkpoint induction and preventing cells with DNA lesions from entering mitosis. In addition to the cell cycle checkpoint response, the ATR-CHK1 pathway is necessary for the activation of crucial proteins that are involved in HRR repair, including BRCA1, BRCA2, and RAD51 [96]. Notably, inhibitors of ATR, ATM, DNA-PKCs, CHK1, and WEE1 kinases have recently entered clinical trials, and several compounds have promising activity against TNBC (Table 4).

A Phase I trial that evaluated cisplatin plus the ATR inhibitor berzosertib in a cohort of 35 patients with mTNBC reported an ORR in 38.9% of cases, indicating encouraging antitumor activity and tolerability of this regimen [98]. A related clinical trial that is testing berzosertib in combination with radiation therapy for chemoresistant TNBC is ongoing (NCT04052555). Ceralasertib, another ATR-selective inhibitor, is currently being tested in combination with chemotherapy or olaparib or *anti*-PD-L1 antibody durvalumab in a modular Phase I study in patients with advanced cancers (NCT02264678). In this study, 35% of patients with metastatic, BRCA-mutated, HER2-negative BC who were treated with the combination of ceralasertib and olaparib achieved a confirmed response, suggesting the potential efficacy of this combination [99]. These observations are being further tested in the ongoing multicenter Phase II VIOLETTE trial (NCT03330847), in which patients with advanced TNBC are randomized to receive olaparib plus ceralasertib or olaparib plus the WEE1 inhibitor AZD1775 or olaparib alone.

UCN-01 was the first defined CHK1 inhibitor that was tested in clinical settings, but this compound also inhibits other serine-threonine protein kinases, including PDK1, CDK2, and CDK4/6 [100]. The combination of UCN-01 and irinotecan was evaluated in a Phase II trial in patients with metastatic TNBC, but the study reported a poor response rate and inconsistent CHK1 inhibition *in vivo* [101]. Prexasertib, a second-generation CHK1/2 inhibitor that selectively binds CHK1 and has minor activity against CHK2, demonstrated only a modest effect as monotherapy in a Phase II study [102]. Treatment with prexasertib plus the dual PI3K/mTOR inhibitor samotolisib exerted antitumor activity in preclinical models and preliminary efficacy in patients with advanced tumors and TNBC, but this combination was associated with significant toxicity in clinical settings [103]. GDC-0425, a new selective inhibitor of CHK1, was recently evaluated in combination with gemcitabine for refractory solid tumors and demonstrated a manageable safety profile in a Phase I trial, suggesting that further studies are necessary [104].

Adavosertib is a first-in-class selective inhibitor of WEE1 kinase. Overexpression of cyclin E1 (*CCNE1*) gene was identified as a biomarker of adavosertib sensitivity in preclinical models of TNBC [105]. These observations were validated in a biomarker analysis of a Phase I trial that tested adavosertib in advanced cancers [106]. A new Phase II clinical trial that is evaluating adavosertib monotherapy for advanced refractory solid tumors with cyclin E amplification is currently ongoing (NCT03253679). Adavosertib was also tested in combination with cisplatin in patients with mTNBC in a Phase II trial [107]. The combined therapy did not meet the prespecified ORR cutoff in this study (26% *vs.* 30%). However, tumors in patients who achieved clinical benefit showed enriched infiltration by T-cells, suggesting that adavosertib may augment antitumor immunity. Additional studies are needed to validate these findings.

Altogether, the results of recent clinical trials suggest that the therapeutic targeting of DDR signaling in TNBC may extend beyond PARP inhibitors. Notably, BRCA1/2-mutated TNBC cells that are resistant to PARP inhibition increasingly depend on ATR, CHK1, and WEE1 for their survival [108]. Thus, the inhibition of these kinases suggests new possibilities for overcoming resistance to PARP inhibitors in triple-negative

Table 4
Trials of agents targeting DNA damage response kinases involving patients with TNBC. PR – partial response, SD – stable disease, CBR – clinical benefit rate, ORR – objective response rate, mTNBC – metastatic TNBC, AR – androgen receptor, HRR – homologous recombination repair.

Trial (NCT identifier)	Phase	Drug	Kinase targets	Patients	TNBC patients (n)	Treatment	Results	Ref.
NCT02157792	I	Berzosertib	ATR	Advanced TNBC	35	Berzosertib plus cisplatin	Preliminary ORR 38.9%	[98]
NCT04052555	I	Berzosertib	ATR	Chemotherapy-resistant TNBC	n/a	Berzosertib plus radiation therapy	N/A	-
NCT02264678	I	Ceralasertib	ATR	Advanced solid tumors	25 TNBC with no alterations in HRR (HRR-wt) 37 patients with BRCA-mutated HER-2 BC	Ceralasertib plus olaparib	No responses in HRR-wt groups 35% (n = 13) of confirmed responses in the BRCA-mutated group	[99]
NCT00031681	I/II	UCN-01	CHK1/2	mTNBC	25	UCN-01 plus irinotecan	ORR 4% CBR 12%	[101]
NCT02124148	I	Prexasertib Samotolisib	CHK1/2 PI3K, mTOR	Advanced TNBC	16	Prexasertib plus samotolisib	ORR in TNBC subgroup 25%	[103]
NCT02203513	II	Prexasertib	CHK1/2	mTNBC/recurrent TNBC	9	Prexasertib	PR n = 1, SD n = 4, ORR 11.1%	[102]
NCT01359696	I	GDC-0425	CHK1/2	Advanced solid tumors	8	GDC-0425	1/8 patients had PR	[104]
NCT03012477	II	Adavosertib	WEE1	mTNBC	34	Adavosertib plus cisplatin	ORR 26%, median PFS 4.9 mo [95% CI 2.3–5.7]	[107]

tumors. Additional studies are required to evaluate the clinical efficacy and safety profile of new DDR-targeting agents and identify optimal combination strategies.

9. Future perspectives for kinase-targeted therapies in TNBC

The discovery of actionable targets across distinct TNBC molecular subtypes has opened new possibilities for the treatment of this heterogeneous disease. The recently proposed TNBC precision treatment paradigm assumes that tumors are first classified into molecular subtypes with the enrichment of certain actionable genomic alterations. Next, subtype-specific therapeutic targets and biomarkers are detected to adjust the treatment regimen [109]. Kinase inhibitors may serve as promising therapeutic tools for subtype-based targeted therapies, as already demonstrated by many preclinical and clinical studies that are discussed above. Nevertheless, identifying feasible biomarkers to classify TNBC into molecular subtypes and guide treatment decisions is still an unmet clinical need that should be addressed by further research.

The major limitations of kinase inhibitor-based therapies include undesired side effects and the emergence of therapeutic resistance. Treatment-related toxicities are largely attributable to the off-target effects of ATP-competitive inhibitors, such as the inhibition of other kinases, enzymes, and ATP-dependent proteins (e.g., ion channels and chromatin remodeling complexes) [110]. Drug resistance can be driven by several unique mechanisms that include acquired mutations of the targeted kinases and the adaptive kinome reprogramming with activation of the compensatory signaling pathways [111]. The inhibitors that target many signaling cascades simultaneously (e.g., RTKs inhibitors) demonstrate high potency and may effectively reduce the possibility of developing drug resistance. These agents, however, can lead to significant side effects due to interference with multiple biologic pathways that play important roles in normal cellular function. Therapies with two or more selective drugs that inhibit different kinases provide the opportunity to target adaptive kinome responses more precisely. Nevertheless, the implementation of effective combination strategies poses many challenges, such as identifying the synergistic drug interactions, appropriate selection of patients, and managing possible overlapping toxicities [112]. Future investigations into the mechanisms underlying drug resistance across TNBC molecular subtypes may facilitate the design of novel therapeutic strategies and rational combinations of targeted agents, chemotherapy, and immunotherapy. Moreover, the new types of kinase-targeting drugs, including allosteric inhibitors, covalent inhibitors, or Proteolysis targeting chimeras (PROTACs), can potentially be used to overcome the limitations of traditional small-molecule inhibitors. Many of these agents demonstrated remarkable specificity and durable target inhibition in preclinical studies, and therefore they should be further investigated to address kinase targets in TNBC [110,113].

Finally, the current therapeutic landscape of TNBC focuses on a relatively small number of molecular targets and signaling pathways with well-known oncogenic functions. Bioinformatic screens have indicated that many understudied kinases are overexpressed in cancer and likely drive tumor progression. Thus, they should be considered clinically relevant therapeutic targets [114]. Accordingly, several potential kinase targets have been recently validated in preclinical TNBC models as novel oncogenes that promote tumor growth, metastasis, and drug resistance, including CK1, CK2/TNIK/DYRK1, MELK, MLK4, NEK2, RON, PIM1, and others (Table 5) [115–122]. Additional studies that seek to identify expression patterns and functions of these kinases across TNBC subtypes are warranted, which may prompt the clinical development of novel small-molecule inhibitors for personalized therapy.

Table 5
The emerging kinase targets for precision therapy of TNBC.

Kinase target	Studies in animal models	Ref.
CK1	- CK1 promotes Wnt/β-catenin signaling in breast cancer xenograft models - CK1 knock-down impairs the growth of TNBC cell line-derived xenografts - Selective CK1 inhibitor (SR-3029) suppresses the growth of TNBC cell line-derived xenografts and chemoresistant PDXs	[122]
CK2/TNIK/DYRK1	- Multikinase inhibitor targeting CK2, TNIK and DYRK1 kinases (108600) suppresses the growth of TNBC cell line-derived xenografts and PDXs - 108600 suppresses the growth of chemoresistant PDXs in combination with paclitaxel - 108600 and paclitaxel synergize to inhibit established TNBC metastases in the mouse model	[118]
MELK	- MELK knock-down inhibits the growth of basal breast cancer cell line-derived xenografts but has little effect on tumors derived from luminal cancer cells - MELK inhibitor (OTSSP167) inhibits the growth of basal breast cancer cells xenografts, but no luminal cancer cells xenografts	[115]
MLK4	- MLK4 knock-down inhibits the growth of TNBC cell line-derived xenografts - MLK4 knock-down sensitizes TNBC cell line-derived xenograft tumors to chemotherapy	[116, 117]
NEK2	- NEK2 inhibitors (INH1 and CMP3a) sensitize TNBC cell line-derived xenografts to chemotherapy - CMP3a sensitizes PDXs with high NEK2 expression to chemotherapy	[119]
PIM1	- The pan–PIM kinase inhibitor (AZD1208) reduces tumor growth in xenograft models of TNBC - AZD1208 sensitizes TNBC cell line-derived xenografts to chemotherapy	[121]
RON	- RON promotes tumor growth and metastasis of xenografts and genetically engineered mouse models of TNBC - RON-targeting ADCs eradicate xenografts derived from TNBC cancer stem cells with CD44+/CD24- phenotype	[120]

10. Conclusions

Significant progress in our understanding of the molecular heterogeneity of TNBC has led to the development of new therapeutic strategies with demonstrated activity in clinical trials, including PARP inhibitors, immunotherapies, and ADCs. Given recent findings, kinase inhibitors may provide additional significant advances in TNBC therapy, particularly in targeting distinct molecular subtypes with the enrichment of specific, actionable alterations. The results of ongoing and future clinical trials of kinase-targeting agents will further guide the clinical development of precision therapies against TNBC.

Author contribution statement

DM, AM conceptualization; DM, AM literature analysis; DM, AM writing original draft & editing.

Declaration of competing interest

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

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MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance

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Chemoresistance constitutes a major challenge in the treatment of triple-negative breast cancer (TNBC). Mixed-Lineage Kinase 4 (MLK4) is frequently amplified or overexpressed in TNBC where it facilitates the aggressive growth and migratory potential of breast cancer cells. However, the functional role of MLK4 in resistance to chemotherapy has not been investigated so far. Here, we demonstrate that MLK4 promotes TNBC chemoresistance by regulating the pro-survival response to DNA-damaging therapies. We observed that MLK4 knock-down or inhibition sensitized TNBC cell lines to chemotherapeutic agents in vitro. Similarly, MLK4-deficient cells displayed enhanced sensitivity towards doxorubicin treatment in vivo. MLK4 silencing induced persistent DNA damage accumulation and apoptosis in TNBC cells upon treatment with chemotherapeutics. Using phosphoproteomic profiling and reporter assays, we demonstrated that loss of MLK4 reduced phosphorylation of key DNA damage response factors, including ATM and CHK2, and compromised DNA repair via non-homologous end-joining pathway. Moreover, our mRNA-seq analysis revealed that MLK4 is required for DNA damage-induced expression of several NF-κB-associated cytokines, which facilitate TNBC cells survival. Lastly, we found that high MLK4 expression is associated with worse overall survival of TNBC patients receiving anthracycline-based neoadjuvant chemotherapy. Collectively, these results identify a novel function of MLK4 in the regulation of DNA damage response signaling and indicate that inhibition of this kinase could be an effective strategy to overcome TNBC chemoresistance.

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INTRODUCTION

Triple-negative breast cancer (TNBC) is a subtype of breast cancer characterized by the absence of estrogen and progesterone receptors and the lack of HER2 amplification or overexpression. It accounts for 15–20% of all invasive breast cancers and is associated with an inferior prognosis compared with other breast cancer subtypes [1]. In the absence of known druggable molecular drivers, anthracyclines and taxanes-based chemotherapy is the mainstay of systemic treatment for TNBC [2]. Approximately 30% of women with TNBC who receive preoperative neoadjuvant chemotherapy (NAC) achieve a pathological complete response (pCR). Achieving pCR at the time of surgery has been correlated with a favorable prognosis in clinical trials [3, 4]. Nevertheless, both intrinsic and acquired resistance to chemotherapy leads to high rates of relapse and poor outcomes in most patients. Therefore, there is a need to identify novel molecular targets that could be exploited to overcome TNBC chemoresistance.

Accumulating evidence suggests that the development of resistance to genotoxic chemotherapy can be caused by aberrant regulation and overexpression of different components of the DNA repair pathways in cancer cells [5]. Ataxia telangiectasia mutated (ATM) kinase is one of the central kinases involved in the cellular response to DNA double strand breaks (DSBs) induced by chemotherapy. Following induction of DSBs, ATM is activated through auto- or trans- phosphorylation at Ser1981 and phosphorylates numerous targets to initiate DNA damage response (DDR), which may involve cell cycle arrest, repair of DNA lesions, and transcriptional reprogramming [6–8]. Many reports linked increased ATM and DDR signaling with the survival of cancer cells after chemotherapy, rendering this pathway an attractive target for overcoming cancer chemoresistance [9–14]. However, despite recent advancements in our understanding of ATM signaling, the complex mechanisms involved in its activation and functional role in resistance to chemotherapy are not yet fully resolved.

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Mixed-Lineage Kinase 4 (MLK4) is a member of the Mixed-Lineage Kinases (MLKs) family of serine/threonine kinases. Large-scale genomic and transcriptomic data indicated that the MLK4 gene (*MAP3K21/KIAA1804*) is frequently mutated and overexpressed in different types of human cancer [15]. Nonetheless, the distinct functions of MLK4 in cancer cell biology and tumor progression remain poorly understood. Intriguingly, the previous studies described both prooncogenic and tumor-suppressive functions of MLK4 [16]. It was demonstrated that MLK4 negatively regulates MAPK signaling pathways and impairs the invasive potential of ovarian cancer cells [17, 18]. Contrary to these findings, we and others showed that MLK4 directly phosphorylates MEK1 and MKK4/7 to activate ERK and JNK pathways in melanoma and colorectal cancer cells [19–21]. The study by Kim et al. indicated that MLK4 induces a mesenchymal phenotype and aggressive growth of glioblastoma cells in an NF-κB-dependent manner [22]. Similarly, we found that MLK4 activates the NF-κB pathway in TNBC, which leads to the high metastatic and invasive potential of breast cancer cells [23]. Here, we investigate the functional role of MLK4 in chemoresistance in TNBC. Our findings link MLK4 with DDR pathway activity upon treatment with DNA-damaging chemotherapy, and thus highlight MLK4 as an attractive target to combat chemoresistance.

MATERIALS AND METHODS

Cell lines and reagents

HCC1806, MDA-MB-436 cell lines were a kind gift from CRUK Manchester Institute, UK. SUM149PT cell line was purchased from BioIVT. HEK293T and MCF10A cell lines were purchased from ATCC. U2OS cell line was a kind gift from the National Cancer Institute, Frederick, MD, USA. HCC1806 cells were cultured in RPMI-1640 supplemented with 10% FCS, 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate. MDA-MB-436 cells were cultured in RPMI-1640 with 25 mM HEPES supplemented with 10% FCS, 1% penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 10 µg/ml insulin. SUM149PT cells were cultured in Ham's F12 medium with 25 mM HEPES supplemented with 10% FCS, 1% penicillin/streptomycin, 1 µg/ml hydrocortisone and 10 µg/ml insulin. HEK293T and U2OS cells were cultured in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate. MCF10A cells were grown in DMEM/F12 supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml insulin, 1% penicillin/streptomycin. Cell lines were authenticated by short tandem repeat profiling by ATCC Service at the beginning of the research and in December 2020. Cell lines were screened for mycoplasma regularly. Doxorubicin hydrochloride, etoposide, neocarzinostatin and KU-60019 were purchased from Sigma Aldrich. Recombinant human IL-6 was purchased from Peprotech. CEP-5214 was a kind gift from Dr. John Brognard, NCI, Frederick, MD, USA.

siRNA and plasmid transfection

JetPRIME (Polyplus Transfections) was used for plasmids and siRNA transfections according to the manufacturer's instructions. MLK4 silencing siRNA smart-pool (siRNA 1–4) and control siRNA (siGENOME Non-Targeting siRNA #5) were purchased from Dharmacon (Supplementary Table S1). MLK4-WT vector was obtained from GeneCopoeia, cloned and mutations (KA = kinase active and KD = kinase dead) were introduced as previously described [20]. pDRGFP and CBASceI vectors were gifts from Maria Jasin (Addgene plasmid # 26475 and # 26477) [24]. pimEJ5GFP was a gift from Jeremy Stark (Addgene plasmid # 44026) [25].

AnnexinV apoptosis assay

Cells were transfected with MLK4-targeting siRNA or control siRNA and after 24 h doxorubicin and etoposide were added at indicated concentrations. After 48 h of treatment, cells were harvested and stained using the AnnexinV-FITC apoptosis detection kit (R&D Biosystems and Invitrogen), according to the manufacturer's instructions. Flow cytometry analysis was performed with LSR II Fortessa (BD Biosciences).

Cell viability assays

HCC1806 and SUM149PT doxycycline-inducible cell lines were seeded into six-well plates and incubated with 1 µg/ml doxycycline to induce MLK4 knock-down. MCF10A cells were transfected with either MLK4-targeting or control siRNA. Subsequently, cells were incubated with doxorubicin and etoposide at indicated concentrations. After 48 h of treatment, cells were fixed with 4% PFA and stained with 0.5% crystal violet solution prepared in 25% methanol. Wells were thoroughly washed and air-dried. For quantification, 2 ml of 10% acetic acid was added to each well, plates were incubated for 20 min with shaking and absorbance values were read at 590 nm using the microplate reader Synergy II (BioTek).

Comet assay

Comet assays were performed using CometAssay Kit (Trevigen) following the manufacturer's instructions. Pictures were taken with a Nikon Eclipse Fluorescent microscope. OPENCOMET plugin for Fiji was used for image analysis.

Analysis of DSB repair by reporter assays

The efficacy of DNA double-strand break repair was measured using GFP-based reporter assays as described previously [24, 25]. Briefly, cells were transfected with either pDR-GFP or pimEJ5-GFP vector. The transfected cells were selected for puromycin resistance for at least two weeks. Selected cell lines were transfected with MLK4-targeting siRNA or control siRNA. The next day, cells were transfected with the SceI endonuclease expression vector (pCBA-SceI) to induce DSBs. 48 h after the induction of DSBs, cells were analyzed by flow cytometry (LSR II Fortessa (BD Biosciences). The percentage of GFP-positive cells was used as an indication of HR and NHEJ efficiency. pGFPMax vector (Lonza) was used as a positive control to monitor the transfection efficiency.

Mouse xenografts and in vivo studies

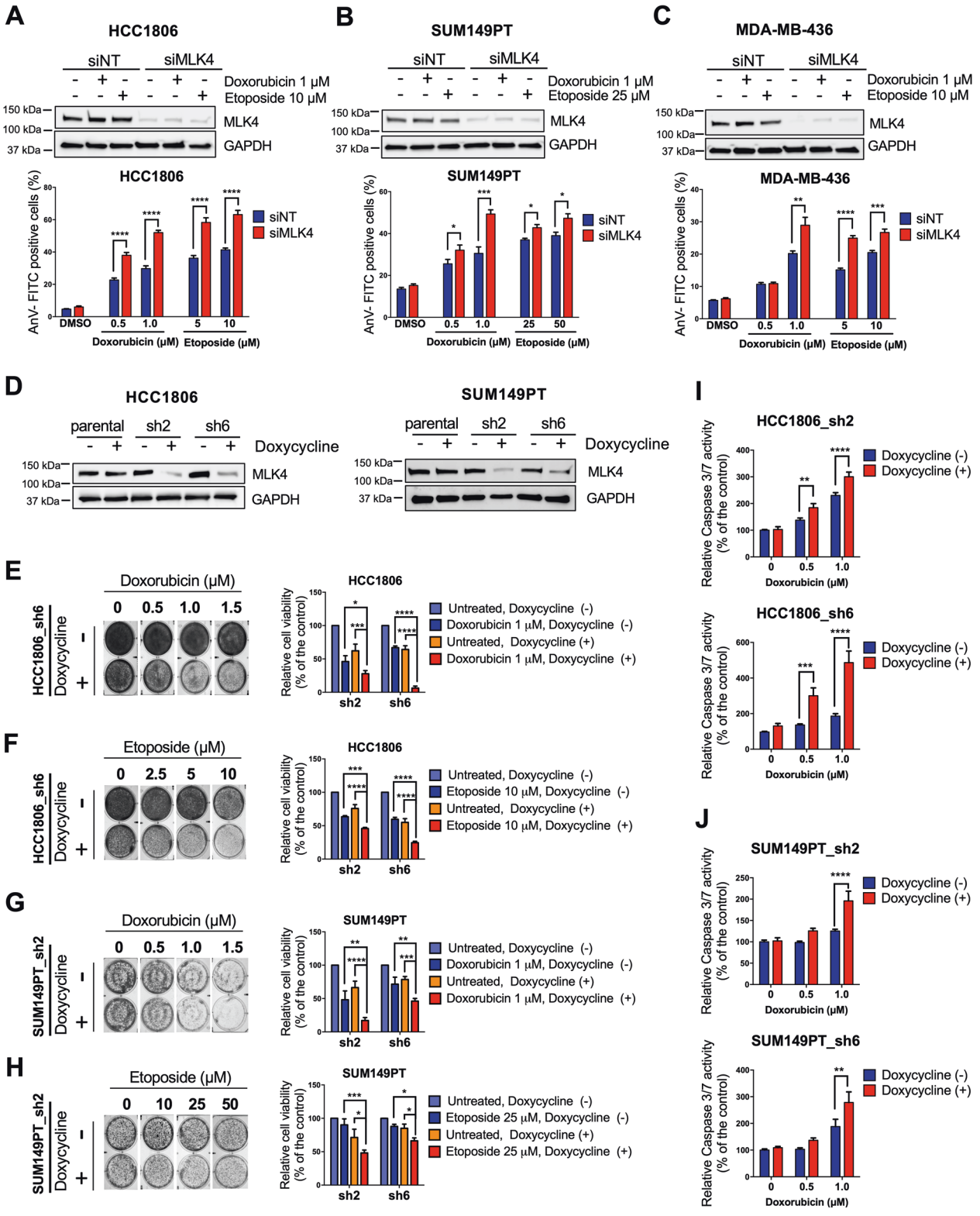
All procedures were approved by the Local Ethics Committee at the University of Warsaw (1035/2020) and carried out in accordance with the requirements of EU (Directive 2010/63/EU) and Polish (Dz. U. poz. 266/15.01.2015) legislation. 8 to 14-week-old RAG2$^{-/-}$ female mice were injected into mammary fat pads with 3×10^6 HCC1806_sh6 cells with Matrigel in proportion 1:1. Mice were allocated randomly into cages and doxycycline was administered in drinking water one day after injections. Doxorubicin or equal volumes of 0.9% NaCl were administered intraperitoneally when the average tumor size reached 100 mm^3. The next doses were administered at 5-day intervals. The tumor growth was monitored two times/week. Tumor volume was calculated based on caliper measurements, using the formula: tumor volume $= (D \times d^2 \times \pi)/6$, where D is the bigger measurement, and d is the smaller measurement. Mice were culled after 4 weeks post injection. Tumors were resected, weighed, and processed for further analyses.

Library preparation and next-generation sequencing

HCC1806 cells were transfected in triplicates with non-targeting control siRNA or MLK4-targeting siRNA. 24 h after transfection, cells were either left untreated or treated with 1 µM doxorubicin for additional 24 h. Next, RNA was isolated using the RNAeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Libraries were prepared using NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs) and KAPA RNA HyperPrep Kit (Kapa Biosciences). The quality of the obtained libraries was tested using Bioanalyzer-2100 and High Sensitivity DNA kit (Agilent). Nucleic acid quantity in the libraries was measured by qPCR using the Kapa Library Quantification kit (Kapa Biosciences). Pair-end sequencing was performed with the NovaSeq 6000 S1 Reagent Kit (200 cycles, Illumina) using NovaSeq 6000 instrument (Illumina). mRNA-seq data processing is described in the Supplementary Information file. All mRNA-seq data have been deposited at GEO DataSets (GSE174692).

Statistical analysis

All experiments were performed at least three times, unless otherwise indicated. For flow cytometry data analysis Flow Jo v10.6.1 software (TreeStar) or BD FACSDiva software (BD Biosciences) were used. Statistical analyses were carried out with GraphPad Prism 7 software. Data are expressed as the mean ± the standard error of the mean (SEM). P-value <0.05 was considered significant: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. For the experiments where the comparison was performed between more than two groups, the statistical significance was assessed by one-way ANOVA followed by Tukey post-hoc tests or two-way ANOVA. For the experiments where two groups were compared, the statistical significance was determined by



unpaired Student's *t*-test. Analysis of the drugs' interaction was performed using Combenefit software, as described previously [26].

The lists of antibodies and primers used in this study are included in Supplementary Table S2 and Supplementary Table S3, respectively. For additional methods, please see the Supplementary Information File.

RESULTS

MLK4 loss or inhibition sensitizes TNBC cells to chemotherapy in vitro

To study the role of MLK4 in response to chemotherapeutic drugs treatment, we used TNBC cell lines with high endogenous

Fig. 1 MLK4 knock-down increases sensitivity of triple-negative breast cancer cells to doxorubicin and etoposide. A–C HCC1806, SUM149PT and MDA-MB-436 cells were transfected using siRNA against MLK4 (siMLK4) or non-targeting siRNA control (siNT). After 24 h, cells were treated with doxorubicin and etoposide at indicated concentrations for an additional 48 h. Next, cells were stained with AnnexinV-FITC, and analyzed by flow cytometry. Error bars indicate ±SEM from two or three independent experiments, performed in triplicates. Significance was calculated using an unpaired two-tailed *t*-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. **D** Cell lines with doxycycline-inducible MLK4 knock-down were generated from HCC1806 and SUM149PT parental cells using lentiviral vectors. Silencing of MLK4 was confirmed by immunoblotting. **E–H** HCC1806_sh2, HCC1806_sh6, SUM149PT_sh2 and SUM149PT_sh6 cell lines were incubated with doxycycline to induce MLK4 knock-down and subsequently cells were treated with doxorubicin and etoposide at indicated concentrations for 48 h. Following treatment, cells viability was assessed by crystal violet staining and quantified by absorbance measurements. Representative plates after crystal violet staining are shown on the left. Error bars indicate ±SEM from three independent experiments ($n = 3$). Significance was calculated using one-way ANOVA followed by Tukey multiple comparisons test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. **I–J** MLK4-silenced and control cells were treated with doxorubicin at increasing concentrations and the activity of caspases 3/7 was measured using bioluminescence assay. Error bars indicate ±SEM from three independent experiments, performed in triplicates ($n = 9$). Significance was calculated using an unpaired two-tailed *t*-test, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

expression of this kinase––HCC1806, SUM149PT, and MDA-MB-436 [23]. Cells were transfected with MLK4-targeting smart-pool or control siRNA and either left untreated or treated with increasing concentrations of topoisomerase II poisoning chemotherapeutics––doxorubicin and etoposide. Downregulation of MLK4 markedly increased apoptosis of TNBC cells upon treatment with both drugs, as revealed by the measurements of AnnexinV-positive cells using flow cytometry (Fig. 1A–C, Supplementary Fig. S1A–C). MLK4 knock-down did not cause apoptosis in untreated cells, which agrees with our previous observations, confirming that MLK4 loss alone is not sufficient to induce TNBC cells death [23]. To exclude the possible off-target effects of MLK4-targeting siRNA smart-pool, we validated MLK4 knock-down using each of the four individual siRNAs, which all decreased the viability of HCC1806 cells in the presence of doxorubicin (Supplementary Fig. S2A, B). To further study the involvement of MLK4 in TNBC chemoresistance, we generated HCC1806 and SUM149PT cell lines with doxycycline-inducible MLK4 knock-down system, using two different lentiviral shRNA vectors––sh2 and sh6 (Fig. 1D). Following MLK4 downregulation, both cell lines exhibited decreased viability in the presence of doxorubicin and etoposide (Fig. 1E–H). The reduction in cell viability resulted from chemotherapeutics-induced apoptosis, as indicated by the higher activity of caspases 3 and 7 in MLK4-depleted cells upon doxorubicin treatment (Fig. 1I, J). To exclude the possibility that the observed effects are related to the use of doxycycline itself, we treated parental cell lines, HCC1806 and SUM149PT, with doxycycline and chemotherapeutics. Incubation with doxycycline did not decrease the viability of HCC1806 and SUM149PT parental cell lines upon treatment with doxorubicin or etoposide (Supplementary Fig. S3A-D) and did not increase apoptosis induction in response to chemotherapy (Supplementary Fig. S3E, F), suggesting that the observed effects were due to MLK4 silencing. Conversely to MLK4 knock-down, the induced overexpression of this kinase led to an increased viability of HCC1806 cells following doxorubicin treatment (Supplementary Fig. S4A, B), indicating that high MLK4 expression protects TNBC cells from chemotherapy-induced cell death.

Following our observation that knock-down of MLK4 increased sensitivity of TNBC cell lines to chemotherapy, we aimed to investigate if pharmacological inhibition of this kinase could also improve the efficacy of doxorubicin against TNBC cells. Since no small molecule inhibitors specifically inhibiting MLK4 kinase have been described so far, we studied other commercially available compounds that could be used to target MLK4-mediated signaling. CEP-5214, a pan-VEGF-R and MLK1-3 inhibitor [27], showed potent inhibitory activity against MLK4, as demonstrated by an in vitro kinase assay and further confirmed by the inhibition of ERK and JNK phosphorylation in HEK293T cells transiently transfected with MLK4 (Fig. 2A, B). Notably, treatment with CEP-5214 potentiated doxorubicin cytotoxicity against HCC1806 and SUM149PT cell lines, and this effect was synergistic (Fig. 2C–F). The combination treatment significantly enhanced apoptosis induction in both cell lines tested, as compared to the treatment with doxorubicin and CEP-5214 alone (Fig. 2G, H). These data confirm that MLK4 is a druggable kinase and provide evidence that depletion or inhibition of MLK4 sensitizes TNBC cells to chemotherapy. We next tested whether the combination of MLK4 silencing and chemotherapy affects non-malignant cells. Knock-down of MLK4 in breast epithelial cell line MCF10A, which is characterized by relatively low endogenous expression of this kinase, did not result in a decreased cell viability in the presence of doxorubicin and etoposide (Supplementary Fig. S5A, B). Furthermore, MLK4 silencing did not increase apoptosis induction and caspases 3/7 activation in MCF10A cells upon treatment with chemotherapy (Supplementary Fig. S5C, D). Finally, treatment with CEP-5214 did not enhance doxorubicin cytotoxicity against MCF10A cells (Supplementary Fig. S5E, F). These results indicated that MLK4 loss or inhibition did not potentiate the toxic effects of chemotherapy against normal cells.

MLK4 knock-down increases chemosensitivity of TNBC cells in 3D cell culture and in vivo models

3D-cultured breast cancer cells forming mammospheres reflect tumor growth in vivo more closely than traditional two-dimensional culture, and therefore, may provide more accurate models to study cancer biology and therapy [28]. Thus, we tested if MLK4 loss could sensitize TNBC cells grown as mammospheres to doxorubicin treatment. We observed increased activation of caspases 3 and 7 in mammospheres formed by MLK4-depleted HCC1806_sh6 and SUM149PT_sh2 cells treated with doxorubicin (Fig. 3A, B). Next, we used a xenograft-based approach to test the effects of MLK4 loss on doxorubicin sensitivity of TNBC tumors in vivo. Consistent with our previous results, the growth of HCC1806_sh6 xenograft tumors was abrogated by MLK4 silencing [23]. The combination of MLK4 knock-down with doxorubicin treatment showed a further significant reduction in tumor growth (Fig. 3C, D). Taken together, our data suggest that MLK4 loss introduces a therapeutic vulnerability by sensitizing tumors towards cytotoxic chemotherapy in vivo.

MLK4 depletion leads to an increased accumulation of doxorubicin-induced DNA damage in TNBC cells

The major mechanism of action of topoisomerase II poisons involves the induction of DNA double-strand breaks (DSB) that, if not repaired, result in cell death [29]. Formation of the DNA DSBs induces phosphorylation of H2AX at Ser139 (γH2AX), which is a sensitive biomarker for DNA damage [30]. We found that MLK4-deficient HCC1806_sh6 and SUM149PT_sh2 cell lines showed increased H2AX phosphorylation after 24–48 h of treatment with doxorubicin (Fig. 4A, B), as well as enhanced formation of doxorubicin-induced nuclear γH2AX foci (Fig. 4C, D). MLK4 knock-down also resulted in significantly higher levels of doxorubicin-induced DNA DSBs as determined by comet assay

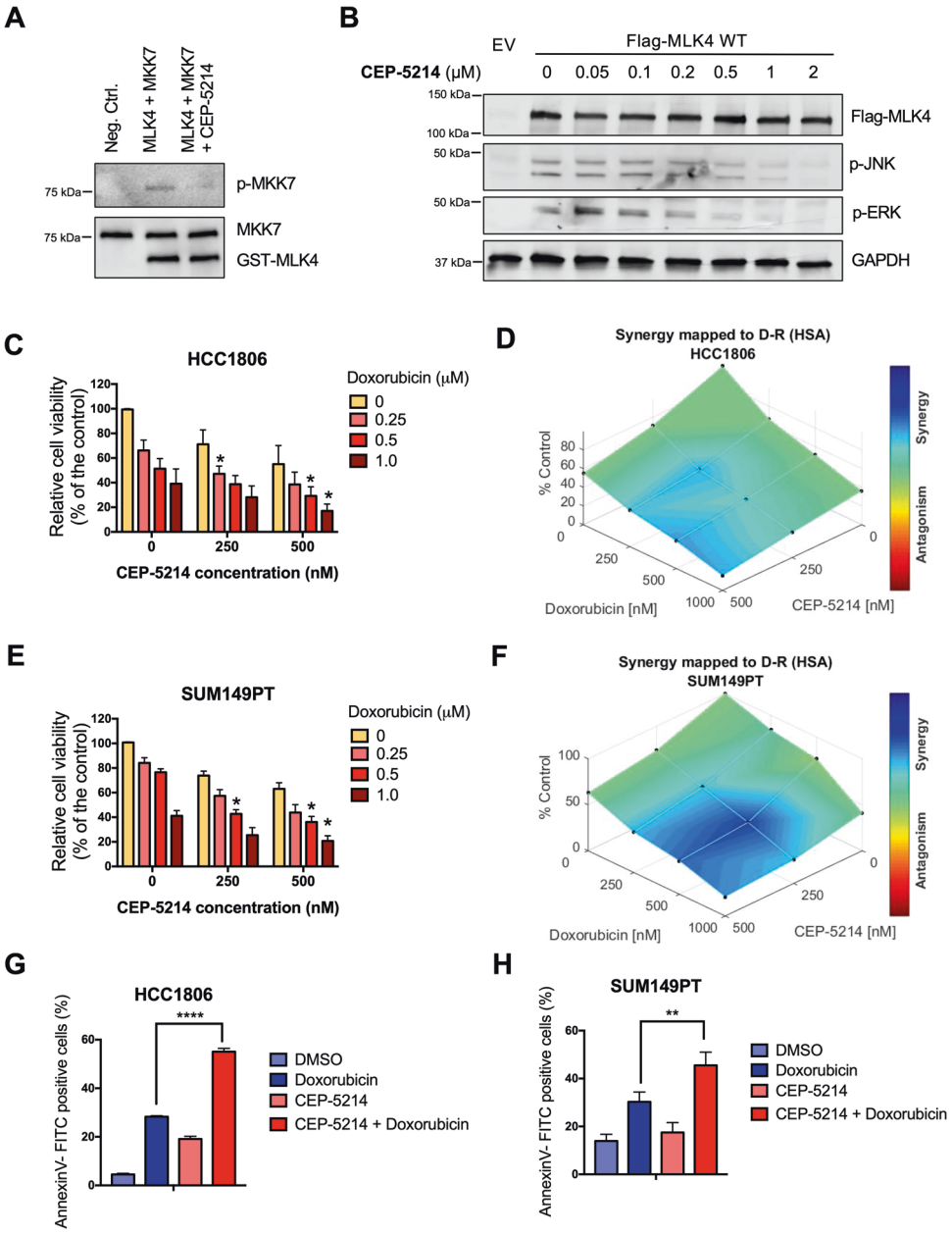


Fig. 2 MLK4 inhibition sensitizes TNBC cells to chemotherapy. A Kinase-inactive MKK7 and purified GST-MLK4 kinase domain (isolated from baculovirus-infected insect cells) were subjected to in vitro kinase assay in the presence or absence of CEP-5214 inhibitor. **B** HEK293T cells were transiently transfected with MLK4-WT vector or with the empty vector and incubated with increasing concentrations of CEP-5214 for 1 h. Next, whole cell lysates were collected and analyzed by immunoblotting. **C**–**F** HCC1806 (C-D) and SUM149PT (E-F) cells were incubated with CEP-5214 or DMSO for 72 h and doxorubicin for 48 h. After treatment, cells viability was assessed by crystal violet staining and quantified by absorbance measurements. Error bars indicate ±SEM from three independent experiments. Analysis of combination efficacy and synergy was performed using HSA model with Combenefit software, $*p < 0.05$. **G**–**H** HCC1806 and SUM149PT cells were incubated with CEP-5214 at concentrations 500 nM and 250 nM, respectively or DMSO for 72 h and doxorubicin for 48 h. Next, cells were stained with AnnexinV-FITC, and measured by flow cytometry. Error bars indicate ±SEM from two independent experiments, performed in triplicates. Significance was calculated using one-way ANOVA followed by Tukey multiple comparisons, $**p < 0.01$, $****p < 0.0001$.

(Fig. 4E). Finally, the analysis of HCC1806_sh6 xenograft tumors by immunofluorescent staining revealed that MLK4 knock-down was associated with persistent H2AX phosphorylation in tumor tissues after the treatment with doxorubicin in vivo (Fig. 4F, G). Collectively, these results indicate that MLK4 loss leads to excessive accumulation of DNA damage induced by chemotherapeutics and contributes to cancer cells death and chemosensitivity.

MLK4 regulates ATM activation and downstream DNA damage response signaling in TNBC cells

Next, to determine cellular mechanisms responsible for the identified MLK4-dependent chemoresistance in TNBC, we performed a quantitative phosphoproteomic analysis of control and MLK4-depleted cells, either untreated or treated with doxorubicin (Fig. 5A). We found that the doxorubicin-induced phosphorylation of several core DNA damage response components, including ATM,

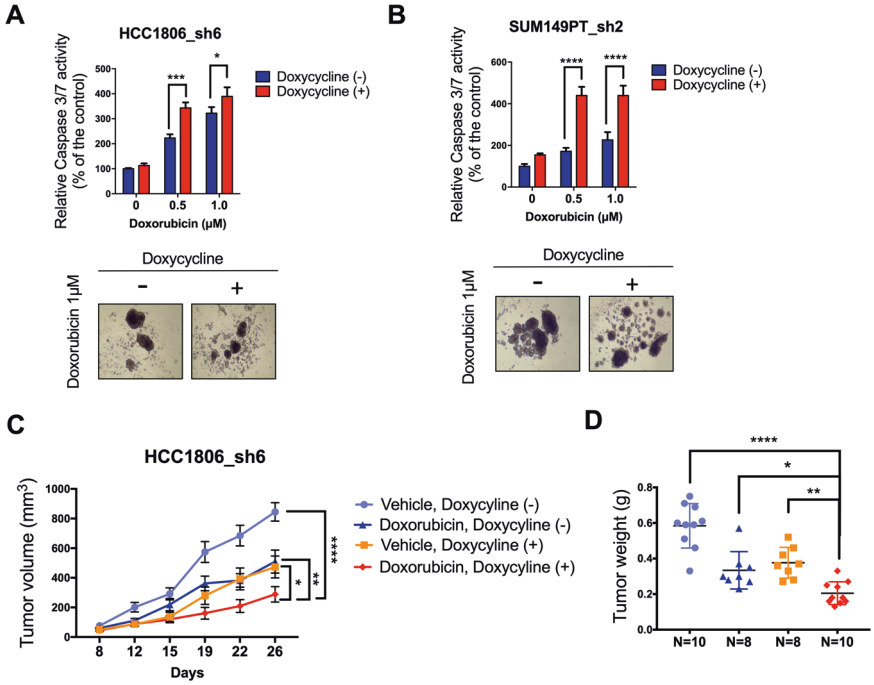


Fig. 3 MLK4 promotes chemoresistance in 3D cell culture and in vivo models. A–B MLK4 knock-down was induced by doxycycline in HCC1806_sh6 and SUM149PT_sh2 cells grown in non-adherent conditions and subsequently cells were treated with doxorubicin at indicated concentrations for 48 h. The activity of caspases 3/7 was measured using bioluminescence assay. Representative pictures of mammospheres upon doxorubicin treatment are shown. Error bars indicate ±SEM from three independent experiments, performed in triplicates ($n = 9$). Significance was calculated using an unpaired two-tailed *t*-test, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. **C** HCC1806_sh6 cells were injected into mammary fat pads of RAG2$^{-/-}$ mice. Doxycycline was administered one day after the injection to induce MLK4 knock-down. Mice were treated with doxorubicin (4 mg/kg, i.p.) or saline at 5-day intervals, starting from day 7 of the experiment. Tumors were measured twice a week. Error bars indicate ±SEM ($n = 10$ for control group, $n = 8$ for doxorubicin-treated group, $n = 8$ for doxycycline-treated group, $n = 10$ for combination treatment group). Statistical comparison was performed using two-way ANOVA, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. **D** Weight of tumors resected at the end of the study. Significance was calculated using one-way ANOVA followed by Tukey multiple comparisons test, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

TRIM28 (KAP-1), MDC1 and TP53BP1, was impaired in cells lacking MLK4 (Fig. 5B, Supplementary Table S4). Western blot analysis of HCC1806_sh6 and SUM149PT_sh2 cells exposed to doxorubicin at different time points confirmed that ATM phosphorylation at Ser1981 was diminished upon MLK4 knock-down, particularly after 6–8 h of treatment with doxorubicin (Fig. 5C–F). Additionally, we observed a decreased phosphorylation of CHK2, an ATM downstream effector kinase, suggesting that MLK4 knock-down compromised ATM signaling in TNBC cells (Fig. 5C, E). These observations were validated by the analysis of protein lysates from HCC1806_sh6 and SUM149PT_sh2 cells treated with DNA DSBs-inducing radiomimetic agent––neocarzinostatin (NCS), which also revealed a decreased phosphorylation of ATM after 6 h of treatment in MLK4-deficient cells (Supplementary Fig. S6A, B). To confirm the involvement of MLK4 in ATM activation, we generated HCC1806 cells with a permanent CRISPR/Cas9-mediated deletion of the MLK4 gene. Consistent with shRNA-mediated knock-down, HCC1806 cells with MLK4 knock-out showed decreased ATM activation after the treatment with doxorubicin (Fig. 5G). At the same time, ATM phosphorylation at Ser1981 was completely rescued by the overexpression of MLK4 wild-type protein in these cells (Fig. 5G). Furthermore, the observed effect is likely to be kinase-dependent, as overexpression of MLK4 kinase-active mutant, but not MLK4 kinase-dead mutant, increased phosphorylation of ATM in MLK4 knock-out cells (Fig. 5H).

DNA DSBs are repaired by two major pathways, namely by non-homologous end joining (NHEJ) and homologous recombination (HR). ATM signaling has been shown to coordinate these DNA repair pathways and modulate their activity at multiple stages in a context-dependent manner [31, 32]. Thus, we sought to determine whether MLK4 knock-down impairs the repair of DSBs by one or both DNA repair pathways. We generated HEK293T and HCC1806 cell lines harboring plasmid-based GFP reporter specific for HR- or NHEJ-mediated DSBs repair (pDR-GFP and pimEJ5-GFP, respectively) [24, 25]. Using these cell lines, we were able to evaluate the efficiency of HR and NHEJ based on the number of GFP positive cells measured by flow cytometry. We found that MLK4 knock-down decreased the efficiency of NHEJ-mediated DSBs repair but only slightly reduced HR-mediated DNA repair efficiency in both cell lines (Fig. 5I, J). Although HCC1806 cells do not harbor deleterious BRCA1/2 mutations and our reporter assays suggested that they are proficient in performing HR-mediated DSBs repair, previous studies reported their sensitivity to some PARP inhibitors [33, 34]. This raises the possibility that HR might be at least partially impaired in this TNBC cell line due to defects other than BRCA1/2 mutations. Therefore, we validated our reporter assays results using additional cancer cell line model – U2OS, that does not have deleterious mutations in p53 and BRCA1/2 genes and is characterized by the largely intact function of the two major DNA DSBs repair pathways [35]. Using U2OS-pDR and U2OS-pimEJ5 cells, we further showed that MLK4 knock-down led to a decreased NHEJ-mediated DSBs repair without significantly affecting HR repair efficiency (Supplementary Fig. S7A, B). Taken together, our findings indicate that MLK4 is required for ATM activation and efficient DSBs repair, primarily via NHEJ pathway.

MLK4 is required for DNA damage-induced transcriptional activation of NF-κB target genes

To gain additional insights into the molecular mechanisms involved in MLK4-mediated chemoresistance, we performed gene

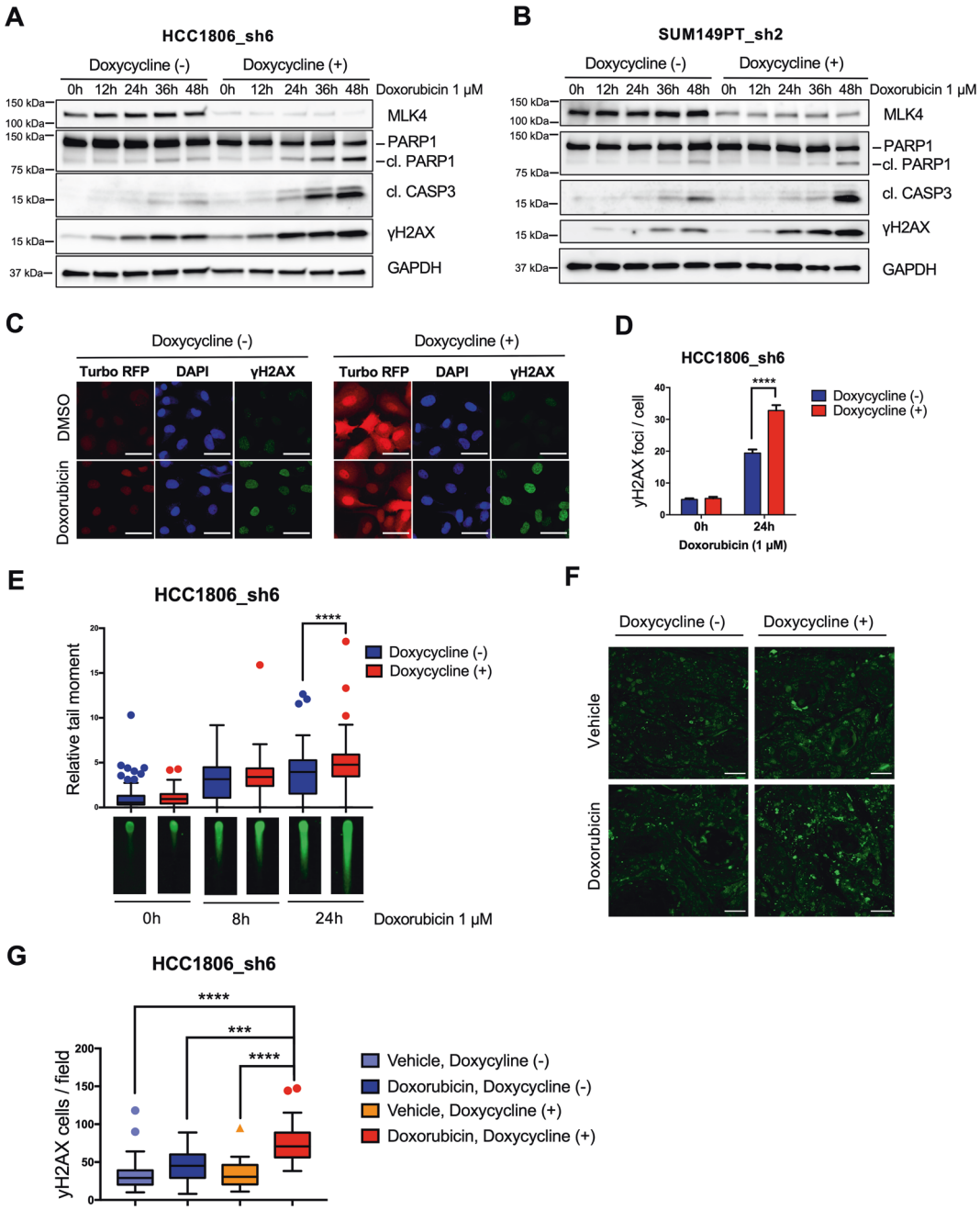
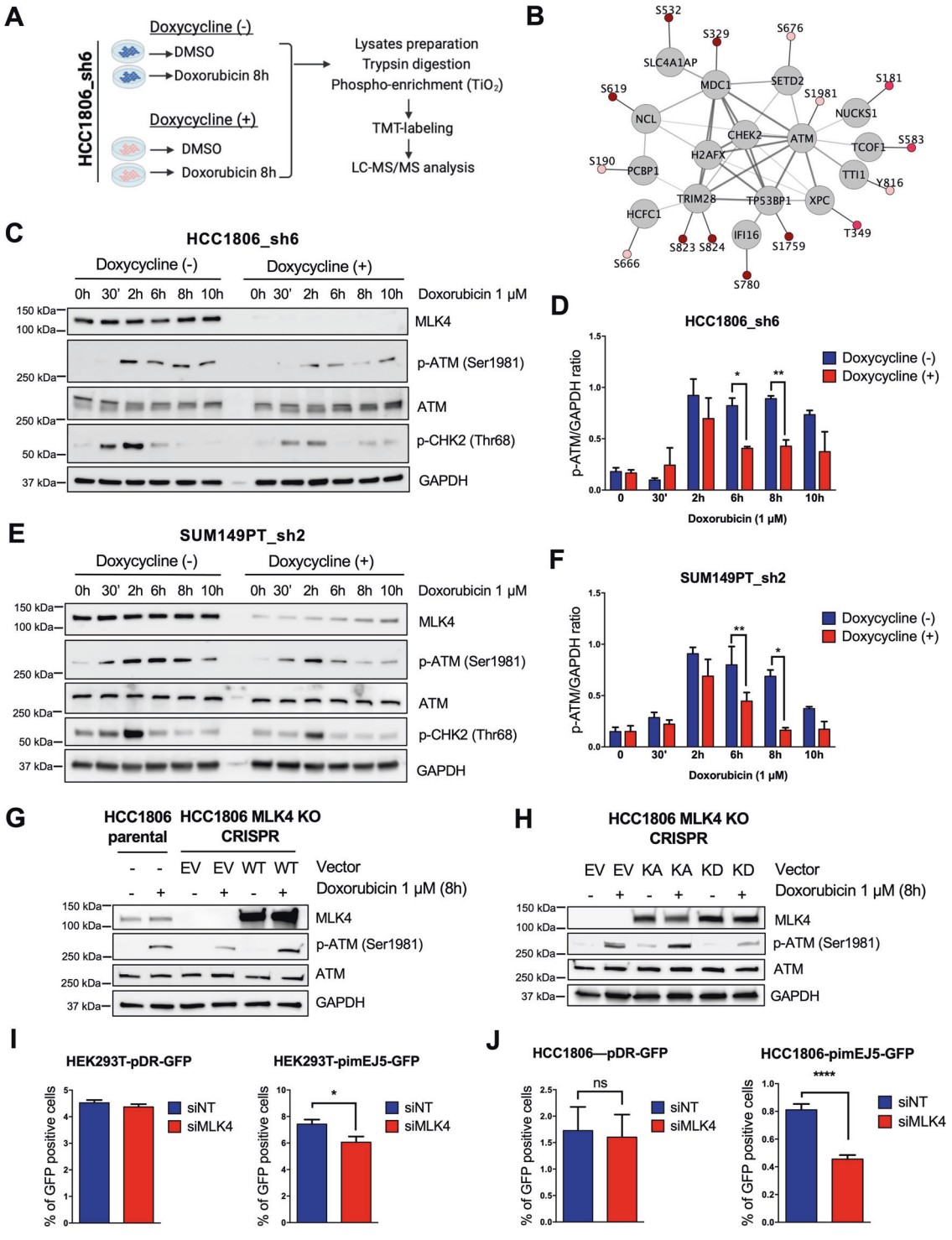


Fig. 4 MLK4 loss results in an accumulation of doxorubicin- induced DNA damage in TNBC cells. A–B MLK4 knock-down was induced by doxycycline in HCC1806_sh6 and SUM149PT_sh2 and subsequently cells were treated with doxorubicin for the indicated time. Following the treatment, whole cell lysates were collected and analyzed by immunoblotting. The persistent phosphorylation of γH2AX in MLK4-depleted cells was associated with apoptotic cell death, as suggested by caspase 3 activation and PARP1 cleavage. **C** HCC1806_sh6 cells were incubated with doxycycline to induce MLK4 knock-down, treated with doxorubicin for 24 h and stained for γH2AX [green] and DAPI [blue]. The expression of turbo-RFP [red] is induced along with the expression of MLK4-targeting shRNA. Scale bars 50 μm. **D** Nuclear foci of γH2AX were quantified in at least 50 cells in each group per experiment using FIJI. Error bars indicate ±SEM from three independent experiments. Significance was calculated using an unpaired two-tailed *t*-test, $^{****}p < 0.0001$. **E** HCC1806_sh6 cells were incubated with doxycycline to induce MLK4 knock-down and treated with doxorubicin for 8 h or 24 h. After the treatment, the extent of DNA damage was analyzed by neutral comet assay. Relative comet tail moment was quantified in at least 50 cells in each group per experiment using ImageJ Open Comet plugin. Error bars indicate ±SEM from three independent experiments. Significance was calculated using an unpaired two-tailed *t*-test, $^{****}p < 0.0001$. **F** HCC1806_sh6 xenograft tumors were harvested and stained for γH2AX [green]. Scale bars 50 μm. **G** γH2AX-positive cells in tumor sections were quantified using FIJI. Significance was calculated using one-way ANOVA followed by Tukey multiple comparisons test, $^{***}p < 0.001$, $^{****}p < 0.0001$.

expression profiling by mRNA-seq in HCC1806 cells transfected with control or MLK4-targeting siRNAs and treated with either doxorubicin for 24 h or vehicle only. Principal component analysis (PCA) confirmed a high degree of reproducibility of our mRNA-seq data and showed that both MLK4 knock-down and doxorubicin treatment led to significant transcriptome-wide alterations in HCC1806 cells (Supplementary Fig. S8A). Differential gene expression analysis revealed that approximately 25% of transcripts



regulated by doxorubicin were unique to either MLK4-depleted or control cells, which suggested that MLK4 knock-down affects global transcriptomic changes in response to doxorubicin treatment (Supplementary Fig. S8B).

When comparing the overlap of transcripts that were upregulated by doxorubicin treatment in control cells (upregulated: siNT + doxorubicin versus siNT) with transcripts whose induction was reduced by MLK4 loss (downregulated: siMLK4 + doxorubicin versus siNT + doxorubicin), we identified a subset of 101 differentially regulated genes (Fig. 6A, B). Among these genes, we found numerous NF-κB targets, including several cytokines and paracrine factors. Functional enrichment analysis of genes downregulated following MLK4 depletion in doxorubicin-treated cells identified cytokine activity and receptor signaling as key downstream targets of MLK4 (Fig. 6C). Furthermore, Gene Set Enrichment Analysis (GSEA) showed that NF-κB signature was significantly downregulated in MLK4-depleted cells upon doxorubicin treatment (Fig. 6D). ATM activation and subsequent phosphorylation of its

Fig. 5 MLK4 knock-down impairs ATM activation and DNA damage repair in TNBC cells. A Workflow summary for multiplexed, quantitative phosphoproteomic analysis of HCC1806_sh6 cells. MLK4 knock-down was induced by the addition of doxycycline to the cell culture medium. Next, cells were treated with doxorubicin or DMSO for 8 h. Following the treatment, whole cell lysates were collected, samples were digested and alkylated prior to phosphopeptide enrichment on TiO2 beads, addition of 10-plex tandem mass tag (TMT) labels and analysis of phosphopeptides using LC-MS/MS. **B** Network of selected proteins related to DNA damage with phosphosites increasing upon doxorubicin in control ($q < 0.15$, positive log2 [fold-change]) but not in MLK4-depleted HCC1806_sh6 cells. Color fills represent the significance of the change in control cells upon doxorubicin treatment (dark red: $q < 0.05$, red: $q = 0.05–0.1$, light red: $q = 0.1–0.15$). **C–F** MLK4 knock-down was induced by doxycycline in HCC1806_sh6 and SUM149PT_sh2 cells, which were next treated with doxorubicin for the indicated time. Following the treatment, whole cell lysates were collected and analyzed by immunoblotting. Densitometry and quantitative analysis of immunoblotting results was performed from at least 3 independent experiments. GAPDH was used to normalize the band's intensity. Error bars indicate ±SEM. Significance was calculated using an unpaired two-tailed *t*-test, $*p < 0.05$, $**p < 0.01$. **G** HCC1806 CRISPR-MLK4 knock-out cells were transfected with MLK4-WT vector to rescue MLK4 expression or with the empty vector. Following the transfection, HCC1806 CRISPR-MLK4 knock-out cells and HCC1806 parental cells were treated with doxorubicin for 8 h. After the treatment, whole cell lysates were collected and analyzed by immunoblotting. **H** HCC1806 CRISPR-MLK4 knock-out cells were transfected with MLK4 KA (kinase active), MLK4 KD (kinase dead) and control empty vector (EV). Following the transfection, cells were treated with doxorubicin for 8 h. Next, whole cell lysates were collected and analyzed by immunoblotting. **I–J** HEK293T-pDR-GFP, HEK293T-piMEJ5-GFP, HCC1806-pDR-GFP and HCC1806-pimEJ5-GFP cell lines were generated from parental cells lines by transfection of the appropriate vectors and subsequent selection with puromycin for over 14 days. The cells stably expressing reporter vectors were transfected with MLK4-targeting siRNA (siMLK4) or control non-targeting siRNA (siNT) along with pSCE-CbaI endonuclease expressing vector. After 72 h, the activity of homologous recombination (pDR-GFP vector expressing cells) and non-homologous end joining (pimEJ5-GFP vector expressing cells) DNA repair pathways was assessed by measuring the percentage of GFP-positive cells using flow cytometry. Error bars indicate ±SEM from three independent experiments performed in triplicates ($n = 9$). Significance was calculated using an unpaired two-tailed *t*-test, $*p < 0.05$, $****p < 0.0001$.

downstream substrates have been shown to play a pivotal role in adaptive transcriptional response following DNA damage [7, 36–38]. Thus, we curated a signature of ATM-dependent transcriptional alterations based on the previously published expression data from ATM-depleted TNBC cells [37]. Interestingly, we observed that genes previously found to be downregulated by ATM depletion were also negatively enriched in MLK4-deficient cells upon doxorubicin treatment in our experiments (Fig. 6D). To validate mRNA-seq results, we analyzed the expression of selected genes in MLK4-silenced and control HCC1806 cells by qRT-PCR. In agreement with our mRNA-seq data, the expression of several NF-κB-regulated cytokines (*IL-6, IL-8, CXCL1, CXCL6, IL-12A, TNFSF15*) was induced upon the treatment with doxorubicin in control cells, while the induction of these genes was compromised by MLK4 depletion (Fig. 6E). To establish whether ATM activation was necessary for the upregulation of the indicated NF-κB target genes by doxorubicin, we examined the expression of selected cytokines in HCC1806 cells treated with ATM inhibitor (KU-60019), doxorubicin or a combination of both. ATM inhibition led to a similar dampening of doxorubicin-induced transcriptional changes as MLK4 silencing (Fig. 6F), suggesting that the impaired induction of NF-κB target genes in MLK4-deficient cells is associated with a compromised ATM activation. Moreover, treatment with KU-60019 significantly enhanced doxorubicin cytotoxicity against HCC1806 and SUM149PT cells (Supplementary Fig. S9A, B).

ATM regulates NF-κB transcription in response to DNA damage through the phosphorylation of NEMO at Ser85, which aids in transmitting nuclear ATM signaling to activate NF-κB in the cytoplasm [39]. Upon activation, NF-κB-p65 translocates to the nucleus, where it regulates gene expression and promotes cell survival [40]. In agreement with our gene expression analyses, we observed reduced NEMO phosphorylation and decreased NF-κB-p65 nuclear translocation in MLK4-depleted cells upon treatment with doxorubicin (Supplementary Fig. S9C, D). Collectively, our results indicate that MLK4 is required for ATM activation that leads to downstream phosphorylation of NEMO and subsequent induction of NF-κB nuclear translocation in response to DNA damage.

MLK4 loss impairs secretion of the pro-survival IL-6 in response to DNA-damaging chemotherapy

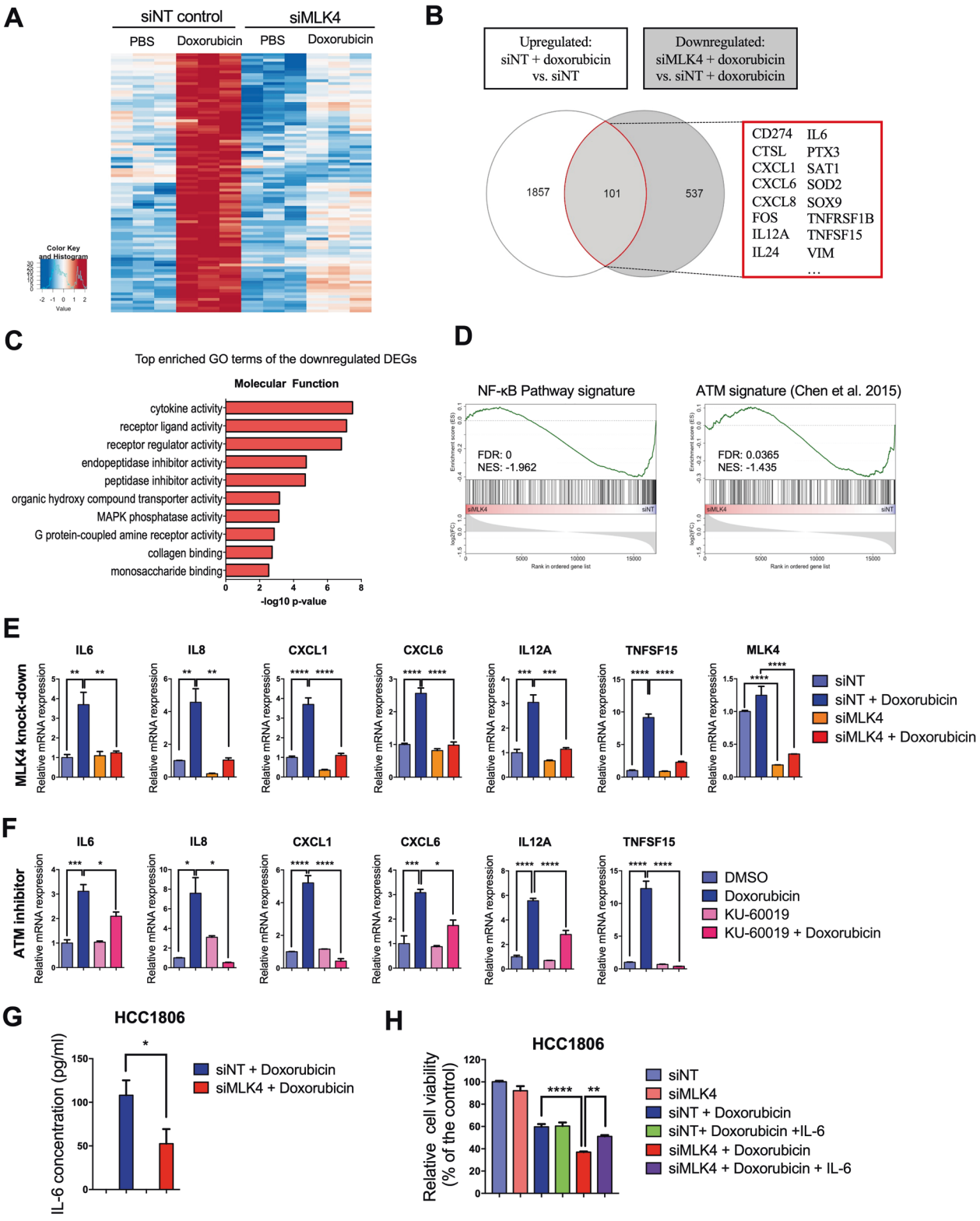
Our mRNA-seq data revealed that MLK4 knock-down diminished the doxorubicin-induced upregulation of several cytokines, which have been previously described to promote drug resistance in an autocrine/paracrine manner, including IL-6, IL-8 and CXCL1 [41–43]. Since IL-6-mediated autocrine signaling has been recently linked to multidrug resistance in TNBC, we verified if reduced IL-6 gene expression upon MLK4 knock-down contributes to increased chemosensitivity of HCC1806 cells. We confirmed a decreased secretion of IL-6 cytokine in MLK4-deficient cells upon treatment with doxorubicin using ELISA (Fig. 6G). Furthermore, we found that the addition of exogenous IL-6 to the cell culture medium increased viability of MLK4-depleted cells upon doxorubicin treatment, thus partially rescuing the effects of MLK4 knock-down (Fig. 6H). Together, these findings suggest that MLK4 function is required for the expression of NF-κB target genes to promote the survival of TNBC cells following chemotherapy.

High expression of MLK4 is associated with poor prognosis in patients receiving anthracycline-based neoadjuvant chemotherapy

Finally, we aimed to explore the prognostic impact of high MLK4 gene expression in TNBC patients treated with neoadjuvant chemotherapy. We analyzed the gene expression and survival data from the TOP trial, in which patients with ER-negative tumors were treated with anthracycline monotherapy [44]. Our analysis indicated that high MLK4 mRNA expression in pre-treatment biopsies was associated with worse overall survival (Fig. 7A). These results suggest that high MLK4 expression in tumor tissue could be related to an unfavorable response to NAC and poor prognosis in TNBC patients.

DISCUSSION

The treatment of TNBC is limited by a lack of actionable targets and aggressive phenotype that is often refractory to cytotoxic chemotherapy. We recently found that MLK4 gene is amplified and highly expressed in the TNBC clinical samples and cell line models [23]. We also demonstrated that this kinase promotes proliferation, migration, and invasive potential of breast cancer cells [23]. However, the functional role of MLK4 in cancer chemoresistance has not been previously investigated. Here, we report our findings supporting the involvement of MLK4 in resistance of TNBC to clinically used chemotherapeutic agents. We demonstrated that MLK4 loss enhanced apoptosis induction and reduced viability of TNBC cell lines upon treatment with doxorubicin and etoposide in vitro, and these results were further recapitulated in breast cancer xenograft model. Notably, MLK4 knock-down alone did not cause cell death or DNA damage accumulation, which agrees with our previous data [23]. These



observations provide a rationale for using MLK4 inhibitors in combination with DNA-damaging chemotherapies in the treatment of TNBC.

ATM is the primary kinase involved in the cellular response to DNA DSBs, and its loss or inhibition sensitizes cancer cells to chemotherapy [10, 14]. Recently, Colomer et al. described that ATM is directly activated by the nuclear form of IKKα kinase downstream of BRAF and TAK1, and inhibition of both IKKα and BRAF impaired DNA repair mechanisms, sensitizing metastatic tumors to DNA-damaging agents in vivo [11]. These results highlight the importance of upstream kinases in DDR regulation and point towards novel therapeutic strategies for overcoming cancer chemoresistance. We showed that MLK4 loss compromises ATM activation and inhibits phosphorylation of several DDR

Fig. 6 MLK4 loss interferes with the induction of NF-κB target genes and compromises adaptive transcriptional response of TNBC cells to chemotherapy. A Heatmap depicting the transcriptome-wide effects of MLK4 depletion and doxorubicin treatment. Shown are significantly [pvalue adj.<0.01] upregulated [log2 FC > 0.75, red] or downregulated [log2 FC < 0.75, blue] genes. **B** Venn diagram comparing the overlapping genes which are upregulated after doxorubicin treatment and significantly downregulated in MLK4-depleted cells compared to control cells after treatment with doxorubicin. The genes whose induction by doxorubicin is reduced by MLK4 loss are highlighted on the right. **C** GO enrichment analysis in MLK4-depleted vs. control cells treated with doxorubicin. **D** GSEA in MLK4-depleted vs. control cells treated with doxorubicin. The list of ATM-dependent genes was curated based on RNA-seq data from Chen et al. [37]. **E** HCC1806 cells were transfected with MLK4-targeting siRNA (siMLK4) or control non-targeting siRNA (siNT). Following the transfection, cells were treated with doxorubicin for 24 h. After the treatment, RNA was isolated and relative gene expression of several NF-κB target genes was analyzed by qRT-PCR. Error bars indicate ±SEM from three experiments ($n = 3$). Significance was calculated using one-way ANOVA followed by Tukey multiple comparisons test, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. **F** HCC1806 cells were pre-treated with ATM inhibitor KU-60019 or DMSO for 1 h, and subsequently cells were treated with doxorubicin for 24 h. After the treatment, RNA was isolated and relative gene expression of indicated NF-κB target genes was analyzed by qRT-PCR. Error bars indicate ±SEM from three experiments. Significance was calculated using one-way ANOVA followed by Tukey multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. **G** Following the transfection with either MLK4-targeting (siMLK4) or non-targeting control siRNA (siNT), cells were treated with doxorubicin for 24 h and the concentration of IL-6 in cell culture medium supernatants was measured using ELISA. Error bars indicate ±SEM from three experiments. Significance was calculated using an unpaired two-tailed *t*-test, $^{*}p < 0.05$. **H** MLK4-depleted and control cells were incubated with doxorubicin alone (0.5 μM) or doxorubicin and IL-6 (10 ng/ml) for 48 h. Following the treatment, cells viability was assessed by crystal violet staining and quantified by absorbance measurements. Error bars indicate ±SEM from three experiments performed in triplicates ($n = 9$). Significance was calculated using one-way ANOVA followed by Tukey multiple comparisons test, $^{**}p < 0.01$, $^{****}p < 0.0001$.

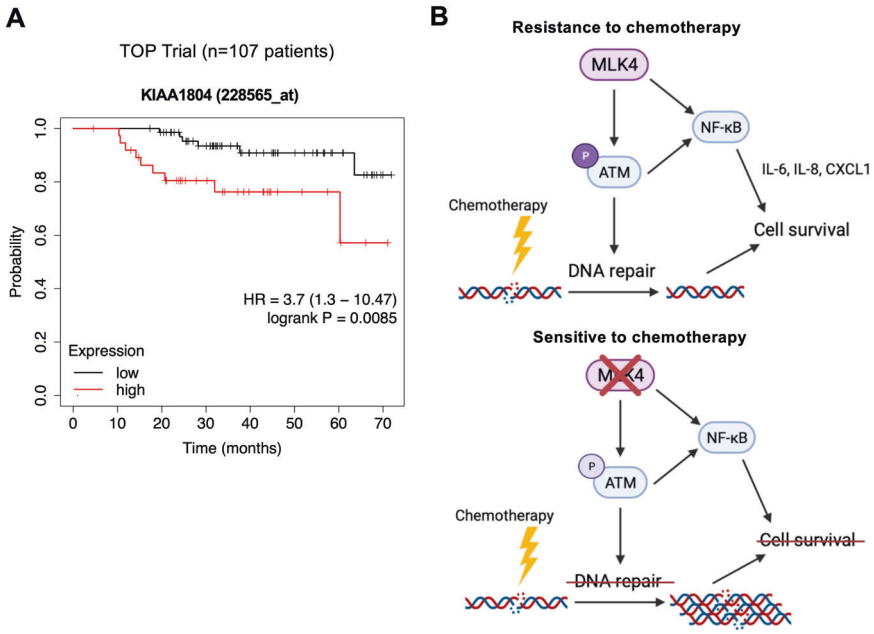


Fig. 7 High MLK4 expression predicts poor prognosis in patients treated with anthracycline-based neoadjuvant chemotherapy. A Probability of overall survival in breast cancer patients from neoadjuvant TOP trial based on MLK4 mRNA expression in tumor tissue, evaluated using pre-treatment biopsies. Patients with estrogen receptor (ER)-negative tumors were treated with anthracycline (epirubicin) monotherapy. The OS was analyzed using the KMplotter tool, with auto-selected best cutoff. Graphic illustrations were obtained from Kmplot.com. **B** Schematic illustration of the proposed role of MLK4 in TNBC chemoresistance.

components, including: CHK2, KAP1, TP53BP1 and MDC1. Moreover, we demonstrated that MLK4 depletion attenuates downstream DNA repair via the NHEJ pathway, which seems to be the major pathway involved in repairing doxorubicin-induced DNA DSBs [45]. Thus, we propose that MLK4 plays a role in ATM activation to facilitate efficient DNA repair and contributes to TNBC survival and chemoresistance. There are several possible mechanisms through which MLK4 may impact ATM activation. MLK4 can directly interact with ATM and phosphorylate its residues, including Ser1981 or other phosphorylation sites essential for ATM activation [11, 46]. Alternatively, additional kinases downstream of MLK4 might be involved. For instance, IKKα/β, which are MLK4 substrates, were demonstrated to phosphorylate ATM in response to DNA damage and promote NHEJ-mediated DNA repair [11, 47]. Therefore, future studies are needed to elucidate the exact mechanisms by which MLK4 regulates ATM function and DDR signaling networks.

Currently, many drugs targeting various DDR components are in preclinical and clinical development. PARP inhibitors have been established as important new therapies for breast cancer patients with inherited BRCA1/2 mutations [48]. ATM inhibitors are now under investigation in phase I clinical trials in patients with glioblastoma and other advanced tumors in combination with radio- and chemotherapies [49]. However, the potential side-effects of prolonged treatment with ATM inhibitors should also be considered, as ATM inhibition sensitizes cells to genotoxic insults in general, raising the concern of normal tissue toxicity [13]. We show that MLK4 acts as a novel, druggable regulator of DDR. Notably, MLK4 loss or inhibition does not potentiate cytotoxic effects of chemotherapy against normal breast epithelial cells in vitro, indicating that the further development of MLK4 inhibitors is warranted and may prove beneficial in cancer therapy. Moreover, our results suggest that MLK4 loss impairs NHEJ-mediated DSBs repair, and therefore may cause synthetic

lethality in HR-deficient tumors due to the inhibition of the remaining DSBs repair pathway. This can be potentially relevant in the context of TNBC therapy, as this subtype of breast cancer is characterized by a relatively high frequency of HR-defects [50, 51].

Numerous studies have linked increased NF-κB activation and autocrine/paracrine signaling with breast cancer chemoresistance [12, 42, 43, 52, 53]. ATM plays a critical role in the induction of NF-κB transcription in response to genotoxic stress, primarily via an established nuclear-to-cytosolic signaling pathway involving NEMO, the regulatory subunit of the IKK complex [39, 54, 55]. Following MLK4 silencing, we observed lower levels of phosphorylated ATM and NEMO, impaired nuclear translocation of NF-κB, and decreased expression of pro-survival NF-κB target genes upon treatment with chemotherapy. These observations suggest that MLK4 activates NF-κB transcription indirectly via regulation of ATM-NEMO-IKK pathway upon DNA damage (Fig. 7B). Nevertheless, MLK4 also regulates NF-κB signaling by direct upstream activation of IKK, independently of DNA damage, which has been previously described in glioma and breast cancer [22, 23]. Thus, it is likely that MLK4 affects NF-κB transcriptional activity via several mechanisms in a context-dependent manner.

In summary, we demonstrate for the first time that MLK4 kinase confers chemoresistance in TNBC. We present evidence that MLK4 is involved in regulating DNA damage response signaling and contributes to chemotherapy-induced NF-κB activation, which facilitates the survival of TNBC cells. Our results indicate that MLK4 targeting can be used as a novel strategy in the treatment of chemoresistant tumors.

DATA AVAILABILITY

The authors declare that all data supporting the findings of this study are available within the article and Supplementary File. mRNA-seq data have been deposited at GEO DataSets (GSE174692). Additional data or reagents are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

DM and AAM designed and supervised the study, provided funding, performed cell culture and in vivo experiments, analyzed the data. DM wrote the manuscript with the supervision of AAM. DN contributed to the study design, provided funding and performed in vivo experiments. ML, AS, AM, DD, AM, and AB performed cell culture experiments and analyzed the data. LMS, AG, PJ and EIS performed microscopy experiments and analyzed the data. MKP generated MLK4 knock-out cell lines.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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Supplementary Information

MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance

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Content:

Supplementary Methods

Generation of doxycycline-inducible cell lines

Parental HCC1806 and SUM149PT were used to generate cells with doxycycline-inducible knock-down of MLK4, as described previously (1). To generate lentivral stock, HEK293T cells were transfected with TRIPZ shRNA_2 and shRNA_6 targeting MLK4 (Supplementary Table S1), purchased from Dharmacon. Cells were transduced with lentiviral stocks and subsequently selected with puromycin. To induce knock-down of MLK4, cells were incubated with doxycycline (Sigma) at 1 μg/ml concentration for 2-4 days.

CRISPR/Cas9-mediated knock-out of MLK4

MLK4 CRISPR/Cas9 knock-out cells were generated using InvitrogenTM TrueGuideTM Synthetic gRNA, according to the manufacturer's instructions. Briefly, HCC1806 cells were transfected with gRNA and CAS9 protein (Thermofisher Scientific), using Lipofectamine CRISPRMAX reagent. Following transfection, the single cell clones were expanded and selected for MLK4 knock-out.

Anchorage-dependent 2D Cas 3/7 Glo apoptotic assay

HCC1806 and SUM149PT doxycycline-inducible cell lines were seeded into 6-well plates and incubated with 1 μg/ml doxycycline to induce MLK4 knock-down. MCF10A cells were transfected with either MLK4-targeting or control siRNA. Subsequently, cells were collected and seeded on a 96-well plate ($1x10^4$ cells/well) in triplicates. The following day doxorubicin at indicated concentrations was added to the wells. After 24 hours, activity of caspases 3/7 was measured using Caspase-Glo 3/7 bioluminescent assay (Promega), according to the manufacturer's instructions. For the luminescence measurement, microplate reader Synergy II (Bio Tek) was used.

Anchorage-independent 3D Cas 3/7 Glo apoptotic assay

HCC1806 and SUM149PT doxycycline-inducible cell lines were seeded on 96-well ultra-low attachment plates (Corning) at a density of 5x103 cells/well. The following day doxycycline was added to the wells at concentration 1 μg/ml to induce MLK4 knock-down and cells were incubated for an additional 48 hours. Subsequently, doxorubicin was added to the wells, and after 48 hours of treatment, activity of caspases 3/7 was measured using Caspase-Glo 3/7 bioluminescent assay as described above.

Protein lysates preparation and immunoblotting

For the whole cell lysates preparation, cells were lysed with RIPA (Sigma) lysis buffer supplemented with protease and phosphatase inhibitor tablets (Roche). For the isolation of nuclear proteins, cells were lysed using Nuclear Extraction Kit (Abcam), according to the manufacturer's instructions. Proteins were resolved by SDS-PAGE gel electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked in 5% fat-free milk in TBST buffer and incubated overnight at 4 ˚C with indicated primary antibodies (Supplementary Table S2), and then for 1h with appropriate horseradish

peroxidase–labeled secondary antibodies (Bio-Rad). The blots were developed with Clarity and Clarity Max substrates (Bio-Rad) and visualized using Amersham Imager 680. Images were analyzed using ImageJ.

Immunofluorescent staining and confocal microscopy

For *in vitro* experiments, HCC1806 cells were seeded onto coverslips and incubated with 1 μg/ml doxycycline to induce MLK4 knock-down. Subsequently, cells were washed with PBS and fixed with 4% PFA in PBS for 20 min. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 15 min, washed and blocked with 10% FBS in PBS for 1 h. The primary yH2AX antibody (Supplementary Table S2) was added in 5% FBS in PBS and incubated in a humid chamber at 4°C overnight. Cells were washed and incubated with secondary antibody in PBS (Goat anti-Rabbit IgG, Alexa Fluor 488, Invitrogen). Next, the cells were washed, and coverslips were mounted on microscope slides using ProLong Diamond Antifade Mountant with DAPI (Thermofisher Scientific). For evaluation of xenograft tumors, tissues were dissected, fixed with 4% PFA and cryoprotected with 30% sucrose in PBS for 24 h. Next, samples were transferred to O.C.T. (Sakura Tissue-Tek) and frozen in −30 °C isopentane. Sections were obtained using a Leica CM1860 cryostat. Frozen free-floating slices were washed with PBST. Antigen retrieval was performed using sodium citrate buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0). Next, slices were blocked in 5% goat serum, incubated with primary antibodies (Supplementary Table S2) and secondary antibodies (Goat anti-Rabbit IgG DyLight 405, Invitrogen). Finally, slices were assembled using Fluromount G. All images were captured with Axio Imager Z2 LSM 700 Zeiss Confocal Microscope. Image analysis was performed using FIJI or ImageJ.

***In vitro* kinase assay**

Human glutathione S-transferase (GST)-tagged MLK4 kinase domain expressed using baculovirus expression system (Carna Biosciences) was incubated with kinase-inactive MKK7 (Carna Biosciences) in the absence or presence of inhibitor. Kinase assay was performed with 200 mM ATP at 30 ˚C for 30 min. Following addition of 4x reduced SDS sample buffer, proteins were resolved by SDS–PAGE and analyzed by western blotting.

LC-MS/MS phosphoproteomic analysis

Cell lysates were obtained in different experimental conditions as indicated. Lysates were digested with 8 μg of trypsin overnight at 37ºC overnight. Next, samples were purified using SEP-PAK cartridges and subjected to phosphopeptides enrichment using TiO_2 beads (GL Sciences). Phospho-enriched samples were analyzed by LC-MS/MS. Mass spectrometry measurements were performed at the Mass Spectrometry Laboratory at the Institute of Biochemistry and Biophysics PAS. Chromatographic separation was performed on an Evosep EV1106 analytical column. Each sample was measured in duplicate. An Evosep One nano-LC system (Evosep) was coupled to a FAIMS interface-equipped Exploris 480 mass spectrometer via an Flex ion source (Thermo Fisher Scientific). The Exploris 480

was operated in data-dependent mode with survey scans acquired at a resolution of 60,000 at m/z 200. Up to 25 of the most abundant isotope patterns with charges 2-6 from the survey scan were selected with an isolation window of 1.6 m/z and fragmented by higher-energy collision dissociation (HCD) with normalized collision energies of 30%, while the dynamic exclusion was set to 20 s. The maximum ion injection times for the survey scan and the MS/MS scans (acquired with a resolution of 30,000 at m/z 200) were set to Auto. The normalized ion target value for MS was set to 300% and for MS/MS to Standard. To increase the resolution for TMT reporter ions the TurboTMT algorithm was applied.

The data were processed with MaxQuant v. 1.6.10.43 and the peptides were identified from the MS/MS spectra searched against the UniprotKB Human Proteome using the built-in Andromeda search engine. Peptide N-terminal/Lys TMT and Cys carbamidomethylation were set as a fixed modification and Ser/Thr/Tyr phosphorylation, Met oxidation, and Asn/Gln deamidation were set as variable modifications. For in silico digests of the reference proteome, cleavages of arginine or lysine followed by any amino acid were allowed (trypsin/P), and up to two missed cleavages were allowed. The FDR was set to 0.01 for peptides, proteins, and sites. Match between runs was enabled and second peptides disabled. Other parameters were used as pre-set in the software. Corrected (based on the correction factors provided by the manufacturer for the TMT batch utilized) of phosphorylation sites' TMT intensities were loaded into Perseus v. 1.6.10.0. Standard filtering steps were applied to clean up the dataset: reverse (matched to decoy database) and potential contaminants (from a list of commonly occurring contaminants included in MaxQuant) were removed. TMT intensities for the phosphorylation sites were log2 transformed, modification site table expanded, and sites with less than 1 valid value filtered off. Gaussian distribution of log2 transformed TMT intensities were confirmed by histogram analysis. Normalization was then applied by subtracting median values in each TMT channel. Student t-testing (permutation-based FDR with 250 randomizations = 0.05, S0 = 0.1, 2-sided, paired) was performed on the dataset to return sites in which phosphorylation levels were statistically significantly changed in response to MLK4 KD and/or drug treatment.

RNA isolation and qRT-PCR

RNA was isolated using the RNAeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration was determined by the absorbance measurements using DS-11 Series Spectrophotometer/Fluorometer (DeNovix). cDNA was synthesized from 400 ng of total RNA using AMV Reverse Transcriptase (EurX). Quantitative real-time PCR was carried out using SYBR Green I (Bio-Rad) as a fluorophore. All qPCR reactions were performed on 96-well plates (Axygen), using Light Cycler 480 instrument (Roche). Samples were assayed in duplicates, and the obtained CT values were used to calculate relative gene expression using the $2^{-\Delta CT}$ method. For each gene, the expression was normalized to the expression of B2M and actin housekeeping genes. The primers used in this study are listed in Supplementary Table S3.

RNA-seq data processing

For data analysis, raw sequences were trimmed according to quality using Trimmomatic using default parameters, except MINLEN, which was set to 50. Trimmed sequences were mapped to human reference genome provided by ENSEMBL, (version grch38_snp_tran) using Hisat2 default parameters. Optical duplicates were removed using MarkDuplicates tool from GATK with default parameters except OPTICAL_DUPLICATE_PIXEL_DISTANCE set to 12000. Mapped reads were associated with transcripts from GRCh38 database with default parameters except –stranded set to "reverse". Differentially expressed genes were selected using DESeq2 package. Fold change was corrected using apeglm. Overrepresentation of Gene Ontology (The Gene Ontology Consortium 2019) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) categories among the top 5% of genes (according to p-value) was assessed with clusterprofiler package. Significance of association between the top 5% of genes and KEGG pathways was further tested with pathway regulation score. GSEA analysis was performed with phenoTest package. All mRNA-seq data have been deposited at GEO DataSets (GSE174692).

Supplementary Figure Legends and Figures

Figure S1. A-C, Following the transfection with MLK4-targeting or control siRNA, cells were treated with doxorubicin and etoposide for 48 h. Next, cells were stained with AnnexinV-FITC, and analyzed by flow cytometry. The representative histograms showing AnnexinV–FITC staining are shown for HCC1806, SUM149PT and MDA-MD-436 cell lines.

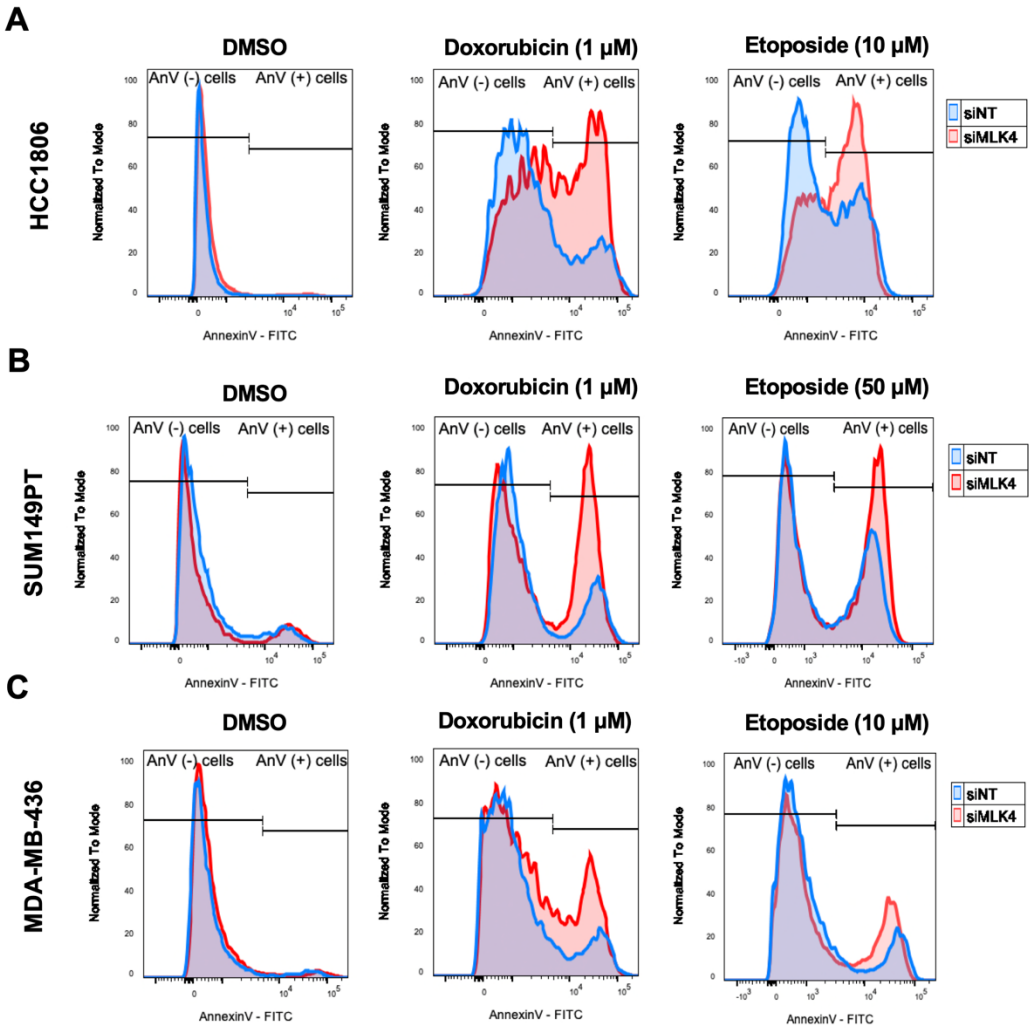


Figure S2. A, HCC1806 were transfected with MLK4-targeting siRNA smart-pool (siMLK4-sp) or with four individual MLK4-targeting siRNAs (siMLK4 1-4). The knock-down efficacy was assessed by immunoblotting. Cells transfected with non-targeting control siRNA (siNT) were used as a control. **B,** After the treatment, cell viability was assessed by crystal violet staining and quantified by absorbance measurements. Error bars indicate ±SEM from two independent experiments performed in triplicates (N=2, n=6). Significance was calculated using one-way ANOVA followed by Tukey multiple comparisons test, *p<0.05, **p<0.01.

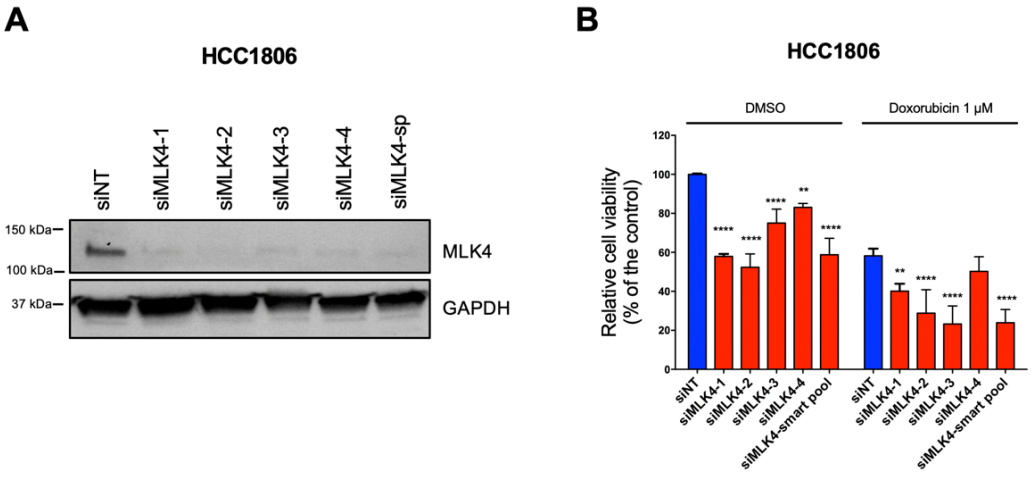


Figure S3. A-D, HCC1806 and SUM149PT parental cell lines were incubated with doxycycline and subsequently cells were treated with doxorubicin (A-B) or etoposide (C-D) for 48 h. Following treatment, cells viability was assessed by crystal violet staining and quantified by absorbance measurements. Error bars indicate ±SEM from three independent experiments, performed in triplicates. Significance was calculated using one-way ANOVA followed by Tukey multiple comparisons test, ns: not significant. **E-F,** HCC1806 and SUM149PT parental cell lines were incubated with doxycycline and subsequently treated with doxorubicin at increasing concentrations for 24 h. The activity of caspases 3/7 was measured using bioluminescence assay. Error bars indicate ±SEM from three independent experiments, performed in triplicates. Significance was calculated using an unpaired two-tailed *t*-test.

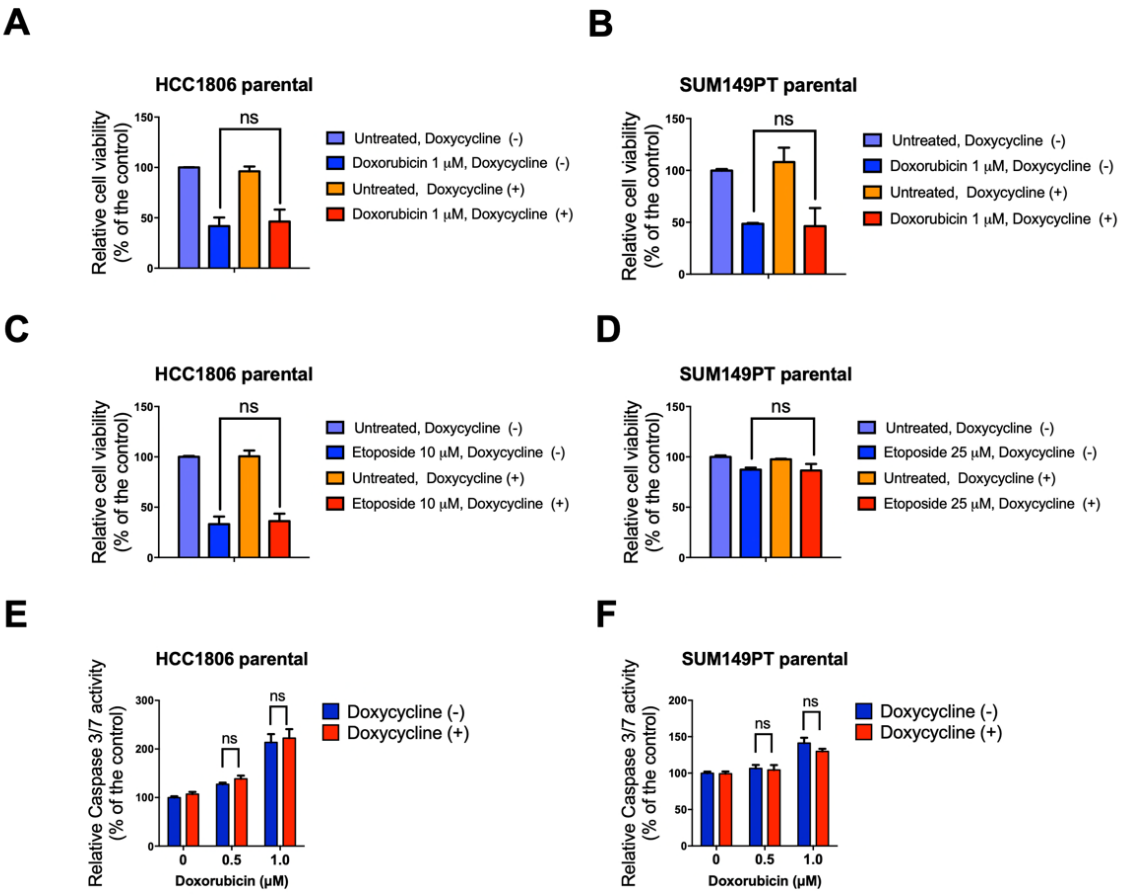


Figure S4. A, Cell lines with doxycycline-inducible MLK4 overexpression were generated from HCC1806 parental cells using lentiviral vectors. MLK4 overexpression was confirmed by immunoblotting. **B** Cells were incubated with doxycycline to induce MLK4 expression and subsequently were treated with doxorubicin at indicated concentrations. Following treatment, cell viability was assessed by crystal violet staining and quantified by absorbance measurements. Error bars indicate ±SEM from three independent experiments (n=3). Significance was calculated using an unpaired two-tailed *t*-test, **p<0.01.

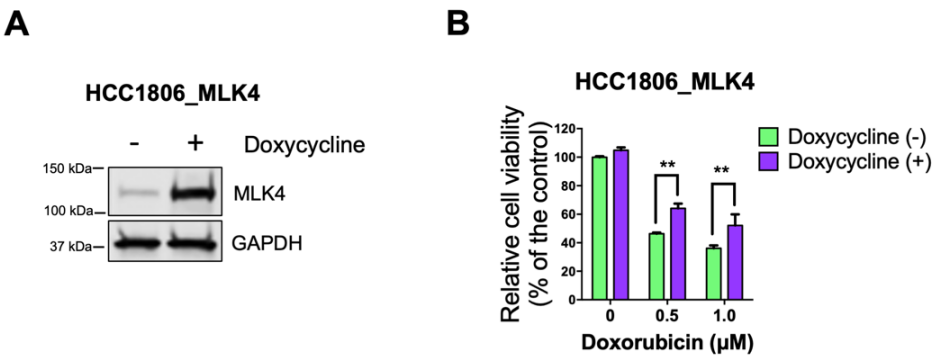


Figure S5. A, MCF10A cells were transfected using siRNA against MLK4 (siMLK4) or non-targeting siRNA control (siNT). MLK4 expression was analyzed by immunoblotting. Lysates from SUM149PT cells were run in parallel as a reference with high MLK4 expression. **B,** Following the transfection with either MLK4-targeting (siMLK4) or non-targeting control siRNA (siNT), cells were incubated with doxorubicin and etoposide at indicated concentrations for 48 h. After the treatment, cell viability was assessed by crystal violet staining and quantified by absorbance measurements. Error bars indicate ±SEM from three independent experiments (n=3). Significance was calculated using an unpaired two-tailed *t*-test. **C,** MLK4-depleted and control cells were incubated with doxorubicin and etoposide at indicated concentrations for 48 h. Next, cells were stained with AnnexinV-FITC, and analyzed by flow cytometry. Error bars indicate ±SEM from three or four independent experiments, performed in triplicates. Significance was calculated using an unpaired two-tailed *t*-test. **D,** MLK4-silenced and control cells were treated with doxorubicin at increasing concentrations and the activity of caspases 3/7 was measured using bioluminescence assay. Error bars indicate ±SEM from three independent experiments, performed in duplicates (n=6). Significance was calculated using an unpaired two-tailed *t*-test. **E,** MCF10A cells were incubated with CEP-5214 (250 nM) or DMSO for 72 h and doxorubicin for 48 h. After treatment, cells viability was assessed by crystal violet staining and quantified by absorbance measurements. Error bars indicate ±SEM from three independent experiments. Significance was calculated using one-way ANOVA followed by Tukey multiple comparisons. **F,** MCF10A cells were incubated with CEP-5214 (250 nM) or DMSO for 72 h and doxorubicin for 48 h, then stained with AnnexinV-FITC, and measured by flow cytometry. Error bars indicate ±SEM from two independent experiments, performed in triplicates (n=6). Significance was calculated using one-way ANOVA followed by Tukey multiple comparisons. For the statistical comparisons - ns: not significant.

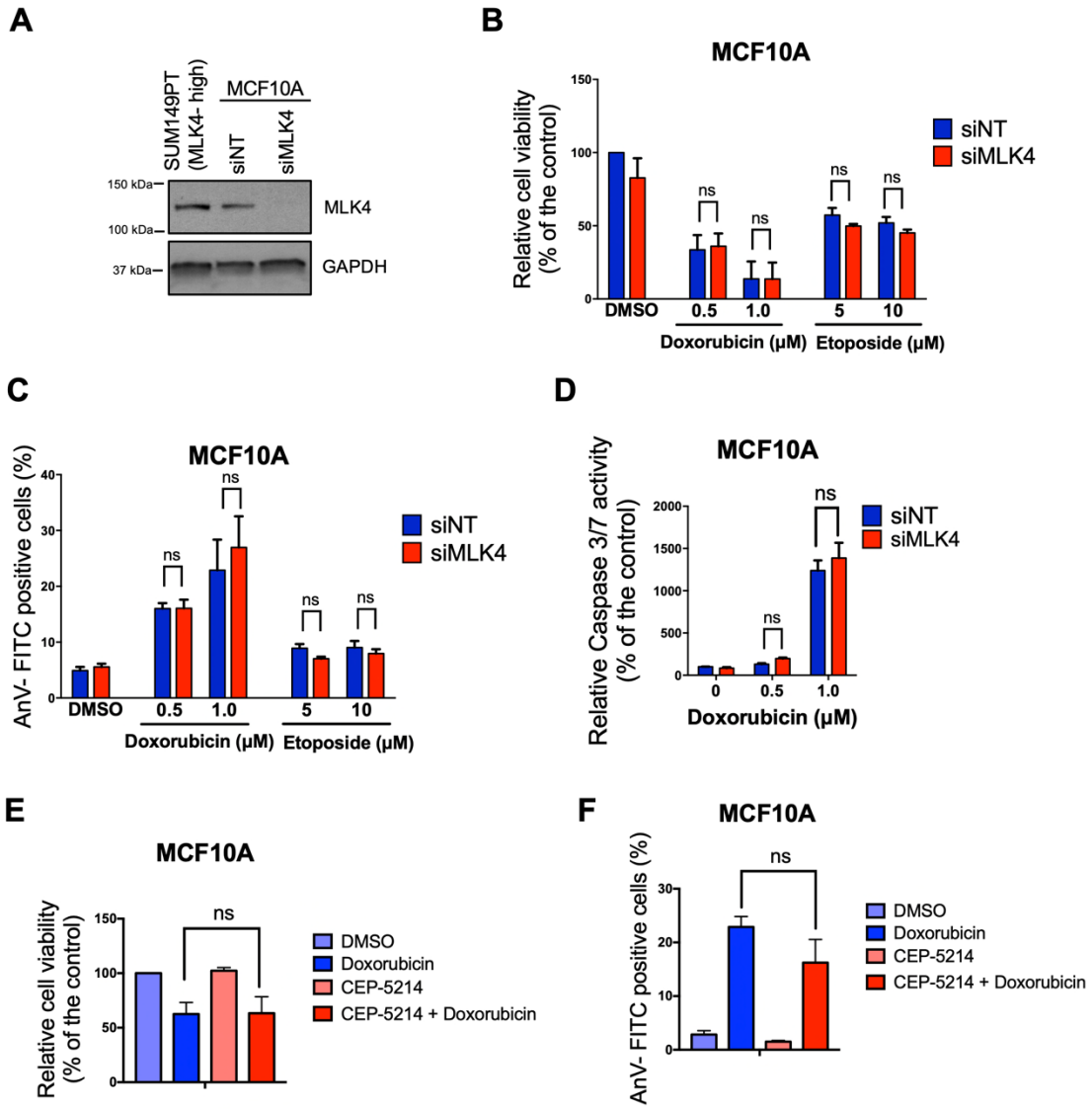

A
SUM149PT (MLK4- high)
MCF10A
siNT
siMLK4
150 kDa
100 kDa
37 kDa
MLK4
GAPDH
B
MCF10A
Relative cell viability (% of the control)
siNT
siMLK4
ns
DMSO
0.5
1.0
5
10
Doxorubicin (μM)
Etoposide (μM)
C
MCF10A
AnV- FITC positive cells (%)
D
MCF10A
Relative Caspase 3/7 activity (% of the control)
E
MCF10A
Relative cell viability (% of the control)
DMSO
Doxorubicin
CEP-5214
CEP-5214 + Doxorubicin
F
MCF10A
AnV- FITC positive cells (%)

Figure S6. A-B, MLK4 knock-down was induced in HCC1806_sh6 and SUM149PT_sh2 cell lines by doxycycline, and cells were subsequently treated with neocarzinostatin at concentration of 100 ng/ml for indicated time. After the treatment, whole cell lysates were collected and analyzed by immunoblotting.

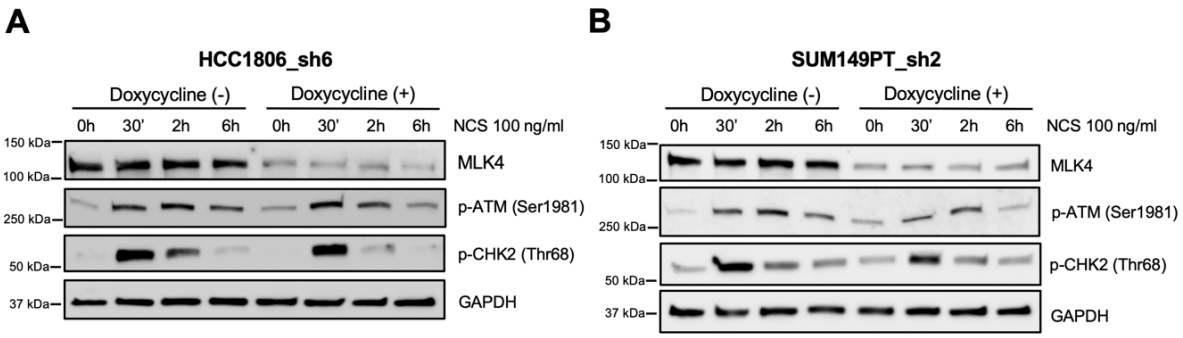


Figure S7. A, MLK4 knock-down in U2OS cells transfected with MLK4-targeting siRNA (siMLK4). Cells transfected with non-targeting siRNA (siNT) were used as a control. **B,** U2OS-pDR-GFP and U2OS-pimEJ5-GFP cell lines were generated from parental cells lines by transfection of the appropriate vectors and subsequent selection with puromycin for over 14 days. The cells stably expressing reporter vectors were transfected with MLK4-targeting siRNA (siMLK4) or control non-targeting siRNA (siNT) along with pSCE-CbaI endonuclease expressing vector. After 72 h, the activity of homologous recombination (pDR-GFP vector expressing cells) and non-homologous end joining (pimEJ5-GFP vector expressing cells) DNA repair pathways was assessed by measuring the percentage of GFP-positive cells using flow cytometry. Error bars indicate ±SEM from three independent experiments performed in triplicates (n=9). Significance was calculated using an unpaired two-tailed *t*-test ****p<0.0001.

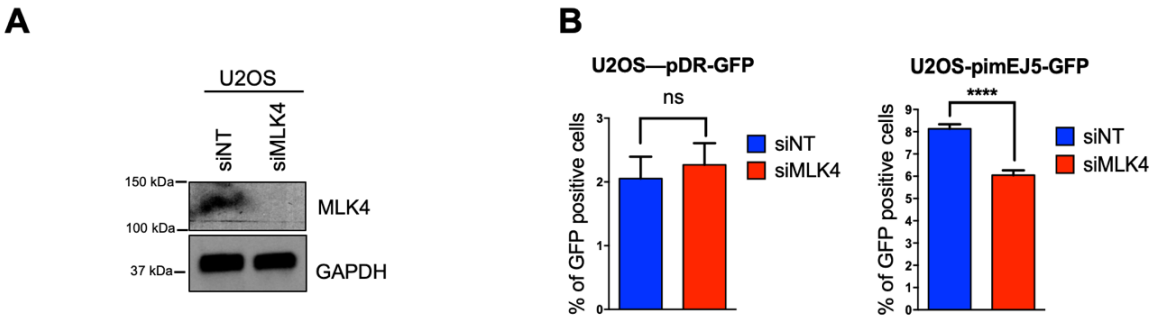


Figure S8. A, Principal component analysis plot obtained after RNA-seq of HCC1806 transfected with MLK4-targeting siRNA or control siRNA, either treated with DMSO or doxorubicin for 24 h. **B,** Venn diagram comparing numbers of transcripts significantly up (UP) or down-regulated (DW) by doxorubicin treatment in each dataset. In control cells, 1958 transcripts were upregulated and 2159 down regulated by doxorubicin. Among these transcripts, 1541 were found to be commonly upregulated in both groups, while 558 and 416 transcripts were uniquely upregulated in MLK4-depleted and control cells, respectively.

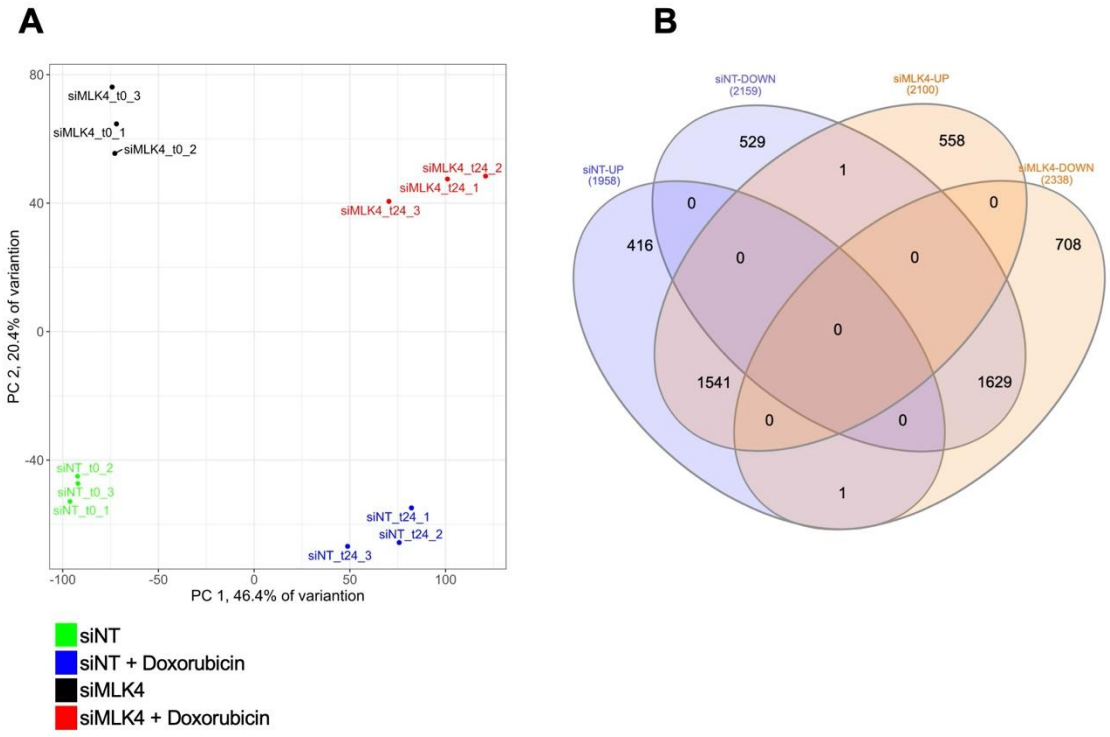
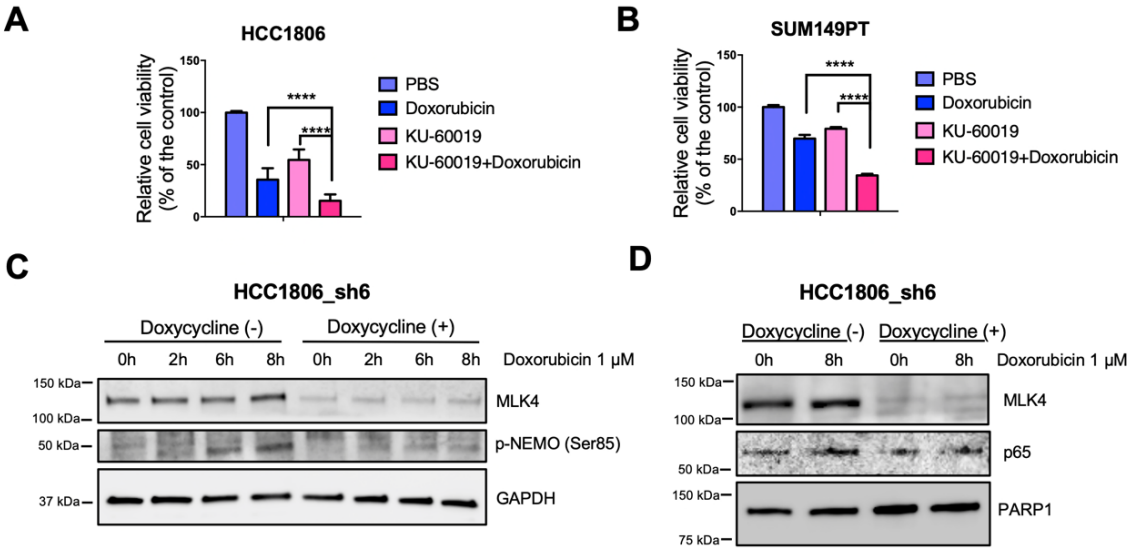


Figure S9. A-B, HCC1806 and SUM149PT cells were treated with doxorubicin, ATM inhibitor: KU-60019 or combination of both for 48h. After the treatment, cell viability was assessed by crystal violet staining and quantified by absorbance measurements. Error bars indicate ±SEM from two independent experiments, performed in triplicates. Significance was calculated using one-way ANOVA followed by Tukey multiple comparisons, ****p<0.0001. **C,** MLK4 knock-down was induced by doxycycline in HCC1806_sh6 cell line, and subsequently cells were treated with doxorubicin for indicated time. Following the treatment, whole cell lysates were collected and analyzed by immunoblotting. **D,** Nuclear extracts were isolated from MLK4-depleted and control HCC1806_sh6 cells and analyzed by immunoblotting.



Supplementary Tables

Table S1. List of siRNA and shRNA sequences.

siRNA	Sequence
siMLK4 #1	GGAAAGAUGCUCAGAGAGAUU
siMLK4 #2	AGGAGAAGCCCAAGGUAAAUU
siMLK4 #3	AGAAGAAACGAGAGGGAAUUU
siMLK4 #4	AGAACAGAUUGCAAAGAAAUU
shRNA	**Sequence**
sh_2	ATCAGAATGTTAAGTTCCC
sh_6	TCTTGATACACTACAATCA

Table S2. List of antibodies used in this study.

Antibody	Species	Source	Catalog no.	Dilution
p-NEMO	Rabbit	Abcam	ab63551-100	1:1000
MLK4	Rabbit	Bethyl	A302-610A	1:1000
ATM	Rabbit	Bethyl	A300-299A	1:100
GAPDH	Rabbit	Cell Signaling Tech.	2118	1:1000
PARP	Rabbit	Cell Signaling Tech.	9532	1:1000
Cl. CASP3	Rabbit	Cell Signaling Tech.	96645	1:500
γH2AX	Rabbit	Cell Signaling Tech.	9718	1:1000
p-ATM (Ser1981)	Rabbit	Cell Signaling Tech.	5883	1:1000
ATM	Rabbit	Cell Signaling Tech.	2873	1:1000
p-CHK2 (Thr68)	Rabbit	Cell Signaling Tech.	2197	1:1000
p65	Rabbit	Cell Signaling Tech.	8242	1:1000
p-JNK	Rabbit	Cell Signaling Tech.	4671	1:1000
p-ERK	Rabbit	Cell Signaling Tech.	4377	1:1000
p-MKK7	Rabbit	Cell Signaling Tech.	4171	1:1000
MKK7	Rabbit	Cell Signaling Tech.	4172	1:1000
GST	Rabbit	Cell Signaling Tech.	2624	1:1000
Flag	Mouse	Sigma	F3165	1:1000

Table S3. List of primers used in this study.

Gene	Forward Primer (5'-3')	Reverse Primer (3'-5')
MLK4	CATGAGGAGGCCTTCGTG	CGCCAACCCAAAATCTGTAA
B2M	TGGAGGCTATCCAGCGTACT	CGGATGGATGAAACCCAGAC
β-actin	CATCCTCACCCTGAAGTACC	AGCCTGGATAGCAACGTACAT
IL-6	GACCCAACCACAAATGCCA	GTCATGTCCTGCAGCCACTG
IL-8	CTGGCCGTGGCTCTCTTG	CCTTGGCAAAACTGCACCTT
IL-12A	ACCACTCCCAAAACCTGC	CCAGGCAACTCCCATTAG
CXCL1	AGCTTGCCTCAATCCTGCATCC	TCCTTCAGGAACAGCCACCAGT
CXCL6	GGGAAGCAAGTTTGTCTGGACC	AAACTGCTCCGCTGAAGACTGG
TNFSF15	AAGGACAGGAGTTTGCACCTTCA	AAGTGCTGTGTGGGAGTTTGTCT

Table S4. Quantitative phosphoproteomics results (as separate file).

Supplementary References

1. A. A. Marusiak *et al.*, “Upregulation of MLK4 promotes migratory and invasive potential of breast cancer cells,” *Oncogene*, vol. 38, no. 15, Art. no. 15, Apr. 2019, doi: 10.1038/s41388-018-0618-0.

Podsumowanie i wnioski

Pierwszy artykuł wchodzący w skład cyklu to praca przeglądowa, w której przedstawiłem dotychczasowe badania kliniczne i przedkliniczne opisujące skuteczność terapeutyczną drobnocząsteczkowych inhibitorów kinaz u chorych na TNBC [32]. W publikacji zaprezentowałem kompleksową analizę opublikowanej literatury oraz trwających badań klinicznych w oparciu o bazę danych www.clinicaltrials.gov. Ponadto, wykonałem analizę mechanizmów działania wybranych inhibitorów drobnocząsteczkowych w oparciu o bazę danych https://www.icoa.fr/pkidb oraz zbadałem częstość występowania mutacji somatycznych i profilu ekspresji genów w poszczególnych podtypach TNBC w kontekście zastosowania terapeutycznego wybranych inhibitorów [49,50]. Na podstawie kompleksowego przeglądu literatury i wykonanych analiz zaproponowałem nowe kierunki badań przedklinicznych i klinicznych, które mogą pozwolić na opracowanie skutecznych terapii z wykorzystaniem inhibitorów kinaz. W niniejszym artykule podkreśliłem, że potrójnie ujemne raki piersi stanowią bardzo heterogenną grupę nowotworów, różniących się od siebie cechami biologicznymi i odpowiedzią na leczenie. Konieczne wydaje się zatem odpowiednie dostosowanie terapii ukierunkowanych molekularnie w oparciu o podtyp molekularny nowotworu. Ponadto, wskazałem, iż dotychczasowe badania skupiały się na terapiach ukierunkowanych na stosunkowo niewielką grupę kinaz o dobrze poznanych funkcjach (m.in. PI3K, AKT, MEK, EGFR, VEGFR). W celu opracowania nowych, racjonalnych strategii leczenia uzasadnione jest zatem poszukiwanie nowych kinaz, które mogą być celem terapii w potrójnie ujemnych nowotworach piersi. W niniejszym artykule wskazałem kinazy, które mogą okazać się atrakcyjnym celem terapeutycznym, do których należą m.in. CK1, MELK, MLK4 oraz PIM1. Uzasadnione są dalsze badania mające na celu lepsze poznanie funkcji wybranych kinaz oraz poszukiwanie optymalnych terapii skojarzonych wykorzystujących drobnocząsteczkowe inhibitory tych kinaz w połączeniu z chemioterapią oraz immunoterapią.

Podsumowując, przedstawiona praca to pierwszy opublikowany artykuł, który systematyzuje obecne możliwości i perspektywy dotyczące wykorzystania inhibitorów kinaz w terapii TNBC.

Drugi artykuł wchodzący w skład cyklu to przeprowadzone przeze mnie oryginalne badania, które pozwoliły na kompleksowe scharakteryzowanie roli kinazy białkowej MLK4 w regulacji chemiooporności TNBC [48]. Przeprowadzona przeze mnie analiza danych transkryptomicznych, wykazała, że zwiększona ekspresja mRNA dla kinazy MLK4 w nowotworze wiąże się z gorszą odpowiedzią na chemioterapię i niekorzystnym rokowaniem w grupie chorych poddawanych chemioterapii z wykorzystaniem antracyklin w badaniu TOP

Trial [51]. Następnie zaobserwowałem, że kinaza MLK4 promuje chemiooporność komórek ludzkich linii TNBC w modelach *in vitro* oraz mysim modelu ksenoprzeszczepienia *in vivo*. Wykazałem, że wyciszenie ekspresji genu dla MLK4 oraz zablokowanie funkcji katalitycznej tej kinazy przy pomocy drobnocząsteczkowych inhibitorów uwrażliwiało komórki nowotworowe na klinicznie stosowane chemioterapeutyki, nie nasilając jednocześnie cytotoksycznego działania chemioterapii względem kontrolnych komórek nienowotworowych. Dane te wskazują na to, że zahamowanie funkcji katalitycznej kinazy MLK4 może okazać się skuteczną strategią przełamywania chemiooporności u dużej części chorych na potrójnie ujemnego raka piersi.

W kolejnych doświadczeniach wykazałem, że aktywna katalitycznie forma kinazy MLK4 reguluje proces naprawy podwójnych pęknięć nici DNA indukowanych przez chemioterapeutyki poprzez aktywację kinazy ATM, odgrywającej kluczową rolę w tym procesie. Wykorzystując odpowiednie wektory reporterowe zaobserwowałem, że w komórkach z wyciszoną ekspresją genu dla MLK4 dochodzi do upośledzenia naprawy DNA w mechanizmie niehomologicznego łączeniu końców (*ang. non-homologous end joining, NHEJ*) [52,53]. Następnie zaobserwowałem, że komórki z wyciszoną ekspresją genu dla MLK4 charakteryzowały się obniżoną aktywacją szlaku sygnałowego ATM-NEMO-NF-κB oraz zmniejszoną ekspresją cytokin zależnych od szlaku NF-κB (m.in. IL-6, IL-8, CXCL1), promujących chemiooporność [14,15].

Odkrycia te bez wątpienia mają wysoce nowatorski charakter, gdyż dotyczą one niekanonicznych i do tej pory nieznanych funkcji kinazy MLK4. Należy również podkreślić uniwersalne znaczenie opisanych przeze mnie zależności, ponieważ wewnątrzkomórkowe szlaki naprawy uszkodzeń DNA odgrywają istotną rolę w procesie rozwoju chemiooporności wielu nowotworów [13]. Uzyskane przeze mnie wyniki wskazują, że inhibitory kinazy MLK4 w połączeniu z chemoterapią mogą stanowić racjonalną kombinację leków o potencjalnym zastosowaniu terapeutycznym. Opracowanie nowych, wysoce specyficznych inhibitorów tej kinazy stanowi pierwszy krok w kierunku kolejnych badań przedklinicznych i klinicznych, które mogą otworzyć nowe możliwości w leczeniu TNBC.

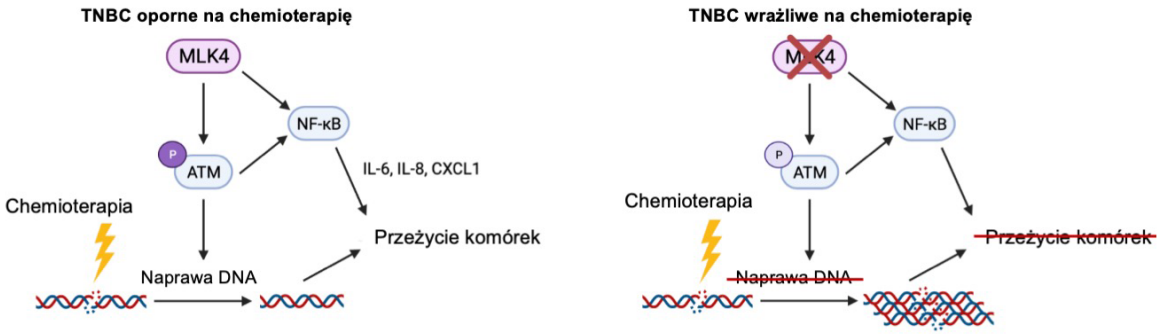
Szczegółowe wnioski wynikające z pracy doktorskiej:

- Dotychczasowe badania kliniczne III fazy wykazywały umiarkowaną skuteczność inhibitorów kinaz w monoterapii i w skojarzeniu z chemioterapią. Prawdopodobne przyczyny umiarkowanego sukcesu klinicznego tych leków wynikają ze znacznej

heterogenności w obrębie podtypów molekularnych TNBC oraz stosunkowo niewielkiej liczby kinaz, które mogą być celem terapii.

- Konieczne wydaje się poszukiwanie nowych kinaz, które mogą okazać się celem leczenia w TNBC. Wśród potencjalnych celów terapeutycznych należy wymienić kinazy PIM1, MLK3, MLK4, MELK oraz CK1.
- Wysoka ekspresja kinazy MLK4 w bioptatach pobranych od chorych na TNBC poddawanych chemioterapii neoadjuwantowej wiąże się z gorszą odpowiedzią na zastosowane leczenie.
- Wyciszenie ekspresji genu dla kinazy MLK4 w komórkach TNBC uwrażliwia je na klinicznie stosowane chemioterapeutyki (doksorubicyna, etopozyd). Po wyciszeniu ekspresji genu MLK4 w komórkach zaobserwowałem nasiloną indukcję apoptozy oraz akumulację uszkodzeń DNA w porównaniu do komórek kontrolnych.
- Inhibitor drobnocząsteczkowy CEP-5214 hamujący aktywność kinazową MLK4 wykazuje synergistyczne działanie w kombinacji z doksorubicyną wobec komórek linii TNBC.
- Analiza proteomiczna oraz badanie ekspresji genów metodą sekwencjonowania następnej generacji wykazały, że komórki linii TNBC z wyciszoną ekspresją genu MLK4 charakteryzują się zmniejszoną aktywacją mechanizmów naprawy DNA i upośledzoną aktywacją czynników transkrypcyjnych z rodziny NF-κB w porównaniu do komórek kontrolnych.
- MLK4 promuje aktywację kinazy ATM i fosforylację innych białek zaangażowanych w proces naprawy DNA. W komórkach linii TNBC z wyciszoną ekspresją genu MLK4 zaobserwowałem upośledzoną naprawę podwójnych pęknięć nici DNA w mechanizmie NHEJ.
- MLK4 promuje aktywację szlaku ATM-NEMO i aktywację NF-κB-zależnych cytokin (IL-6, IL-8, CXCL1), które promują przeżycie komórek linii TNBC w odpowiedzi na chemioterapię w mechanizmie parakrynnym/autokrynnym.

Podsumowując, przedstawiony cykl publikacji opisuje dotychczasowe badania z wykorzystaniem drobnocząsteczkowych kinaz w terapii TNBC oraz wskazuje, że zahamowanie aktywności kinazy MLK4 może okazać się nową, racjonalną strategią przełamywania chemiooporności tych nowotworów (Ryc. 3).



Rycina 3 - Zidentyfikowana nowa rola kinazy MLK4 w regulacji chemiooporności TNBC poprzez modulację odpowiedzi komórek na uszkodzenie DNA. Opracowano na podst. Mehlich i wsp. 2021.

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M. Buyse, J.-M. Nogaret, Y. Qi, F. Symmans, L. Pusztai, V. D'Hondt, M. Piccart-Gebhart, C. Sotiriou, Multifactorial approach to predicting resistance to anthracyclines, J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 29 (2011) 1578–1586. https://doi.org/10.1200/JCO.2010.31.2231.

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UCHWAŁA NR 1035/2020

z dnia 8 września 2020 r.

I Lokalnej Komisji Etycznej do spraw doświadczeń na zwierzętach w Warszawie

§ 1

Na podstawie art. 48 pkt. 1 ustawy z dnia 15 stycznia 2015r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. poz. 266) zwanej dalej „ustawą" po rozpatrzeniu wniosku pt **„Wpływ wyciszenia kinazy MLK4 na uwrażliwienie komórek potrójnie ujemnego raka piersi na chemioterapię"** z dnia **28.08.2020 r.** złożonego przez **Centrum Nowych Technologii Uniwersytetu Warszawskiego ul. Banacha 2c, 02-097 Warszawa** zaplanowanego przez **dr Annę Marusiak** lokalna komisja etyczna

WYRAŻA ZGODĘ

Na przeprowadzenie doświadczeń na zwierzętach w zakresie wniosku.

§ 2

W wyniku rozpatrzenia wniosku o którym mowa w § , Lokalna Komisja Etyczna ustaliła, że:

1. Wniosek należy przypisać do kategorii**: A. Badania podstawowe**
2. Najwyższy stopień dotkliwości proponowanych procedur to: **UMIARKOWANY**
3. Doświadczenia będą przeprowadzane na gatunkach lub grupach gatunków: **Mysz domowa RAG2-/- 8-12 tygodni 55 os.**
4. Doświadczenia będą przeprowadzane przez: **Anna Marusiak, Dominika Nowis, Dawid Mehlich**
5. Doświadczenie będzie przeprowadzane w terminie[1]:**01.10.2020-30.12.2022 r.**
6. Doświadczenie będzie przeprowadzone w ośrodku: w:
7. Doświadczenie będzie przeprowadzone poza ośrodkiem w: **Zwierzętarni Zakładu Immunologii WUM ul. Banacha1B (budynek CBP) 02-091 Warszawa**
8. Użyte do procedur zwierzęta dzikie zostaną odłowione przez: -
9. Doświadczenie nie zostanie poddane ocenie retrospektywnej

[1] Nie dłużej niż 5 lat

§ 3

Uzasadnienie:

Komisja oceniła wniosek zgodnie z kryteriami zawartymi w art. 47.1 ustawy z dnia 15 stycznia 2015r o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. poz. 266). Po zapoznaniu się z problematyką zawartą we wniosku komisja stwierdza, że pod względem etycznym przedstawiony wniosek spełnia zasady dopuszczenia doświadczeń na zwierzętach. Na podstawie art. 107 § 4 ustawy z dnia 14 czerwca 1960r – Kodeks postępowania administracyjnego z późniejszymi zmianami (Dz. U. 2017 poz. 1257) odstąpiono od sporządzenia uzasadnienia decyzji, gdyż uwzględnia ona w całości żądanie strony.

Niniejsza uchwała wchodzi w życie z dniem wydania i jest ważna do 30.12.2022 r.

§ 4

Integralną część niniejszej uchwały stanowi kopia wniosku, o którym mowa w § 1

(Pieczęć lokalnej komisji etycznej)

I LOKALNA KOMISJA ETYCZNA
ds. Doświadczeń na Zwierzętach
przy Wydziale Biologii UW
ul. Ilji Mieczynikowa 1, 02-096 Warszawa
tel. 022 5541028, e-mail: ike1waw@biol.uw.edu.pl

Podpisy przewodniczącego komisji

.....

Pouczenie:

Zgodnie z art. 33 ust. 3 i art. 40 ustawy w zw. z art. 127 § 1 i 2 oraz 129 § 2 ustawy z dnia z dnia 14 czerwca 1960 r. Kodeks postępowania administracyjnego (Dz. U. 2017, poz. 1257 – t.j.; dalej KPA) od uchwały Lokalnej Komisji Etycznej strona może wnieść, za jej pośrednictwem, odwołanie do Krajowej Komisji Etycznej do Spraw Doświadczeń na Zwierzętach w terminie 14 od dnia doręczenia uchwały.

Na podstawie art. 127a KPA w trakcie biegu terminu do wniesienia odwołania strona może zrzec się prawa do jego wniesienia, co należy uczynić wobec Lokalnej Komisji Etycznej, która wydała uchwałę. Z dniem doręczenia Lokalnej Komisji Etycznej oświadczenia o zrzeczeniu się prawa do wniesienia odwołania przez ostatnią ze stron postępowania, decyzja staje się ostateczna i prawomocna.

Otrzymuje:

1) Użytkownik,
2) Organizacja społeczna dopuszczona do udziału w postępowaniu (jeśli dotyczy)
3) a/a

Użytkownik kopie przekazuje:

- Osoba planująca doświadczenie
- Zespół ds. dobrostanu

Warszawa, 07.02.23r
(miejscowość, data)

Agnieszka Borowiec
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Wykonanie doświadczeń w modelach komórkowych raka piersi i analizę danych.
Mój udział procentowy w przygotowaniu publikacji określam jako 0.5 %.

Wkład Dawida Mehlich w powstawanie publikacji określam jako 51%,
obejmował on: zaprojektowanie badania, metodyki, nadzorowanie prac badawczych, zapewnienie finansowania badań, przeprowadzenie doświadczeń w modelach komórkowych i modelach zwierzęcych, analizę danych i interpretację wyników, napisanie manuskryptu, edycję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Dawida Mehlich.

Agnieszka Borowiec
(podpis oświadczającego)

Warszawa 3.02.13 (miejscowość, data)

Dagmara Dymerska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Wykonanie doświadczeń w modelach komórkowych raka piersi i analizę danych.
Mój udział procentowy w przygotowaniu publikacji określam jako 0,5 %.

Wkład Dawida Mehlich w powstawanie publikacji określam jako 51%,
obejmował on: zaprojektowanie badania, metodyki, nadzorowanie prac badawczych, zapewnienie finansowania badań, przeprowadzenie doświadczeń w modelach komórkowych i modelach zwierzęcych, analizę danych i interpretację wyników, napisanie manuskryptu, edycję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Dawida Mehlich.

Dymerska
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Gdańsk, 9.02.2023

Adam Gorczyński
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Wykonanie obrazowania mikroskopowego i analiza danych.
Mój udział procentowy w przygotowaniu publikacji określam jako 0.5 %.

Wkład Dawida Mehlich w powstawanie publikacji określam jako 51%,
obejmował on: zaprojektowanie badania, metodyki, nadzorowanie prac badawczych, zapewnienie finansowania badań, przeprowadzenie doświadczeń w modelach komórkowych i modelach zwierzęcych, analizę danych i interpretację wyników, napisanie manuskryptu, edycję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Dawida Mehlich.

Adam Gorczyński
.....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Gdańsk, 31.01.2023.
.....(miejscowość, data)

Ewa Iżycka - Świeszewska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Wykonanie obrazowania mikroskopowego i analiza danych.
Mój udział procentowy w przygotowaniu publikacji określam jako 0.5 %.

Wkład Dawida Mehlich w powstawanie publikacji określam jako 51%,
obejmował on: zaprojektowanie badania, metodyki, nadzorowanie prac badawczych, zapewnienie finansowania badań, przeprowadzenie doświadczeniach w modelach komórkowych i modelach zwierzęcych, analizę danych i interpretację wyników, napisanie manuskryptu i jego edycję.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Dawida Mehlich.

Zakład Patologii i Neuropatologii
Gdański Uniwersytet Medyczny
prof. dr hab. Ewa Iżycka-Świeszewska
kierownik

Ewa Iżycka-Świeszewska
.....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Gdańsk, 31.01.2023 (miejscowość, data)

Paweł Jabłoński
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Wykonanie obrazowania mikroskopowego i analiza danych.
Mój udział procentowy w przygotowaniu publikacji określam jako 0.5 %.

Wkład Dawida Mehlich w powstawanie publikacji określam jako 51%,
obejmował on: zaprojektowanie badania, metodyki, nadzorowanie prac badawczych, zapewnienie finansowania badań, przeprowadzenie doświadczeń w modelach komórkowych i modelach zwierzęcych, analizę danych i interpretację wyników, napisanie manuskryptu, edycję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Dawida Mehlich.

Paweł Jabłoński
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 31.01.2023 (miejscowość, data)

Michał Łomiak
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Wykonanie doświadczeń w modelach komórkowych raka piersi i analizę danych.
Mój udział procentowy w przygotowaniu publikacji określam jako 7 %.

Wkład Dawida Mehlich w powstawanie publikacji określam jako 51%,
obejmował on: zaprojektowanie badania, metodyki, nadzorowanie prac badawczych, zapewnienie finansowania badań, przeprowadzenie doświadczeń w modelach komórkowych i modelach zwierzęcych, analizę danych i interpretację wyników, napisanie manuskryptu, edycję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Dawida Mehlich.

M. Łomiak

(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 03.02.23 (miejscowość, data)

Anna Marusiak
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. " Kinase inhibitors for precision therapy of triple-negative breast cancer: Progress, challenges, and new perspectives on targeting this heterogeneous disease " oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

analiza literatury, edycja manuskryptu

Mój udział procentowy w przygotowaniu publikacji określam jako 20%.

Wkład Dawida Mehlich w powstawanie publikacji określam jako 80%,
obejmował on: przygotowanie tematu pracy, analizę literatury, wykonanie analiz dotyczących mechanizmów działania inhibitorów drobnocząsteczkowych, analizę dostępnych danych klinicznych, napisanie manuskryptu, edycję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Dawida Mehlich.

A. Marusiak

(podpis oświadczającego)

Warszawa, 03.02.23 (miejscowość, data)

Anna Marusiak
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Zaprojektowanie badania, nadzorowanie prac badawczych, zapewnienie finansowania badań, przeprowadzenie doświadczeń w modelach komórkowych i modelach zwierzęcych, analizę danych i interpretację wyników, edycję manuskryptu.
Mój udział procentowy w przygotowaniu publikacji określam jako 29 %.

Wkład Dawida Mehlich w powstawanie publikacji określam jako 51%,
obejmował on: zaprojektowanie badania, metodyki, nadzorowanie prac badawczych, zapewnienie finansowania badań, przeprowadzenie doświadczeń w modelach komórkowych i modelach zwierzęcych, analizę danych i interpretację wyników, napisanie manuskryptu, edycję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Dawida Mehlich.

A. Marusiak

(podpis oświadczającego)

Warszawa, 30.1.2023 (miejscowość, data)

Alicja Mazan
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Wykonanie doświadczeń w modelach komórkowych raka piersi i analizę danych.
Mój udział procentowy w przygotowaniu publikacji określam jako 2 %.

Wkład Dawida Mehlich w powstawanie publikacji określam jako 51%,
obejmował on: zaprojektowanie badania, metodyki, nadzorowanie prac badawczych, zapewnienie finansowania badań, przeprowadzenie doświadczeń w modelach komórkowych i modelach zwierzęcych, analizę danych i interpretację wyników, napisanie manuskryptu, edycję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Dawida Mehlich.

Alicja Mazan
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa 03.03.23 (miejscowość, data)

Anna Mehlich
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Wykonanie doświadczeń w modelach komórkowych raka piersi i analizę danych.
Mój udział procentowy w przygotowaniu publikacji określam jako 0.5 %.

Wkład Dawida Mehlich w powstawanie publikacji określam jako 51%,
obejmował on: zaprojektowanie badania, metodyki, nadzorowanie prac badawczych, zapewnienie finansowania badań, przeprowadzenie doświadczeń w modelach komórkowych i modelach zwierzęcych, analizę danych i interpretację wyników, napisanie manuskryptu, edycję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Dawida Mehlich.

Anna Mehlich

(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 31.01.2023 ..... (miejscowość, data)

Dominika Nowis
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Przeprowadzenie doświadczeń w modelach zwierzęcych, nadzorowanie prac badawczych, zapewnienie finansowania badań.

Mój udział procentowy w przygotowaniu publikacji określam jako 5 %.

Wkład Dawida Mehlich w powstawanie publikacji określam jako 51%,

obejmował on: zaprojektowanie badania, metodyki, nadzorowanie prac badawczych, zapewnienie finansowania badań, przeprowadzenie doświadczeń w modelach komórkowych i modelach zwierzęcych, analizę danych i interpretację wyników, napisanie manuskryptu, edycję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Dawida Mehlich.

Laboratorium
Medycyny Doświadczalnej
prof. dr hab. med. Dominika Nowis
.....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Brisbane, 6.02.2023

Monika Prełowska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Wykonanie doświadczeń w modelach komórkowych raka piersi i analizę danych.
Mój udział procentowy w przygotowaniu publikacji określam jako 0.5 %.

Wkład Dawida Mehlich w powstawanie publikacji określam jako 51%,
obejmował on: zaprojektowanie badania, metodyki, nadzorowanie prac badawczych, zapewnienie finansowania badań, przeprowadzenie doświadczeń w modelach komórkowych i modelach zwierzęcych, analizę danych i interpretację wyników, napisanie manuskryptu, edycję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Dawida Mehlich.

Prełowska

.....

(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa 30.01.23r.
.....(miejscowość, data)

Aleksandra Sobiborowicz-Sadowska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Wykonanie doświadczeń w modelach komórkowych raka piersi i analizę danych.
Mój udział procentowy w przygotowaniu publikacji określam jako 2 %.

Wkład Dawida Mehlich w powstawanie publikacji określam jako 51%,
obejmował on: zaprojektowanie badania, metodyki, nadzorowanie prac badawczych, zapewnienie finansowania badań, przeprowadzenie doświadczeń w modelach komórkowych i modelach zwierzęcych, analizę danych i interpretację wyników, napisanie manuskryptu, edycję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Dawida Mehlich.

A. Sobiborowicz-Sadowska
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 31-01-2023

dr Łukasz M. Szewczyk
Centrum Nowych Technologii
Uniwersytetu Warszawskiego
Stefana Banacha 2c
02-097 Warszawa

OŚWIADCZENIE

Jako współautor pracy pt. "MLK4 regulates DNA damage response and promotes triple-negative breast cancer chemoresistance" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
Wykonanie obrazowania mikroskopowego i analizę danych.
Mój udział procentowy w przygotowaniu publikacji określam jako 0.5 %.

Wkład Dawida Mehlich w powstawanie publikacji określam jako 51%,
obejmował on: zaprojektowanie badania, metodyki, nadzorowanie prac badawczych, zapewnienie finansowania badań, przeprowadzenie doświadczeń w modelach komórkowych i modelach zwierzęcych, analizę danych i interpretację wyników, napisanie manuskryptu, edycję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Dawida Mehlich.

Łukasz M. Szewczyk

(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników