A Functional Cancer Genomics Screen Identifies a Druggable Synthetic Lethal Interaction between $MSH3$ and $PRKDC$
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, and non-homologous end joining (NHEJ; ref. 2). MMR removes nucleotides that were mis-paired during replication, as well as insertion and deletion loops, which result from slippage during the replication of repetitive sequences. The NER pathway is used to repair helix-distorting lesions, whereas small chemical modifications of bases are removed by the BER machinery (2).

Specifically in response to DNA double-strand breaks (DSB), mammalian cells use 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 noncatalytic 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). Homologous recombination, 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 homologous recombination process, is DSB end resection to create a single-stranded 3’-overhang, which becomes rapidly coated with the single-strand-binding protein replication protein A (RPA) and provides a substrate for activation of the proximal DDR kinase ATR (6). During the ensuing steps of the homologous recombination process, RPA is replaced by RAD51, which is recruited to ssDNA in a BRCA1/BRCAl2/PALB2-dependent fashion (7). Once loaded onto ssDNA, RAD51 mediates the core reactions of the homologous recombination process, namely homology searching, strand exchange, and Holliday junction formation (5).

The homologous recombination pathway is indispensable for the maintenance of genomic integrity, and patients with heterozygous germline mutations in different homologous recombination 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 homologous recombination genes, including BRCA1, BRCA2, ATM, CHEK2, RAD50, RAD51C, and others, are recurrently somatically mutated in various different cancer entities (7).

Intriguingly, homologous recombination 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 PARP1 and the high-penetration 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 homologous recombination gene ATM and the NHEJ kinase gene PRKDC (12–14). Here, we propose that it is the homologous recombination 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 used 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 that 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

To identify genomic aberrations that are associated with non-oncogene addiction to the NHEJ kinase DNA-PKcs, we screened the specific DNA-PKcs inhibitor KU60648 (IC_{50} = 19 nmol/L; ref. 15) against a panel of 94 genomically well-annotated cancer cell lines (Supplementary Table S1), which covered a broad spectrum of cancer entities, histologic subtypes (Supplementary Fig. S1A, inset), and cancer-associated genomic aberrations (Fig. 1A and B). We used the BRD4-NUT-fused cell line HCC2249 as a positive control (Fig. 1A and Supplementary Table S1), 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 to 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 CellTiter-Glo assay.

To identify genomic aberrations that are associated with DNA-PKcs addiction, we systematically linked compound activity in 67 cell lines to mutation status of 1,319 cancer-associated genes on DNA-PKcs dependence (Supplementary Fig. S2). Although displaying strong effects (GI_{50} ratio > 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 clustered significantly with compound activity (Supplementary Fig. S2). In agreement with our initial hypothesis, a Gene Ontology (GO)-based analysis (23) revealed that disabling mutations in DNA repair genes were significantly (P = 2.98 × 10^{−12}) enriched in DNA-PKcs inhibitor-sensitive cell lines (Fig. 1A, inset). In particular, we observed an accumulation of homologous recombination genes, for which mutations were significantly associated with KU60648 sensitivity. The initial steps of the homologous recombination 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 of each process (Fig. 1C). These data suggest that homologous recombination deficiency in general represents a cancer-associated condition that displays synthetic lethality with the NHEJ kinase DNA-PKcs.

To our surprise, mutations in the MMR gene MSH3 (P = 9.53 × 10^{−4}) emerged among the most significant predictors of compound activity (Fig. 1B). Further analysis revealed that MSH3 mutations gained strongly in significance (P = 7.43 × 10^{−9}) and activity effect (GI_{50} ratio, 5.3) when restricted to protein structure–damaging microdeletions in the two longest mononucleotide/trinucleotide repeat sequences of the MSH3 coding sequence (Fig. 1A and B and Supplementary Figs. S2 and S3A). Intriguingly, frameshift mutations in A_{8} stretches occur frequently as somatic alterations of MSH3 in microsatellite-instable (MSI) colorectal cancer (24–27). Concordantly, we detected such mutations in our cancer cell line panel; 10 of 67 cell lines displayed microdeletions in the two longest mononucleotide/trinucleotide repeat sequences (Fig. 1A and Supplementary Fig. S3A). Together, our observations strongly suggest that DNA-PKcs inhibition might emerge as a novel therapeutic principle for the targeted treatment of MSH3-defective cancer.

In our initial screen, we noticed that only two of a total of three BRCA1-mutant cell lines were classified as KU60648-sensitive (H1838 and H1563). To molecularly dissect this diverse...
Non-oncogene Addiction to DNA-PKcs in Homologous Recombination–Defective Cancers

pattern of compound sensitivity, we subjected the BRCA1\textsuperscript{mut} cell lines H2347, H1563, and H1838 to Sanger sequencing of all 23 BRCA1 exons (Supplementary Fig. S3B–S3D) and found that only the H1563 cell line carried homozygous BRCA1 mutations. Intriguingly, KU60648-sensitive H1838 cells did not show homozygous BRCA1 mutations. However, they carried an MSH3 mutation (Fig. 1A), which likely rationalizes their KU60648 sensitivity.

**DNA-PKcs Inhibition Induces Apoptosis in MSH3-Mutant Cells**

The CellTiter-Glo assays revealed that MSH3-mutant cell lines displayed a robust DNA-PKcs inhibitor response ($P_{\text{max}} = 7.81 \times 10^{-6}$, t-test; Supplementary Fig. S1C). To address the characteristics of the apparent reduction of cell viability that we observed in MSH3-mutant cells, we next quantified KU60648-induced cell-cycle arrest and apoptosis using immunoblotting (Fig. 2A) and flow cytometry (Fig. 2B and Supplementary Fig. S4). To this end, cell-cycle profiles of a panel of six sensitive (HCC44, H1838, H1703, H2030, H1563, and HCC2429) and three resistant (H2347, HCC1359, and H1915) cell lines were longitudinally monitored at seven distinct time points under 1 \textmu mol/L exposure of KU60648 (0, 6, 12, 24, 48, 72, and 96 hours). H1563 cells carrying homozygous protein-damaging BRCA1 mutations (Supplementary Fig. S3B–S3D), as well as HCC2429 cells that

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Figure 1. Biologic activity of the DNA-PKcs inhibitor KU60648 associates with mutations in DNA repair genes. A, GI\textsubscript{50}s of the DNA-PKcs inhibitor KU60648 are plotted for 67 cancer lines (bottom). Aberration frequencies (Cancer Cell Line Encyclopedia database) of 13 genes in KU60648-sensitive (GI\textsubscript{50} < 400 nmol/L; green) versus KU60648-resistant (red) cell lines are compared by the Fisher exact test (right). For each cell line, the corresponding GI\textsubscript{50} ratio-sensitivity associations. 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 exact test, y-axis) is plotted against the ratio of average GI\textsubscript{50} values of mutant versus wild-type 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 (HR)–mediated DNA repair pathway. Protein interactions are represented schematically; alterations, which are associated with DNA-PKcs inhibitor sensitivity, are highlighted in red. Str, short tandem repeat.
carry a **BRD4–NUT** 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 Supplementary Fig. S4, all nine cell lines displayed an early prominent loss of the S-phase populations (6 and 12 hours), followed by a subsequent decrease of cells with 4N DNA content (24 hours). Intriguingly, KU60648-resistant cell lines showed a remarkable reconstitution to the initial cell-cycle profiles of untreated cells (Supplementary Fig. S4), 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 (Supplementary Fig. S4), corroborating the persistent cell-cycle arrest observed in our flow cytometry experiments (Fig. 2A, top). In contrast, the **MSH3**-proficient control lines H2347, H1568, HCC1359, and H1915 displayed continued cyclin A2 expression after 48-hour treatment with 1 μmol/L of KU60648 (Fig. 2A, bottom).

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, and HCT116 and the distinct cellular response patterns, we next used immunoblotting (Fig. 2A). Exposure of the **MSH3**-mutant cell lines HCC44, H1838, and HCT116, as well as the positive control HCC2429 to 1 μmol/L KU60648 (48 hours), 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, top). In contrast, the **MSH3**-proficient control lines H2347, H1568, HCC1359, and H1915 displayed continued cyclin A2 expression after 48-hour treatment with 1 μmol/L of KU60648 (Fig. 2A, bottom).

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**Figure 2.** Functional and genetic validation of KU60648 activity across **MSH3**-mutated and **Msh3**-deficient cell lines. **A,** induction of apoptosis after 72-hour exposure to KU60648 (0, 0.1, 0.5, and 1 μmol/L) in 16 cancer lines was assessed by flow cytometry (annexin-V/PI double-positive populations). Error bars, SDs of three independent experiments; significance was calculated by t test. * values that are significantly superior to control. Inset, exemplary dot plots of annexin-V/PI double-positive apoptotic cell populations. **B,** protein expression of cleaved caspase-3, cyclin A2, γH2AX, and β-actin was assessed in HCC2429 [**BRD4–NUT**], HCC44 [**MSH3**−/−], H1838 [**MSH3**−/−], HCT116 [**MSH3**−/−], H2347 [ctrl], H1568 [ctrl], HCC1359 [ctrl], and H1915 [ctrl] cells after 48-hour treatment with the DNA-PKcs inhibitor KU60648 (0, 0.1, and 1 μmol/L) by immunoblotting. Of note, not all bands were detected at the same membrane due to overlapping protein sizes. **C,** representative morphology (×100 magnification) of the HCC44, HCC2429, H1568, H1703, H1563, H1568, and HCC1359 cell lines 2 weeks after transduction with viruses encoding either control or DNA-PKcs targeting shRNA. **D,** induction of apoptosis by 1 μmol/L treatment with KU60648 (0, 24, 48, 72, and 96 hours) in Msh3−/− (blue) and Msh3+/+ control (gray) murine embryonic fibroblasts as assessed by flow cytometry (annexin-V/PI double-positive populations). Error bars, SD of three independent experiments.
positive control HCC2429 displayed cleavage of caspase-3 after KU60648 treatment (1 μmol/L, 48 hours), indicating execution of apoptosis (Fig. 2A, top). In marked contrast, no caspase-3 cleavage could be observed after KU60648 treatment (1 μmol/L, 48 hours) in the MSH3-proficient control lines H2347, H1568, HCC1359, and H1915 (Fig. 2A, bottom).

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 hours of KU60648 exposure (0, 0.1, 0.5, and 1 μmol/L). We note that the resistant cells had shown full recovery of proliferation after 72 hours of sustained KU60648 treatment (Supplementary Fig. S4). 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 (HCC44, BRD4–NUT, and H1563, BRCA1-mutant). In contrast, even 1 μmol/L 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 DSBinducing 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 three MSH3-mutant cell lines (HCT116, H1838, and HCC44, Supplementary Fig. S5A). We used CellTiter-Glo assays as read-out 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; Supplementary Fig. S5A).

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Next, we compared apoptosis levels under cotreatment with KU60648 (1 μmol/L) and doxorubicin (0, 30, 100, 250, and 1,000 nmol/L) with induction of apoptosis under single-agent therapy. Again, we detected synergistic induction of apoptosis exclusively for MSH3-deficient cell lines at low and variable concentrations of doxorubicin (Supplementary Fig. S5B).

In summary, our experiments underscore the functional relevance of the observations made in our high-throughput cell line-based screen. These data strongly suggest that DNA-PKcs inhibition results in the apoptotic demise of MSH3-mutant cells (Fig. 2A and B and Supplementary Fig. S4). 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 (Supplementary Fig. S6).

**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 and D and Supplementary Figs. S6–S8). 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 (Supplementary Fig. S6). 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 and HCC2429), 120 hours following viral short hairpin RNA (shRNA) delivery (Supplementary Fig. S6). 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 morphologic 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 five shRNAs constructs specifically targeting KU80 and transduced four independent cell lines (HCT116 [MSH3mut], HCC44 [MSH3mut], HCC1359 [ctrl], and H1568 [ctrl]) with each of these constructs. We assessed knockdown efficiency by immunoblotting (Supplementary Fig. S7A) to choose two shRNAs (shXRCC5 #1 and shXRCC5 #3) that 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 (Supplementary Fig. S7B and S7C). In contrast, strong cytotoxic effects were detected in MSH3-deficient cells for both constructs targeting KU80 (Supplementary Fig. S7B and S7C). 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 Msh3-deficient murine embryonic fibroblasts (MEF) to validate Msh3 deficiency as a genetic determinant of DNA-PKcs addiction (Supplementary Fig. S8A). In brief, we first assessed KU60648 potency in Msh3mut and Msh3+/− MEFs using CellTiter-Glo assays under the same conditions as in our initial screen. As shown in Supplementary Fig. S8B, Msh3-deficient MEFs were significantly more sensitive to DNA-PKcs inhibition than their isogenic Msh3-proficient counterparts (P = 9.33 × 10−3). To further characterize the nature of this response, we assessed KU60648-induced apoptosis by immunoblotting and flow cytometry (Fig. 2D and Supplementary Fig. S8C). We observed caspase-3 cleavage (Supplementary Fig. S8C) 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 and Supplementary Fig. S8C). Together, genetic repression of PRKDC in MSH3-mutant cells, on the one hand (Fig. 2C and Supplementary Fig. S6), and pharmacologic inhibition of DNA-PKcs activity in
Msh3-deficient cells, on the other hand (Fig. 2D and Supplementary Fig. S8), cross-validated the proposed synthetic lethal interaction between these two genes. Furthermore, the cytotoxic effects, which we observed in MSH3-mutant cells under suppression of KU80 expression (Supplementary Fig. S7), might suggest a generalizable synthetic lethal interaction between the homologous recombination and NHEJ pathways.

**MSH3-Defective Cell Lines Display a Defect in Homologous Recombination-Based DSB Repair**

Our initial screen revealed that alterations in homologous recombination-mediated DSB repair are associated with DNA-PKcs inhibitor sensitivity (Fig. 1 and Supplementary Figs. S1 and S2). Thus, we next asked whether homologous recombination 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+/- MEFs) and MSH3-defective (HCC44, H1838, HCT116, RL95-2, H1703, and Msh3−/− MEFs) cells with the DSB-inducing topoisomerase II inhibitor etoposide (0.1 μmol/L, 1-hour pulse) to induce tractable DSBs. As BRCA1 loss-of-function had previously been shown to result in severely impaired homologous recombination-mediated DSB repair (7, 11), we also studied the BRCA1-mutant H1563 cell line (Fig. 3 and Supplementary Fig. S9A).

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 hours following removal of etoposide (Fig. 3A and B and Supplementary Fig. S9A), whereas no nuclear γH2AX foci could be detected in untreated controls. However, 72 hours after etoposide removal, these foci had disappeared in all cell lines (Fig. 3A and B and Supplementary Fig. S9A), suggesting that both MSH3-proficient and MSH3-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 and C and Supplementary Fig. S9A), which are a hallmark feature of ongoing homologous recombination-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 Supplementary Fig. S9A, the MSH3- and BRCA1-proficient H1568 and HCC1359, as well as wild-type MEFs, displayed prominent nuclear RAD51 foci 4 hours after the removal of etoposide, suggesting functional homologous recombination-mediated DSB repair. Nuclear RAD51 foci were not detectable in these cell lines 72 hours after etoposide removal, suggesting complete DSB repair. In marked contrast, RAD51 foci could not be detected within a 72-hour time frame 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 × 10−3; Fig. 3A and C and Supplementary Fig. S9A). These data strongly suggest that the homologous recombination-mediated repair of etoposide-induced DSBs is substantially impaired in MSH3-mutant cells. Similar effects have been previously described for BRCA1 deficiency (Fig. 3A and C; ref. 11).

**Homologous Recombination Deficiency Rationalizes the Synthetic Lethality between MSH3 and PRKDC**

Given the substantial homologous recombination defect that we had observed in MSH3-mutant cells (Fig. 3 and Supplementary Fig. S9A), we next hypothesized that pharmacologic 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 homologous recombination-proficient (H1568, HCC1359, and Msh3+/- MEFs) and homologous recombination-defective (HCC44 [MSH3mut], H1838 [MSH3mut], HCT116 [MSH3mut], RL95-2 [MSH3mut], H1703 [MSH3mut], Msh3−/− MEFs, and H1563 [BRCA1mut]) cells by applying an etoposide pulse (0.1 μmol/L, 1 hour) in the absence or continued presence of 0.5 μmol/L of KU60648. Cells were protected from premature apoptosis by the addition of the irreversible pan-caspase inhibitor Z-VAD (10 μmol/L), which was applied together with etoposide. Similar to the experiments detailed in Fig. 3, we performed immunofluorescence to detect nuclear γH2AX and RAD51 foci (Fig. 4 and Supplementary Fig. S9B). Recruitment of RAD51, the core component of the homologous recombination machinery, to DSBs requires prior resection of DNA ends to generate RPA-coated 3′ 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 ssDNA. As shown in Fig. 4A–C and Supplementary Fig. S9B, 4 hours after etoposide removal, prominent γH2AX foci could be detected in all cell lines, in both the absence or presence of KU60648. No γH2AX foci could be detected in the absence of KU60648 72 hours following etoposide removal. Similarly, we observed no γH2AX foci in the homologous recombination-proficient cells 72 hours 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 hours following etoposide removal in the homologous recombination-defective cells that were continuously exposed to KU60648. Intriguingly, the staining patterns for RPA1 foci were identical to those observed for γH2AX foci (Fig. 4A, D, and E and Supplementary Fig. S9B). The persistent presence of RPA1 foci in homologous recombination-defective cells that were treated with KU60648 strongly suggests that DSBs are resected in these cells. However, the continued presence of γH2AX foci indicates that DSBs cannot be repaired in homologous recombination-defective cells when NHEJ is pharmacologically abrogated. In summary, our immunofluorescence data lend strong support to the hypothesis that MSH3 mutations result in a homologous recombination defect. Impaired homologous recombination proficiency renders MSH3-mutant cells dependent on functional DNA-PKcs-mediated NHEJ 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
Figure 3. MSH3-mutant or MSH3-deficient cells display a robust homologous recombination defect. A, DNA DSB repair kinetics were monitored (0, 4, and 72 hours) after short (1 hour) exposure to a low-dose (0.1 μmol/L) etoposide pulse. Representative immunofluorescence images [green, γ-H2AX or RAD51 nuclear foci; blue, 4′,6-diamidino-2-phenylindole (DAPI) counterstain] are shown for HCC44 [MSH3mut], H1838 [MSH3mut], HCT116 [MSH3mut], RL95-2 [MSH3mut], H1563 [BRCA1mut], and H1568 [ctrl] cancer cell lines, as well as Msh3−/− and Msh3wt/wt MEFs. B, box plot diagrams display the quantification of γ-H2AX stains for the experiment shown in A in 9 independent cancer cell lines. Significance values were derived from comparing γ-H2AXfoci-positive cell counts (4 hours) by t test; n = 3. C, box plot diagrams representing the quantification of RAD51 foci for the experiment shown in A in nine independent cancer cell lines. Significance levels were determined by t test; n = 3.
Figure 4. Homologous recombination–defective MSH3-mutant cells fail to repair DNA DSBs when DNA-PKcs is pharmacologically repressed. A, DNA DSB repair kinetics were monitored (4 and 72 hours) after short (1 hour) exposure to low-dose (0.1 μmol/L) pulses of etoposide and permanent DNA-PKcs inhibition (1 μmol/L KU60648). Representative immunofluorescence images [green, γ-H2AX, RAD51, or RPA1 foci; blue, 4′,6-diamidino-2-phenylindole (DAPI) counterstain] are shown for HCC44 [MSH3mut], H1838 [MSH3mut], HCT116 [MSH3mut], RL95-2 [MSH3mut], H1563 [BRCA1mut], and H1568 [ctrl] cancer cell lines, as well as Msh3−/− and Msh3wt/wt MEFs. 

A–E, γ-H2AX (B and C) and RPA1 (D and E) stains (0, 4, 48, and 72 hours) 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 biologic replicates) are plotted against time after etoposide exposure (x-axis) for KU60648-sensitive (red) and KU60648-resistant (blue) cell lines. Quartiles are shown as envelopes (dashed lines).
investigate whether endogenous DNA damage, specifically in homologous recombination–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 homologous recombination–defective MSH3-mutant cells under DNA-PKcs inhibition in vitro (Figs. 1 and 2) motivated us to further assess its efficiency as a single agent in vivo. To this end, we used an NMRI nu/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 and Supplementary Fig. S10A–S10F). For negative controls, we used either KRAS-mutant A549 cells or MYC/HRASG12V double-transduced Msh3-proficient MEFs (Fig. 5A–E and Supplementary Fig. S10A–S10F).

As described previously (14), we administered 40 mg/kg doses of the DNA-PKcs inhibitor KU60648 twice daily (intra-peritoneal injection). Intriguingly, we observed a substantial (final tumor volume, 31.5%) and significant ($P = 3.1 \times 10^{-4}$; Fig. 5A and Supplementary Fig. S10A and S10E) 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 and Supplementary Fig. S10B and S10F). 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/HRASG12V double-transduced MEFs displayed a more aggressive phenotype than the HCT116/
A549–driven tumors (Fig. 5C and D and Supplementary Fig. S10C and S10D). Thus, tumor volumes could be followed for only 7 days before control animals had to be sacrificed. Despite this highly aggressive growth behavior, therapy with KU60648 resulted in stable disease (final tumor volume, 109%) of Mdb3−/− MEF-driven lesions, whereas we did not observe any significant therapeutic effect of KU60648 on Mdb3−/− MEF-driven tumors (Fig. 5C and D and Supplementary Fig. S10C and S10D). In summary, our results strongly recommend DNA-PKcs as a promising drug target for the rational design of personalized therapies for homologous recombination–defective neoplastic disease. Specifically, the therapeutic effect on Msh3−/− MEFs and tumors derived from these cells confirms that loss-of-function mutations in MSH3 are genetic and functional predictors of DNA-PKcs inhibitor activity in vivo.

**DISCUSSION**

**Alterations in Homologous Recombination 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 “mutator phenotype” (33–35). For instance, cancer genome resequencing data suggest that approximately 50% of high-grade serous ovarian carcinomas are homologous recombination defective (36).

We have previously reported an actionable synthetic lethal interaction between the homologous recombination gene ATM and the critical NHEJ gene PRKDC (13, 14, 37). In addition, the combined knockout of ATM and 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). On the basis of these observations, we hypothesized that mutations in additional homologous recombination genes might be associated with a similar DNA-PKcs addiction.

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 (GI50). Hence, we amended our interpretation of the cell line screen by Hill coefficients and nearest-neighbor distances (Supplementary Fig. S1). Using this approach, we found that mutations in genes involved in DNA repair (P = 2.98 × 10−12) significantly associated with KU60648 sensitivity. More precisely, we were able to confirm mutations in several genes with a known role in homologous recombination–mediated DSB repair, including BRCA1, BRCA2, ATM, CHEK2, RAD50, SMC2, and PAXIP, to predict DNA-PKcs addiction (Fig. 1 and Supplementary Fig. S2). Intriguingly, the therapeutic response of homologous recombination–defective cancer cells to KU60648 seemed to be independent of TP53 mutation status (Fig. 1A). This observation strongly suggests that DNA-PKcs inhibition in homologous recombination–defective tumors might be a viable therapeutic strategy to selectively target TP53-defective lesions, which are typically resistant against most first-line anticancer agents, such as chemotherapy and radiotherapy.

To our surprise, we identified MSH3 as a strong determinant for KU60648 sensitivity, which we showed to be involved in homologous recombination–mediated DSB repair in follow-up experiments (Figs. 1–4). There is accumulating circumstantial evidence suggesting a role for the MSH2–MSH3 complex in DSB repair (39–43). For instance, RNAi-mediated MSH3 depletion has recently been shown to result in substantially delayed RAD51 loading after 2-Gy ionizing radiation (25). Here, we demonstrate that MSH3 mutation or deficiency is associated with a homologous recombination defect due to impaired RAD51 loading (Fig. 3). More importantly, we link this MSH3 deficiency–associated homologous recombination defect to a druggable DNA-PKcs addiction in vitro (Fig. 2) and 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 pharmacologic DNA-PKcs inhibition in Mdb3−/− MEFs resulted in the induction of massive apoptosis, compared with Mdb3−/− proficient isogenic control cells. Conversely, RNAi-mediated repression of PRKDC resulted in apoptotic demise of MSH3−/− mutant cancer cells, whereas MSH3-proficient control cells were largely unaffected by PRKDC knockdown (Figs. 2 and 6).

Functionally, we demonstrate that MSH3 knockout results in substantially impaired homologous recombination–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 (Figs. 3 and 6). However, pharmacologic 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 (Supplementary Fig. S4). However, only KU60648-resistant cells were able to repair KU60648-induced DNA damage and returned to normal cell-cycle profiles within 48 hours. 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 homologous recombination genes, including BRCA1, BRCA2, ATM, CHEK2, RAD50, SMC2 and PAXIP, might be therapeutically exploited in patients with homologous recombination–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 being 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
Abortive HR

Figure 6. A simplified model for DNA-PKcs addiction in homologous recombination (HR)-defective tumors. DNA DSB repair pathways are extensively rewired in homologous recombination-defective cancer cells to channel DNA repair toward NHEJ. Homologous recombination-defective cells remain capable of repairing DNA DSBs through the error-prone NHEJ pathway (top). Pharmacologic repression of NHEJ-mediated DNA DSB repair results in a failure to resolve DSBs and leads to the accumulation of ssDNA repair intermediates and ultimately to the apoptotic demise of DNA-PKcs inhibitor-treated homologous recombination-defective cells.
mutations in approximately 7% of all samples and 40% of all hypermutated, 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 pharmacologic 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 and stained for RAD51 foci, after short exposure to high-dose etoposide, to identify those patients who are most likely to benefit from a DNA-PKcs inhibitor therapy.

METHODS

Cell Lines and Reagents

All human cell lines were obtained from the American Type Culture Collection (www.atcc.org) and cultured in RPMI or Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% of fetal calf serum (FCS) at 37°C in a humidified incubator supplied with 5% CO2. Cell lines were 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 and doxorubicin), dissolved in water or dimethyl sulfoxide (DMSO), and stored as aliquots at −80°C or −20°C or DMSO twice daily. Perpendicular tumor diameters were assessed daily by a external caliper, and tumor volumes were calculated by the modified ellipsoid formula [V = 1/2 (length × width2)]. After 7 (double-transduced MEFs) or 14 (HCT116, A549) days of therapy, mice were sacrificed and subcutaneous tumors were resected and then fixed in 4% formalin overnight.

For further details, please refer to the Supplementary Methods.

Cell Line–Based Screening

High-throughput cell line-based screening was performed as described previously (47). In brief, cell lines were plated in triplicate into sterile 96-well plates at 1,000 cells per well density and treated with 10 increasing concentrations (range, 1 nmol/L–2 μmol/L) of KU60648 for 96 hours. Relative cell viability was determined by measuring the ATP content (CellTiter-Glo; Promega) and normalizing it using intracellular ATP content of DMSO-treated cells as 100%. We calculated for each gene its sensitivity effect by comparing GI50 values between mutant versus wild-type cell lines. For missense mutations, we predicted their functional effect on global protein structure by the PolyPhen-2 algorithm (49).

Xenograft Mouse Models

All animal procedures were approved by the local animal protection committee and the local authorities. Six- to 10-week-old male NMRI nu/nu mice (CRL:NMRI-FOXN1 NU; Charles River Laboratories) were subcutaneously engrafted with 5 × 106 tumor cells (HCT116, A549) or MYC/RAS double-transduced Msh3−/− or Msh3+/+ MEFs. The DNA-PKcs inhibitor KU60648 was dissolved in PBS at a final concentration of 6 mg/mL for xenograft application.

Upon the formation of palpable subcutaneous tumors, mice received intraperitoneal injections of either KU60648 (40 mg/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 = 1/2 (length × width2)]. After 7 (double-transduced MEFs) or 14 (HCT116, A549) days of therapy, mice were sacrificed and subcutaneous tumors were resected and then fixed in 4% formalin overnight.

Disclosure of Potential Conflicts of Interest

L.C. Heukamp has received honoraria from the speakers’ bureau of Pfizer and Roche. H.C. Reinhardt has received honoraria from the speakers’ bureau of Celgene. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


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