A functional cancer genomics screen identifies a druggable synthetic lethal interaction between MSH3 and PRKDC

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Abstract

Here, we use a large-scale cell line-based approach to identify cancer cell-specific mutations that are associated with DNA-PKcs dependence. For this purpose, we profiled the mutational landscape across 1,319 cancer-associated genes of 67 distinct cell lines and identified numerous genes involved in homologous recombination (HR)-mediated DNA repair, including BRCA1, BRCA2, ATM, PAXIP and RAD50 as being associated with non-oncogene addiction to DNA-PKcs. Mutations in the mismatch repair gene MSH3, which have been reported to occur recurrently in numerous human cancer entities, emerged as the most significant predictors of DNA-PKcs addiction. Concordantly, DNA-PKcs inhibition robustly induced apoptosis in MSH3-mutant cell lines in vitro and displayed remarkable single agent efficacy against MSH3-mutant tumors in vivo. Thus, we here identify a therapeutically actionable synthetic lethal interaction between MSH3 and the NHEJ kinase DNA-PKcs. Our observations recommend DNA-PKcs inhibition as a therapeutic concept for the treatment of human cancers displaying HR-defects.

Significance

We associate mutations in the MSH3 gene, which are frequently detected in microsatellite-instable colon cancer (~40%), with a therapeutic response to specific DNA-PKcs inhibitors. Since potent DNA-PKcs inhibitors are currently entering early clinical trials, we offer a novel opportunity to genetically stratify patients, who may benefit from a DNA-PKcs-inhibitory therapy.
Introduction

In response to DNA damage, cells activate a complex signaling cascade to prevent further cell cycle progression (1). Activation of this signaling pathway, which is commonly referred to as the DNA damage response (DDR), allows time for DNA repair, or, if the lesions are beyond repair capacity, leads to the induction of apoptosis (1). Mammalian cells have evolved at least five partially overlapping DNA repair pathways to correct various types of genotoxic lesions - mismatch repair (MMR), nucleotide-excision repair (NER), base-excision repair (BER), homologous recombination (HR) and non-homologous end joining (NHEJ) (2). MMR removes nucleotides that were mispaired during replication, as well as insertion- and deletion loops, which result from slippage during replication of repetitive sequences. The NER pathway is employed to repair helix-distorting lesions, while small chemical modifications of bases are removed by the BER machinery (2).

Specifically in response to DNA double-strand breaks (DSBs), mammalian cells employ two distinct repair pathways. NHEJ is an error-prone pathway that is preferentially used during early phases of the cell cycle, when no sister chromatid is available (3). During NHEJ, the non-catalytic subunits Ku70 and Ku80 form a heterodimer that binds to the free DNA ends and subsequently recruits the catalytic subunit DNA-PKcs (encoded by *PRKDC*). DNA-PKcs kinase activity is essential for XRCC4- and Lig4-mediated rejoining of the broken DNA ends during NHEJ (4). HR, the second major DSB repair pathway, is largely restricted to the S- and G2-phases of the cell cycle, when a sister chromatid is available to serve as an intact template for DSB repair (5).

One of the early events, necessary for completion of the HR process, is DSB
end resection to create a single-stranded 3'-overhang, which becomes rapidly coated with RPA and provides a substrate for activation of the proximal DDR kinase ATR (6). During the ensuing steps of the HR process, RPA is replaced by Rad51, which is recruited to single-stranded DNA (ssDNA) in a Brca1/Brca2/Palb2-dependent fashion (7). Once loaded onto ssDNA, Rad51 mediates the core reactions of the HR process, namely homology searching, strand exchange, and Holliday junction formation (5).

The HR pathway is indispensable for the maintenance of genomic integrity and patients with heterozygous germline mutations in different HR-genes display a massively increased risk for the development of cancer (7). Most notably, germline mutations in BRCA1, BRCA2 and RAD51C are associated with predisposition to breast and ovarian cancer (8, 9). Furthermore, numerous HR genes, including BRCA1, BRCA2, ATM, CHEK2, RAD50, RAD51C and others, are recurrently somatically mutated in various different cancer entities (7).

Intriguingly, HR-deficiency in BRCA1- or BRCA2-defective cells and tumors was recently shown to represent an actionable alteration in cancer. Specifically, a series of recent studies reported a synthetic lethal interaction between Poly(ADP-ribose) polymerase (PARP1) and the high-penetrance breast and ovarian cancer susceptibility genes BRCA1 and BRCA2 (10, 11). In addition, we and others have recently identified a druggable synthetic lethal interaction between the HR gene ATM and the NEHJ kinase gene PRKDC (12-14). Here, we propose that it is the HR defect that is responsible for the robust non-oncogene addiction to DNA-PKcs that we had previously observed in ATM-mutant cells and tumors (14). Thus, the aim of this study was to
compile a systematic classification of the most frequent mutations in cancer, which are most likely to be associated with an actionable DNA-PKcs addiction. To this end, we employed a combined genomic and chemical vulnerability analysis of 94 cancer cell lines and examined the effect of mutations in 1,319 cancer-associated genes on DNA-PKcs dependence. Furthermore, we functionally confirmed the most striking associations that we identified in high-throughput screening and deciphered mechanisms, which might rationalize these effects. Finally, we provide a detailed examination of our novel therapeutic strategies in vivo.

Results
Activity Profile of KU60648 in a large panel of genomically-annotated cancer cell lines

In order to identify genomic aberrations that are associated with non-oncogene addiction to the NHEJ kinase DNA-PKcs, we screened the specific DNA-PKc inhibitor KU60648 (IC_{50}=19 nM, (15)) against a panel of 94 genomically well-annotated cancer cell lines (Suppl. Table 1), which covered a broad spectrum of cancer entities, histological subtypes (Suppl. Fig. 1A, inset) and cancer-associated genomic aberrations (Fig. 1A, B). We used the Brd4Nut-fused cell line HCC2429 as a positive control (Fig. 1A, Suppl. Table 1), for which we had recognized addiction to DNA-PKcs in follow-up experiments of a previous study (14). Conversely, A375 and A549 cells, which we had previously shown to be resistant against the DNA-PKcs inhibitor KU60648, were included to benchmark DNA-PKcs independence (14). Cell
viability was assessed using high-throughput luminescence-based measurements of relative cellular ATP content by CellTiterGlo® (CTG) assay.

To identify genomic aberrations, which are associated with DNA-PKcs addiction, we systematically linked compound activity in 67 cell lines to mutation status of 1.319 cancer-associated genes (16-20) (Fig. 1B, C, Suppl. Fig. 1, 2). We analyzed Hill’s coefficients and nearest-neighbor-distances in order to identify 400nM as the threshold concentration for KU60648 sensitivity (Suppl. Fig. 1B). We employed this threshold, in order to classify cell lines into KU60648-sensitive and -resistant groups. Comparing for each gene its mutation frequency between the two cohorts (Fisher’s exact test), we identified 121 genes, for which mutations significantly ($p < 0.05$) co-clustered with KU60648 sensitivity, as well as MAP3K1 mutations, which emerged as the only significant ($p = 0.048$) genomic marker predicting KU60648 resistance (Fig. 1B). We note that mutations in PRKDC were strongly associated with KU60648 resistance, suggesting that the compound was indeed targeting DNA-PKcs.

We initially hypothesized that NHEJ abrogation through DNA-PKcs inhibition should selectively eradicate HR-defective cells. Coherent with this notion, mutations in the HR genes BRCA2 ($p=1.06\times10^{-4}$), RAD50 ($p=3.99\times10^{-3}$), CHEK2 ($p=5.97\times10^{-3}$) and PAXIP ($p=1.33\times10^{-4}$, encoding PTIP), as well as the Fanconi anemia pathway component FANCD2 ($p=5.97\times10^{-3}$) emerged as lesions that significantly co-clustered with KU60648 sensitivity. Mutations in different components of the PTIP/MLL3/MLL4 complex, which plays a critical role in RAG-mediated cleavage and repair during V(D)J recombination (21), as well as HR-mediated DSB repair (22), emerged as highly significant...
determinants of DNA-PKcs dependence (Fig. 1B). Specifically, frameshift mutations in *MLL3* (encoding the PTIP-interacting protein MLL3) \((p=9.32 \times 10^{-4})\) were identified as significant predictors for non-oncogene addiction to the NHEJ kinase DNA-PKcs. Furthermore, mutations in *ATM* and *BRCA1* were also associated with KU60648 sensitivity (Suppl. Fig. 2). While displaying strong effects \((GI_{50} \text{ ratio } \geq 3.5)\), these associations did not meet significance criteria, either due to the low mutation frequency of these genes in the examined cancer lines (e.g. *BRCA1*, \(n = 3\)) or due to dissimilar effects on compound activity depending on the respective mutation type (e.g. *ATM*); exclusively frameshift mutations of the latter co-clustered significantly with compound activity (Suppl. Fig. 2). In agreement with our initial hypothesis, a gene ontology-based analysis \((23)\) revealed that disabling mutations in DNA repair genes were significantly \((p=2.98 \times 10^{-12})\) enriched in DNA-PKcs inhibitor-sensitive cell lines (Fig. 1A, inset). In particular, we observed an accumulation of HR genes, for which mutations were significantly associated with KU60648 sensitivity. The initial steps of the HR pathway can be arbitrarily clustered into three distinct processes, namely focus nucleation, DSB resection and Rad51 loading (Fig. 1C); our screen revealed mutations in critical components in all of them (Fig. 1C). These data suggest that HR deficiency in general represents a cancer-associated condition that displays synthetic lethality with the NHEJ kinase DNA-PKcs.

To our surprise, mutations in the mismatch repair gene *MSH3* \((p=9.53 \times 10^{-4})\) emerged amongst the most significant predictors of compound activity (Fig. 1B). Further analysis revealed that *MSH3* mutations gained strongly in significance \((p=7.43 \times 10^{-6})\) and activity effect \((GI_{50} \text{ ratio: } 5.3)\), when restricted
to protein structure-damaging micro-deletions in the two longest mono-/ trinucleotide repeat sequences of the \textit{MSH3} coding sequence (Fig. 1A, B, Suppl. Fig. 2, 3A). Intriguingly, frameshift mutations in \textit{A\_} stretches occur frequently as somatic alterations of \textit{MSH3} in microsatellite-instable colorectal cancer (24-27). Concordantly, we detected such mutations in our cancer cell line panel; 10 out of 67 cell lines displayed micro-deletions in the two longest mono-/ trinucleotide repeat sequences (Figure 1A, Suppl. Fig. 3A). Together, our observations strongly suggest that DNA-PKcs inhibition might emerge as a novel therapeutic principle for the targeted treatment of \textit{MSH3}-defective cancer.

In our initial screen, we noticed that only two of a total of three \textit{BRCA1}-mutant cell lines were classified as KU60648-sensitive (H1838, H1563). To molecularly dissect this diverse pattern of compound sensitivity, we subjected the \textit{BRCA1}\textsuperscript{mut} cell lines H2347, H1563, H1838 to Sanger sequencing of all 23 \textit{BRCA1} exons (Suppl. Fig. S3B, C, D) and found that only the H1563 cell line carried homozygous \textit{BRCA1} mutations. Intriguingly, KU60648-sensitive H1838 cells did not show homozygous \textit{BRCA1} mutations. However, they carried an \textit{MSH3} mutation (Fig. 1A), which likely rationalizes their KU60648 sensitivity.

**DNA-PKcs inhibition induces apoptosis in \textit{MSH3}-mutant cells**

CTG assays revealed that \textit{MSH3}-mutant cell lines displayed a robust DNA-PKcs inhibitor response ($p_{\text{max}} = 7.81 \times 10^{-6}$, t-test) (Suppl. Fig. 1C). To address the characteristics of the apparent reduction of cell viability that we observed in \textit{MSH3}-mutant cells, we next quantified KU60648-induced cell cycle arrest
and apoptosis using immunoblotting (Fig. 2A) and flow cytometry (Fig. 2B, Suppl. Fig. 4). To this end, cell cycle profiles of a panel of 6 sensitive (HCC44, H1838, H1703, H2030, H1563, HCC2429) and three resistant (H2347, HCC1359, H1915) cell lines were longitudinally monitored at seven distinct time points under 1μM exposure of KU60648 (0, 6, 12, 24, 48, 72 and 96hrs). H1563 cells carrying homozygous protein-damaging BRCA1 mutations (Suppl. Fig. 3B-D), as well as HCC2429 cells that carry a Brd4Nut fusion were included as positive controls. Upon completion of drug exposure, cells were stained with propidium iodide (PI) and relative cellular DNA content was assessed by flow cytometry. As shown in Suppl. Fig. 4, all nine cell lines displayed an early prominent loss of the S-phase populations (6 and 12 hrs), followed by a subsequent decrease of cells with 4N DNA content (24hrs). Intriguingly, KU60648-resistant cell lines showed a remarkable reconstitution to the initial cell cycle profiles of untreated cells (Suppl. Fig. 4), strongly suggesting full cell cycle checkpoint recovery and restart of proliferation. In marked contrast, all KU60648-sensitive cells had a persistent loss of S- and G2/M populations (Suppl. Fig. 4), rationalizing the results of our CTG-based screen (Fig. 1A, Suppl. Fig. 1C). To validate these distinct cellular response patterns, we next employed immunoblotting (Fig. 2A). Exposure of the MSH3-mutant cell lines HCC44, H1838, HCT116, as well as the positive control HCC2429 to 1μM KU60648 (48hrs) resulted in a substantial loss of S-phase cyclin A2 expression, corroborating the persistent cell cycle arrest observed in our flow cytometry experiments (Fig. 2A, upper panel). In contrast, the MSH3-proficient control lines H2347, H1568, HCC1359 and H1915 displayed
continued cyclin A2 expression after 48-hour treatment with 1μM of KU60648 (Fig. 2A, lower panel).

We next asked whether the KU60648-sensitive cell lines also displayed signs of apoptotic cell death. Therefore, we used immunoblotting to detect cleaved caspase-3, as a marker for apoptosis. Consistent with the results of our initial screen, the sensitive cell lines HCC44, H1838, HCT116 and the positive control HCC2429 displayed cleavage of caspase-3 after KU60648 treatment (1μM, 48hr), indicating execution of apoptosis (Fig. 2A, upper panel). In marked contrast, no caspase-3 cleavage could be observed after KU60648 treatment (1μM, 48hr) in the MSH3-proficient control lines H2347, H1568, HCC1359 and H1915 (Fig. 2A, lower panel).

To further validate KU60648-induced apoptosis with an independent assay, we used flow cytometry (Fig. 2B) and stained cells with annexin-V and PI after 72 hr of KU60648 exposure (0, 0.1, 0.5 and 1μM). We note that resistant cells had shown full recovery of proliferation after 72 hr of sustained KU60648 treatment (Suppl. Fig. 4). As indicated by the appearance of a large annexin-V/PI double-positive population in the MSH3-mutant cells following KU60648 exposure, the reduced viability that we observed in the initial screen was likely attributable to massive apoptosis (Fig. 2B). Similar effects were observed in the positive control lines (HCC2429, Brd4Nut and H1563, BRCA1-mutant). In contrast, even 1μM of KU60648 did not result in any substantial apoptosis in MSH3-proficient control cells (p = 0.011) (Fig. 2B).

As KU60648 treatment has been reported to induce DSBs, we next asked whether the DSB-inducing anthracycline drug doxorubicin might synergistically enhance KU60648 activity in MSH3-mutant cells. For this
purpose, we examined the effect of 120 different concentration combinations of KU60648 and doxorubicin on 3 MSH3-mutant (HCT116, HCC44, H1838) and 3 control lines (H1915, H1568, HCC1359) (Suppl. Fig. 5A). We used Cell Titer Glo assays as readout for cellular viability after 48 and 96 hours of compound exposure. For each concentration, we compared the observed residual viability with the expected viability, assuming additive effects of both compounds (Bliss independence) (Suppl. Fig. 5A). We found that doxorubicin was active on most cells examined, without discriminating between MSH3-deficient and -proficient lines. However, the effect of doxorubicin (50nM - 250 nM) was synergistically enhanced by co-treatment with KU60648 (~1μM) for MSH3-deficient cell lines only; additive effects of both compounds were observed for the MSH3-proficient controls (Suppl. Fig. 5A).

We next compared apoptosis levels under co-treatment with KU60648 (1μM) and doxorubicin (0, 30, 100, 250, 1000nM) with induction of apoptosis under single-agent therapy. Again, we detected synergistic induction of apoptosis exquisitely for MSH3-deficient cell lines at low and variable concentrations of doxorubicin (Suppl. Fig. 5B).

In summary, our experiments underscore the functional relevance of the observations made in our high-throughput cell line-based screen. These data strongly suggest DNA-PKcs inhibition results in the apoptotic demise of MSH3-mutant cells (Fig. 2A, B, Suppl. Fig. 4). The cytotoxic effect of KU60648 on MSH3-mutant cells can be further increased by combination with low concentrations of doxorubicin. However, this combination is less discriminative between MSH3-deficient and -proficient cells. (Suppl. Fig. 6).
Genetic validation of the apparent synthetic lethality between *MSH3* and *
PRKDC*

As studies with ATP-competitive inhibitors are frequently hampered by off-
target effects, we next performed genetic experiments to functionally confirm
DNA-PKcs (encoded by *PRKDC*) as the target of KU60648 in *MSH3*-mutant
and *BRCA1*-defective cell lines (Fig. 2C, D, Suppl. Fig. 6, 7, 8). We used
RNA-interference (RNAi) to deplete DNA-PKcs in four sensitive and two
resistant control lines (Fig. 2C). Knockdown efficiency was confirmed by
immunoblotting (Suppl. Fig. 6). Confirming that repression of DNA-PKcs
expression, leads to the induction of apoptosis in *MSH3*-mutant settings, we
reproducibly detected cleavage of caspase-3 in KU60648-sensitive cell lines
(HCC44, HCC2429), 120 hours following viral shRNA delivery (Suppl. Fig. 6).
Next, we assessed the effects of RNAi-mediated repression of *PRKDC* in
colony formation assays (Fig. 2C). Two weeks after viral RNAi delivery, we
observed complete eradication of KU60648-sensitive HCC44, H1703,
HCC2429, and H1563 lines, whereas KU60648-resistant H1568 and
HCC1359 lines did not exhibit any signs of morphological change (Fig. 2C).
We next examined whether other components of the NHEJ pathway might be
similarly synthetically lethal with *MSH3*. DNA damage repair by NHEJ is
initiated by a complex formed by the proteins Ku70 (encoded by *XRCC6*) and
Ku80 (encoded by *XRCC5*). Hence, we compiled 5 shRNA constructs
specifically targeting Ku80 and transduced 4 independent cell lines (HCT116
[*MSH3*mut], HCC44 [*MSH3*mut], HCC1359 [cntrl], H1568 [cntrl]) with each of
these constructs. We assessed knockdown efficiency by immunoblotting
(Suppl. Fig. 7A), in order to choose two shRNAs (shXRCC5 #1, shXRCC5 #3), which effectively silenced expression of Ku80.

In colony formation assays, we observed mild growth-arrestive effects of Ku80 depletion in the MSH3-proficient cell lines, indicating that Ku80 is critical for cell survival in general (Suppl. Fig. 7B, C). In contrast, strong cytotoxic effects were detected in MSH3-deficient cells for both constructs targeting Ku80 (Suppl. Fig. 7B, C). Therefore, shRNA-mediated suppression of an independent NHEJ protein had similar cytotoxic effects in MSH3-deficient cells as knockdown of PRKDC.

Next, we compared the KU60648 response of Msh3-proficient and -deficient murine embryonic fibroblasts (MEFs) to validate Msh3 deficiency as a genetic determinant of DNA-PKcs addiction (Suppl. Fig 8A). In brief, we first assessed KU60648 potency in Msh3<sup>wt/wt</sup> and Msh3<sup>-/-</sup> MEFs using CTG assays under the same conditions as in our initial screen. As shown in Suppl. Fig. 8B, Msh3-deficient MEFs were significantly more sensitive to DNA-PKcs inhibition than their isogenic Msh3-proficient counterparts (<i>p = 9.33 \times 10^{-5}</i>). To further characterize the nature of this response, we assessed KU60648-induced apoptosis by immunoblotting and flow cytometry (Fig. 2D, Suppl. Fig. 8C). We observed caspase-3 cleavage (Suppl. Fig. 8C) and the appearance of an annexin-V/PI double-positive population (Fig. 2D) in Msh3-deficient MEFs, strongly suggesting that DNA-PKcs inhibition results in apoptosis in Msh3-defective settings. In stark contrast, neither caspase-3 cleavage, nor appearance of an annexin-V/PI double-positive, apoptotic population could be detected in Msh3-proficient MEFs (Fig. 2D, Suppl. Fig. 8C). Together, genetic repression of PRKDC in MSH3-mutant cells on the one hand (Fig. 2C, Suppl.
Fig. 6) and pharmacological inhibition of DNA-PKcs activity in Msh3-deficient cells on the other hand (Fig. 2D, Suppl. Fig. 8) cross-validated the proposed synthetic lethal interaction between these two genes. Further, the cytotoxic effects, which we observed in MSH3-mutant cells under suppression of Ku80 expression (Suppl. Fig. 7), might suggest a generalizable synthetic lethal interaction between the HR and NHEJ pathways.

**MSH3-defective cell lines display a defect in homologous recombination-based DSB repair**

Our initial screen revealed that alterations in HR-mediated DSB repair are associated with DNA-PKcs inhibitor sensitivity (Fig. 1, Suppl. Fig. 1, 2). Thus, we next asked whether HR defects were responsible for the DNA-PKcs addiction that we observed in MSH3-mutant cells. To this end, we transiently incubated MSH3-proficient (H1568, HCC1359 and Msh3\textsuperscript{wt/wt} MEFs), and -defective (HCC44, H1838, HCT116, RL95-2, H1703 and Msh3\textsuperscript{-/-} MEFs) cells with the DSB-inducing topoisomerase II inhibitor etoposide (0.1μM, 1hr pulse) to induce tractable DSBs. As BRCA1 loss-of-function had previously been shown to result in severely impaired HR-mediated DSB repair (7, 11), we also studied the BRCA1-mutant H1563 cell line (Fig. 3, Suppl. Fig. 9A).

To examine the DSB repair kinetics in our cell line panel, we used indirect immunofluorescence to monitor the persistence of etoposide-induced γH2AX nuclear foci as an established marker for unrepaired DSBs (1). We observed robust formation of γH2AX nuclear foci in all cell lines that were analyzed 4 hr following removal of etoposide (Fig. 3A, B, Suppl. Fig. 9A), whereas no
nuclear γH2AX foci could be detected in untreated cells. However, 72 hr after etoposide removal, these foci had disappeared in all cell lines (Fig. 3A, B and Suppl. Fig. 9A), suggesting that both MSH3-proficient and -defective, as well as BRCA1-mutant cells were capable of repairing etoposide-induced DSBs. In a parallel set of experiments, we stained these cells with an antibody detecting Rad51 nuclear foci (Fig. 3A, C and Suppl. Fig. 9A), which are a hallmark feature of ongoing HR-mediated DSB repair (11). Of note, BRCA1-deficient cells were previously shown to lack Rad51 foci formation in response to DSB-inducing agents (7, 11). As shown in Fig. 3 and Suppl. Fig. 9A the MSH3- and BRCA1-proficient H1568 and HCC1359, as well as wildtype MEFs displayed prominent nuclear Rad51 foci 4 hr after removal of etoposide, suggesting functional HR-mediated DSB repair. Nuclear Rad51 foci were not detectable in these cell lines 72 hr after etoposide removal, suggesting complete DSB repair. In marked contrast, Rad51 foci could not be detected within a 72 hr timeframe after etoposide removal in either the MSH3-defective (HCC44, H1838, HCT116, RL95-2, H1703 and Msh3-/- MEFs), or the BRCA1-mutant H1563 cells (p = 2.3 x 10^{-3}) (Fig. 3A, C, Suppl. Fig. 9A). These data strongly suggest that the HR-mediated repair of etoposide-induced DSBs is substantially impaired in MSH3-mutant cells. Similar effects have been previously described for BRCA1-deficiency (Fig. 3A, C and (11)).

**HR-deficiency rationalizes the synthetic lethality between MSH3 and PRKDC**

Given the substantial HR defect that we had observed in MSH3-mutant cells (Fig. 3, Suppl. Fig. 9A), we next hypothesized that pharmacological NHEJ
abrogation through DNA-PKcs inhibition might lead to the generation of persistent unrepaired DSBs in these cells. To directly test this hypothesis, we induced DSBs in HR-proficient (H1568, HCC1359 and Msh3<sup>wt/wt</sup> MEFs), and -defective (HCC44 [MSH3mut], H1838 [MSH3mut], HCT116 [MSH3mut], RL95-2 [MSH3mut], H1703 [MSH3mut], Msh3<sup>-/-</sup> MEFs and H1563 [BRCA1mut]) cells by applying an etoposide pulse (0.1μM, 1hr) in the absence or continued presence of 0.5μM KU60648. Cells were protected from premature apoptosis by addition of the irreversible pan-caspase inhibitor Z-VAD (10 μM), which was applied together with etoposide. Similar to the experiments detailed in Figure 3, we performed immunofluorescence to detect nuclear γH2AX and Rad51 foci (Fig. 4, Suppl. Fig. 9B). Recruitment of Rad51, the core component of the HR machinery, to DSBs requires prior resection of DNA ends to generate RPA-coated 3’-single-stranded DNA (ssDNA) overhangs (28). To monitor the occurrence of 3’-ssDNA repair intermediates in our cell line panel, we thus also included a set of experiments in which we monitored nuclear RPA1 foci, as a marker for single-stranded DNA (ssDNA). As shown in Fig. 4A-C and Suppl. Fig. 9B, 4 hr after etoposide removal prominent γH2AX foci could be detected in all cell lines, both in the absence or presence of KU60648. No γH2AX foci could be detected in the absence of KU60648 72 hr following etoposide removal. Similarly, we observed no γH2AX foci in the HR-proficient cells 72 hr after etoposide pulse treatment, even when KU60648 was present. In stark contrast and consistent with a severe DSB repair defect, γH2AX foci could be visualized 72 hr following etoposide removal in the HR-defective cells that were continuously exposed to KU60648. Intriguingly, the staining patterns for RPA1 foci were identical to
those observed for $\gamma$H2AX foci (Fig. 4A, D, E and Suppl. Fig. 9B). The persistent presence of RPA1 foci in HR-defective cells that were treated with KU60648 strongly suggests that DSBs are resected in these cells. However, the continued presence of $\gamma$H2AX foci indicates that DSBs cannot be repaired in HR-defective cells, when NHEJ is pharmacologically abrogated. In summary, our immunofluorescence data lend strong support to the hypothesis that MSH3-mutations result in an HR-defect. Impaired HR-proficiency renders MSH3-mutant cells dependent on functional DNA-PKcs-mediated NHEJ in order to repair DSBs.

In vivo validation of DNA-PKcs as an actionable target in MSH3-defective tumors

Human tumors frequently display an activated DDR, likely as a result of stalled replication forks and DSBs (29-31). These genotoxic lesions are thought to be the molecular equivalent of oncogene-induced replicative stress and unscheduled replication firing (29, 32). This presence of genotoxic stress in otherwise untreated human neoplastic lesions, led us to investigate whether endogenous DNA damage, specifically in HR-defective tumors, might offer a therapeutic window for the use of DNA-PKcs inhibitors, in vivo. The strong and robust effects that we observed in HR-defective MSH3-mutant cells under DNA-PKc inhibition in vitro (Fig. 1, 2), motivated us to further assess its efficiency as a single agent in vivo. To this end, we employed an NMRInu/nu xenograft mouse model to study therapeutic drug response, in vivo. In brief, we subcutaneously engrafted nude mice either with KRAS-driven, MSH3-mutant HCT116 cells or with MYC/HRASG12V double-transduced Msh3-
deficient MEFs (Fig. 5A-E, Suppl. Fig. 10A-F). As negative controls we used either KRAS-mutant A549 cells or MYC/HRAS<sup>G12V</sup> double-transduced isogenic Msh3-proficient MEFs (Fig. 5A-E, Suppl. Fig. 10A-F).

As described previously (14), we administered 40mg/kg doses of the DNA-PKcs inhibitor KU60648 twice daily (intraperitoneal injection). Intriguingly, we observed a substantial (final tumor volume: 31.5%) and significant (p = 3.1x10<sup>-4</sup>) (Fig. 5A, Suppl. Fig. 10A, E) tumor volume shrinkage for HCT116-driven tumors under KU60648 therapy within 14 days, whereas A549 control tumors were completely resistant and even showed continued volume gains under KU60648 therapy (Fig. 5B, Suppl. Fig. 10B, F). In order to further compare the tumor proliferation rate between both therapy groups, we stained tumor samples with Ki67-specific antibodies after 14-day therapy (Fig. 5E). Shrinkage of HCT116 tumors translated into complete eradication of the Ki67-positive cell fraction under KU60648 therapy. In marked contrast, the Ki67 staining of A549 tumors remained stable between control and therapy groups, indicating their maintained proliferation under therapy (Fig. 5E).

Tumors driven by MYC/HRAS<sup>G12V</sup> double-transduced MEFs displayed a more aggressive phenotype than the HCT116/A549-driven tumors (Fig. 5 C, D, Suppl. Fig. 10C, D). Thus, tumor volumes could only be followed for seven days before control animals had to be sacrificed. In spite of this highly aggressive growth behavior, therapy with KU60648 resulted in stable disease (final tumor volume: 109%) of Msh3<sup>−/−</sup> MEF-driven lesions, while we did not observe any significant therapeutic effect of KU60648 on Msh3<sup>wt/wt</sup> MEF-driven tumors (Fig. 5C, D, Suppl. Fig. 10C, D). In summary, our results strongly recommend DNA-PKcs as a promising drug target for rational design.
of personalized therapies of HR-defective neoplastic disease. Specifically the therapeutic effect on \textit{Msh3}-knockout MEFs and tumors derived from these cells confirms that loss-of-function mutations in \textit{MSH3} are genetic and functional predictors of DNA-PKcs inhibitor activity, \textit{in vivo}.

\textbf{Discussion}

\textbf{Alterations in HR signaling are associated with DNA-PKcs addiction}

Eukaryotic cells have evolved a plethora of DNA repair pathways, which together function to maintain genomic integrity of multicellular organisms (2). Perhaps not surprisingly, inactivating mutations in these DNA repair pathways are commonly observed in human tumors and are thought to fuel a \textit{`mutator phenotype'} (33-35). For instance, cancer genome re-sequencing data suggest that \textasciitilde{}50\% of high-grade serous ovarian carcinomas are HR-defective (36).

We have previously reported an actionable synthetic lethal interaction between the HR gene \textit{ATM} and the critical NHEJ gene \textit{PRKDC} (13, 14, 37). In addition, the combined knockout of \textit{ATM} and \textit{PRKDC} was recently shown to result in embryonic lethality at E7.5 in mice (12). Intriguingly, E7.5 is a developmental stage at which embryonic cells are hypersensitive to DNA damage (12). Based on these observations, we hypothesized that mutations in additional HR genes might be associated with a similar DNA-PKcs addiction.

In order to systematically decipher additional genetic aberrations that are associated with DNA-PKcs addiction, we first linked large-scale sequencing data (16) to high-throughput KU60648 activity profiling across 67 cancer cell
lines (Fig. 1). As reported recently (38), potency and selectivity of several compounds are underestimated, if analysis is restricted to their half maximal growth inhibitory concentrations (GI_{50}). Hence, we amended our interpretation of the cell line screen by Hill’s coefficients and nearest-neighbor distances (Suppl. Fig. 1). Using this approach, we found that mutations in genes involved in DNA repair ($p = 2.98 \times 10^{-12}$) significantly associated with KU60648 sensitivity. More precisely, we were able to confirm mutations in several genes with a known role in HR-mediated DSB repair, including \textit{BRCA1, BRCA2, ATM, CHEK2, RAD50, SMC2} and \textit{PAXIP}, to predict DNA-PKcs addiction (Fig. 1, Suppl. Fig. 2). Intriguingly, the therapeutic response of HR-defective cancer cells to KU60648 appeared to be independent of \textit{TP53} mutation status (Fig. 1A). This observation strongly suggests that DNA-PKcs inhibition in HR-defective tumors might be a viable therapeutic strategy to selectively target \textit{TP53}-defective lesions, which are typically resistant against most frontline anticancer agents, such as chemo- and radiation therapy.

To our surprise, we identified \textit{MSH3} as a strong determinant for KU60648 sensitivity, which we showed to be involved in HR-mediated DSB repair in follow-up experiments (Fig. 1-4). There is accumulating circumstantial evidence suggesting a role for the Msh2/Msh3 complex in DSB repair (39-43). For instance, RNAi-mediated \textit{MSH3} depletion has recently been shown to result in substantially delayed Rad51 loading after 2Gy ionizing radiation (25). Here, we demonstrate that \textit{MSH3} mutation or deficiency is associated with an HR defect due to impaired Rad51 loading (Fig. 3). More importantly, we link this \textit{MSH3}-deficiency-associated HR defect to a druggable DNA-PKcs addiction \textit{in vitro} (Fig. 2) and \textit{in vivo} (Fig. 5).
Therapeutically targeting the synthetic lethal interaction between *MSH3* and *PRKDC*

We genetically validated the synthetic lethal interaction between *MSH3* and *PRKDC* that emerged from our initial screen (Fig. 2). To this end, we showed that pharmacological DNA-PKcs inhibition in *Msh3* knockout MEFs resulted in the induction of massive apoptosis, compared to *Msh3*-proficient isogenic control cells. Conversely, RNAi-mediated repression of *PRKDC* resulted in apoptotic demise of *MSH3*-mutant cancer cells, while *MSH3*-proficient control cells were largely unaffected by *PRKDC* knockdown (Fig. 2, 6).

Functionally, we demonstrate that *Msh3* knockout results in substantially impaired HR-mediated DSB repair due to delayed Rad51 loading (Fig. 3). However, *MSH3*-deficiency did not completely abrogate etoposide-induced DSB repair. In fact, *MSH3*-defective cells remained capable of repairing etoposide-induced genotoxic lesions, likely through recruitment of alternative DSB repair pathways, such as NHEJ (Fig. 3, 6). However, pharmacological inhibition of the essential NHEJ kinase DNA-PKcs completely prevented etoposide-induced DSB repair and led to the generation of ssDNA repair intermediates, which have previously been shown to represent a chromatin structure that triggers apoptosis (6, 14, 44). Moreover, we observed early loss of S-phase for all cell lines under KU60648 treatment (Suppl. Fig. 4). However, only KU60648-resistant cells were able to repair KU60648-induced DNA damage and returned to normal cell cycle profiles within 48 hr. Together, these observations mechanistically rationalized the massive induction of apoptosis that we detected by immunoblotting and flow cytometry (Fig. 2).
Clinical Perspective

Our data reported here strongly suggest that the synthetic lethal interaction between the NHEJ kinase DNA-PKcs and multiple HR genes, including BRCA1, BRCA2, ATM, CHEK2, RAD50, SMC2 and PAXIP, might be therapeutically exploited in patients with HR-defective neoplastic disease. Thus, it might be desirable to include genetically-stratified patient cohorts into next generation clinical trials with DNA-PKcs inhibitors, such as CC-115, a dual mTOR/DNA-PKcs inhibitor, currently evaluated in phase I trials (45).

A recent study conducted a genome-scale analysis of 276 colorectal tumors and identified somatic MSH3 loss-of-function mutations in ~7% of all samples and 40% of all hypermutated, microsatellite-instable (MSI) tumors (46). Thus, disabling MSH3 mutations are present in a substantial fraction of colorectal cancer, which represents one of the most common cancer entities in the Western world. To the best of our knowledge, this is the first study that discovers a molecular liability in MSH3-mutant neoplastic disease that is amenable for pharmacological intervention both in vitro and in vivo. Thus, our findings might have direct therapeutic impact on the clinical care of patients suffering from MSH3-mutant MSI colorectal cancer. Furthermore, biopsies retrieved from MSI colorectal cancers should be both sequenced to determine MSH3 status, as well as stained for Rad51 foci, after short exposure to high-dose etoposide, in order to identify those patients, who are most likely to benefit from a DNA-PKcs inhibitor therapy.
Materials and methods

Cell lines and reagents

All human cell lines were obtained from ATCC (www.atcc.org) and cultured in RPMI or DMEM media, supplemented with 10% of FCS at 37°C in a humidified incubator supplied with 5% CO₂. Their identity was authenticated by genotyping (SNP 6.0 arrays, Affymetrix) and all cell lines were tested for infection with mycoplasma (MycoAlert, Lonza).

Compounds were purchased from Axon Medchem (KU60648) or Sigma Aldrich (etoposide, doxorubicin), dissolved in water or DMSO and stored as aliquots at -80°C or -20°C. Two independent lots were tested for each compound.

Retroviral packaging constructs pMDg and pMDg/p were a kind gift from T. Benzing. Plasmids containing shRNA targeting PRKDC (V2HS 233593) were obtained from J. B. Lazoro and retroviral plasmids for double transduction of MEFs (pBabe-MYC and pBabe-HRAS^{G12V}) were kindly provided by T. Brummelkamp. Plasmids containing shRNA targeting Ku80 (TRCN10468, TRCN18363, TRCN288701, TRCN295856, TRCN307986) were purchased from Sigma Aldrich.

Cell line-based screening

High-throughput cell line-based screening was performed as described previously (47). In brief, cell lines were plated in triplicates into sterile 96-well plates at 1.000 cells/well density and treated with 10 increasing concentrations (range: 1nM – 2μM) of KU60648 for 96 hr. Relative cell viability was determined by measuring the ATP-content (CellTiter-Glo,
Promega, USA) and normalizing it to the untreated control. Measurements were repeated, if half-maximal inhibitory concentrations (GI50) of triplicates differed by more than 10% or if the average GI50 value was lower than 400nM.

**Genetic compound activity prediction**

For calculation of GI50, concentration-viability curves were interpolated by logistic functions (R package “ic50” (48)). For each concentration (range: 150nM – 1μM) we calculated the distance to its nearest neighbor in the KU60648 screening activity profile, in order to infer a suitable GI50 threshold. We used this threshold to classify cells into KU60648-sensitive and -resistant lines.

We next annotated all cell lines, for which sequencing data were available in the CCLE database (16) (MAF files), by its protein coding mutations. We tested for each gene whether mutations were more frequent in the KU60648-sensitive cohort than in the group of resistant cell lines by Fisher’s exact test. Additionally, we calculated for each gene its sensitivity effect by comparing GI50 values between mutant vs. wildtype cell lines. For missense mutations, we predicted their functional effect on global protein structure by PolyPhen-2 algorithm (49).

**Xenograft mouse models**

All animal procedures were approved by the local animal protection committee and the local authorities. 6-10 week old male NMRInu/nu mice (CRL:NMRI-FOXN1 NU, Charles River) were subcutaneously engrafted with 5x10^6 tumor cells (HCT116, A549) or Myc/Ras double-transduced Msh3^-/- or
$Msh3^{wt/wt}$ murine embryonic fibroblasts (MEFs). The DNA-PKcs inhibitor KU60648 was dissolved in PBS at a final concentration of 6mg/ml for xenograft application.

Upon formation of palpable subcutaneous tumors, mice received intraperitoneal injections of either KU60648 (40mg/kg) or PBS twice daily. Perpendicular tumor diameters were assessed daily by an external caliper and tumor volumes were calculated by the modified ellipsoid formula ($V = \frac{1}{2} (\text{Length} \times \text{Width}^2)$). After 7 (double-transduced MEFs) or 14 (HCT116, A549) days of therapy, mice were sacrificed, subcutaneous tumors were resected and fixed in 4% formalin over night.

For further details we refer to Supplementary Methods.
References


Figure Legends

Figure 1. Biological activity of the DNA-PKcs inhibitor KU60648 associates with mutations in DNA repair genes.

A. Half-maximal growth inhibitory concentrations (GI<sub>50</sub>) of the DNA-PKcs inhibitor KU60648 are plotted for 67 cancer lines (lower panel). Aberration frequencies (CCLE database) of 13 genes in KU60648-sensitive (GI<sub>50</sub> < 400nM, green) vs. KU60648-resistant (red) cell lines are compared by Fisher’s exact test (right panel). For each cell line, the corresponding histology (upper panel), as well as the mutational status for 13 selected genes are shown (grid plot). Inset. GO-TERM-based analysis of damage-associated genes by Fisher’s exact test.

B. Volcano plot representation of a systematic association of mutations in 1,319 genes with DNA-PKcs inhibitor sensitivity (KU60648) across a panel of 94 cancer cell lines. For each gene, significance (Fisher’s exact test, y-axis) is plotted against the ratio of average GI<sub>50</sub> values of mutant vs. wildtype cell lines (y-axis). Circle sizes are proportional to the number of mutant cell lines included in the screen. Insets I-III are magnified views of ratio-sensitivity associations.

C. Functional clustering of sensitivity-associated mutations into the homologous recombination-mediated DNA repair pathway. Protein interactions are represented schematically; alterations, which are associated to DNA-PKcs inhibitor sensitivity, are highlighted in red.

Figure 2. Functional and genetic validation of KU60648 activity across MSH3-mutated and -deficient cell lines.
A. Induction of apoptosis after 72-hour exposure to KU60648 (0, 0.1, 0.5 and 1μM) in 16 cancer lines was assessed by flow cytometry (annexinV/PI double-positive populations). Error bars represent standard deviations of three independent experiments; significance was calculated by t-test. Values, which are significantly superior to control, are indicated (*). **Inset.** Exemplary dot plots of annexinV/PI double-positive apoptotic cell populations. 

B. Protein expression of cleaved-caspase-3, cyclin A2, γ-H2AX and β-actin was assessed in HCC2429 [Brd4Nut], HCC44 [MSH3mut], H1838 [MSH3mut], HCT116 [MSH3mut], H2347 [ctrl], H1568 [ctrl], HCC1359 [ctrl] and H1915 [ctrl] cells after 48-hour treatment with the DNA-PKcs inhibitor KU60648 (0, 0.1 and 1μM) by immunoblotting. Of note, not all bands were detected at the same membrane due to overlapping protein sizes. C. Representative morphology (100x magnification) of HCC44, HCC2429, H1568, H1703, H1563, H1568 and HCC1359 cells two weeks after transduction with viruses encoding either control or DNA-PKcs targeting shRNA. D. Induction of apoptosis by 1μM treatment with KU60648 (0, 24, 48, 72 and 96 hours) in Msh3−/− (blue) and Msh3wt/wt control (gray) MEFs as assessed by flow cytometry (annexinV/PI double-positive populations). Error bars display standard derivation of three independent experiments.

**Figure 3. MSH3-mutant or -deficient cells display a robust HR defect.**

A. DNA double-strand break repair kinetics were monitored (0hrs, 4hrs, 72hrs) after short (1hr) exposure to a low-dose (0.1μM) etoposide pulse. Representative immunofluorescence images (green: γ-H2AX or Rad51 nuclear foci, blue: DAPI counterstain) are shown for HCC44 [MSH3mut].
H1838 [MSH3\textsuperscript{mut}], HCT116 [MSH3\textsuperscript{mut}], RL952 [MSH3\textsuperscript{mut}], H1563 [BRCA1\textsuperscript{mut}] and H1568 [ctrl] cancer cell lines, as well as Msh3\textsuperscript{−/−} and Msh3\textsuperscript{wt/wt} MEFs. B. Box plot diagrams display quantification of γ-H2AX stains for the experiment shown in A. in 9 independent cancer cell lines. Significance values were derived from comparing γ-H2AX foci\textsuperscript{pos} cell counts (4hrs) by t-testing, n=3. C. Box plot diagrams representing the quantification of Rad51 foci for the experiment shown in A. in 9 independent cancer cell lines. Significance levels were determined by t-test, n=3.

Figure 4. HR-defective MSH3-mutant cells fail to repair DNA double-strand breaks, when DNA-PKcs is pharmacologically repressed. A. DNA double-strand break repair kinetics were monitored (4hrs, 72hrs) after short (1hr) expose to low-dose (0.1μM) pulses of etoposide and permanent DNA-PKcs inhibition (1μM KU60648). Representative immunofluorescence images (green: γ-H2AX, Rad51 or RPA1 foci, blue: DAPI counterstain) are shown for HCC44 [MSH3\textsuperscript{mut}], H1838 [MSH3\textsuperscript{mut}], HCT116 [MSH3\textsuperscript{mut}], RL952 [MSH3\textsuperscript{mut}], H1563 [BRCA1\textsuperscript{mut}] and H1568 [ctrl] cancer cell lines, as well as Msh3\textsuperscript{−/−} and Msh3\textsuperscript{wt/wt} MEFs. B, C, D, E. γ-H2AX (B, C) and RPA1 (D, E) stains (0, 4, 48, 72 hrs) of the experiment shown in A. were quantified (normalized to the maximum of each cell line) and interpolated by a generalized Hubbert function. Medians (y-axis) of interpolation curves (n=9 independent cell lines, n=3 biological replicates) are plotted against time after etoposide exposure (x-axis) for KU60648 sensitive (red) and resistant (blue) cell lines. Quartiles are shown as envelopes (dashed lines).
Figure 5. DNA-PKcs is a therapeutically amenable target in vivo

A-D. NMR<sup>nu/nu</sup> nude mice were engrafted with KRAS<sup>G13D</sup>-driven HCT116 [MSH3<sup>mut</sup>] (A), KRAS<sup>G12S</sup>-driven A549 [KRAS<sup>mut</sup>] (B) cancer cells, as well as MYC/RAS-transduced Msh3<sup>-/-</sup> (C) and Msh3<sup>wt/wt</sup> control (D) MEFs. Upon formation of palpable subcutaneous tumors, mice received intraperitoneal injections of KU60648 (red, 40 mg/kg, twice daily) or vehicle solution (blue) for 14 (A, B) or 7 (C, D) days, respectively. The volume of each tumor was determined through daily measurements with an external caliper and normalized to initial tumor volume. Relative tumor volumes (y-axis) are plotted against therapy time (x-axis). Error bars display standard derivation of at least seven independent tumors in each group. E. Tumors formed by HCT116 [MSH3<sup>mut</sup>] (top) or A549 [KRAS<sup>mut</sup>] (bottom) cells were stained with Ki67-specific antibodies after 14-day therapy with control (left) or KU60648 (right). Representative images (20x magnification) of at least five independent tumors are shown for each group.

Figure 6. A simplified model for DNA-PKcs addiction in HR-defective tumors.

DNA double-strand break repair pathways are extensively re-wired in HR-defective cancer cells to channel DNA repair towards NHEJ. HR-defective cells remain capable of repairing DNA double-strand breaks through the error-prone NHEJ pathway (upper panel). Pharmacological repression of NHEJ-mediated DNA double-strand break repair results in a failure to resolve double-strand breaks and leads to the accumulation of single-stranded DNA.
repair intermediates and ultimately to the apoptotic demise of DNA-PKcs inhibitor-treated HR-defective cells.
**Figure 3**

**A**

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**etoposide pulse**

**B**

![Box plot of γH2AX positive cells for resistant and sensitive cells over time.](image41)

**C**

![Box plot of Rad51 positive cells for resistant and sensitive cells over time.](image42)
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A. HCT116 [MSH3<sup>+</sup>]

B. A549 [KRAS<sup>mut</sup>]

C. MEF MSH3<sup>+/−</sup>

D. MEF MSH3<sup>++</sup>

E. Control versus KU60648

Image E shows immunohistochemical staining for MSH3 in HCT116 and A549 cells treated with control or KU60648.
Abortive HR

Abortive HR

Functional DSB repair

NHEJ dependence

DSB

DNA-PKcs inhibitor

Intercepted NHEJ

Catastrophic DSB repair
A functional cancer genomics screen identifies a druggable synthetic lethal interaction between MSH3 and PRKDC

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