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Ausnutzung molekularer Defekte in der *DNA damage response ATM*-mutierter Zellen durch gezielte Therapie einer starken *non-oncogene addiction* von *PRKDC*

Exploiting molecular defects in the DNA damage response of *ATM* mutated cells via therapeutic targeting of a robust non-oncogene addiction to *PRKDC*

Inaugural-Dissertation zur Erlangung der Doktorwürde

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Glossary

ATM: *Ataxia telangiectasia-mutated.*

AT: Ataxia telangiectasia.

BRCA1: Breast cancer 1.

BRCA2: Breast cancer 2.

druggable: Referring to a molecular species, such as a protein, that has the structural and functional properties suggesting that low-molecular-weight therapeutic compounds can be developed that specifically interact with and perturb its function (85, 108).

DDR: DNA damage response.

DNA: Deoxyribonucleic acid.

DNA-PKcs: DNA-dependent protein kinase, catalytic subunit; encoded by *PRKDC*.

DSB: Double-strand break.

dsDNA: Double-stranded DNA.

E3 ubiquitin ligase: Together with ubiquitin-activating (E1s) enzymes and ubiquitin-conjugating enzymes (E2s), they perform ubiquitylation, targeting proteins for degradation by the 26S proteasome (71).

EGFR: Epidermal growth factor receptor. The epidermal growth factor receptor is member of the ErbB (HER) family receptor tyrosine kinases (31). EGFR regulates cell growth and differentiation and is mutated in many human malignancies (31).

genotoxic: Referring to an agent that is capable of damaging the genome, i.e., is mutagenic (73, 108).

HR: Homologous recombination. Type of DNA repair for double stranded breaks which is largely restricted to the S- and G_2-phases of the cell cycle using sequences in a sister chromatid as repair template and thus is error-free (15).

KRAS: Kirsten Rat Sarcoma virus. *KRAS* is one of the most frequently activated oncogenes, with 17 - 25% of all human tumors harboring an activating mutation in the gene (60). The encoded protein KRAS is a

GDP/GTP-binding protein that in its wildtype form acts as a self-inactivating signal transducer (60).

NHEJ: Nonhomologous endjoining. Type of DNA repair for double stranded breaks consisting of fusion of two dsDNA ends involving the protein DNA-PK (consisting of Ku70, Ku80 and DNA-PKcs). The joining of the two ends is not informed or directed by sequences in a sister chromatid or homologous chromosome. Therefore, it is error prone (108).

NSCLC: Non-small cell lung cancer.

oncogene: A gene that, upon alteration by DNA-damaging agents or viral genomes, can acquire the ability to induce cancer (108).

p53: tumor suppressor encoded by the tumor suppressor gene *TP53*. Because of its outstanding role in the DNA damage response it is described as “the guardian of the genome” (62).

PARP1: Poly(ADP-ribose) polymerase 1.

shRNA: Short hairpin RNA.

SSB: Single-strand break.

ssDNA: Single-stranded DNA.

TSG: Tumor suppressor gene. A gene, which is responsible for constraining cell proliferation. The partial or complete inactivation of such a gene, occurring in either germ line or the genome of a somatic cell, leads to an increased likelihood of cancer development (108).

Introduction

Cancer

Cancer is a heterogeneous group of diseases, which can arise from nearly every type of tissue (108). It is characterized by an uncontrolled hyperproliferation of cells (108). The cancerous cells can invade other tissues *per continuitatem* or spread into more distant organs via the lymphatic system or the blood stream, the latter referred to as metastasis (108). In contrast, benign tumors lack these two invasive properties (85, 108).

In 2008, an estimated 12.7 million people suffered from cancer and an estimated 7.6 million people died due to a malignancy, worldwide (52). In economically developed countries, cancer is the leading cause of death (52). In economically developing countries, the incidence of cancer is only half of the incidence in the developed world, but as a consequence of later diagnosis and limited access to medical services, survival is worse and here cancer is the second leading cause of death (52). Not only because of the aging society, but also because of widespread cancer-promoting behaviors, notably smoking, physical inactivity and Western diet, the incidence of cancer increased continuously during the last decades (9). With a predicted incidence of 22.2 million cases worldwide the incidence of cancer will be nearly the double by 2030 (9).

Traditionally, four distinct treatment strategies for cancer have been pursued, namely surgery (mostly for limited disease), immunotherapy, radiation and chemotherapy (5, 14, 108). The latter two are thought to induce genotoxic stress, ultimately leading to the demise of cancer cells (5, 81, 108).

Due to a lack of knowledge and understanding of the molecular nuts and bolts of this disease, cancer has been treated mostly with relatively unspecific drugs for a long time (14). Most of these drugs, often referred to as "classical chemotherapy", have been found by their capability of harming rapidly dividing tissues by causing genotoxic stress (14, 56, 81). But as rapid proliferation is a trait that is not specific for cancerous cells, also healthy tissues with high cell turnover, such as bone-marrow haematopoietic precursors and

gastrointestinal mucosal epithelial cells, get damaged (56, 81, 108). This leads to serious and dose-limiting side effects, like myelosupression or uncontrollable diarrhea, respectively (81). Furthermore, chemotherapeutics often have characteristic tissue-specific side effects due to their molecular mechanism of action or chemical properties. The mitotic inhibitor *vincristine* is inhibiting the assembly of microtubules (81). Microtubules are not only indispensable for mitosis but also for the transport of proteins from the nucleus of an axon to its periphery (81). By disturbing this transport, *vincristine* typically can cause peripheral neuropathy (81). Other prominent examples with tissue-specific side effects include *anthracyclines,* which can lead to cardiomyopathy, *cyclophosphamide,* which can cause hemorrhagic cystitis, *bleomycin,* causing pulmonary fibrosis and *cytarabine*, which is toxic for the cerebellum (56, 81).
Another striking disadvantage of classical chemotherapy is its great potential to induce secondary malignancies, due to its mutagenic mechanism of action (20, 29, 59). Thus, newly developed anti-cancer drugs not only need to be effective against cancerous cells, but also have to come with an acceptable side-effect profile. By understanding the individual steps, which lead to the malignant transformation of incipient cancer cells, we might develop molecularly-tailored strategies to either reverse these steps or to exploit the altered phenotype of cancerous cells by uncovering tumor-specific liabilities (44).

Tumorigenesis

Tumorigenesis is a multistep mutational process, which includes genetic and epigenetic changes ultimately leading to an increased activity of oncogenes (via gain of function and/or overexpression) and/or a decreased activity of tumor suppressor genes (via loss of function and/or silencing) (44, 69, 82).
These changes provide the mutated cells with a set of traits that are essential for the uncontrolled growth of cancer cells, the so-called "hallmarks of cancer" (44, 45). These hallmarks include different alterations: 1) mitogen independence, 2) immune evasion, 3) apoptosis resistance and escape from growth-inhibitory signals, 4) continuous angiogenesis, as well as 5) tissue invasion and metastasis (44). Altogether, these hallmarks result in the

phenotype of cancer (44). New technologies, which allowed an unprecedented DNA sequence analysis of individual tumor genomes, made it possible, to shed light onto the complex genetic alterations, which ultimately result in cancerous growth (24, 40, 41, 54, 78, 97). Today, we can distinguish a certain number of high frequency "driver" mutations, which mediate the hallmark features of cancer cells, from a larger number of less important passenger mutations, which do not contribute to cancerous growth (24, 40, 66). By sequencing a large number of tumor genomes, we gained insight into the nature and frequency of driver mutations and the involved signaling pathways (24, 40, 41, 54, 69, 78, 97, 113). It appears that there is a limited number of high frequency mutations, which fall into the category of driver mutations, even though the precise number is still under debate (69). With an average of 50 to 80 mutations in an analysis of colon- and breast tumors, only less than 15 could be classified as driver mutations (69). This certain number of driver lesions seems to affect a comparable number of core signaling pathways, leading to tremendous changes in signal transduction resulting in the cancerous phenotype (69).

Driver mutations can be classified into two different groups: they either can result in the gain of function and/or overexpression of oncogenes, or in the loss of function or silencing of tumor suppressor genes (44, 69).

As we have learned from studies of mouse models of human cancers, the continuous expression of certain oncogenes e.g. *HRAS, KRAS, EGFR* or *Myc* is often essential for tumor maintenance (17, 32, 49, 94). Here, *KRAS*-driven lung adenocarcinomas may serve as an example. In transgenic mice, which express mutant *KRAS4b*^{G12D} in the presence of doxycycline in alveolar type II pneumocystes, we can observe formation of adenomas and adenocarcinomas of the lungs following doxycycline administration which hence results in expression of *KRAS4b*^{G12D} within two months (32). However, after withdrawal of doxycycline, causing a rapid fall in the concentration of *KRAS4b*^{G12D}, the tumor burden is dramatically decreased by three days and tumors are undetectable by one month (32). Analogous, transgenic mice expressing mutant *EGFR* (*EGFR*^{L858R} or *EGFR*$^{\Delta L747–S752}$) in type II pneumocytes under the control of a doxycycline-inducible promoter develop lung adenomocarcinomas after two weeks of induction of mutant *EGFR* (79). Withdrawal of doxycycline, which reduces the transgene expression, or treatment with *erlotinib*, a

reversible tyrosine kinase inhibitor of EGFR, causes rapid tumor regression, demonstrating that mutant EGFR is required for tumor maintenance (79). This dependency of tumors on continuous expression of oncogenes led to the term *oncogene addiction* (111).

Similar findings have been made for tumor suppressor genes (111). Ventura *et al.* designed mice carrying a reactivatable *TP53* knock out allele by inserting a transcription–translation stop cassette flanked by *LoxP* sites (LSL) in the first intron of the endogenous wild-type *TP53* locus (105). Mice homozygous for $TP53^{LSL/LSL}$ are functionally equivalent to *p53* null animals and therefore are cancer-prone (105). $p53^{LSL/LSL}$ mice were crossed with mice carrying a Cre-recombinase-Oestrogen-Receptor-T2 allele targeted to the ubiquitously expressed *ROSA26* locus (*R26.Cre-*ER^{T2}) (105). Via the administration of tamoxifen, *Cre recombinase* can enter the nucleus and restore the function of p53 by permitting the recombination of genomic *LoxP* sites and thus eliminating the previously mentioned stop cassette (105). Depending on the tissue of origin, the restoration of *TP53*-function in mice following the formation of neoplastic lesions lead to tumor regression through apoptosis (lymphomas) or senescence followed by clearance through the immune system (sarcomas) (105). Similar findings were reported by others (70, 115). This dependency of tumors on continuous suppression of tumor suppressor gene activity led to the term *tumor suppressor gene hypersensitivity* (111).

As a consequence, oncogene addiction and tumor suppressor gene hypersensitivity got into the focus of intensive investigation as attractive drug targets for the treatment of cancer (111).

Targeting kinase oncogenes already has been proven to be relatively easy as many kinases are directly druggable by ATP-competetive kinase inhibitors (27, 89, 94). Pharmacological inhibition of *driver lesions*, such as the BCR-ABL fusion protein by imatinib, the amplified or mutant EGF receptor by erlotinib or gefitinib or HER2 by trastuzumab already have become therapeutic options (27) (89, 94).

Patients primary sensible to these compounds often develop secondary drug resistance after a certain time of treatment. However, there are some mutations responsible for secondary drug resistances, which are seen with high frequency. In about 50% of non-small cell lung cancer (NSCLC) patients who show initial response to the EGFR-inhibitors gefitinib or erlotinib and

subsequently progress, secondary drug resistance occurs due to a single missense mutation, *T790M,* within the *EGFR*-kinase domain (37). This mutation prevents the binding of gefitinib or erlotinib to the EGFR-kinase domain (37). With the irreversible EGFR/HER-2 kinase inhibitor neratinib it is possible to overcome the drug resistance following the *T790M* mutation *in vitro* (37) and another irreversible EGFR/HER-2 kinase inhibitor, afatinib, has been proven to prolong progression free survival in NSCLC patients following occurence of the *T790M* mutation (73). With the development of lapatinib in trastuzumab resistance and dasatinib in imatinib resistance it also has been possible to design second generation substances for *Her2* and *BCR/ABL* mutated malignancies, which are already in clinical use (12, 103).

In contrast, decreased levels of tumor suppressor activity, e.g. of *TP53* or *ATM,* have been thought to be "non-druggable" for a long time. Depending on the underlying molecular or genetic mechanism, decreased levels of functional tumor suppressors can already be restored in certain special cases. MDM2 is a ubiquitin ligase, which binds and negatively regulates p53 (104). The *MDM2* gene is overexpressed in many human malignancies, leading to decreased levels of p53 (104). Vassilev *et al.* identified a group of small-molecule inhibitors of the MDM2-p53 interaction, called *nutlins*, which prevent the interaction of p53 with its negative regulator MDM2, thus leading to an accumulation of p53 resulting in antitumor activity *in vitro* and *in vivo (104)*.

In case of loss of function mutation of a tumor suppressor, gene delivery could restore the lost tumor suppressor function. But, as the integration of a shuttle vector might be associated wit insertional mutagenesis, which can lead to the formation of malignancies, such as lymphomas, this strategy still remains in its infancy (90). However, using the principle of synthetic lethality is a very elegant and feasible way to target cells with loss of function mutations in tumor suppressor genes specifically by taking advantage of the disturbed pathways instead of trying to restore them.

Synthetic lethality

The concept of synthetic lethality has first been described by Theodosius Dobzhanzki, who experimented with *Drosophila melanogaster* in 1946 (25).

Two genes *A* and *B* are said to engage in a synthetic lethal interaction, if the mutation of either one of them is tolerated by the affected cell, while simultaneous mutations in genes *A* and *B* are lethal (56).

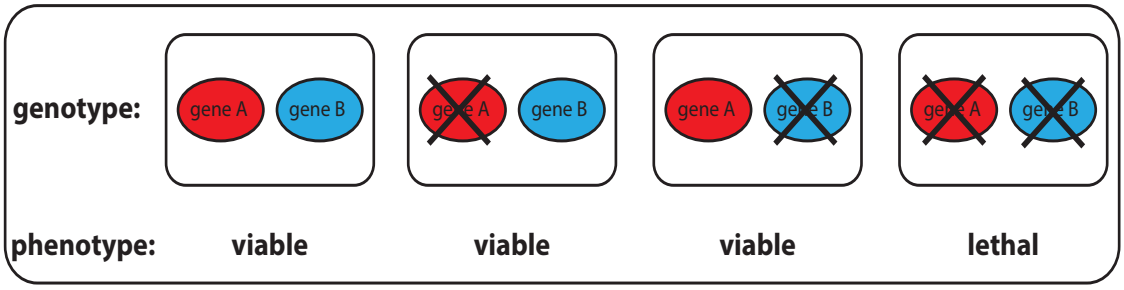

Figure 1. Concept of synthetic lethality: Mutation of either *gene A* or *B* is tolerated, while simultaneous mutations of both genes are lethal.

This situation offers an interesting perspective for the development of novel cancer therapies. Driver mutations leading to cancerous growth frequently result in an extensive rewiring of signal transduction pathways, which ultimately produces tumor cell-specific dependencies on otherwise non-essential genes (56). Druggable protein-products of genes that are in a synthetic lethal interaction with primarily non-druggable driver mutations (e.g. loss of function mutations in TSG such as *ATM*, *TP53*, *BRCA1/2* or gain of function mutations in oncogenes such as *KRAS* or *MYC*) should represent ideal drug-targets for the development of new anti-cancer therapies (56, 82).

By exploiting the concept of synthetic lethality for non-druggable driver lesions, it might be possible to target these driver mutations indirectly (56, 82). Furthermore, pharmacological inhibition of the protein-product of a gene, which is in a synthetic lethal interaction with a driver lesion should kill cancerous cells with high selectivity as healthy cells lack the cancer cell-specific driver-lesion (56, 82).

The probably most advanced example of synthetic lethality in anticancer therapies is the use of Poly(ADP-ribose) polymerase 1 (PARP1)-inhibitors, such as olaparib, in *BRCA1*- or *2*-deficient malignancies. The TSGs *BRCA1* and *BRCA2* are involved in the DSB-repair via homologous recombination (HR) and mutations of either one of them are found in hereditary breast and ovarian cancers as well as in sporadic tumors, such as pancreatic cancer (8, 35). Today, there is no viable therapeutic option to directly target the loss of function of *BRCA1/2* (8, 35, 82).

PARP1 is one of the key enzymes involved in base excision repair, which repairs base modifications prior to S-phase entry (11, 30). PARP1-inhibition leads to the formation of single strand breaks (SSBs) (11, 30). During S-phase these SSBs get encountered by DNA replication forks, leading to replication fork collapse and double strand break (DSB) formation (11, 30). In healthy cells, the newly synthesized sister chromatid can serve as a template for HR to repair the DSB (11, 30). However, due to their inherent HR defect, *BRCA1*- or -*2*-mutated cancer cells fail to repair those replication associated DSBs correctly and inhibition of PARP1 in these cells results in chromosomal instability, cell cycle arrest and subsequent apoptosis (11, 30).

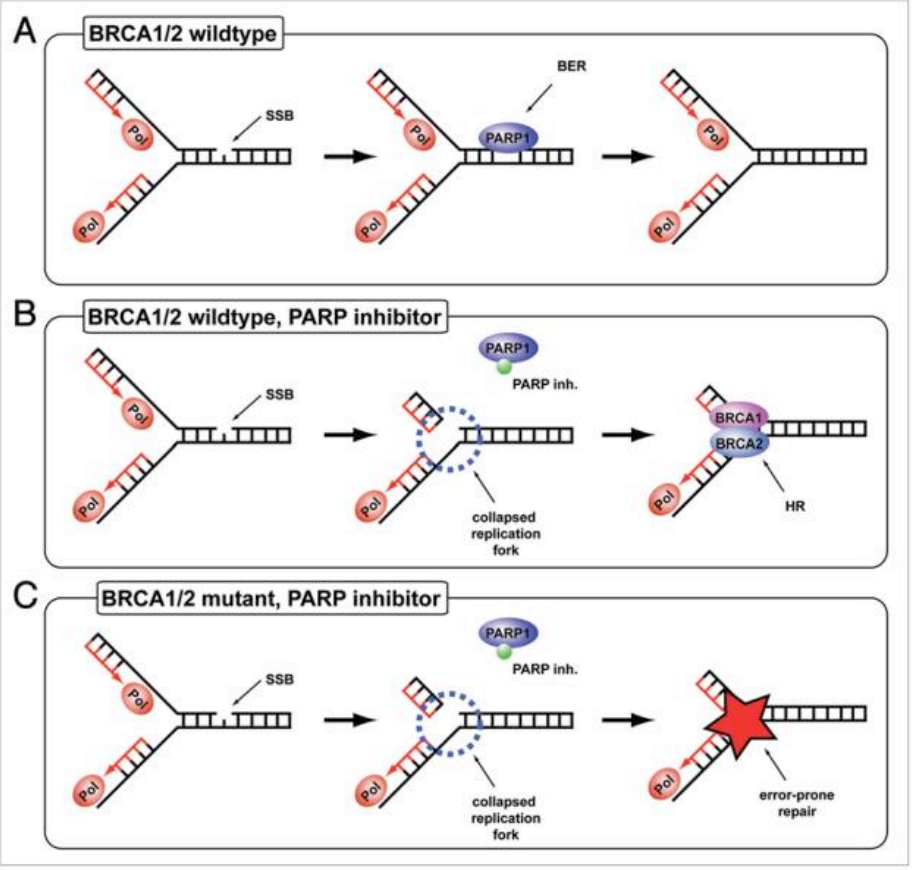

Figure 2. Targeted treatment of *BRACA1/2*-mutated cancer via inhibition of PARP1. (A) In healthy cells, base modifications get repaired using PARP1 dependent base excision repair prior to S-phase. (B) Pharmacological PARP1 inhibition results in unrepaired SSBs, leading to replication fork arrest associated with a DSB. In BRCA proficient cells the newly synthesized sister chromatid serves as a template for error-free HR of the DSB. (C) In BRCA-deficient cells HR of PARP1 inhibitor induced DSBs is interrupted and cells must rely on error-prone DNA repair pathways, such as NHEJ, resulting in genomic instability and subsequent cell death.
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As the surrounding healthy tissue possesses functional *BRCA1/2*, it can perform HR and repair the DSB and thus cope with PARP-inhibition (30). Taken together, the exploitation of the synthetic lethality between *BRCA1/2*

and *PARP1* via PARP1-inhibition offers an opportunity to indirectly target the loss of function of *BRCA1/2* with high specificity.

Here, we focused on the development of novel therapeutic strategies for neoplasms carrying loss of function mutations in the prominent tumor suppressor gene *ATM*.

ATM

The multiple functions of ATM

Ataxia telangiectasia-mutated (*ATM*) is known to be mutated in the autosomal recessive disease Ataxia telangiectasia (A-T). A-T patients carry homozygous mutations in *ATM,* resulting in cerebellar ataxia, cellular and humoral immune defects, progeric changes of the skin, including telangiectasia, endocrine disorders, gonadal abnormalities and an increased sensitivity to ionizing radiation (IR) (72). Most important, A-T patients suffer from genetic instability with an extraordinarily high incidence of cancer, especially for the development of lymphomas (252- and 750-fold increased risk for caucasians and african americans, respectively) (72, 74, 102).

Today, we know that the ATM protein is one of the main actors involved in DNA damage response (DDR) signaling, mediating cell cycle arrest, DNA repair and/or induction of apoptosis (23). ATM is a serine/threonine kinase, which preferentially selects a (L)-S-Q-(E) or (L)-T-Q-(E) motif and belongs to the PI3K protein family (57, 95). This protein family, with PI3K as its founding member, can be further divided into four classes. ATM belongs to class IV, the phosphatidylinositol 3-kinase (PI3K)-related protein kinases (PIKK) (57, 95). Today, besides ATM there are five other known human PIKKs: mammalian target of rapamycin (mTOR), suppressor of morphogenesis in genitalia (SMG-1), transformation/transcription domain-associated protein (TRRAP), ataxia- and Rad3-related (ATR) and DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) (57, 65). Besides ATM, also ATR and DNA-PKcs are involved in the DDR (57, 65).

The genome of human nucleated cells is attacked by constant endogenous and exogenous genotoxic stress, which leads to the formation of many thousand DNA lesions per day (23). There are many different types of DNA lesions, but the DSB is arguably the most harmful. DSBs are caused by both, exogenous and endogenous agents, including IR, radiomimetic chemicals, as well as reactive oxygen species, naturally formed in the cell's metabolism (46). Another source of DSBs are specialized cellular processes, such as recombination during meiosis and processes of the immune system, including the V(D)J-recombination and immunoglobulin class switching (46). Lastly, SSBs can be converted into DSBs when the replication fork reaches a damaged template during DNA synthesis (see above) (46). The DSB is the most rare type of DNA lesion, but still, there is an estimated rate of 10 to 50 DSBs per cell division (43, 106).

Prior to mitosis, cells progress through a series of highly conserved cell cycle checkpoints to ensure a correct transmission of the genome to both daughter cells (48). In response to DNA damage, these checkpoints get activated to prevent further cell cycle progression (48). This allows time to either repair existing lesions or, if the lesion is beyond the capacity of DNA repair, to initiate apoptosis (48). Following encounter of a DSB, ATM is the core kinase orchestrating these distinct cell fate decision processes (23).

Cell cycle checkpoints

A dividing cell is running sequentially through four different phases of the cell cycle, G_1, S, G_2 and M. To guarantee accurate transmission of fully replicated and undamaged DNA, a cell has to pass three checkpoints before entering mitosis, the G_1/S, intra-S and G_2/M checkpoints (109). As shown in Figure 3, ATM serves as a master regulator initiating these cell cycle checkpoints in response to DSBs (23, 95).

Even if we know many of the involved factors today, with every new discovery the picture is getting more complex and there are more questions arising (23, 95).

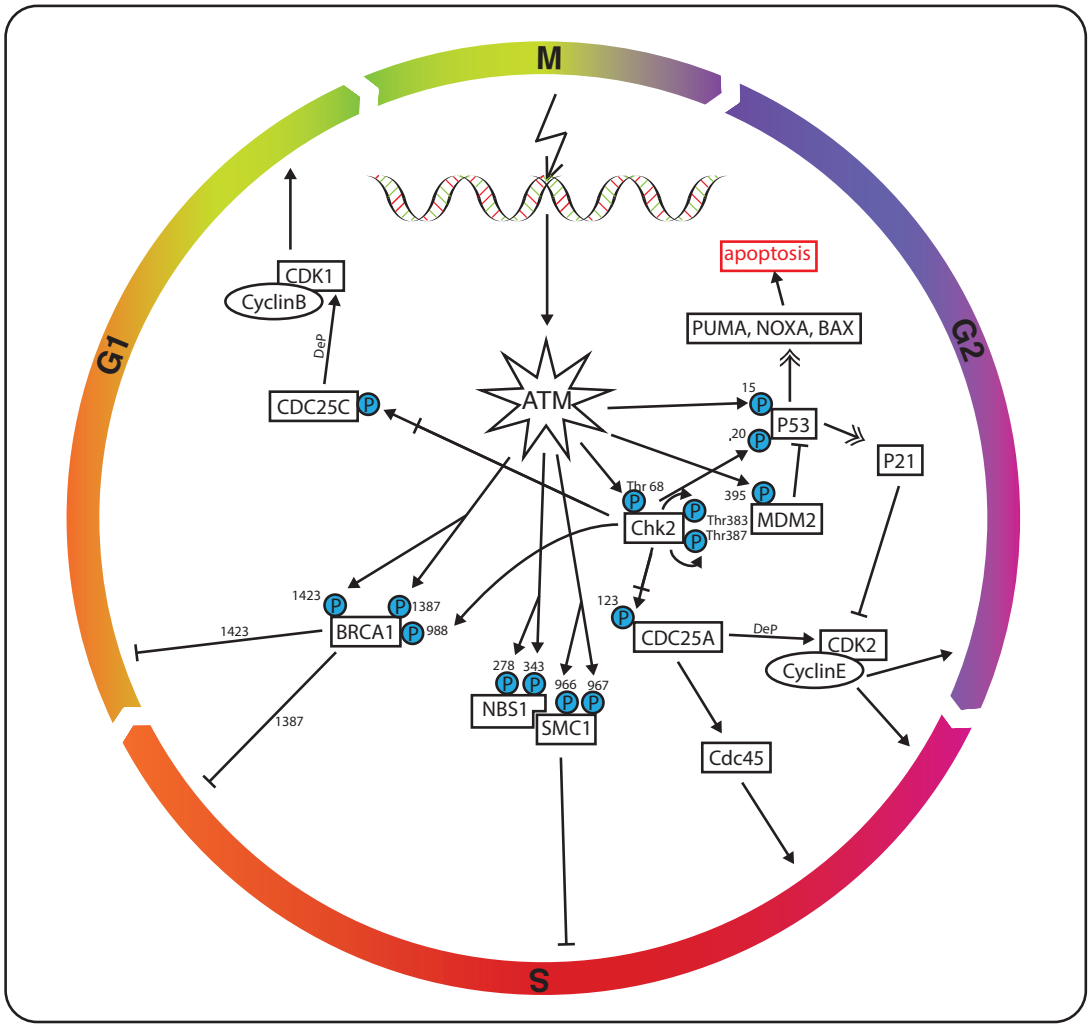

Figure 3. Simplified model of ATM mediated induction of cell cycle arrest and apoptosis in response to DSBs. Arrows indicate stimulation; line through an arrow indicates inhibitory phosphorylation; T-shaped lines mark inhibition; P marks phosphorylation; DeP marks dephosphorylation; the number refers to the position of the phosphorylated residue; phosphorylated residues are serines, unless marked with Thr for Threonin.

Apoptosis

If DNA damage is excessive, apoptosis can be initiated (110). Various studies have shown the ATM-Chk2-p53 axis to be the critical pathway linking the presence of genotoxic lesions with the induction of apoptosis (13, 53, 77). Following its activation, ATM phosphorylates p53 directly on serine-15, which enhances its activity as a transcription factor and prevents interaction with MDM2 (63, 95). In addition, ATM directly phosphorylates its downstream effector kinase Chk2 on threonine-68 within a serine/threonine cluster,

ultimately leading to the activation of Chk2 (63, 84, 95). Active Chk2 in turn posphorylates p53 on serine-20, further preventing the interaction between p53 and MDM2 (23, 63, 95). MDM2 is a negative regulator of p53, which normally binds and thereby inactivates p53 as a transcription factor and also serves as an E3 ubiquitin ligase of p53, which leads to the nuclear export and degradation of p53 (93). MDM2 also gets directly phosphorylated by ATM on serine-395, which abrogates the nuclear export of the MDM2-p53 complexes (63, 95). Together, these events lead to the accumulation and a higher level of activity of p53 (23, 95). Accumulated and activated p53 eventually promotes cell death via the transactivation of pro-apoptotic genes, such as *PUMA, NOXA and BAX* (63). Alternatively, p53 can also lead to sustained cell cycle arrest as shown in figure 3. How p53 finally makes its choice between cell cycle arrest and apoptosis is a sophisticated process, including activities from other signal-transduction pathways and still needs to be further investigated (83, 87).

DSB-repair

Mammalian cells employ two different pathways to repair DSBs. Non-homologous end joining (NHEJ) is an error-prone DSB repair pathway that is preferentially used during early phases of the cell cycle, when no sister chromatid is available for homologous recombination-mediated repair (46, 47). During NHEJ the non-cataytic 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*) (64, 100). DNA-PKcs kinase activity is essential for XRCC4 and Lig4-mediated re-joining of the broken ends during the NHEJ process (22, 64, 99, 100). The second major DSB repair pathway, homologous recombination (HR), is largely restricted to the S- and G_2-phases of the cell cycle, when a sister chromatid is available as a template for DSB repair (15). 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 by the single strand-binding protein RPA and provides a substrate for activation of the proximal DDR kinase ATR (18). During ensuing steps of the HR process, RPA is replaced by Rad51, which mediates the core reactions of HR - homology searching, strand exchange, and Holliday

junction formation (15). There is strong evidence for a role of ATM in HR-mediated DSB repair, with less pronounced effects on NHEJ (10, 16, 21, 61, 68, 75, 86, 96, 114, 116). Cells derived from A-T patients show a subtle, but distinct defect in DSB repair, which is due to impaired assembly and functioning of the RAD51-associated protein complexes in the HR arm of DSB repair (75, 95, 116). Recruitment of Rad51 to DSBs requires resection of DNA ends to generate RPA coated 3′-single-stranded overhangs (50). This resection process and hence the Rad51 focus formation has been shown to be ATM-dependent (1, 50, 76). Recent reports further refined the current view of the role of ATM in HR-mediated DSB repair. ATM has been shown to be required for the HR-dependent DSB repair component in G_2. This notion is further supported by the observation that IR-induced sister chromatid exchanges in G_2 require ATM (7, 19, 51). Lastly, ATM appears to specifically mediate HR-dependent DSB repair in heterochromatin (HC). Indeed, ATM directly phosphorylates the heterochromatin-building factor KAP-1. This KAP-1 phosphorylation is critical to allow HR-mediated repair in HC areas and KAP-1-depletion is able to rescue the ATM repair defect in G_1- and G_2 (38, 39, 51). Thus, the apoptosis-evading effect of ATM-deficiency in human neoplasias likely comes at the cost of a reduced ability to repair chemotherapy-induced DSB lesions via error-free HR.

Why is ATM mutation a problem?

Intriguingly, *ATM* is not only mutated in A-T patients, but also in various human cancer entities, including lung, breast, pancreatic and ovarian cancer, as well as different Non-Hodgkin lymphomas (3, 8, 24, 26, 82, 88).

By screening blood samples of B-cell chronic lymphatic leukemia (B-CLL) patients for frequent genomic aberrations, Döhner *et al.* found, that a deletion of *11q22-23*, which is known to harbor the gene locus of *ATM*, has been the second most frequent cytogenetic aberration seen in approximately 33% of these patients (26). By correlating the aberrations with patient survival, they could show, that overall survival in patients carrying a CLL clone with deletion *11q22-23* has been significantly worse than in those with a normal karyotype or a deletion on the long arm of chromosome 13 (26). Only the prognosis of

patients with a deletion of chromosome *17p13*, known to harbor the gene locus of *TP53*, has been worse (26). The finding that *ATM* deletion in patients with B-CLL is a frequent mutation and associated with short survival and increased probability of disease progression could be confirmed by others (3, 88). Austen *et al.* have gone further by dividing the patient groups with a deletion in *11q22-23* into two subgroups, regarding the *ATM*-status of the remaining allele (3). Here, there was a trend that patients with a remaining mutated *ATM* allele ($ATM^{del/mut}$) had a shorter survival compared with those with one remaining wildtype copy of *ATM* ($ATM^{del/wt}$) (3). They also found a significant higher frequency of resistance to chemotherapeutic drugs in the $ATM^{del/mut}$ subset compared to the $ATM^{del/wt}$ CLLs (3).

Jiang *et al.* could recently demonstrate, that a loss of *ATM* or a decrease in its concentration, results in a dramatically increased resistance against genotoxic chemotherapy in *TP53* wildtype settings (53). They also could verify these results by analyzing the 10-year-survival of chemotherapy-treated breast cancer patients (53). Here, patients with mutant *ATM* and wildtype *TP53* had a significantly reduced survival compared to patients with wildtype *ATM* and wildtype *TP53* (53). Jiang *et al.* further measured the transcriptional levels of both, pro-apoptotic and cell cycle arrest-mediating p53 target genes, in *ATM* wiltype and *ATM* knockdown cells in the presence of functional p53 (53). Here, the transcriptional levels of the pro-apoptotic genes *PUMA* and *Noxa* have been significantly reduced in *ATM* knockdown cells following doxorubicin treatment (53). In stark contrast, the up-regulation of the cell cycle mediating genes *p21* and *Gadd45α* in response to doxorubicin treatment has been nearly unimpaired in *ATM*-depleted cells compared to their *ATM*-wildtype counterparts (53). These findings strongly indicate a specific role of *ATM* in the induction of p53-dependent apoptosis, whereas the induction of a p53-dependent cell cycle arrest seems to be independent of *ATM* (53).

By performing an expression analysis on a large panel of human epithelial tumor specimens Jiang *et al.* found an aberrant expression of *ATM* and *TP53* in about 9% and 29%, respectively (53). These data are well supported by another study by Ding *et al.* who performed large-scale sequencing of 188 lung adenocarcinomas (24). Here, mutations in *ATM* and *TP53* have been seen in about 8% and 36% of the 188 tumors, respectively (24).

Altogether, mutations in *ATM* in tumors with a wildtype *TP53* are seen at high frequency. The selective decapitation of the pro-apoptotic p53 response via the mutation of *ATM* results in a dramatic resistance to chemotherapy and is associated with poor prognosis implicating a great need for the development of therapies targeting ATM-mutated malignancies.

Synthetic lethality between ATM and DNA-PKcs

A first finding leading into the direction of a targeted therapy for *ATM*-mutated malignancies was made by Gurley *et al.*. In 2001 they reported a synthetic lethality between simultaneous *ATM* knock out (encoding for the kinase ATM) and *PRKDC* mutation (encoding for the kinase DNA-PKcs) in murine embryos during embryogenesis (42).

Based on the critical roles for *ATM* and *PRKDC* for HR and NHEJ, respectively, Jiang *et al.* proposed a synthetic lethal interaction between the two in form of an addiction on NHEJ and thus DNA-PKcs not only during embryogenesis, but also in ATM deficient malignancies (53). They could demonstrate hyperphosphorylation of DNA-PKcs on threonine 2606 (corresponding to human threonine 2609) in ATM-deficient MEFs, following doxorubicin treatment and doxorubicin treated *PRKDC/ATM* double-knockdown tumors showed dramatically increased cell-death when compared with ATM single-knockdowns (53). This effect could also been shown pharmacologically, using a DNA-PKcs-inhibitor and Jiang et al. emphasized on the further investigation of these findings as a potential targeted therapy for patients bearing an ATM-deficient tumor (53).

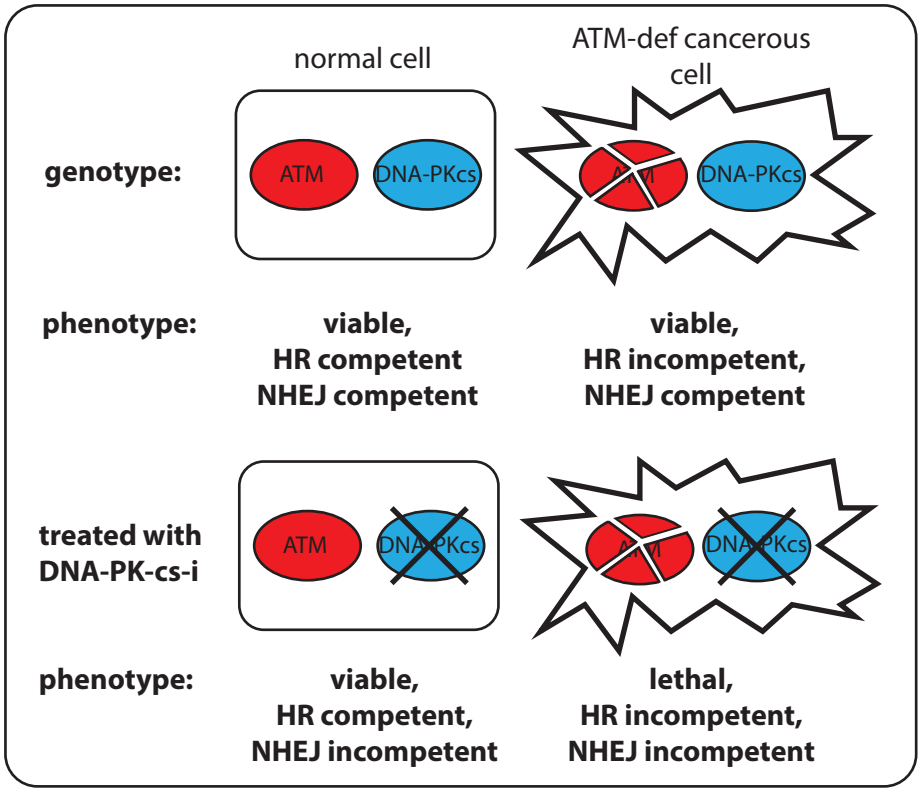

Figure 4. Proposed synthetic lethality between ATM and DNA-PKcs. Following pharmacological inhibition of DNA-PKcs resulting in abrogation of NHEJ, cells can still rely on ATM-dependent HR to repair naturally occurring DSBs. Thus, the phenotype is viable. ATM-deficient cancer cells are unable to perform HR. Pharmacological abrogation of NHEJ should result in genomic instability ultimately leading to death of the cells.

Publication

RESEARCH ARTICLE

CANCER

Therapeutic Targeting of a Robust Non-Oncogene Addiction to *PRKDC* in *ATM*-Defective Tumors

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When the integrity of the genome is threatened, cells activate a complex, kinase-based signaling network to arrest the cell cycle, initiate DNA repair, or, if the extent of damage is beyond repair capacity, induce apoptotic cell death. The ATM protein lies at the heart of this signaling network, which is collectively referred to as the DNA damage response (DDR). ATM is involved in numerous DDR-regulated cellular responses—cell cycle arrest, DNA repair, and apoptosis. Disabling mutations in the gene encoding ATM occur frequently in various human tumors, including lung cancer and hematological malignancies. We report that ATM deficiency prevents apoptosis in human and murine cancer cells exposed to genotoxic chemotherapy. Using genetic and pharmacological approaches, we demonstrate in vitro and in vivo that ATM-defective cells display strong non-oncogene addiction to DNA-PKcs (DNA-dependent protein kinase catalytic subunit). Further, this dependence of ATM-defective cells on DNA-PKcs offers a window of opportunity for therapeutic intervention: We show that pharmacological or genetic abrogation of DNA-PKcs in ATM-defective cells leads to the accumulation of DNA double-strand breaks and the subsequent CtBP-interacting protein (CtIP)–dependent generation of large single-stranded DNA (ssDNA) repair intermediates. These ssDNA structures trigger proapoptotic signaling through the RPA/ATRIP/ATR/Chk1/p53/Puma axis, ultimately leading to the apoptotic demise of ATM-defective cells exposed to DNA-PKcs inhibitors. Finally, we demonstrate that DNA-PKcs inhibitors are effective as single agents against ATM-defective lymphomas in vivo. Together, our data implicate DNA-PKcs as a drug target for the treatment of ATM-defective malignancies.

INTRODUCTION

In response to DNA damage, cells activate a signaling cascade to prevent further cell cycle progression. Activation of this DNA damage response (DDR) network allows time for DNA repair or, if the lesions are beyond repair capacity, leads to the induction of apoptosis (*1*). The proximal DDR kinase ATM, which is mutated in the human cancer–prone disorder ataxia-telangiectasia (A-T), is a master regulator of three essential DDR processes—cell cycle regulation, DNA repair, and apoptosis. ATM affects the different cellular outcomes through the phosphorylation of numerous substrates, including H2AX, MDC1, Nbs1, Chk2, p53, and MDM2 (*2*, *3*). *ATM* is frequently mutated in various sporadic human cancers, and biallelic loss of *ATM* is associated with chemotherapy resistance and poor survival (*4*–*10*). It has been recently shown that ATM is required for the induction of p53-driven apoptosis after genotoxic chemotherapy (*10*). Thus, ATM deficiency is likely a selected genomic aberration in cancer because it protects from p53-driven apoptosis. Beyond mediating apoptosis, ATM also plays a critical role in DNA double-strand break (DSB) repair. Mammalian cells use two distinct DSB repair pathways. Nonhomologous end joining (NHEJ) is an error-prone DSB repair pathway that is preferentially used during early phases of the cell cycle, when no sister chromatid is available (*11*). During NHEJ, the noncatalytic subunits Ku70 and Ku80 form a heterodimer that binds to the free DNA ends and subsequently recruits DNA-PKcs (DNA-dependent protein kinase catalytic subunit). DNA-PKcs kinase activity is essential for XRCC4- and Lig4-mediated rejoining of the broken ends during NHEJ (*12*). The second major DSB repair pathway, homologous recombination (HR), is largely restricted to the S and G_2 phases of the cell cycle, when a sister chromatid is available as a template for DSB repair (*13*). One of the early events necessary for completion of the HR process is DSB end resection to create a 3′ single-stranded overhang, which becomes rapidly coated with RPA and provides a substrate for activation of the proximal DDR kinase ATR (*14*). During the ensuing steps of the HR process, RPA is replaced by Rad51, which mediates the core reactions of HR, namely, homology searching, strand exchange, and Holliday junction formation (*13*). There is strong evidence for a role of ATM in HR-mediated DSB repair, with less pronounced effects on NHEJ (*15*–*18*). Cells derived from A-T patients show a DSB repair defect, which is due to impaired assembly and functioning of the RAD51-associated protein complexes in the HR arm of DSB repair (*16*, *18*, *19*). Recruitment of Rad51 to DSBs requires resection of DNA ends to generate RPA-coated 3′ single-stranded overhangs. This resection process and the resulting Rad51 focus formation are ATM-dependent (*20*–*22*). ATM is required for the HR-dependent DSB repair component in G_2, as supported by the observation that ionizing radiation–induced sister chromatid exchanges in G_2 require ATM (*23*–*25*). Thus, the apoptosis-evading effect of ATM deficiency in human neoplasias likely comes at the cost of a reduced ability to repair chemotherapy-induced DSB lesions via error-free HR.

Because ATM-deficient human tumors frequently display chemotherapy resistance (*4*, *6*–*10*), one might speculate that chemotherapy-

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induced DSBs are repaired in ATM-deficient cancer cells to ensure long-term survival. It is likely that alternative, error-prone DSB repair pathways, such as NHEJ, compensate for the HR defect in ATM-deficient cells. Consistent with the idea that NHEJ might serve as a backup mechanism for failed HR-mediated DSB repair, mice deficient for both *ATM* and *PRKDC* (encoding DNA-PKcs) display early embryonic lethality (*26*), whereas animals lacking either *ATM* or *PRKDC* are viable (*27*, *28*). Here, we aimed to characterize DNA-PKcs as a drug target for the treatment of inherently chemotherapy-resistant ATM-defective neoplastic disease.

RESULTS

ATM-defective cancer cells display DNA-PKcs addiction

We have recently shown that ATM depletion renders murine cells and tumors resistant to genotoxic chemotherapy, mimicking the effects of disabling *ATM* mutations in human patients (*10*). We further showed that DNA-PKcs repression in ATM-depleted murine embryonic fibroblasts increases their sensitivity to DSB-inducing chemotherapy (*10*). However, it remains unclear whether ATM-defective human cancer cells display a similar addiction to DNA-PKcs and whether DNA-PKcs is indeed a druggable target for the treatment of ATM-defective cancers. Finally, the molecular details of the apparent synthetic lethal interaction between *ATM* and *PRKDC* remain elusive.

To test whether ATM-defective cancer cells display DNA-PKcs addiction, we investigated HT144 and H1395 cells. The human melanoma cell line HT144 carries a homozygous *GG* to *AA* substitution at codon 2845 in *ATM*, resulting in a premature stop codon (*29*). In addition, this cell line carries a homozygous $BRAF^{V600E}$ mutation. As an ATM-proficient control for this cell line, we used $BRAF^{V600E}$-driven A375 melanoma cells. The human non–small cell lung cancer (NSCLC) cell line H1395 carries an *A* to *G* substitution at codon 2666 of *ATM*, resulting in a Thr to Ala mutation in the ATM Ser/Thr kinase domain (*30*). As an ATM-proficient control for this cell line, we used A549 NSCLC cells. All four cell lines are p53-proficient. We assessed the effects of DNA-PKcs inhibition using the DNA-PKcs inhibitor KU-0060648. Cells were treated for 24 hours with KU-0060648 (0.5 μM), the DSB-inducing topoisomerase II inhibitor etoposide (10 μM), or a combination of both compounds (Fig. 1A). Apoptosis was assessed by flow cytometry after cells had been stained with antibodies to cleaved caspase-3. Etoposide induced widespread apoptosis in the ATM-proficient cell lines A375 (90.6 ± 6.8%) and A549 (54.6 ± 5.4%) (Fig. 1A), whereas ATM-defective HT144 and H1395 cells were resistant against etoposide with only 14.2 ± 2.5% and 15.0 ± 2.6% of apoptotic cells after 24 hours, respectively. When these cell lines were exposed to KU-0060648, we observed a clear segregation on the basis of their *ATM* status. ATM-proficient cells showed only marginally increased apoptosis, compared to the non-treated controls (Fig. 1A), whereas the ATM-defective cell lines displayed a marked apoptotic response after 24 hours of exposure to KU-0060648. Specifically, HT144 cells showed 44.3 ± 10.3% and H1395 cells displayed 49.6 ± 10.2% apoptotic cells compared to less than 3% of apoptotic cells in the respective untreated controls (Fig. 1A). Combination treatment with etoposide plus KU-0060648 had no additional significant effect on the apoptotic response of ATM-defective cells compared to either drug alone (Fig. 1A). Similar effects were observed when we repeated these experiments and replaced

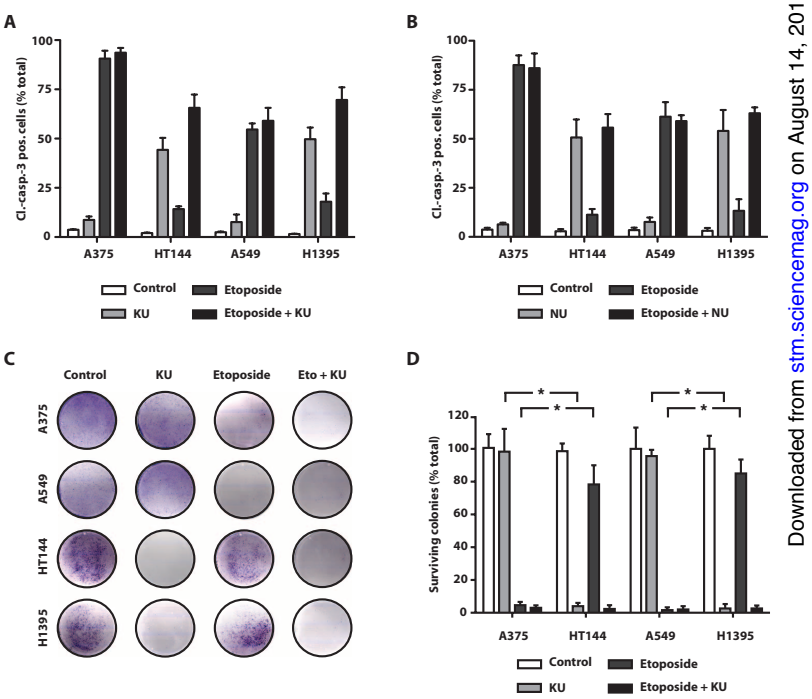

Fig. 1. Non-oncogene addiction to DNA-PKcs in ATM-defective human cancer cells. (**A**) ATM-proficient (A375 and A549) and ATM-defective (HT144 and H1395) cells were grown to assess their response to etoposide (10 μM), the DNA-PKcs inhibitor KU-0060648 (KU) (0.5 μM), or a combination treatment. After 24 hours, cells were harvested, and cleaved caspase-3 staining was analyzed by flow cytometry to assess the percentage of apoptotic cells (bars indicate means ± SEM, $n = 12$). (**B**) To exclude off-target effects of KU-0060648, we treated cells as in (A) with the exception that KU-0060648 was replaced by NU7441 (NU) as an alternative DNA-PKcs inhibitor (bars indicate means ± SEM, $n = 12$). (**C**) Clonogenic survival assay. ATM-proficient (A375 and A549) and ATM-defective (HT144 and H1395) cells were treated with 10 μl of phosphate-buffered saline (PBS) (vehicle control) or exposed to etoposide (10 μM), KU-0060648 (0.5 μM), or a combination treatment for 12 hours, washed, and replated at 5000 cells per 10-cm dish. Fourteen days later, colonies were stained and counted. (**D**) Quantification of the experiments described in (C) [bars indicate means ± SEM ($n = 3$), normalized to untreated control]. *$P < 0.05$, two-tailed Student's t test.

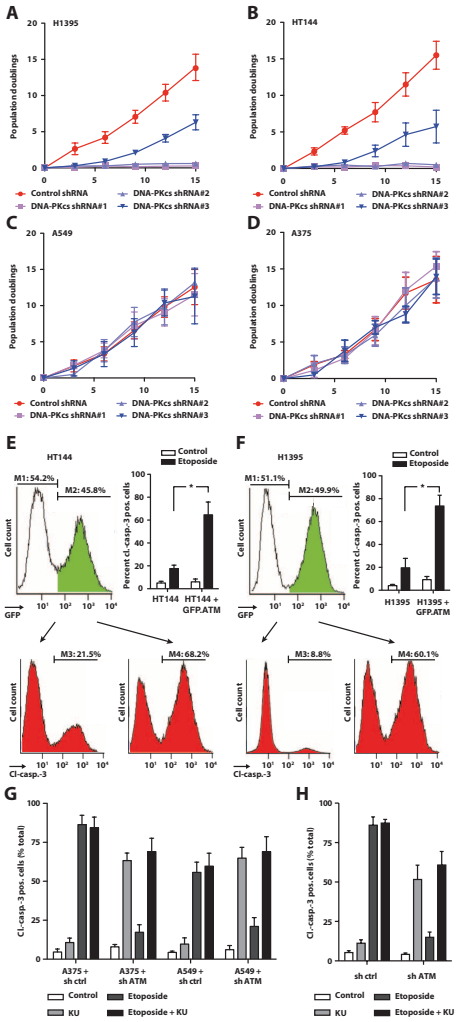

Fig. 2. Genetic validation of the synthetic lethal interaction between *ATM* and *PRKDC*. (**A** to **D**) Effect of a series of DNA-PKcs–targeting shRNAs with varying degrees of knockdown efficiency. ATM-defective H1395 (A) and HT144 (B) cells, as well as ATM-proficient A549 (C) and A375 (D) cells, were transduced with control shRNAs or three distinct DNA-PKcs–targeting shRNAs, and population doublings were recorded. Experiments shown in (A) to (D) were performed at $n = 8$ (bars indicate means ± SEM). (**E** and **F**) ATM-defective HT144 (E) and H1395 (F) cells were transiently transfected with a plasmid encoding Flag.ATM and GFP. Twenty-four hours later, cells were treated with etoposide (10 μM) for 24 hours and harvested, and apoptosis was assessed by flow cytometry. GFP coexpression was used to separate ATM-complemented cells from the parental cells [top left panels in (E) and (F)]. Gate M1 indicates GFP-negative cells, and gate M2 indicates GFP-expressing cells. The bottom panels in (E) and (F) show histogram plots of the parental (left) and ATM-complemented (right) HT144 and H1395 cells. Gates M3 and M4 indicate the fraction of cleaved caspase-3–positive parental and ATM-complemented cells, respectively. Quantification of the individual experiments is shown at the top right of (E) and (F). Bars indicate means ± SEM ($n = 8$). *$P < 0.05$, two-tailed Student's t test. (**G**) A375 and A549 cells were infected with lentiviruses encoding ATM-targeting or luciferase control shRNAs. Cells were left untreated or exposed to etoposide (10 μM), KU-0060648 (KU) (0.5 μM), or a combination treatment for 24 hours before apoptosis was quantified using flow cytometry. The experiments shown in (G) were performed at $n = 8$ (bars indicate means ± SEM). (**H**) *Eμ:MYC;ARF*$^{-/-}$-driven cells were infected with retroviruses encoding ATM-targeting or luciferase control shRNAs. Upon puromycin selection, cells were left untreated or exposed to etoposide (10 μM), KU-0060648 (0.5 μM), or a combination treatment for 24 hours before apoptosis was quantified using flow cytometry (bars indicate means ± SEM, $n = 9$).

KU-0060648 with NU7441 (0.5 μM) as an alternative DNA-PKcs inhibitor (Fig. 1B).

To validate these experiments, we used colony survival assays. Cells were left untreated and exposed to etoposide, KU-0060648, or a combination treatment consisting of etoposide plus KU-0060648 for 12 hours (Fig. 1C). Surviving colonies were assayed 14 days after reseeding subsequent to completion of the different treatments. ATM-defective cells displayed etoposide resistance compared to their ATM-proficient counterparts, which is reflected in significantly more surviving colonies in HT144 and H1395 cells (Fig. 1, C and D). In contrast, ATM-defective cells appeared to critically depend on DNA-PKcs signaling for their survival, even in the absence of exogenous genotoxic stress. Treatment with KU-0060648 as a single agent resulted in a significant decrease in the number of surviving colonies in HT144 and H1395 cells compared to untreated controls. This is in contrast to their ATM-proficient counterparts, which show essentially no difference in the number of surviving colonies when comparing control cells to those exposed to KU-0060648 (Fig. 1D). These data suggest that ATM deficiency is associated with marked etoposide resistance in human cancer cells. Furthermore, the observation that DNA-PKcs inhibition promotes apoptosis in ATM-defective cells suggests that these cells are DNA-PKcs–dependent.

Because studies with adenosine triphosphate–competitive inhibitors are frequently hampered by off-target effects, we next performed genetic studies to further assess the DNA-PKcs dependence of ATM-defective cancer cells. To examine whether DNA-PKcs was required for survival in ATM-proficient and ATM-defective human cancer cells, we used RNA interference (RNAi) to deplete DNA-PKcs and examined population doubling rates upon knockdown (Fig. 2, A to D). Cells were infected with lentiviruses delivering short hairpin RNAs (shRNAs) against luciferase (control) or DNA-PKcs. We used three DNA-PKcs–targeting shRNAs with different degrees of knockdown efficiency, essentially allowing us to analyze an allelic series of DNA-PKcs expression levels (fig. S1). DNA-PKcs depletion completely prevented further proliferation of ATM-defective H1395 and HT144 cells when we used the two most potent shRNAs (#1 and #2) (Fig. 2, A and B). When we tested the effects of an shRNA with a less strong knockdown efficiency (#3), proliferation of both H1395 and HT144 cells was markedly reduced, but not completely abolished, compared to cells expressing control shRNA. In contrast, DNA-PKcs repression in ATM-proficient cells did not significantly reduce the population doubling rates in these cells, indicating that DNA-PKcs is not essential in ATM-proficient settings (Fig. 2, C and D).

We next performed a set of complementation experiments in which we compared the effect of KU-0060648 in the ATM-defective parental cell lines H1395 and HT144 and their ATM-complemented counterparts. Cells were transfected with a plasmid driving the expression of ATM and green fluorescent protein (GFP) (fig. S2). We chose conditions under which 40 to 50% transfection efficiency was reached. Transfected cultures were exposed to etoposide for 12 hours, and apoptosis was analyzed by flow cytometry. Coexpression of GFP and ATM allowed the separate gating of ATM-defective (GFP-negative) and ATM-complemented (GFP-positive) cells (Fig. 2, E and F). In keeping with our hypothesis, we found that complementation of ATM-defective and inherently chemotherapy-resistant HT144 and H1395 cells significantly enhanced their apoptotic response to etoposide (Fig. 2, E and F, bottom panels).

If ATM deficiency was indeed responsible for the etoposide resistance and DNA-PKcs addiction that we observed in HT144 and H1395 cells, one might expect ATM depletion to produce a similar phenotype in initially ATM-proficient cells. To test this, we infected A375 and A549 control cells with lentiviruses expressing ATM-targeting shRNAs and analyzed the apoptotic response of these cells to KU-0060648, etoposide, or a combination treatment (Fig. 2G and fig. S3). Consistent with our hypothesis, we found that ATM depletion rendered A375 and A549 cells resistant to etoposide and exquisitely sensitive to DNA-PKcs inhibition (Fig. 2G). To further prove our hypothesis that ATM deficiency is associated not only with resistance against genotoxic chemotherapy but also with DNA-PKcs dependence, we turned to murine *Eμ:MYC;ARF*$^{-/-}$-driven B–non-Hodgkin's lymphoma (B-NHL) cells (Fig. 2H and fig. S4). To address the effect of ATM depletion in an otherwise isogenic system, we infected *Eμ:MYC;ARF*$^{-/-}$-driven lymphoma cells with retroviruses expressing ATM-targeting shRNA and compared the effects of KU-0060648, etoposide, or a combination treatment. In agreement with our initial experiments, we found that ATM-proficient control cells were highly sensitive to etoposide, although this sensitivity was completely abolished when ATM was depleted (Fig. 2H). Furthermore, ATM-depleted *Eμ:MYC;ARF*$^{-/-}$ lymphoma cells became DNA-PKcs–addicted because KU-0060648 exposure induced massive apoptosis in these cells (Fig. 2H). This DNA-PKcs addiction of ATM-depleted cells was likely not due to DNA-PKcs overexpression in ATM-depleted cells. When we performed immunoblotting to analyze DNA-PKcs expression levels and activation (as monitored by an antibody detecting phospho-Thr^{2647}), we found that knockdown of ATM did not result in increased DNA-PKcs expression, but in increased DNA-PKcs activity, even in the absence of genotoxic stress (fig. S5). Together, these data indicate that ATM deficiency is associated with resistance against genotoxic chemotherapy, likely through an abrogation of p53-driven apoptosis. On the other hand, this apoptosis evasion appears to come at the cost of a non-oncogene addiction to DNA-PKcs, which could be targeted with DNA-PKcs inhibitors.

ATM-defective cells fail to repair DSBs when DNA-PKcs is inhibited

We next aimed to mechanistically characterize the DNA-PKcs addiction of ATM-defective cells. Beyond mediating apoptosis (*19*), ATM has also been shown to be an important driver of HR-mediated DSB repair (*15*–*18*). However, because ATM-defective cancers appear to be largely resistant against genotoxic chemotherapy, these HR-defective malignancies may use alternative DSB repair pathways, such as DNA-PKcs–dependent NHEJ, to repair DSBs. If this was the case, one might expect to observe the prolonged persistence of unrepaired DSBs in ATM-defective cells that had been exposed to DNA-PKcs inhibitors. To test this, we used immunofluorescence to monitor the persistence of etoposide-induced γH2AX and 53BP1 nuclear foci in ATM-proficient and ATM-deficient cells exposed to KU-0060648 or vehicle control. We chose to monitor γH2AX and 53BP1 foci because both are established markers of DSBs (*31*, *32*).

We treated ATM-proficient (A375 and A549) and ATM-deficient (HT144 and H1395) cells for 20 min with a low-dose etoposide pulse (0.1 μM) to induce DSBs. In a parallel experiment, cells were pretreated with KU-0060648 for 1 hour before addition of etoposide. KU-0060648 remained present in the medium after etoposide removal. In both experiments, cells were protected from premature apoptosis by addition of the irreversible pan-caspase inhibitor Z-VAD (10 μM), which was applied together with etoposide. Consistent with the induction of DSBs by etoposide, we detected similar numbers of γH2AX and 53BP1 foci in all cell lines 1 hour after etoposide removal (Fig. 3, A to D). Furthermore, there were no detectable differences in the repair kinetics of all cell lines, when the number of γH2AX/53BP1 foci–positive cells was assessed at 72 and 96 hours. However, marked differences in the DSB repair kinetics could be observed in ATM-proficient and ATM-deficient cells that were treated with KU-0060648. ATM-proficient cells showed no evidence of increased γH2AX or 53BP1 foci at 72 and 96 hours compared to vehicle-treated controls. This picture was different in ATM-defective cells treated with KU-0060648. Both HT144 and H1395 cells displayed persistent γH2AX and 53BP1 foci even 96 hours after etoposide removal, when DNA-PKcs was inhibited (Fig. 3, A to D). These observations are in line with a DSB repair defect being present in cells lacking both ATM and DNA-PKcs activity.

ATM- and DNA-PKcs–defective cancer cells generate RPA-coated single-stranded DNA intermediates

We next aimed to further characterize the DSB repair defect in ATM-defective cells. Recruitment of Rad51, the core component of the HR machinery, to DSBs requires resection of DNA ends to generate RPA-coated 3′ single-stranded DNA (ssDNA) overhangs. To assess whether ATM-defective cells that were exposed to KU-0060648 initiated early steps of the HR process, we submitted ATM-proficient and ATM-deficient cells to the same treatment regimen as in Fig. 3 to monitor the occurrence and persistence of nuclear RPA foci, markers of ssDNA repair intermediates (*33*). All four cell lines displayed prominent RPA foci 1 hour after removal of etoposide, regardless of ATM status and independent of the presence or absence of a DNA-PKcs inhibitor (Fig. 4, A and B). At 72 and 96 hours after etoposide exposure, the ATM-proficient cells (A375 and A549) remained largely RPA foci–negative, paralleling their lack of γH2AX and 53BP1 foci and indicative of completed DSB repair at these late time points. There was no difference among the ATM-competent cells that were exposed to either KU-0060648 or vehicle. Furthermore, RPA foci were largely undetectable in ATM-defective HT144 and H1395 cells at 72 and 96 hours, when no DNA-PKcs inhibitor was present. In contrast, when ATM-defective cells were treated with an etoposide pulse and KU-0060648, large RPA foci were detectable in these cells 72 and 96 hours after etoposide removal. These data indicate that DSBs not only persist for extended periods in ATM-defective cells that are exposed to DNA-PKcs inhibitors but also are extensively modified in these cells to yield RPA-coated ssDNA structures.

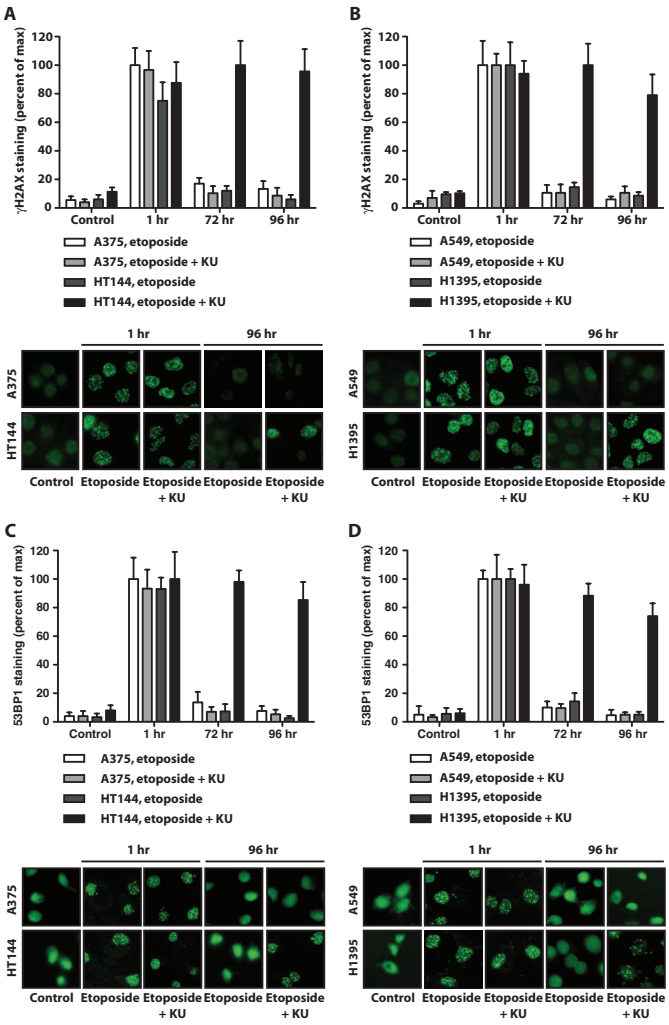

Fig. 3. DSB repair defect resulting from DNA-PKcs inhibition in ATM-defective cells. (**A** and **B**) Treatment of ATM-defective cells with KU-0060648 (KU) results in persistent DSBs as indicated by persistent nuclear γH2AX staining. ATM-proficient (A375) and ATM-defective (HT144) human melanoma cells (A), as well as ATM-proficient (A549) and ATM-defective (H1395) human NSCLC cells (B), were exposed to a low-dose etoposide pulse (0.1 μM, 20 min) and harvested 1, 72, and 96 hours later. Control cells were left untreated. In a parallel experiment, cells were pretreated with KU-0060648 (0.5 μM) for 1 hour before addition of etoposide. Top panels show a quantification of these experiments (n = 9; bars indicate means ± SEM). Bottom panels depict representative original immunofluorescence data. (**C** and **D**) Exposure of ATM-defective HT144 and H1395 cells to KU-0060648 results in persistent DSBs as indicated by persistent 53BP1 nuclear foci. A375, A549, HT144, and H1395 cells were treated as in (A) and (B) and stained with antibodies detecting 53BP1. Top panels show a quantification of these experiments (n = 7, bars indicate means ± SEM). Bottom panels depict representative original immunofluorescence data.

RPA-coated ssDNA triggers activation of the ATR/Chk1/p53/Puma axis

We next asked whether ssDNA repair intermediates might induce proapoptotic signaling in ATM-defective cells. Because both the HT144 and H1395 cell lines are p53-proficient, we reasoned that the KU-0060648–induced apoptosis of these cells might be p53-dependent. Numerous kinases can activate p53, including ATM, DNA-PKcs, and ATR (*34*). Intriguingly, RPA-coated ssDNA recruits ATR through binding of its regulatory subunit ATRIP to RPA (*14*). We reasoned that the RPA-coated ssDNA that we had observed in ATM-defective cells treated with KU-0060648 might trigger an ATR-dependent p53 activation, ultimately promoting p53-driven apoptosis. To test this, we used immunoblotting to assess the activation status of the ATR/Chk1/p53 signaling axis in ATM-proficient and ATM-deficient cells that were treated with etoposide, KU-0060648, or vehicle control. ATR activation was monitored with an antibody detecting a phospho-epitope on Thr^{1989}. Chk1 activation was assessed with an antibody to phospho-Ser^{317}. p53 activation was assessed with an antibody detecting total (stabilized) p53 and an antibody directed against phospho-Ser^{20}, the residue targeted by Chk1. Using these assays, we found the ATR/Chk1/p53 axis to be activated in the ATM-defective cell lines 24 hours after addition of KU-0060648 (Fig. 5A). Consistent with the primary resistance of these ATM-defective cells, we failed to detect any activation of the ATR/Chk1/p53 axis after etoposide treatment. A strikingly different activation pattern of the ATR/Chk1/p53 axis emerged in ATM-proficient cell lines. Twenty four hours after etoposide, these cells displayed prominent activation of ATR, Chk1, and p53, whereas KU-0060648 treatment did not result in any substantial activation of the ATR/Chk1/p53 axis (Fig. 5A). These data suggest that DNA-PK inhibition leads to ATR/Chk1-dependent p53 activation in ATM-defective cells.

To assess the outcome of this p53 accumulation, we used quantitative polymerase chain reaction (qPCR) to monitor the expression of the p53 target genes *PUMA*, *BAX*, *BAK*, *GADD45A*, and *RPRM*. KU-0060648 treatment markedly increased mRNA expression of the pro-apoptotic p53 target gene *PUMA* in ATM-defective cells (Fig. 5, B and C). In contrast, etoposide treatment did not result in a significant change in the expression level of any of the investigated p53 target genes. These observations agree with a primary resistance of ATM-defective cells to DNA damage–induced apoptosis through the p53 pathway. However, this resistance appears to be overcome by exposure of ATM-defective cells to KU-0060648, suggesting that repression of DNA-PK activity in ATM-defective cells leads to an ATR/Chk1-dependent activation of the proapoptotic p53/Puma axis, likely as a result of aberrant ATR activation downstream of RPA-coated ssDNA repair intermediates.

We next asked whether interception of signaling through the ATR/Chk1/p53/Puma axis would abolish

KU-0060648–induced apoptosis in ATM-defective cells. To this end, we exposed ATM-proficient and ATM-deficient cells to either etoposide, etoposide plus KU-0060648, or etoposide plus KU-0060648 plus the Chk1 inhibitor AZD-7762 (200 nM) for 12 hours. Addition of either KU-0060648 or KU-0060648 plus AZD-7762 did not significantly alter the degree of apoptosis in ATM-proficient cells when compared to the effects of etoposide alone (fig. S6, A and B). In contrast, exposure of ATM-defective cells to a combination treatment consisting of etoposide plus KU-0060648 led to the robust induction of apoptosis. Addition of AZD-7762 to this regimen led to a precipitous drop in the percentage of apoptotic cells, suggesting that signaling through the ATR/Chk1 axis is involved in mediating apoptosis in DNA-PKcs inhibitor–treated ATM-defective cells (fig. S6, C and D).

To further interrogate the contribution of the ATR/Chk1/p53/Puma axis in mediating KU-0060648–induced apoptosis in ATM-defective cells, we next assessed the effects of Puma repression in this setting. ATM-proficient and ATM-deficient cells were infected with lentiviruses encoding either control or Puma-targeting shRNAs. Cells were then treated with KU-0060648, etoposide, or a combination of both compounds for 24 hours before apoptosis was assessed. As expected, depletion of Puma resulted in marked resistance of ATM-proficient cells to etoposide or combination treatment with etoposide plus KU-0060648 (fig. S7, A and B). Loss of Puma also repressed the apoptotic response of ATM-deficient HT144 and H1395 cells treated with KU-0060648 or a combination of etoposide plus KU-0060648 (fig. S7, C and D). These data lend further support to our hypothesis that signaling through the ATR/Chk1/p53/Puma axis is involved in mediating KU-0060648–dependent apoptosis in ATM-defective cells.

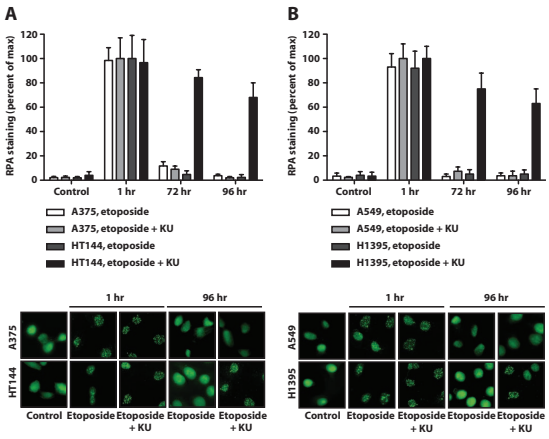

Fig. 4. RPA-coated ssDNA intermediates in ATM-defective cells without functional DNA-PKcs. (**A** and **B**) ATM-proficient (A375) and ATM-defective (HT144) melanoma cells, as well as ATM-proficient (A549) and ATM-defective (H1395) NSCLC cells, were exposed to a low-dose etoposide pulse (0.1 μM, 20 min) and harvested 1, 72, and 96 hours later. Control cells were left untreated. In a parallel experiment, cells were pretreated with KU-0060648 (KU) (0.5 μM) for 1 hour before addition of etoposide. RPA foci were visualized using indirect immunofluorescence. Top panels show a quantification of these experiments (n = 12; bars indicate means ± SEM). Bottom panels depict representative original immunofluorescence data.

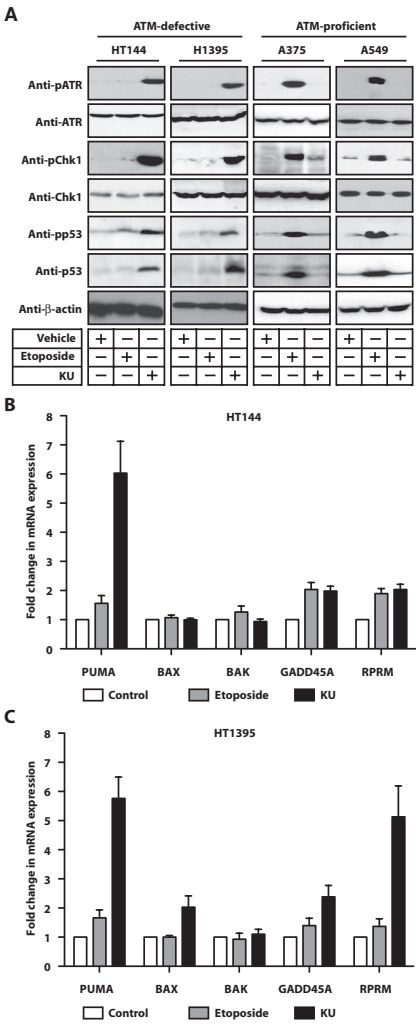

Fig. 5. Activation of apoptosis through ATR in ATM-defective cells treated with DNA-PKcs inhibitors. (**A**) ATM-proficient (A375 and A549) and ATM-deficient (HT144 and H1395) cells were left untreated, exposed to etoposide (10 μM), or treated with KU-0060648 (KU) (0.5 μM). Cells were harvested after 24 hours and analyzed by immunoblotting using the indicated antibodies. β-Actin staining served as a loading control. (**B** and **C**) To assess the functional consequence of the p53 accumulation depicted in (A), we used qPCR to monitor the expression level of the p53 target genes *PUMA*, *BAX*, *BAK*, *GADD45A*, and *RPRM* in ATM-defective HT144 (B) and H1395 (C) cells after exposure to either etoposide (10 μM) or KU-0060648 (0.5 μM). Cells were treated as in (A), harvested, and analyzed by qPCR. Expression levels of the indicated p53 target genes were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (bars indicate means ± SEM, n = 4).

Repression of CtBP-interacting protein abolishes 3′-ssDNA repair intermediates and prevents apoptosis

CtBP-interacting protein (CtIP) is required for the resection of DNA ends to generate RPA-coated 3′-ssDNA overhangs (*35*, *36*). There is accumulating evidence suggesting a two-step model for DSB resection where CtIP and the Mre11/Rad50/Nbs1 complex cooperate to initiate resection before the exonuclease Exo1 continues the resection process to generate the 3′-ssDNA tails required for the HR process (*37*). Our data suggested that the generation of RPA-coated ssDNA repair intermediates triggered ATR/Chk1 activation, ultimately promoting p53-dependent *PUMA* induction and subsequent apoptosis of ATM-defective cells treated with KU-0060648. We hence speculated that repression of CtIP might prevent the generation of ssDNA intermediates and thus might preclude activation of the proapoptotic ATR/Chk1/p53/Puma axis in ATM-defective cells treated with KU-0060648. To test this, we compared the dynamics of nuclear RPA foci formation in ATM-defective HT144 and H1395 cells expressing either control or CtIP-targeting shRNAs (fig. S8). As shown in fig. S9A, CtIP depletion significantly reduced the number of RPA foci–positive HT144 and H1395 cells 1 hour after etoposide removal and almost completely blocked the generation of RPA foci at 72 and 96 hours. As described by others, we found that shRNA-mediated depletion of CtIP caused hypersensitivity toward etoposide (fig. S9B) (*38*). CtIP depletion also resulted in a significant ($P < 0.05$) reduction of KU-0060648 toxicity when applied alone or in combination with etoposide (fig. S9B). These results point toward a critical role for CtIP not only in mediating the resection of DSBs in DNA-PK inhibitor–treated ATM-defective cells but also in promoting subsequent activation of apoptosis.

ATM-defective chronic lymphocytic leukemia cells display DNA-PKcs addiction

Deletions on the long arm of chromosome 11 (harboring the *ATM* gene located at *11q22.3–11q23.1*) are found in about 20% of patients with chronic lymphocytic leukemia (CLL) and identify a subgroup with poor outcome (*9*). CLL with *del(11q)* can be further divided into two subgroups based on the integrity of the residual *ATM* allele. Patients with biallelic *ATM* alterations display defective responses to cytotoxic chemotherapeutics in vitro and a poorer clinical outcome (*4*). Hence, we next aimed at validating our findings in primary CLL cells derived from patients with either *del(11q)* or a wild-type configuration on *11q*. Patients were stratified as being wild type or *del(11q)* by clinical-grade fluorescence in situ hybridization (FISH) analysis (Fig. 6, A and B). Primary CLL cells were seeded onto a feeder layer of CD40 ligand–expressing NIH 3T3 cells before treatment with KU-0060648, etoposide, or a combination of both compounds. Wild-type CLL cells were highly sensitive to etoposide but resistant against KU-0060648 (Fig. 6). Addition of KU-0060648 to the etoposide regimen did not significantly enhance the response of the ATM-proficient CLL cells. In contrast, *del(11q)* CLL cells were exclusively sensitive to KU-0060648 but largely resistant to etoposide (Fig. 6). These data suggest that DNA-PK inhibition might be a useful strategy to treat chemotherapy-resistant, ATM-defective CLLs.

DNA-PKcs is a valid target for the therapy of ATM-defective lymphoma

To validate our observations in vivo, we used the *Eμ:MYC;ARF*$^{-/-}$-driven B-NHL model (*10*). Lymphoma cells derived from *Eμ:MYC;ARF*$^{-/-}$ mice were infected with lentiviruses encoding either luciferase control or ATM-specific shRNAs. C57BL/6J recipient mice were transplanted with 1.5×10^6 transduced lymphoma cells. Upon lymphoma manifestation, treatment with either KU-0060648, etoposide, or a combination of KU-0060648 plus etoposide was initiated. Untreated control animals carrying either luciferase shRNA– or ATM shRNA–expressing lymphomas were used to monitor the natural course of the disease. The entire cohort of animals bearing untreated control tumors succumbed to their disease within 25 days after initial manifestation (Fig. 7A). The overall survival of animals carrying control shRNA–expressing lymphomas could be significantly enhanced when these animals were treated with etoposide (Fig. 7A). KU-0060648 treatment did not produce a significant extension in overall survival of these animals. Furthermore, addition of KU-0060648 to the etoposide regimen did not result in significant additional survival gains beyond

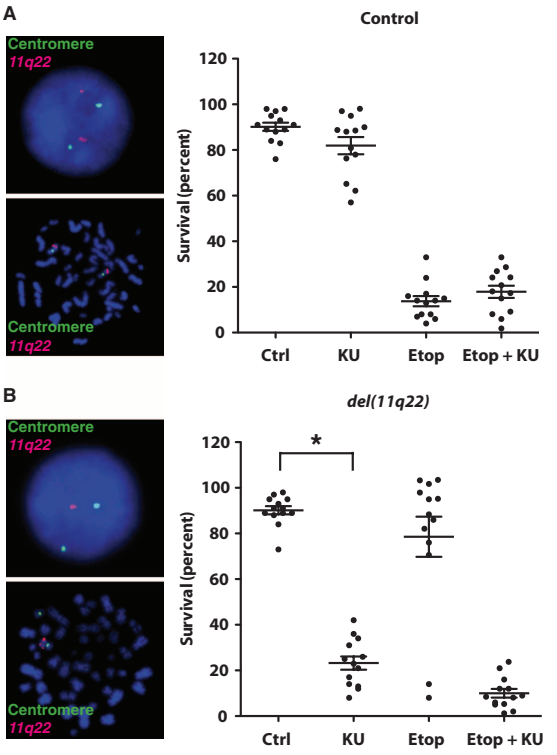

Fig. 6. Etoposide resistance and DNA-PKcs addiction in CLL cells carrying *del(11q)*. (**A** and **B**) CLL patients were stratified as wild type on *11q22* ($n = 13$) or *del(11q)* ($n = 13$) through FISH analysis (left panels). Primary CLL cells freshly isolated from 26 patients were seeded onto a feeder layer of CD40 ligand–expressing NIH 3T3 cells before treatment with KU-0060648 (KU) (0.5 μM), etoposide (10 μM), or a combination of both compounds. After 24 hours, cells were harvested and incubated with 7-aminoactinomycin D (7AAD) and fluorescein isothiocyanate–labeled annexin V and then analyzed by flow cytometry. Survival was quantified as the percentage of 7AAD^{low}/annexin V^{low} cells (bars indicate means ± SEM, $n = 9$). *$P < 0.05$, two-tailed Student's t test.

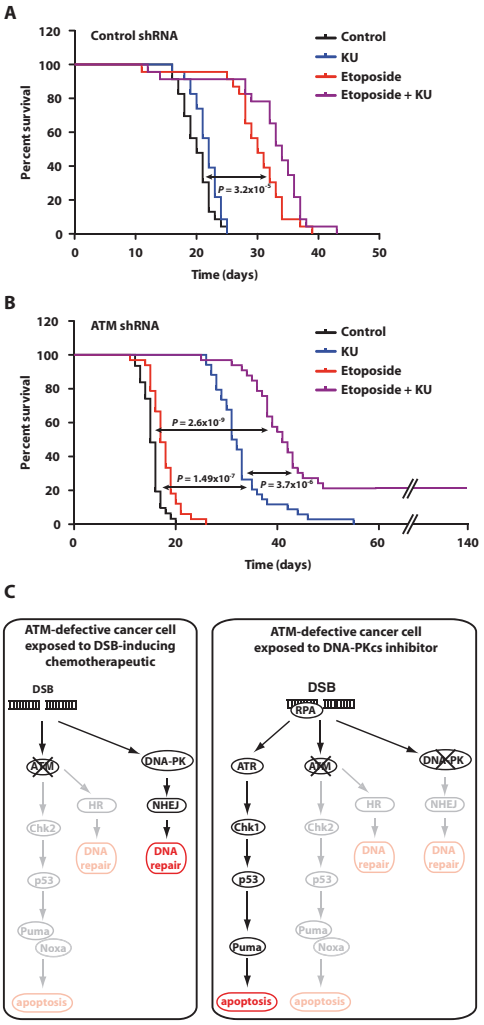

Fig. 7. Validation of DNA-PKcs as a drug target for the treatment of ATM-defective B-NHL in vivo. (**A** and **B**) RNAi-mediated suppression of ATM in Eμ-*MYC*;*ARF*$^{-/-}$-driven lymphomas confers etoposide resistance and DNA-PKcs dependence in vivo. Lymphoma cells were transduced with luciferase shRNA– or ATM shRNA–expressing retroviruses and injected into isogenic C57BL/6 recipient mice. Upon lymphoma manifestation, animals were treated with one course of KU-0060648 (KU) [blue lines in (A) and (B)], etoposide [red lines in (A) and (B)], or a combination of both compounds [purple lines in (A) and (B)] or left untreated [black lines in (A) and (B)]. Overall survival is shown in Kaplan-Meier format. Recording of survival was initiated on day 1 of each treatment regimen. In total, 23 mice in each treatment cohort carrying luciferase shRNA–expressing lymphomas and 34 animals in each treatment cohort carrying ATM shRNA–expressing lymphomas were included. Statistically significant survival differences are indicated (two-tailed Student's *t* test). (**C**) Proposed mechanism of DNA-PKcs addiction of ATM-defective cancer cells and the therapeutic targeting of the synthetic lethal interaction between *ATM* and *PRKDC*.

those that were achievable with etoposide. In contrast, survival of untreated animals bearing ATM-depleted lymphomas was slightly reduced compared to untreated control lymphomas, likely reflecting a more aggressive phenotype. Furthermore, ATM-depleted lymphomas were resistant to etoposide, with no significant survival gains compared to the untreated cohort. ATM-depleted lymphomas were highly sensitive to KU-0060648, leading to significant ($P < 0.05$) survival gains when compared with untreated and etoposide-treated animals. Combination treatment with KU-0060648 and etoposide prolonged the survival of ATM-depleted lymphoma-bearing animals even further, and a plateau was reached at about 20% (Fig. 7B). This plateau persisted for up to 140 days after initiation of treatment, suggesting that a cure rate of 20% might be achievable with a single course of KU-0060648 plus etoposide in this model. These data strongly suggest that DNA-PKcs inhibitors either alone or in combination with DSB-inducing genotoxic agents might be a valuable strategy to target ATM-defective human cancers.

DISCUSSION

Loss of ATM is associated with addiction to DNA-PKcs

Loss of ATM in neoplastic disease is associated with resistance against genotoxic therapies (*4*, *7*–*10*, *39*, *40*). This resistance has been attributed to a functional interception of the ATM/Chk2/p53 DDR signaling axis, which relays the presence of genotoxic lesions to an apoptotic cellular outcome (*10*, *40*) (Fig. 7C). However, ATM not only mediates apoptosis but also plays a critical role in HR-mediated DSB repair (*16*, *18*, *19*). Thus, apoptosis resistance is associated with reduced HR-driven DSB repair capacity in ATM-defective neoplastic disease. The observation that ATM-defective cells not only proliferate but also are resistant against genotoxic chemotherapy suggests that these cells can repair DSBs. Mammalian cells exploit two major DSB repair pathways—the ATM-dependent HR pathway and DNA-PKcs–mediated NHEJ. We show that ATM-defective cells rely on functional DNA-PKcs signaling for their survival, even in the absence of exogenously induced DNA damage. Our data suggest that the NHEJ pathway is a backup pathway for DSB repair in ATM-defective HR-impaired cells. Thus, although isolated loss of *ATM* appears to protect cancer cells from genotoxic stress by blunting the proapoptotic p53 response, it renders these cells exquisitely susceptible to DNA-PKcs inhibition. Our data are reminiscent of those seen in a synthetic lethal interaction between *BRCA1/2* and *PARP1* (*41*, *42*). Cells exposed to a PARP1 inhibitor accumulate DSBs, likely as a result of impaired base excision repair. Although these lesions are typically resolved through HR-dependent DSB repair, *BRCA1/2*-defective cells, as a result of their inherent HR defect, fail to repair PARP1 inhibitor–induced DSBs, ultimately resulting in cell death (*41*, *42*).

Targeting the synthetic lethal interaction between *ATM* and *PRKDC*

We focused on the exploitability of DNA-PKcs inhibitors for the treatment of ATM-defective cancers. We showed that both cancer-associated *ATM* mutations and ATM depletion resulted in DNA-PKcs dependence. To validate DNA-PKcs as a drug target for the treatment of ATM-defective human cancers, we used two distinct DNA-PKcs inhibitors, namely, KU-0060648 [a dual DNA-PKcs and phosphatidylinositol 3-kinase (PI3K) inhibitor (*43*)] and NU7441 [a DNA-PKcs inhibitor with only weak activity against PI3K (*44*)]. Both compounds displayed cytotoxic activity specifically in ATM-defective cells. Given

that both compounds display at least some degree of activity against PI3K as an off-target effect, it is conceivable that the effects we observed were at least partially due to PI3K inhibition. However, we believe that the contribution of PI3K inhibition to the cytotoxic effects of KU-0060648 and NU7441 is only marginal because RNAi-mediated DNA-PKcs depletion in ATM-defective human cancer cells mimicked the cytotoxic effects of KU-0060648 and NU7441. Furthermore, DNA-PKcs depletion produced dose-dependent effects, with potent shRNAs resulting in complete prevention of cell proliferation, whereas a less efficient shRNA allowed minimal residual growth of ATM-defective cells. Together, these data indicate that activity against DNA-PKcs mediates the cytotoxic effects of KU-0060648 and NU7441 in ATM-defective cells.

Clinical perspective

Disabling *ATM* mutations occur in about 10% of human tumors (*5*, *10*). Recently, two large CLL genome resequencing projects analyzing distinct patient cohorts have been published (*45*, *46*). One cohort only included treatment-naïve patients (*45*), whereas the second cohort also included pretreated patients (*46*). Consistent with our hypothesis that ATM deficiency is associated with resistance against frontline genotoxic chemotherapy, the number of *ATM* mutations was lower in the untreated cohort [4 of 105 patients (*45*)] than in the cohort that included pretreated patients [8 of 91 patients, 5 of whom were in the pretreated group (*46*)]. These independent observations suggest that *ATM* mutations accumulate in therapy-refractory CLL patients. Therapeutic options are currently very limited for these patients because they typically do not qualify for allogeneic transplantation. Thus, it will be interesting to test DNA-PKcs inhibitors in CLL patients who have been stratified on the basis of their *ATM* status. One such molecule might be CC-115, a dual mammalian target of rapamycin (mTOR)/DNA-PKcs inhibitor, currently in phase 1 clinical trials (*47*). Additional tumors in which ATM is frequently inactivated include head and neck squamous cell carcinoma and mantle cell lymphoma (*48*–*50*). Given the availability of DNA-PKcs inhibitors that are in clinical testing, as well as the various human malignancies with high rates of ATM inactivation, there will be ample opportunity to validate our findings in human patients with ATM-defective neoplastic disease.

MATERIALS AND METHODS

Lymphoma model

C57BL/6J recipient mice were anesthetized with isoflurane, and 1.5×10^6 *Eμ:MYC;ARF*$^{-/-}$ lymphoma cells were injected intravenously. Lymphoma cells had been isolated from the spleen of *Eμ:MYC;ARF*$^{-/-}$ lymphoma-bearing animals. Lymphoma burden was monitored by palpation of the axillary and brachial lymph nodes. Upon the appearance of substantial tumor burden (palpable lesions with a diameter of >0.5 cm, usually 11 to 13 days after injection), mice were exposed to the indicated treatments. KU-0060648 was administered at 10 mg/kg, twice daily, on days 1 to 4, and etoposide was given at 20 mg/kg, once daily, on days 1 to 4. Overall survival was measured as an end point of the current study. Experiments were approved by the local animal care committee of the University of Cologne.

Statistics

Values reported represent means ± SEM. *P* values were calculated with GraphPad Prism, with $P < 0.05$ considered significant. Experiments were done 3 to 12 times, and the particular statistical analyses used in the experiments are noted in the figure captions. Statistics were performed to illustrate significance between groups where $n \geq 3$.

Cell culture methods, virus production, immunoblotting, immunofluorescence, clonogenic survival assay, fluorescence-activated cell sorting and FISH analyses, and all reagents are described in detail in the Supplementary Materials and Methods.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. shRNA-mediated knockdown of DNA-PKcs.
Fig. S2. ATM complementation of HT144 and H1395 cells.
Fig. S3. shRNA-mediated knockdown of ATM in A375 and A549 cells.
Fig. S4. shRNA-mediated knockdown of ATM in *Eμ:MYC;ARF*$^{-/-}$ lymphoma cells.
Fig. S5. ATM depletion in *Eμ:MYC;ARF*$^{-/-}$ lymphoma cells leading to DNA-PKcs hyperactivation.
Fig. S6. Induction of apoptosis in DNA-PKcs inhibitor–treated ATM-defective cells rescued through Chk1 inhibition.
Fig. S7. Induction of apoptosis in DNA-PKcs inhibitor–treated ATM-defective cells rescued by suppressing Puma.
Fig. S8. shRNA-mediated knockdown of CtIP in ATM-defective HT144 and H1395 cells.
Fig. S9. Prevention of apoptosis by CtIP repression in DNA-PKcs inhibitor–treated ATM-defective cells.

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Discussion

Loss of ATM promotes resistance against chemotherapy and is associated with molecular addiction DNA-PKcs

Due to the functional interception of the ATM/Chk2/p53/Puma DDR signaling axis, which mediates apoptosis in the presence of genotoxic lesions, loss of ATM is associated with resistance against genotoxic chemotherapies (3, 26, 53, 58, 88, 98, 101). However, ATM is also known to play a major role in HR-mediated DSB repair, which is essential for an error-free repair of DSBs during late S- and G_2-phase, when an intact sister chromatid is present as a template (15). Thus, the resistance to apoptosis is accompanied by a reduced capacity of HR for DSB repair (75, 95, 116). To compensate this defect in HR in order to survive after genotoxic therapies, cells must rely on another DSB repair pathway. We could demonstrate, that this pathway is NHEJ, which is dependent on DNA-PKcs function (85). To further validate whether there is a synthetic lethal interaction between ATM and DNA-PKcs, we have shown that ATM mutation, as well as ATM depletion result in a robust non-oncogene addiction to DNA-PKcs (85). This opens a therapeutic window for the treatment of ATM defective malignancies via inhibition of DNA-PKcs. We could demonstrate a dose-dependent cytotoxic effect of DNA-PKcs inhibition by the use of different shRNAs with different degrees of DNA-PKcs knockdown efficiency (85). Furthermore, the significance of DNA-PKcs as a drug target could be shown via the use of two distinct, ATP-competitive DNA-PKcs inhibitors (KU-0060648 and NU7441), with both compounds displaying a high cytotoxic specificity for ATM-defective cells (85). We could demonstrate the efficiency of these DNA-PKcs inhibitors in the treatment of *ATM*-deficient malignancies *in vitro* and *in vivo* (85).

Resistance

A common problem in the treatment of cancer with ATP-competitive kinase inhibitors is the development of mutations inducing secondary resistance. These mutations can generally be divided into two groups. (1) There are "on-

target" mutations, which change the configuration of the kinase and intercept the interaction between the kinase and its inhibitor. This process is frequently seen in chronic myeloid leukemia (CML) patients receiving the first-line BCR-ABL kinase inhibitor imatinib (4). With the development of second-generation BCR-ABL kinase inhibitors i.e. dasatinib and nilotinib this resistance could be overcome (4).

Another example for on-target drug resistance is the missense mutation *T790M* within the *EGFR*-kinase domain which frequently occurs in erlotinib treated *EGFR*-mutaded NSCLC (37). Here, the irreversible *EGFR/HER2* inhibitor afatinib has recently been shown to prolong the progression free survival in erlotinib resistant patients in a clinical phase II trial (73).

A similar tumor evolution can be expected in the treatment of ATM-deficient malignancies with DNA-PKcs inhibitors and the development and evaluation of second generation DNA-PKcs inhibitors could become necessary.

(2) Mutations leading to drug-resistance can also be seen "off-target". In patients with tumors bearing loss-of-function mutations in *BRCA1*- or *2* the treatment with PARP1 inhibitiors recently has become a therapeutic option (2, 11, 28, 30, 33, 34). However, in pancreatic and ovarian tumors treated with PARP1 inhibitors, re-gain of function in *BRCA2* via intragenic deletion of small regions carrying the initial disabling frameshift mutation is described, leading to secondary resistance via generation of an HR competent isoform of BRCA2 (28, 91). Analogous to this model, re-gain of function in the ATM gene could lead to secondary resistance against DNA-PKcs inhibition.

Off-target effects

We have been able to demonstrate the efficacy of DNA-PKcs-inhibition in inherently chemotherapy resistant ATM-deficient malignancies *in vitro* and *in vivo* (85). As kinase inhibitors that we used in our study not only inhibit DNA-PKcs, but also have a known low level inhibitory off-target effect against PI3K, it would have been possible, that this effect is due to PI3K-inhibition, at least to a certain extent. By performing RNAi-mediated DNA-PKcs-knock downs with different shRNAs with different knock down efficacy, we could show a significant inhibitory dose-dependent effect on population doubling times of

ATM-deficient tumor cell lines compared to ATM-proficient counterparts (85). This finding may be substantiated by the described lethal phenotype for double knock out mice for DNA-PKcs and ATM, leading to a developmental arrest at ED7.5 (42). Thus, we propose that the effect of PI3K-inhibition is negligible and the observed cytotoxic effect of the DNA-PKcs inhibitors used in our study is indeed due to DNA-PKcs-inhibition.

Enhanced effectiveness of DNA-PKcs inhibition in ATM-deficient malignancies via combinatorial treatment with PARP inhibitors

As mentioned above PARP1 inhibitors have already become a therapeutic option in *BRCA1*- or *2*-deficient malignancies (with *BRCA1*- or *2*-deficiency causing a defect in HR). As a logical consequence PARP1 inhibitors also got tested in ATM-deficient malignancies and there is first evidence that PARP1 inhibition might also be a successful strategy in the treatment of those (112).
The inhibition of PARP1 leads to the conversion of SSBs to DSBs during cell division. As SSBs occur with a frequency of tens of thousands per day (instead of only 10 DSB per day), the combinatorial treatment of ATM-deficient malignancies with PARP1-inhibitors might potentiate the effect of DNA-PKcs inhibition and needs further investigation. A further consequence of our recent study must be the testing of a combinatorial treatment with DNA-PKcs and PARP1 inhibitors in *BRCA1*- or *2*-deficient malignancies, as DNA-PKcs inhibition should also potentiate the effect of PARP1 inhibition.
As both treatments are based on synthetic lethal interactions in HR-defective cells, this combinatorial treatment should selectively kill the cancerous cells by sparing their HR-competent neighbors. Furthermore, this dual kinase inhibition would spare any genotoxic compounds and thus should have a decreased frequency of secondary malignancies compared to established treatment regimens.

Long-term effects of DNA-PKcs inhibition

There are no data about the long-term effects of DNA-PKcs inhibition in humans. Mice treated with the two different DNA-PKcs inhibitors used in our

study did not show any long-term toxicities. However, Gao *et al.* have shown, that mice bearing a targeted homozygous DNA-PKcs null mutation are viable but are not capable to perform normal B and T cell differentiation via V(D)J-recombination and therefore suffer from immunodeficiency (36). V(D)J-recombination is a mechanism of genetic recombination, which assembles immunoglobulin and T cell receptor genes during lymphocyte development (55, 92). This process is responsible for the plethora of different immunoglobulins and T cell receptors, which enable specific responses to an enormous number of different antigens (55, 92). V(D)J-recombination includes a series of controlled DNA breakage and rejoining events, with the latter dependent on functional NHEJ and thus on DNA-PKcs (55, 92).

As most of the V(D)J-recombination takes place during embryogenesis and childhood and as the intermittent administration of a DNA-PKcs inhibitors will not block the V(D)J-recombination at all times, a treatment started in adult patients might not result in a major immunodeficiency as described for DNA-PKcs null mutant mice. Nevertheless there might be a certain degree of immunodeficiency predisposing for infectious diseases and secondary malignancies, especially if a lifelong treatment course with DNA-PKcs inhibitors will turn out to be necessary to successfully keep ATM-deficient malignancies under control.

Clinical Perspective

Shown for different tumor entities, disabling ATM mutations seem to occur in about 10 percent of human malignancies (6, 8, 24, 53, 80, 82). Furthermore, there is strong evidence for a poor prognosis for patients harboring *ATM* mutated tumors (3, 26, 88). The resistance to chemotherapy for tumors with loss of ATM previously described by Jiang and Reinhardt *et al.* could be further sustained by two large CLL re-sequencing projects (53, 80, 107). In one study only patients without any pretreatment were included (80), while the other one also included pretreated patients (107). With a relative mutational rate of under 4 percent in the untreated cohort (4/105 patients) and a relative mutational rate of more than 8 percent in the pretreated cohort (8/91 patients), the frequency of *ATM* mutation has been dramatically higher in the pretreated

group, indicating the accumulation of *ATM* mutations in therapy-refractory CLL patients (80, 107).

As there are only very limited therapeutic treatment options especially for these therapy-refractory CLL patients today, CLL patients stratified by their ATM-status might be an ideal collective to test the efficacy of DNA-PKcs inhibitors in *ATM*-mutated malignancies in men. CC-115 is a dual mTOR/DNA-PKcs inhibitor, which already has entered phase I clinical trials, could be an ideal candidate for the evaluation of DNA-PKcs inhibition these patients (67).

Zusammenfassung

Als Antwort auf genotoxischen Stress aktivieren Zellen eine komplexe, Kinase-basierte Signalkaskade um einen Zellzyklusarrest einzuleiten. In Abhängigkeit vom Umfang des Schadens wird ferner entweder eine Reparatur der DNA oder, bei zu ausgedehnten Schäden, die Apoptose initiiert. Diese Signalkaskade wird im Englischen unter dem Begriff *DNA damage response* (DDR) zusammengefasst. Die Kinase ATM bildet das Zentrum der DDR. Als solches ist sie sowohl am Zellzyklusarrest, der DNA-Reparatur sowie der Einleitung der Apoptose beteiligt. Mutationen, welche die Funktion von ATM beeinträchtigen, werden in unterschiedlichen Tumorentitäten sehr häufig nachgewiesen. Unter ihnen sind sowohl solide Tumoren, wie das Bronchialkarzinom als auch maligne hämatologische Erkrankungen, wie die chronisch lymphatische Leukämie.

Wir konnten zeigen, dass ATM-defiziente humane und murine Tumoren apoptoseresistent gegenüber genotoxischen Therapien sind. Mit Hilfe von genetischen und pharmakologischen Ansätzen konnten wir *in vitro* und *in vivo* eine starke Non-Oncogene Addiction von der Kinase DNA-PKcs in ATM-defekten humanen und murinen Zellen nachweisen. Des Weiteren gelang es zu zeigen, dass diese Abhängigkeit von DNA-PKcs in ATM-defekten Zellen eine Möglichkeit der therapeutischen Intervention bietet. Sowohl eine pharmakologische als auch eine genetische Hemmung von DNA-PKcs führen in ATM-defizienten Tumoren zur Akkumulation von DNA-Doppelstrangbrüchen mit anschließender CtIP-abhängiger Generierung großer einzelsträngiger DNA-Reperaturintermediate. Diese wiederum lösen eine Aktivierung proapoptotischer Signale aus, welche über die RPA/ATRIP/ATR/Chk1/p53/Puma-Achse vermittelt werden. Diese proapoptotischen Signale resultieren in apoptotischem Untergang DNA-PKcs-Inhibitor-exponierter ATM-defizienter Zellen. Zusätzlich konnten wir präklinisch *in vivo* eine deutliche monotherapeutische Wirksamkeit von DNA-PKcs-Inhibitoren gegen ATM-defekte Lymphome zeigen. In Zusammenschau unserer Daten scheint die Kinase DNA-PKcs eine vielversprechende Zielstruktur in der Behandlung ATM-defizienter maligner Erkrankungen zu sein.

Summary

In response to genotoxic stress, cells activate a complex, kinase-based signaling network to arrest the cell cycle, initiate DNA repair, or, if the extend of damage is beyond repair capacity, induce apoptotic cell death. ATM lies at the heart of this signaling network, which is collectively referred to as the DNA damage response (DDR). ATM is involved in all three of these DDR-regulated cellular responses – cell cycle arrest, DNA repair and apoptosis. Disabling ATM mutations occur frequently in various human tumor entities, including lung cancer and hematological malignancies. Here we show that ATM-deficiency protects human and murine cancer cells from apoptosis induced by genotoxic chemoptherapy. Using genetic and pharmacological approaches we then demonstrate *in vitro* and *in vivo* that ATM-defective murine and human cells display a strong non-oncogene addiction to DNA-PKcs signaling. We further show that this dependence of ATM-defective cells on DNA-PKcs offers a window for therapeutic intervention. We show that pharmacological or genetic abrogation of DNA-PKcs in ATM-defective settings leads to the accumulation of DNA double-strand breaks (DSBs) and the subsequent CtIP-dependent generation of large single-stranded DNA (ssDNA) repair intermediates. These ssDNA structures trigger the activation of pro-apoptotic signaling through the RPA/ATRIP/ATR/Chk1/p53/Puma axis, ultimately leading to the apoptotic demise of ATM-defective cells exposed to DNA-PKcs inhibitors. Lastly, we demonstrate that DNA-PKcs inhibitors show remarkable preclinical activity as single agents against ATM-defective lymphomas *in vivo*. Together, our data implicate DNA-PKcs as a novel drug target for the treatment of ATM-defective malignancies.

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Lebenslauf

Mein Lebenslauf wird aus Gründen des Datenschutzes in der elektronischen Fassung meiner Arbeit nicht veröffentlicht.